EXPLORING THE METABOLIC POTENTIAL OF NOVEL MARINE ACTINOBACTERIA

By

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Declaration

I declare that this thesis is my work, and there are no prior submissions of this report elsewhere, and related works by others that serve as a source of knowledge have been duly referenced.

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May 2022

Abstract

Actinobacteria are filamentous and ubiquitous Gram-positive bacteria with diverse potential to produce significant secondary metabolites, that have end biotechnology applications in medicine, agriculture, environmental and energy sectors. Over the years, the isolation of actinobacteria from terrestrial habitats has resulted in the isolation of the same genera thus decreasing the chances of discovering potential novel metabolites. This research study was aimed at isolating novel actinobacteria from marine habitats and exploring their metabolic potential. The marine environment was the focus of this study because it is still underexplored for the isolation of actinobacteria. A culture-dependent approach with selective media was used to isolate marine actinobacteria. Genotypic methods such as 16S rRNA sequencing, next-generation genomic sequencing by Illumina technology and phenotypic methods were used to characterize the isolates belonging to different genera. Our results showed a total number of nine novel actinobacterial species across six genera (*Brachybacterium, Kocuria, Micrococcus, Micromonospora, Streptomyces* and *Salinibacterium*) were isolated including those from previous work done in our laboratory. The small number of actinobacteria isolated could be due to the small aliquots of sample used in the inoculation which might not have captured the true diversity coupled with the fact that actinobacteria represent a small fraction of bacteria in the marine environment. The 16S rRNA gene sequences of the isolates were deposited in the NCBI GenBank database with a unique accession number assigned. ISP-ASW selective media supported the isolation of the most marine actinobacteria. The analysis of the genome sequencing data revealed that the draft genome features of the isolates consisted of assembled genome size which ranged from 3,994,542 bp for NB 16 to 9,849,154 bp for NB 20, and the percentage of G+C content ranged from 62.1% for NB 20 to 72.8% for NB 21 and the total number of gene ranged from 2,416 for NB 19 to 9,567 for NB 18. The analysis of the genomic sequencing data has given an insight into the different classes of BGCs present in the genome of the isolates and a clue to the kind of secondary metabolites that could be screened for from the isolates. A total of 26 biosynthetic gene clusters (BGCs) were detected in

the genome of the isolates as predicted by antiSMASH (Antibiotics and Secondary Metabolite Analysis Shell). The ability of the isolates to produce biosurfactants and extracellular enzymes were screened by phenotypic assays on multi-well agar plates. Our results showed that isolates NB 14, NB 16, NB 19, and NB 20 could produce biosurfactants according to phenotypic assay, TLC (thin layer chromatography) and HPLC-MS (high-performance liquid chromatography-mass spectrometer) analyses. Our phenotypic assay for the ability of the isolates to produce extracellular enzymes revealed that isolates NB 2, NB 15 and NB 16 could produce amylase while isolates NB 2, NB 14, NB 15, NB 16, NB 18 NB 19, NB 20, and FOP 8 produce protease and cellulase enzymes and isolate NB 2 could produce lipase. Genomic sequencing analysis also revealed the presence of cellulase and cellulose-related genes in NB 2, NB 18, and FOP 8. Rare marine actinobacteria have found wide applications in many sectors and this has necessitated their search and isolation in recent years. The phenotypic assay to produce extracellular enzymes is not enough to conclude that these isolates could produce these enzymes. The result needs to be backed up with molecular studies. This study has therefore revealed the different diversity of actinobacteria in the marine environment. This marine environment could be a rich reservoir for the isolation of marine actinobacteria and biosynthesis of important compounds and secondary metabolites.

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Acronyms and abbreviations

16S rRNA	16S ribosomal ribonucleic acid
ACP	Acyl carrier protein
AE	Elution buffer
AGS	Arginine glycerol salt
AIA	Actinomycetes isolation agar
AL	Lysis buffer
AntiSMASH	Antibiotics and Secondary Metabolite Analysis Shell
ASW	Artificial Sea water
AT	Acyltransferase
AW	Wash buffer
BGCs	Biosynthetic gene clusters
BLAST	Basic Local Alignment Search Tool
BP	Base pair
CDSs	Coding sequences
CoA	Acyl-coenzyme A
CMC	Carboxymethyl cellulase
CTP	Cytidine triphosphate
DAG	Diacylglycerol
DNA	Deoxyribonucleic acid

dNTP	Deoxynucleotide triphosphate
EDTA	Ethylenediaminetetraacetic acid
FAS	Fatty acid synthase
G+C	Guanine-Cytosine
GyrA	DNA gyrase A subunit
HPLC-MS	High-performance liquid chromatography-Mass Spectrometer
ISP	International Streptomyces Project
KEGG	Kyoto Encyclopaedia of Genes and Genomes
KS	Ketosynthase
L	DNA ladder
LAP	Linear azol(in)e-containing peptide
m/z	Mass to charge ratio
MEGA	Molecular Evolutionary Genetics Analysis
MF	Molecular formular
MRSA	Methicillin-resistance *Staphylococcus aureus*
MS	Mass spectrometer
MSM	Mineral salts medium
MW	Molecular weight
ncRNA	non-coding ribonucleic acid (ncRNA)

NAGGN	N-acetylglutaminylglutamine amide
NAPAA	Non-alpha poly-amino group acids
NMR	Nuclear magnetic resonance
NCBI	National centre for biotechnology information
NRP	Non-ribosomal peptide
NRPS	Non-ribosomal peptide synthetases
ORFs	Open reading frames
PBS	Phosphate buffered saline
PCP	Peptidyl carrier protein
PCR	Polymerase chain reaction
PGAP	Prokaryotic genome annotation pipeline
PKS	Polyketide synthases
R2A	Reasoner's 2A agar
RAPT	Read assembly and annotation pipeline tool
rDNA	Ribosomal Deoxyribonucleic acid
recA	Recombinase A
REP	Repetitive extragenic palindromic
RF	Retention factor
RiPPs	Ribosomally synthesized and post-translationally modified peptides

RO	Reverse osmosis
RPM	Revolutions per minute
rpoB	RNA polymerase β-subunit
RRE	RiPP recognition element
rRNA	Ribosomal ribonucleic acid
RT	Retention time
SCA	Starch casein agar
SKESA	Strategic K-mer extension for scrupulous assembly
SMA	Skimmed milk agar
SMBGCs	Secondary metabolite biosynthetic gene clusters
TAE	Tris-acetate-EDTA
Taq	*Thermus aquaticus*
TE	Thioesterase
TIC	Total ion chromatogram
tRNA	Transfer ribonucleic acid
TSA	Tryptone soya agar
TSB	Tryptic soy broth
UV	Ultraviolet
VOC	Volatile organic compounds

Chapter one

1.0 Introduction

1.1 Background: Actinobacteria - An overview

Actinobacteria are filamentous and ubiquitous Gram-positive bacteria (Hazarika & Thakur, 2020). They are mainly aerobic, facultatively anaerobic, or anaerobic (Hazarika & Thakur, 2020). They belong to the phylum Actinobacteria and order Actinomycetales with a G+C content of about 70% (Hazarika & Thakur, 2020). However, some members especially freshwater-dwelling have low G+C content (Kavagutti et al., 2019). The word ''Actinomycete'' combines two Greek words; aktis meaning "ray", and mykes, meaning "fungus" (Hazarika & Thakur, 2020; Segaran et al., 2017). Morphologically, they form a transitional link between fungi and bacteria (Barka et al., 2016). *Strepthrotrix foersteri* isolated in 1875 is the first actinobacteria species to exhibit both bacterial and fungal morphology (Sousa & Olivares, 2016). Actinobacteria are currently a separate group in the bacterial domain and constitute an important taxonomic group among the eight major lineages of bacteria (Ventura et al., 2007). They are also ubiquitous and they grow and proliferate in both terrestrial (soil) or aquatic environments (freshwater and marine) and in plants and animals, either as pathogens, parasites or commensals (Hazarika & Thakur, 2020). They are often found at moderate pH levels (Basavaraj et al., 2010; Ramesh & Mathivanan, 2009), though there are also some extreme acidophiles and alkaliphiles (Gohel & Singh, 2012; Poomthongdee et al., 2015; Zenova et al., 2011). They show great diversity in various characteristics, including moisture tolerance (Zenova et al., 2011), habitat, optimal pH, and thermophilicity (Embley &

Stackebrandt, 1994), enhancing their ubiquity. Genomic sequencing of Actinobacteria reveals that the genomes harbour secondary metabolites useful in medicine, agriculture, and biotechnology (Ventura et al., 2007). They comprise interesting groups of microorganisms characterised by their specific features and the biosynthesis of diverse secondary metabolites (Hohmann et al., 2009; Katz and Baltz, 2016). Actinobacteria reproduce either vegetatively by fragmenting their mycelia or asexually by the formation of spore or conidia (Shivlata & Satyanarayana, 2015). During the reproduction cycle, they form either a single spore (monosporic) or a pair of spores (bisporic), or many spores (oligosporic) on aerial mycelium (Shivlata & Satyanarayana, 2015). The actinobacteria with oligosporic spores have their spores arranged in a special form (hooked, straight, or wavy) on the mycelium (Shivlata & Satyanarayana, 2015). Their mycelial morphology often distinguishes members of this group with branched hyphae and the ability to form spores, though not all actinomycetes can produce spores (Embley & Stackebrandt, 1994). A typical life cycle of actinobacteria involves an asexual mode of reproduction (Figure 1.1).

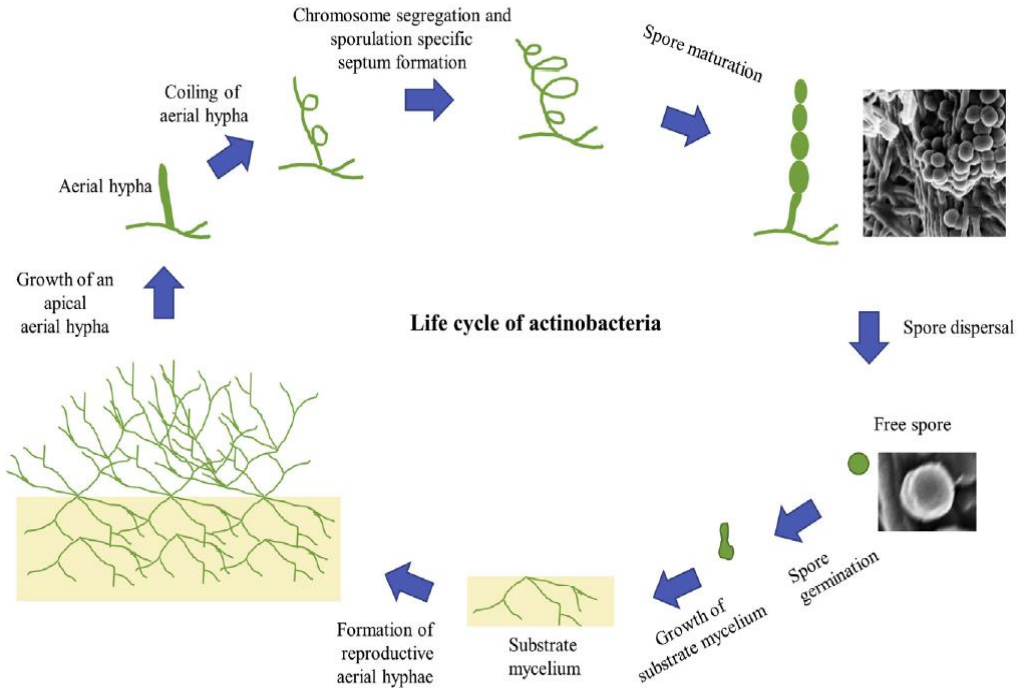

Figure 1. 1: Typical life cycle of actinobacteria showing hyphae and sporulation.
Adapted from (Hazarika & Thakur, 2020)

1.2 Origin: Isolation and Identification of Actinobacteria

Microbiologists used conventional plating techniques to get pure microbial cultures (Bodor et al., 2020). However, this technique does not depict the true nature of microbial diversity in the biosphere as some microbes are not culturable (Austin 2017). Although there is huge microbial diversity on earth, more than 99% of viable and potentially culturable microbes remain unculturable in the laboratory (Locey and Lennon 2016). Only a very small number of microorganisms are culturable by current culture-dependent approaches (Hahn et al., 2019; Hofer, 2018; Pedrós-Alió & Manrubia, 2016). Molecular ecological studies have shown that only small number of prokaryotes in the natural environment have been isolated (Bull et al., 2000; Bull & Stach, 2007; Head et al., 1998). In-vitro cultural conditions may not enhance isolation of target actinobacteria in a given sample (Wade, 2002). This could be because the required (key) nutrients are not present in the culture medium, or the medium might contain some toxic substances, or other organisms present in the sample might produce inhibitory substances against the target organisms (Wade, 2002). The isolation of actinomycetes from their different natural habitats is troublesome due to their slow growing nature in comparison with other fast growing microbes (Hazarika & Thakur, 2020). Due to their slow-growing nature and complex nutritional requirements for each genus, a selective mode of isolation tailored to a particular genus of the group has been the best strategy for their isolation. However, innovative selective isolation and characterisation studies have provided an avenue for isolating previously uncultivated (un-isolated) organisms (Goodfellow, 2010). The ubiquity of actinobacteria makes it difficult to have a single procedure for isolation

because of their diverse nutritional requirements in their respective areas of origin' (habitat). Many different methodologies have been highlighted for the isolation of specific actinobacteria genera (Goodfellow, 2010). Most selective methodologies focus on isolating a particular member of the actinobacterial groups based on the peculiar characteristics of the actinobacteria in that group (Goodfellow, 2010). The isolation of a specific kind of actinobacteria also depends on specific nutritional requirements such as sources of carbon, nitrogen, and complex minerals or trace elements (Hazarika & Thakur, 2020). Selective media such as actinobacteria isolation agar (AIA), humic acid vitamin B agar (HV), yeast extract malt extract agar, Kuster's agar, starch-casein agar, starch nitrate agar, inorganic salt starch agar, glycerol-glycine agar, chitin agar, and International Streptomyces Project (ISP) agar support the specific isolation of Actinobacteria (Hazarika & Thakur, 2020; Küster & Williams, 1964). Amino acids such as L-arginine and glycine act as nitrogen sources and play a very crucial role in the selective isolation of actinobacteria (Kumar & Jadeja, 2016). Since actinobacteria live in a diverse community in their natural habitats, the growth of other organisms such as gram-negative bacteria, other gram-positive bacteria and fungi in their habitat can be inhibited by adding antibiotics to the media (Hazarika & Thakur, 2020). Media for selective isolation are usually supplemented with antibacterial agents, either singly or in combination. Commonly used agents are nystatin, chloramphenicol, rifampicin, nalidixic acid, trimethoprim, tunicamycin, cycloheximide, leucomycin, faridomycin, kanamycin and chlortetracycline at various concentrations (Hazarika & Thakur, 2020). Isolation of actinobacteria is also enhanced by the method of pre-treating

the samples before the actual sample preparation (Baskaran et al., 2011). Traditional enrichment is simply based on trial-and-error methods using different chemical and physical treatments strategies to isolate the uncultured actinobacterial majority (Hug et al., 2018). Physical pre-treatment like the use of radiation, air drying, centrifugation, and heat drying of soil sample at 120^{O}C for 1 hr, enhances the isolation of actinobacteria (Hazarika & Thakur, 2020). Also, selective isolation of actinobacteria is enhanced by effectively treating samples with calcium carbonate and chitin, calcium chloride, phenol, sodium dodecyl sulfate (SDS), yeast extract, germicide, chemotactic agents, and chloramine-T (Hazarika & Thakur, 2020). Figure 1.2 below shows a schematic representation of the isolation and identification of actinobacteria

1.2.1 Identification of Actinobacteria using Molecular Markers

Actinobacteria identification is currently based on 16S rRNA gene sequencing supported by morphological and biochemical parameters (Goodfellow et al., 2012). These techniques have enhanced their taxonomic and phylogenetic relationship (de Jesus Sousa & Olivares, 2016; Salwan & Sharma, 2020). In the identification of actinobacteria, molecular markers such as rpoB, atpD, gyrB, recA, trpB and ssgB, have been used to delineate closely related genera or species (Barka et al., 2016; Girard et al., 2013; Ventura et al., 2007). Actinobacteria are also identified based on their phylogenetic relationship with other microbes by partially sequencing the 16S rRNA and 23S rRNA genes (Verma et al., 2013).

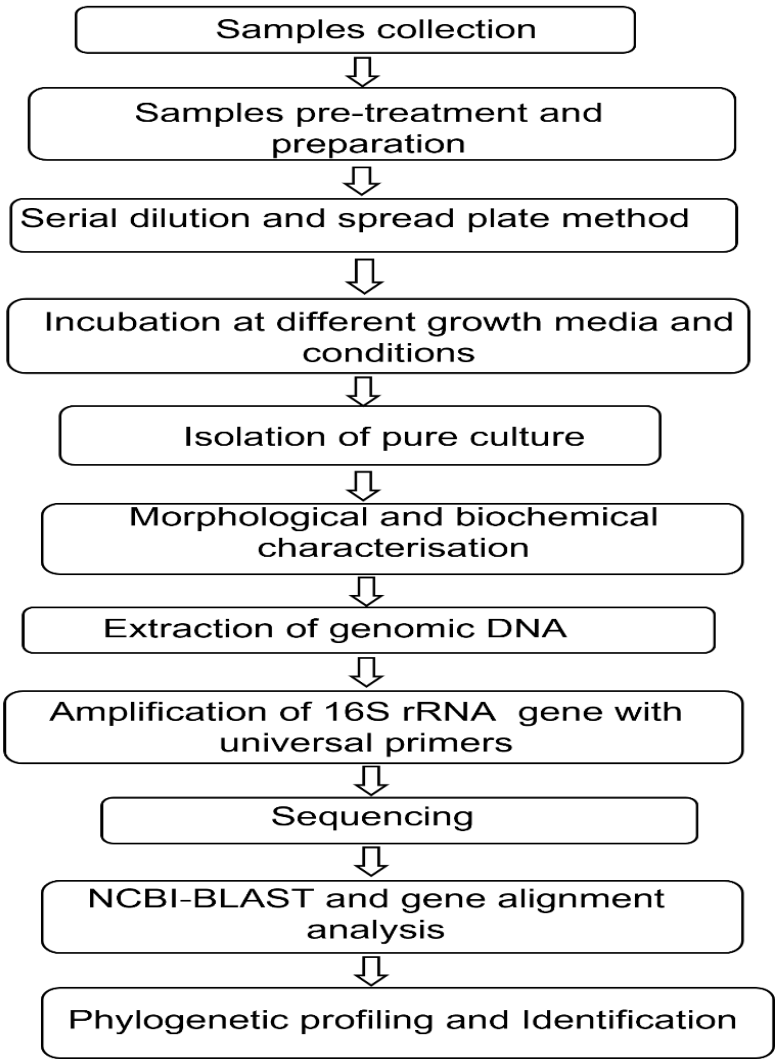

Figure 1. 2: Schematic representation for the isolation and identification of Actinobacteria (adapted from Hazarika & Thakur, 2020)

1.3 Habitat of Actinobacteria

The ubiquitous nature of actinobacteria implies that they are found virtually everywhere in both natural and artificial habitats (Hazarika & Thakur, 2020). Most actinobacteria are saprophytes while others form mutualistic or parasitic relationships with plants and animals (Mayfield et al., 1972; Barka et al., 2016). The ability of actinobacteria to adapt to a different range of ecological environments supports their biodiversity (Barka et al., 2016). Over the years, exploration, and isolation of actinomycetes have been focussed on terrestrial (soil) habitats. This has led to the over-exploitation and re-isolation of the same kind of actinobacteria, especially *Streptomyces*. In recent years, the search for new actinobacteria taxa and possible novel secondary metabolites, including bioactive molecules, has moved towards underexplored, unexplored, and extreme habitats (Hug et al., 2018). Underexplored environment such as oceans (Ma et al., 2017), deserts, mountains (Arasu et al., 2008) and Antarctica (L.-H. Lee et al., 2012), hot springs (L. Liu et al., 2016), endophytes (Silva-Lacerda et al., 2016) and symbionts (Hamm et al., 2017) are attracting attention for the exploration and isolation of novel actinobacteria and possible new metabolites including new antibiotics (Figure 1.3).

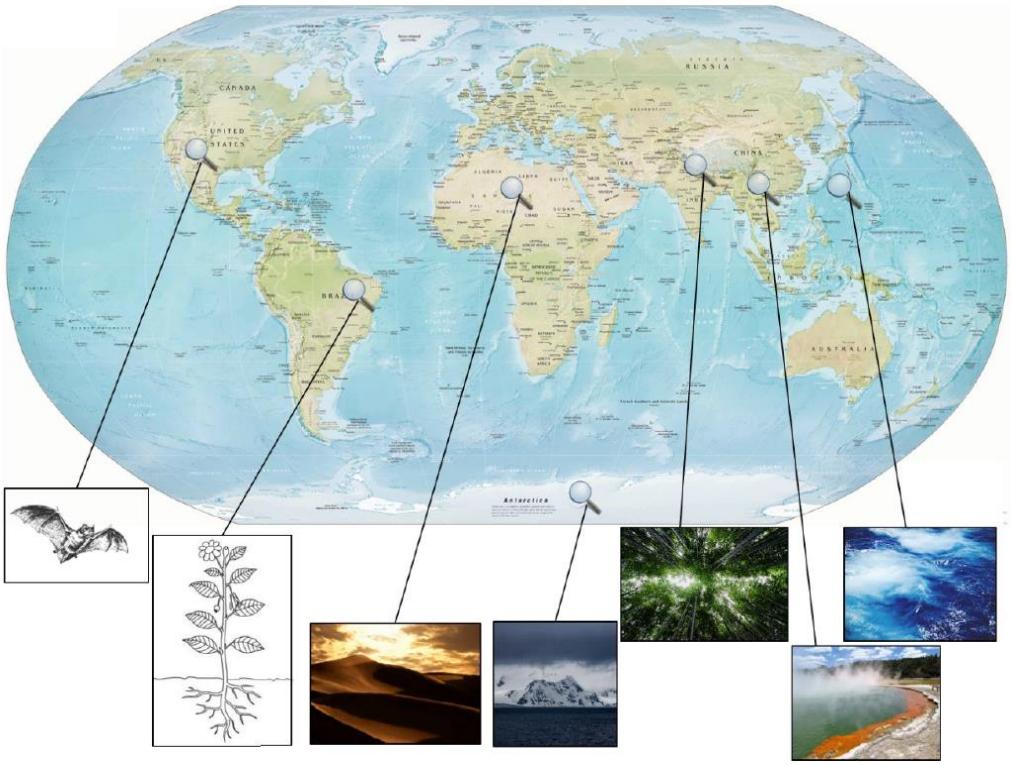

Figure 1. 3: Underexplored environments for actinobacteria.

Adapted from Hug et al., 2018

1.3.1 Marine habitat

The marine environment covers about 70% of the earth's crust. The marine habitat is quite complex in terms of its pressure, salinity, and temperature variation (Fenical, 1993). In the past, the marine habitat was overlooked in the isolation of microbes because of the impression that it contains very few organism as its salinity, temperature, and pH were deemed unfavourable for the growth and proliferation of many organisms (Davies, Adeyemi, and George, 2015). The marine environment is the least explored environment, especially when searching and bioprospecting for bioactive compounds (Lilja, 2013). They are well known habitat for the isolation of actinomycetes (Das, Lyla, and Khan, 2006; Ward and Bora, 2006). *Actinomycetes* are widely distributed in marine environments, and they are obtained from samples collected from different sections such as neuston (upper water layer), sediments, marine sponges, and seaweeds (Bull & Stach, 2007; Goodfellow & Fiedler, 2010). Marine *Actinobacteria* have been described as an emerging source for bioprospecting of secondary metabolites (Kamala et al., 2020). Marine actinomycetes have been cultured from different locations and microenvironments of the marine habitats, but they are predominantly obtained from marine sediments (Bredholdt *et al*., 2007; Duncan *et al*., 2015; Gontang, Fenical, & Jensen, 2007; León *et al*., 2007; Maldonado et al., 2008; Yuan et al., 2014).

1.3.2 Terrestrial habitat

The terrestrial environment is one of the most dominant habitats for Actinobacteria (Hazarika & Thakur, 2020). They are found on soil at a depth of about 2 meters and they have been isolated from different soil

types (Hazarika & Thakur, 2020). The *Streptomyces sp*. are the most isolated actinobacteria from the terrestrial habitat followed by *Nocardia*, and Nocardiopsis (Goodfellow, 1983). The growth and proliferation of actinobacteria in terrestrial habitats is influenced by temperature, pH, and soil moisture (Hazarika & Thakur, 2020). Soil that is rich in organic matter favours the growth of actinobacteria (Hazarika & Thakur, 2020). Screening of actinomycetes isolated from the terrestrial environment has resulted in the production of essential antimicrobials such as antibiotics (amphotericin B, erythromycin, vancomycin), anti-cancer (daunorubicin, bleomycin, mitomycin) and immunosuppressive agents such as rapamycin from these environments (Zotchev, 2012).

1.4 Phenotypes, Taxonomy, and classification of Actinobacteria

Actinobacteria are one of the significant phyla among bacterial domain lineages based on chemotaxonomic, phenotypic, and molecular systematic studies (Hazarika & Thakur, 2020). Actinobacteria has evolved over the years to include six classes, 22 orders, 54 families, 250 genera and more than 3000 species (Hazarika & Thakur, 2020). Members have been classified according to the nature of their spores, pigmentation and morphological and physiological features (de Jesus Sousa & Olivares, 2016). The mycelial morphology of Actinobacteria is rigid with the presence or absence of either straight or aerial mycelium (Salwan & Sharma, 2020). Morphologically, actinobacteria can be coccus, coccobacilli, streptococcus with fragmenting hypha and branched mycelia (Ventura, Canchaya, Tauch, et al., 2007). The production of spores and pigments in most actinobacteria are characteristic features in their taxonomical classification (Barka et al., 2016). Spores from actinobacteria are either produced in single cells or

chains, and they could be flagellated or non-flagellated (Salwan & Sharma, 2020). They have pigments known as melanoid polymers, which contribute significantly to their survival even though they are not required for growth (Barka et al., 2016). Several factors based on extensive studies have been used to separate the actinobacteria from other bacteria. These are the 16S rRNA and 23S rRNA gene analysis (Zhi et al., 2009). The presence of indels or conserved insertions and deletions in specific proteins (such as cytochrome-c oxidase subunit 1, cytidine triphosphate (CTP) synthase and glutamyl-tRNA synthase) (Gao & Gupta, 2005) characterize gene arrangements (Kunisawa, 2007).

1.5 Actinobacterial genome

The genome of actinobacteria is unique and has metabolic potential for the biosynthesis of secondary metabolites (Berdes, 2005). In recent years, genomic sequencing has transformed how we look at bacteria (actinobacteria) and this has greatly influenced the way their genome is explored for their metabolic potential (Bramhachari et al., 2018). Genome sequencing analysis helps to predict BGCs (Biosynthesis gene clusters) responsible for the biosynthesis of secondary metabolites (Thong et al., 2016). The development in genomics, metagenomics and high-throughput screening has given useful genetic information in the exploration of metabolites (Bramhachari et al., 2018). The Joint Genome Institute (JGI) and Integrated Microbial Genomes (IMG) database houses over 4600 actinobacteria genomes (Schorn et al., 2016). In 2018, about 71 actinobacterial genomes were completely sequenced and annotated (Bramhachari et al., 2018). Currently about 4233 actinobacterial genome is sequenced and annotated (Genome List - Genome - NCBI (nih.gov)) (accessed

on 15th January 2022). Previous studies have revealed that genome mining for metabolites could result in the discovery of novel metabolites (Fischbach & Walsh, 2009; Schorn et al., 2016). Genome mining in actinomycetes has discovered numerous cryptic BGCs with huge chances of producing novel metabolites (Gomez-Escribano et al., 2016). Genome mining is also a bioinformatics approach for the discovery of secondary metabolites (Antoraz et al., 2015; Katz & Baltz, 2016). For example, in *Streptomyces*, genome mining has become a powerful tool in unmasking their metabolic potential, (Weber et al., 2015). Some *Streptomyces* with linear chromosomes (Chen et al., 2002) and genome sizes ranging from 6.2 Mb for *S. cattleya* NRRL 8057 (Barbe et al., 2011) to 12.7 Mb for *S. rapamycinicus* NRRL 5491 (Baranasic et al., 2013) have been completely sequenced (Bramhachari et al., 2018). In Streptomyces, about 5% of their genomes are used for the biosynthesis of secondary metabolites (Ikeda et al., 2003). The first actinobacterial genome to be sequenced was *Mycobacterium tuberculosis* H37Rv (Cole et al., 1998). The genome of some other actinobacteria has been completely sequenced (Table 1.1), while the sequencing of others is still in progress (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). Chapter three of this thesis highlights the genomic sequencing of our selected genera.

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Table 1. 1: Some published data of actinobacteria genome

Actinobacterial Species	**Genome Size (bp)**	**No. of ORFs**	**% G+C content**	**No. of rRNA operons**	**No. of tRNAs**	**Reference**
Brachybacterium sp. P6-10-X1	4,385,603	4,045	70.90	9	50	(Zhao et al., 2017)
Streptomyces griseus XylebKG-1	8,566,464	6,851	72.21	5	66	(Schneider et al., 2018)
Streptomyces atratus SCSIO ZH16	9,641,288	9,245	69.50	18	69	(Li et al., 2018)
Mycobacterium tuberculosis TCDC7	4,641,184	4,370	65.55	3	53	(Wang et al., 2020)
Micromonospora sp. DSW705	6,795,311	6,219	72.90	4	51	(Komaki et al., 2016)
Plantactinospora sp. strains BB1	8.492,201	7,322	72.37	6	64	(Contreras-Castro et al., 2018)
Frankia Sp. BMG5.12	7,589,313	6,253	71.67	2	51	(Nouioui et al., 2013)
Salinispora Sp. H7-4	5,258,402	4,789	70.17	3	51	(Ulanova et al., 2020)
Brevibacterium Sp XM4083	4,324,102	3,732	68.02	3	47	(Daniela et al., 2019)
Micrococcus Sp. R8502A1	2,607,861	2,322	72.91	2	49	(Daniela et al., 2019)
Streptomyces spinoverrucosus SNB-032	8,854,993	7,906	70.9	6	70	(Schwarzer et al., 2021)
Gordonia hongkongensis strain EUFUS-Z928	5,329,221	4,987	67.97	8	47	(Sánchez-Suárez et al., 2022)
Verrucosispora sp. Strain CWR15	6,367,494	5,231	71.0	5	49	(Kennedy et al., 2020)
Arthrobacter sp. strain B6	4,663,437	3,956	64.67	6	89	(Xu et al., 2017)
Actinokineospora sp. strain EG49	7,529,476	6,629	72.8	3	50	(Harjes et al., 2014)

Streptomyces hyaluromycini MB-PO13	11,525,033	10,098	71.0	6	78	(Harunari et al., 2018)
Rhodococcus sp. Aw25M09	5,810,111	5,141	62.4	4	46	(Erik et al., 2013)
Stretomyces formicae KY5	9,611,874	8,162	71.38	6	65	(Holmes et al., 2018)
Streptomyces sp. Strain GMY02	8,512,626	7,098	70.4	9	69	(Jaka et al., 2021)
Actinoalloteichus hymeniacidonis HPA 177	6,306,386	5,346	68.08	6	67	(Schaffert et al., 2016)
Streptomyces sp. DUT11	8,027,164	7,745	72.22	9	63	(Xu et al., 2018)
Streptomyces niveus SCSIO 3406	7,990,492	7,088	70.46	6	65	(Zhu et al., 2021)
Nonomuraea sp. SBT364	9,992,837	9338	70.74	7	57	(Horn et al., 2015)
Nocardiopsis sp. SBT366	5,790,753	5,123	72.72	8	57	(Horn et al., 2015)
Micromonospora sp. HK10	6,911,179	6282	73.39	3	58	(Talukdar et al., 2016)
Streptomyces sp. A2-16	9,765,518	8,518	70.88	18	73	(Feng et al., 2022)

1.6 Applications of Actinobacteria

Actinobacteria serve as an important promising source for both primary and secondary metabolites with great application in many different sectors (Figure 1.4) (Ranjani Anandan & Manogaran, 2016; Zotchev, 2012; Hazarika & Thakur, 2020). They are also versatile groups of microbes with the capacity to produce secondary metabolites and some of these metabolites have antimicrobial properties against pathogens (Aouiche et al., 2014; Arasu et al., 2008). Actinomycetes also account for more than half of the currently known bioactive compounds that serve as antibiotics, antitumor agents, anti-cancer, anti-parasitic, anti-fungal, antiviral and immunosuppressive agents (Bérdy, 2005). Actinomycetes produce various bioactive (antimicrobial) compounds, industrial enzymes, and environmental and clinical compounds (Imada, 2005; Atta, Dabour and Desoukey, 2009; Valli *et al.*, 2012). The isolation of new actinomycetes from a new habitat could leads to the discovery of novel secondary metabolites (Adegboye and Babalola, 2013; Adegboye & Babalola, 2012; Dietz & Currie, 1996). Rare actinobacteria are genera of actinobacteria other than *Streptomyces* (Bérdy, 2005) or actinobacteria not isolated with the normal parameters of selective isolation (Baltz, 2006; Lazzarini et al., 2000). Isolation of novel actinomycetes from unexplored area like desert (Kurapovaetal.,2012), marine (Manivasagan & Venkatesan, 2013), and wetlands (Yu et al.,2015) could give a better chance of finding novel metabolites. New and under-explored habitats, including marine habitats, have been postulated to be a rich source of rare actinomycetes, that could potentially be producing novel bioactive compounds (Hong et al., 2009).

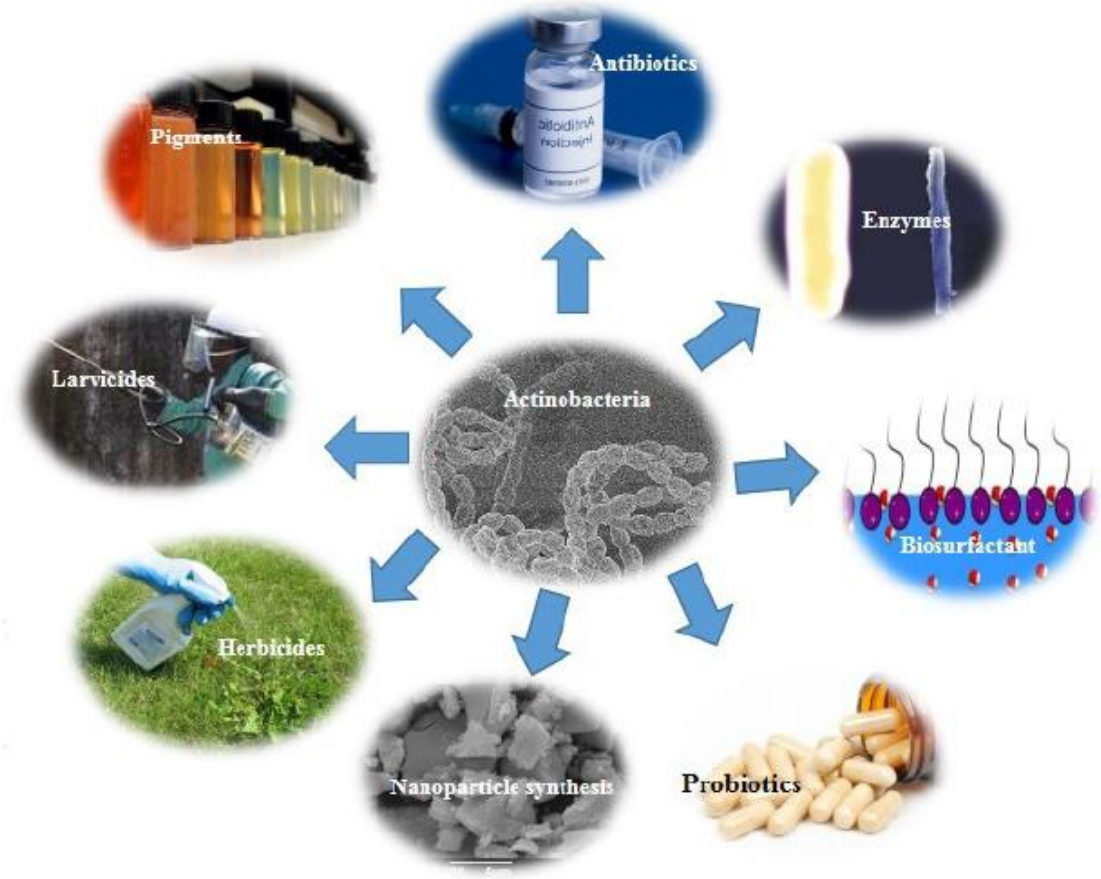

Figure 1. 4: Biotechnological applications of Actinomycetes

Source: (Ranjani Anandan & Manogaran, 2016)

1.7 Secondary metabolites

Actinomycetes have been a source for prospecting for natural compounds (Bérdy, 2012). These compounds have several applications in biotechnology, agriculture, medicine, industries and pharmaceutical companies (Doroghazi & Metcalf, 2013). They are also well recognised for the biosynthesis of essential secondary metabolites. Secondary metabolites play an important roles in the producing organism even though they are not needed for growth and development of the organism (Baltz, 2008; Bennett et al., 2005; Hopwood, 2007; van Keulen & Dyson, 2014). They provide a form of defence (or attack), competition, signalling depending on the environmental circumstances, thus increasing the likelihood of survival in a harsh environment (Brachmann et al., 2013; Rey-Caballero et al., 2017). They are generally produced at the end of log phase and the production depends greatly on the condition of the media (pH, temperature, nutrients, etc.) (Adegboye & Babalola, 2013). Secondary metabolites are usually produced when growth is limited or by the exhaustion of one or more essential nutrients required by the producing organism, such as carbon or nitrogen (Barrios-González & Mejýa, 2008; Sanchez and Demain, 2002). Secondary metabolites are also structurally diverse, and some of them function as antimicrobial agents, toxins, pesticides, ionophores, bioregulators, and those involved in quorum signaling (Adegboye & Babalola, 2013). Biosynthesis of secondary metabolite is usually catalysed by several enzymes, encoded by genes and these genes occur in clusters (Adegboye & Babalola, 2013). These genes which are located together in the genome of an organism are referred to as biosynthetic gene clusters (Harir et al., 2018; Doroghazi & Metcalf,

2013). They contain the necessary genes for the biosynthetic enzymes, regulatory proteins, resistance genes for the production (antimicrobial) of the secondary metabolites and genes for their secretion (Adegboye & Babalola, 2013). The genes clusters, polyketide synthase (PKS) and non-ribosomal peptide synthetase (NRPS) are involved in the biosynthesis of secondary metabolites (Donadio *et al*., 2007). The size of the gene cluster responsible for the synthesis of each secondary metabolite ranges from 10 kb to 100 kb (Adegboye & Babalola, 2013). The genes for the enzymes responsible for the synthesis of other constitutive compounds, such as sugars, amino acids are also located adjacent to the gene cluster (Adegboye & Babalola, 2013). After the core secondary metabolites are biosynthesized, structurally diverse derivatives may then be made either by elongation, synthesis, glycosylation, alkylation, and oxidation (Adegboye & Babalola, 2013). The complete process involving the production and transportation is regulated by transcriptional regulators and transporters (Adegboye & Babalola, 2013; Ichikawa *et al*., 2012). The genes encoding for enzyme transcriptional regulators and transporters are also located next to PKS and NRPS BGCs (Adegboye & Babalola, 2013). The majority of these BGCs (PKS and NRPS) are found in actinobacteria for the biosynthesis of bioactive metabolites (Donadio et al., 2007; Salomon et al., 2004). Apart from actinobacteria, other significant sources of secondary metabolites are Bacilli (Sansinenea & Ortiz, 2011), and *Myxococci* (Gerth et al., 2003; Wenzel & Müller, 2009) and Pseudomonads (Gross & Loper, 2009). Other biosynthetic pathways are the peptide pathway, the hybrid (non-ribosomal peptide polyketide) synthetic pathway, the shikimate pathway, the β-lactam synthetic pathway, and the

carbohydrate pathway (Figure 1.5) (Harir et al., 2018). Genome sequencing and analysis of the resulting data are used to predict the genes for the biosynthesis of metabolites (Thong et al., 2016).

1.7.1 Polyketide synthase (PKS)

Polyketides are a large class of structurally diverse and biologically active secondary metabolites derived from natural sources such as animals, plants, fungi and bacteria (McDaniel et al., 1993). Polyketides are synthesised by polyketide synthases (PKSs), a multi-enzyme complex or system that is highly similar to the fatty acid synthase (FAS) (Curran et al., 2018). This enzyme system consists of acyltransferase (AT), ketosynthase (KS), thioesterase (TE) and other optional domains (Wang et al., 2020). The biosynthesis of polyketides is initiated by loading the starter unit acyl-Coenzyme A (CoA) on the acyl carrier protein (ACP) catalysed by the AT domain (Piel, 2010). PKSs can be classified into types I, II, and III PKSs (Staunton & Weissman, 2001; Zhou et al., 2012). Type I PKSs are multifunctional peptides containing linearly arranged and covalently fused domains (Wang et al., 2020). Type II PKSs are multi-enzyme complexes composed of monofunctional proteins (Wang et al., 2020). They are found predominantly in bacteria and produce diverse aromatic polyketides (Hertweck et al., 2007). They are mainly responsible for producing aromatic polyketides by catalysing iterative Claisen condensation reactions usually using acetate as the starter unit (Waldman & Balskus, 2014). Type III PKSs are mainly found in plants as simple homodimers that use CoA rather than ACP as an anchor for chain extension (Shimizu et al., 2017).

1.7.2 Non-ribosomal peptide synthetases (NRPS)

Non-ribosomal peptide synthetases (NRPSs) are multi-modular enzymes that catalyse the biosynthesis of non-ribosomal peptides (NRPs) (Le Govic et al., 2019). NRPs are secondary metabolites produced by bacteria and fungi and also by higher organisms, such as nudibranchs, but it is thought that they are produced by bacteria residing inside these organisms (Ding & Dai, 2012). NRP represent a diverse group of natural compounds (Dang & Süssmuth, 2017). Structurally, they are linear, branched, circular or macro-circular (Dang & Süssmuth, 2017; Süssmuth & Mainz, 2017). NRPs are biosynthesized by the NRPS enzyme system in a modular manner, and each module is responsible for incorporating a specific amino acid into the peptide backbone (Martínez-Núñez & López, 2016). A module consists of three domains, adenylation (A) domain, a peptidyl carrier protein (PCP) or thiolation (T) domain, and condensation (C) (Drake et al., 2016). NRPSs are classified into a type I NRPS when the modules are arranged on a single protein and type II NRPS when the independent proteins are transient during the biosynthesis of NRP (Hur et al., 2012; Sattely et al., 2008). An NRPS can be as simple as a single modular unit consisting of three domains, though there are also complex and large NRPS that contain about 15 modules with 46 domains giving about 1.8 mega dalton protein complex (Bode et al., 2015; Wang et al., 2014).

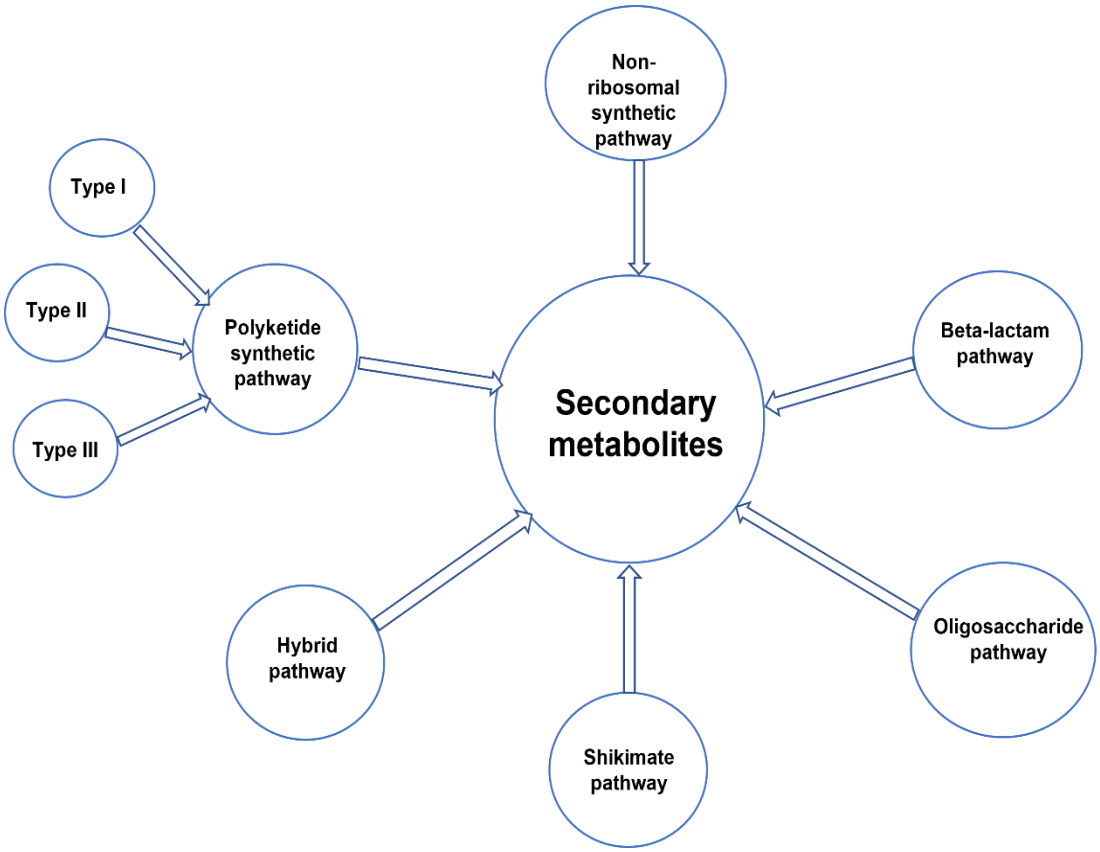

Figure 1. 5: Various pathways responsible in the assembly of secondary metabolites in actinobacteria. Adapted from (Harir et al., 2018)

1.7.3 Bioactive compounds (Antibiotics)

Bioactive compounds are biological metabolites that act on the activity of living organism, tissues, or cells. They are low molecular compounds that exhibit various biological activities against other organisms of either the same or different strains and species (Bull et al., 2005). Actinobacterial strains with bioactivities have mostly come from the soil (Guo et al., 2015). However, they could also be obtained from other habitat especially the marine habitat (Hassan et al., 2015; Xu et al., 2017). Actinobacteria possess the ability to produce different groups of secondary metabolites that have biological activities against multi-drug resistant pathogens. Actinobacteria have been the main focus of numerous research projects in recent years for the discovery of novel antibiotics that are safe, efficient and effective against pathogens (Gram-negative organisms, fungi, viruses and mycobacteria) including multi-drug resistances pathogens (Davies, Adeyemi, & Wang, 2015). Bioactive compounds from actinobacteria have accounted for approximately two-thirds of the naturally occurring antibiotics discovered, making them the single most important source of antibiotics (Subramani & Aalbersberg, 2012). Some antibiotics are produced in simple synthetic media or in complex organic substrates in either mono or co-culturation experiments. Table 1.2 shows a list of some selected bioactive compounds produced by marine actinobacteria.

Table 1. 2: Selected list of bioactive compounds produced by marine *Actinobacteria*

Compound	Source	Biological activity	Reference
1,8-Dihydroxy-2-ethyl-3- methylanthraquinone	*Streptomyces sp.*	Antitumor	(Huang et al., 2006)
1-hydroxy-1-norresistomycin	*Streptomyces chinaensis*	Antibacterial; anticancer	(Gorajana *et al*., 2005; Kock et al., 2005)
2-Allyloxyphenol	*Streptomyces sp.*	Antimicrobial; preservative and oral disinfectant	(Arumugam et al., 2010)
Arenicolides A-C	*Salinispora arenicola*	Mild cytotoxicity	(Jensen et al., 2007)
Arenimycin	*Salinispora arenicola*	Antibacterial; anticancer	(Asolkar et al., 2010)
Bisanthraquinone	*Streptomyces sp.*	Antibacterial	(Socha et al., 2006)
Butenolides	*Streptoverticillium luteoverticillatum*	Antitumor	(F. Li et al., 2005)
Caboxamycin	*Streptomyces sp.*	Antibacterial; anticancer	(Hohmann *et al*., 2009)
Caerulomycins	*Actinoalloteichus cyanogriseus* WH1-2216-6	Cytotoxic, antibacterial	(Fu et al., 2011)
Chinikomycins	*Streptomyces sp.*	Anticancer	(Li *et al*., 2005)
Chloro-dihydroquinones	*Novel actinomycete*	Antibacterial; anticancer	(Soria-Mercado et al., 2005)
Cyanogramides	*ctinoalloteichus cyanogriseus* WH1-2216-6	Multidrug resistance (MDR) reversing activity	(Fu *et al*., 2014)
Cyanosporaside A	*Salinispora pacifica*	Unknown	(Jensen et al., 2007)
Cyclomarine Cyclomarazine	*S. arenicola* CNS-205	Anti-inflammatory	(Schultz et al., 2008)
Daryamides	*Streptomyces sp.*	Antifungal; anticancer	(Asolkar et al., 2010)
Dermacozines, Phenazine derivatives	*Dermacoccus abyssi sp. nov.*, strains MT1.1 and MT1.2	Cytotoxic and antioxidant	(Abdel-Mageed et al., 2010)
Echinomycin	*Streptomyces echinatus*	Antibacterial, antiviral, and antitumor activities	(Foster et al., 1985; Kong et al., 2005)
Fijiolides	*Nocardiopsis* CNS-653	Inhibitor of TNF-a-induced NFkB activation	(Nam et al., 2010)

Fluostatin	*Micromonospora rosaria* SCSIO N160	Antimicrobial	(W. Zhang et al., 2012)
Frigocyclinone	*Streptomyces griseus*	Antibacterial	(Bruntner et al., 2005)
Glaciapyrroles	*Streptomyces sp.*	Antibacterial	(Macherla et al., 2005)
Isomethoxyneihumicin	*Nocardiopsis alba* KM6-1	Cytotoxic	(Fukuda et al., 2017)
Juvenimicin C	*Micromonospora* sp (CNJ-878)	Cancer chemo preventive	(Carlson *et al*., 2013)
Lagumycin B, Dehydrorabelomycin & Phenanthroviridone	*Micromonospora* sp.	Cytotoxic	(Mullowney et al., 2015)
Lajollamycin	*Streptomyces nodosus*	Antibacterial	(Manam et al., 2005)
Levantilide C	*Micromonospora* strain FIM07-0019	Antiproliferative	(Fei et al., 2013)
Levantilides	*Micromonospora* M71-A77	Cytotoxic	(Gärtner et al., 2011)
Lobosamides	*Micromonospora sp.* RL09-050-HVF-A	Antitryposomal	(Schulze *et al*., 2015a)
Lodopyridone	*Saccharomonospora sp.*	Anticancer	(Maloney et al., 2009)
Lucentamycins	*Nocardiopsis lucentensis (strain CNR-712)*	Cytotoxic	(Cho *et al*., 2007)
Lynamicins A-E	*Marinispora* sp	Antimicrobial	(McArthur et al., 2008)
Marinacarbolines,	*Marinactinospora thermotolerans* SCSIO 00652	Antimalarial	(Huang *et al*., 2011)
Marinomycins A-D	*Marinispora*	Antimicrobial; anticancer	(Kwon et al., 2006)
Marinopyrroles A-F	*Streptomyces* spp	Cyotoxicity and antibacterial	(Hughes et al., 2008)
Marthiapeptide A	*Marinactinospora thermotolerans SCSIO 00652*	Antibacterial, Cytotoxic	(Zhou et al., 2012)
Mechercharmycins	*Thermoactinomyces sp.*	Anticancer	(Kanoh et al., 2005)
Nocapyrones	*Nocardiopsis* sp.	Reduced the pro-inflammatory factor	(Kim *et al*, 2013)
Nocarimidazoles	*Nocardiopsis* sp. CNQ115	Weak antibacterial	(Leutou et al., 2015)
Piericidins	*Streptomyces sp.*	Antitumor	(Y. Hayakawa et al., 2007)

Proximicins	*Verrucosispora sp.*	Antibacterial; anticancer	(Fiedler et al., 2008)
Pseudonocardians	Pseudonocardia sp. SCSIO 01299	Antibacterial and cytotoxic	(Li *et al*., 2011)
Resistoflavin methyl ether	*Streptomyces sp.*	Antibacterial; anti-oxidative	(Kock et al., 2005)
Retimycin	*S. arenicola* strain CNT-005.	Cytotoxic	(Duncan et al., 2015)
Saliniketal	*Salinispora arenicola*	Cancer chemoprevention	(Jensen et al., 2007)
Salinipostins	*Salinispora* sp. RL08-036-SPS-B	Antimalarial	(Schulze *et al*., 2015b)
Salinispyrone	*Salinispora pacifica*	Unknown	(Jensen et al., 2007)
Salinoquinones	*Salinispora arenicola* CNS-325.	Cytotoxic	(Murphy et al., 2010)
Salinosporamide A	*Salinispora tropica*	Anticancer; antimalarial	(Jensen *et al*., 2007; Prudhomme *et al*., 2008)
Salinosporamides B & C	*Salinispora tropica*	Cytotoxicity	(Williams et al., 2005)
Sesquiterpene	*Streptomyces sp.*	Unknown	(Wu et al., 2006)
Sporolide A	*Salinispora tropica*	Unknown	(Jensen et al., 2007)
Staurosporinone	*Streptomyces sp.*	Antitumor; phycotoxicity	(Wu et al., 2006)
Streptokordin	*Streptomyces sp.*	Antitumor	(Jeong et al., 2006)
Taromycin	*Saccharomonospora* sp. CNQ-490	Antibacterial	(Yamanaka et al., 2014)
Tirandamycins	*Streptomyces sp.*	Antibacterial	(Carlson et al., 2009)
ZHD-0501	*Actinomadura sp.*	Anticancer	(Han et al., 2005)

1.7.4 Biosurfactants

Biosurfactants are amphiphilic biological compounds with emulsification properties and the ability to reduce surface tensions across polar substances such as oil and water (Smith et al., 2020). Biosurfactants are structurally diverse group of surface-active agents produced by different microorganisms, including bacteria (actinomycetes), yeast, and filamentous fungi from different environmental habitats (Banat et al., 2010; Saharan et al., 2011; Sharma & Singh Saharan, 2014; Singh Saharan et al., 2014). Actinobacteria are good producers of biosurfactants with varied industrial applications Chapter four of this thesis talk more on biosurfactant and bioprospecting and analysis techniques in our isolates

1.7.5 Extracellular Enzymes

Actinobacteria are groups of microorganisms characterized for their metabolic potential (Mukhtar et al., 2017). Production of enzymes by microorganisms including actinobacteria is highly efficient because of their relatively high yields, high scalability, cost-efficiency, and susceptibility to genetic manipulation (Vaijayanthi et al., 2016). Microbial enzymes are generally regarded as safe (GRAS) and they function well at a wide range of temperatures, pH, salinity or other extreme conditions (Mukhtar et al., 2017). Many genera of actinobacteria have been reported to produce different kinds of extracellular enzymes with biotechnological, medical and pharmaceutical applications (Nawani et al., 2013). Genomic sequencing and bioinformatic analysis have made the study of microbial enzyme production through proteomics and metaproteomics methods possible in recent times (Pieper et al., 2013). Actinobacteria have been continuously screened for their ability to produce proteases, cellulases, chitinases,

amylases, xylanases, and other important industrial enzymes (Vaijayanthi et al., 2016). Several genera of Actinobacteria have been reported to produce enzymes with several applications (Table 1.3). Different screening and phenotypic methods are used to prospect for the ability of actinobacteria to produce enzymes. Chapter five of this thesis highlights particular methods for prospecting selected extracellular enzymes production in actinobacteria.

1.7.6 Other metabolites from actinobacteria

Apart from bioactive compounds (antibiotics), biosurfactants and enzymes, actinobacteria also produce siderophores (Salwan & Sharma, 2020; Zeng et al., 2018), Plant growth hormones (Selim et al., 2021; de Jesus Sousa & Olivares, 2016; Wei et al., 2019) and volatile organic compounds (Sherwood et al., 2013) as metabolites. They are also reported to be good producers of other secondary metabolites such as biopesticide agents, antitumor compounds, larvicides, vitamins, antiviral agents, pharmacological compounds, pigments, probiotics, enzyme inhibitors anti-inflammatory compounds and single-cell protein feed (Selim et al., 2021; Harir et al., 2018).

Table 1. 3: Some enzymes produced by Actinobacteria and their industrial application

Enzymes	**Actinobacterial species**	**Uses**	**Industrial application**	**References**
Protease	*Streptomyces galbus, S. pactu, S. hermoviolaceu, Streptomyces ruber, S. lividans, Thermobifida halotolerans, Micromonospora spp*	Detergents Cheese making Clarification-low calorie beer Dehiding Treatment of blood clot	Detergents and cleaning Food Brewing Leather Medicine	(Kumar et al., 2014; Selim et al., 2021; Azzeddine et al., 2013; Mukhtar et al., 2017; Bentley et al., 2002)
Cellulase	*S. actuosus, S. erumpens, S. ruber, Thermobifida fusca, T. halotolerans*	Removal of stains	Deinking, modification of beers, paper, and pulp finishing, spinning of cotton	(Kumar et al., 2014; Selim et al., 2021; Zhang et al., 2011; Yang & Liu, 2004)
Lipase	*S. griseochromogenes, S. exfoliates, Nocardiopsis alba*	Removal of stain Stability of dough and conditioning Cheese flavoring Deinking, cleaning	Detergents and cleaning Baking Dairy Textile	(Kumar et al., 2014; Selim et al., 2021; Gandhimathi et al., 2009)
Xylanase	*S. rameus, Streptomyces spp., Actinomadura sp.*	Conditioning of dough Digestibility Bleach boosting	Baking Animal feeds Paper and pulp	(Kumar et al., 2014; Selim et al., 2021; Brzezinski et al., 1999)
Chitinase	*Thermobifida fusca,* Streptomyces thermoviolaceus, Nocardiopsis prasina	Chitin degradation	Textile and leather	(Kumar et al., 2014; Selim et al., 2021; 2016; Bhattacharya et al., 2007)
Pectinase	*S. fradzae, S. nztrosporeur, Thermomonospora flisca, S. viridochromogenes, S. ydicus*	Clarification, mashing, Retting and degumming of fiber crops	Brewing and Beverage Textile industries	(Kumar et al., 2014; Selim et al., 2021;Jacob et al., 2008)

Amylase	*S. aureofasciculus, S. galilaeus, S. erumpens, Thermobifida fusca,*	Removal of stains volume Deinking, drainage improvement Production of glucose and fructose syrups Removal of starch from wooden fabric	Detergents Baking Paper and pulp Starch industry Textile	(Kumar et al., 2014; Selim et al., 2021; Sharma et al., 2014; Zhang et al., 2011)
Glucos oxidase	*Streptomyces sp.*	Strengthening of dough	Baking	(Kumar et al., 2014; Selim et al., 2021)
Lipoxygenase	*Streptomyces sp.*	Bread whitening	Baking	(Kumar et al., 2014; Selim et al., 2021)
Phytase	*S. ambofaciens, S. lienomycini.*	Phytate digestibility	Animal feed	(Kumar et al., 2014; Selim et al., 2021)
Peroxidase	*Thermomonospora fusca, S. viridosporus*	Removal of excess dye	Textile	(Kumar et al., 2014; Selim et al., 2021)
β-galactosidase	*Streptomyces sp.*	Enzymatic hydrolysis of lactose either from milk/whey or pure lactose	Dairy	(Kumar et al., 2014; Selim et al., 2021)
L-asparaginase	*S. aureofasciculus, S. canus, S. hawaiiensis, S. olivoviridid, S. orientalis, S. plicatus*	Reduce the formation of acrylamide, a carcinogen found in starchy food products	Food industry	(Kumar et al., 2014; Selim et al., 2021)
L-glutaminase	*S. rimosus, S. galbus*	Flavor enhancing agent in food	Food industry	(Kumar et al., 2014; Selim et al., 2021)
Keratinase	*Doretomycetes microsporus*	Animal feed	Poultry industry	(Kumar et al., 2014; Selim et al., 2021; Habbeche et al., 2014)

1.8 Aim and Objectives

This research study aims to explore marine habitat for novel actinobacteria with metabolic potential. The specific objectives to achieve this aim were.

1. Selective isolation and identification of novel actinobacteria from the marine environment.
2. Exploring metabolic potential of marine actinobacteria from previous study in the lab.
3. Genomic sequencing and analysis of selected isolates belonging to different genera.
4. Phenotypic screening, production, and analysis of biosurfactants from the isolates.
5. Phenotypic screening and production of industrial extracellular enzymes from the isolates.

1.9 References

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Chapter Two

2.0 Selective isolation and identification of actinobacteria from marine environment

2.1 Introduction

The oceans (marine habitat) occupy about 70% of the earth's surface, with great microbial diversity that can be a source of different secondary metabolites (Orlova et al., 2015; Subramani & Aalbersberg, 2012). The marine habitat is quite complex in terms of pressure, salinity, and temperature variation (Fenical, 1993). They are one of the most under-explored environments in bioprospecting for secondary metabolites (Lilja, 2013). It has been one of the biggest sources of chemical and biological diversity, and hence research focusing on marine habitats has gained importance in recent times (Ramesh & Mathivanan, 2009). The biodiversity of the marine environment is quite different from those of the terrestrial, where much has been reported (Donia & Hamann, 2003). As with terrestrial habitats, the aquatic habitat contains limited amounts of readily available organic matter, with most sources of carbon being present in complex forms (i.e., cellulose and chitin) (Magarvey et al., 2004). The marine environment has been overlooked in the past probably because of the impression that the ocean contains very few microorganisms due to its harsh condition of salt, temperature and pH which are deemed unfavourable for the growth and proliferation of many microorganisms (Flora et al., 2015). The search and bioprospecting of secondary metabolites from actinomycetes in the past focused on the terrestrial environment (Laatsch, 2010; Solanki et al., 2008). It has been hypothesised that since there are different conditions in the terrestrial and

marine environment, microorganisms in the marine environment could be synthesising metabolites that are enabling them to survive the harsh conditions of the marine environment (Imada *et al.,* 2007; Valli *et al.*, 2012). But culture-independent studies show that marine sediments contain a wide range of unique microbes not present in the terrestrial habitat (Ravenschlag et al., 1999; Stach et al., 2003). They are widely distributed in marine environment, as they have been isolated from different sections of the marine environment such as neuston (upper water layer), waterbody, sediments, sea banks, and marine sponges and seaweeds (Bull & Stach, 2007; Goodfellow & Fiedler, 2010). Though marines actinomycetes have been isolated from various locations and microenvironments of the marine habitats, they are manly isolated from marine sediments (Bredholdt et al., 2007; Duncan et al., 2015; Gontang et al., 2007; León et al., 2007; Maldonado et al., 2008; Yuan et al., 2014). The isolation of actinomycetes from the marine environment has gained considerable attention in recent years (Lane & Moore, 2011; Liu et al., 2010). Marine actinomycetes are challenging to grow in the laboratory with the regular standard culturing techniques due to their special growth requirement compared to their terrestrial counterpart (Zotchev, 2012). However, improvements and enrichment in both the sampling and cultivation techniques allow for isolating valid marine actinomycete genera with different metabolic potentials (Jensen et al., 2005). Although the isolation of actinomycetes from marine sources started a long time ago, it was believed that they were like their terrestrial counterparts (Zotchev, 2012) and that they could have been obtained from wash-off from the terrestrial environment, mainly when samples from such isolations were

obtained from the seashores or banks (Ward & Bora, 2006). Isolation of true marine actinomycetes at that time were obtained from deep-sea sediments, rocks and marine flora and fauna (Zotchev, 2012). True marine actinomycetes are thought to be protected from the influence of the land wash-offs because of the distance from the seashore or ocean bank and other biological barriers (Zotchev, 2012). Specialized sampling techniques, such as modified sediment grab and designer-built bounce corer allow sediment sampling from depths of 2000m (Fenical & Jensen, 2006). The use of divers (Bredholdt et al., 2007), underwater remote-operated vehicles (Bruntner et al., 2005) and neuston sampling devices (Hakvåg et al., 2008) have provided access to essential sampling areas in the marine environment for the isolation of novel diversity of actinomycetes taxa (Zotchev, 2012). These rare marine actinomycetes with different metabolic potentials have attracted significant attention in terms of bioprospecting due to their unique potential to produce compounds of pharmaceutical and biotechnological importance (Azman et al., 2015; Bull & Stach, 2007; Subramani & Aalbersberg, 2013). This chapter exemplifies some of the above strategies in isolation, identification, and characterisation of novel and rare actinobacteria from the marine environment. Our focus in this chapter was to understand the actinobacterial diversity in the marine environment of the United Kingdom comprising the marine sediment, water body and seashore or riverbanks.

2.2 Materials and Methods

2.2.1 Sample collection

Samples comprising of marine soil (sediments), water and seashore soils were collected from Liverpool (53.4084^{o}N, 2.9916^{o}W) and Newcastle (54.9783^{o}N, 1.6178^{o}W) Seas, United Kingdom in May 2018, and June 2019 respectively (figure 2.1). The sediments were collected from the Sea at about 250 feets from the sea bank at a depth of 5 meters with the help of Sea divers. The water was collected from 3 meters from the sea bank. The seashores soils were collected at the sea bank at about 1.5 meters from the water body. The samples were collected in 50 ml centrifuge tubes and were immediately transported to the laboratory and stored at 4^{0}C until further analysis.

2.2.1.1 Sample pre-treatment

The samples were pre-treated by taking 2 ml of the samples (sediment, sand, and water) into a 15 ml centrifuge tube. The tube was vortexed vigorously for 30 minutes. One gram of the marine sediment and soil samples were weighed and transferred into a fresh 15 ml centrifuge tube. Similarly, 1 ml of the water sample was transferred into a 15 ml centrifuge tube. Nine millilitre of freshly prepared ringer's salt solution was added to the samples, and they were vortexed vigorously for 10 minutes. A five-fold serial dilution of the samples was prepared and then incubated at 56^{0}C for 15 minutes before inoculating various selective media.

2.2.1.2 Differential Centrifugation

A 2 ml aliquot each of the samples were placed in a 15 ml centrifuge tube and centrifuged (Eppendorf centrifuge 5810R machine) at 500 revolutions per minute (RPM) for 5 minutes. The supernatant was transferred into a

fresh 15 ml centrifuge tube for subsequent inoculation. The pellets were further centrifuged at 1500 RPM for 5 minutes. The supernatants were moved into a fresh tube while the pellets were further centrifuged at 2000 RPM for 5 minutes. At each stage of the differential centrifugation, a 5-fold serial dilution was prepared for inoculation on to respective selective media.

Figure 2.1: Location of sampling sites for rare marine actinobacteria from Newcastle and Liverpool Seas in United Kingdom. Red dots represent the location of the sampling place in the two seas. Letter A represent sample area in the Newcastle Sea and letter B represent the sample area in the Liverpool Sea

2.2.2 Selective isolation of marine Actinobacteria

Several media were prepared using both reverse osmosis (RO) water and artificial seawater (ASW) by Reagecon, Ireland. Constituents of the media used were obtained from Sigma-Aldrich unless otherwise indicated. Arginine glucose salt (AGS) agar was prepared according to Jihani *et al.*, 2012 by dissolving 1 g of arginine, 12.5 g of glycerol (100%), 1 g of dipotassium hydrogen phosphate (K_2HPO_4), 1 g of Sodium chloride (NaCl), 0.5 g of magnesium sulphate heptahydrate ($MgSO_4.7H_20$) (Thermo Fisher Scientific), 0.01 g of iron (iii) sulphate hexahydrate [$Fe(SO_4)_3.6H_20$], 0.001 g of copper sulphate pentahydrate ($CuSO_4.5H_20$), 0.001 g of zinc sulphate heptahydrate ($ZnSO_4.7H_20$) (Thermo Fisher Scientific), 0.001 g of manganese sulphate monohydrate ($MnS0_4.H_20$), 15 g of agar No. 2 (SLS) into 1000 ml of both RO and ASW water. The pH was adjusted to 7.2. The Reasoner's 2A (R2A) agar was prepared by dissolving 0.5 g of yeast extract, 0.5 g of protease, 0.5 g of Casamino Acids (VWR international LLC), 0.5 g of glucose, 0.5 g of soluble starch, 0.3 g of K_2HPO_4, 0.05 g of $MgSO_4.7H_20$ (Thermos Fisher Scientific), 0.3 g of sodium pyruvate, and 15 g of agar No. 2 Oxoid (SLS) in 1000ml of both RO and ASW water (Magarvey *et al.,* 2004). NaST21Cx agar containing solution A (750 ml of ASW containing 1.0 g of K_2HPO_4 and 10.0 g of Agar) and solution B (250 ml of ASW containing 1.0 g of KNO_3, 1.0 g of $MgSO_4$, 1.0 g of $CaCl_2.2H_2O$, 0.2 g of $FeCl_3$, and 0.1 g of $MnSO_4{\cdot}7H_2O$) according to Magarvey *et al.*, 2004 was prepared. Solutions A and B were autoclaved separately at 121^0C for 15 minutes at 15mmHg and then mixed. The media was tempered to 45^OC and was supplemented with 1.0 ml of trace elements (0.1 g of $FeSO_4.7H_2O$, 0.1 g of $MnCl_2$, and 0.1 of $ZnSO_4$ prepared in 100 ml of RO

water to give a final dilution of 0.0001 g for the respective trace metals) (Drews, 1976). International Streptomyces project media 2 (ISP-2) (Singh et al., 2016) (4 g of yeast extract, 10 g of malt extract, 4 g of glucose, 2 g of calcium carbonate, 12g of agar) was prepared in 1000 ml of artificial seawater (ASW). Difco™ marine agar 2216 from scientific laboratory supply was prepared according to the manufacturer's instructions. International Streptomyces project media 4 (ISP-4)/ inorganic salt starch agar was prepared according to Shirling & Gottlieb, 1966. The composition of the media consisted of 10 g of soluble starch, 1 g of dipotassium phosphate, 19 g of magnesium sulphate heptahydrate, 1 g of sodium chloride, 2 g of ammonium sulphate, 2 g of calcium carbonate and trace minerals such as 0.001 g of ferrous sulphate heptahydrate, 0.001g of manganese chloride heptahydrate, 0.001 g of zinc sulphate heptahydrate, 20 g of agar and 1000ml of ASW and ISP 5 (Shirling & Gottlieb, 1966) consisting of 1 g of L- asparagine, 1 g of dipotassium phosphate, 20 g of agar, 10 ml of glycerol and 1 ml of trace salt solution (0.001 g of Ferrous sulphate heptahydrate, 001 of manganese chloride tetrahydrate and 0.001 g of zinc sulphate heptahydrate) were prepared. Starch Casein Agar (SCA) consisting of 10 g of soluble starch, 2 g of K_2HPO_4, 2 g of KNO_3, 0.3 g of casein, 0.05 g of $MgSO_4.7H_2O$, 0.02 g of $CaCO_3$, 0.01 g of $FeSO_4.7H_2O$, 15 g of agar and 1000 ml of both RO and artificial sea water. The pH was adjusted to 7.0±0.1. Actinomycetes isolation agar (AIA) consisting of 2.0 g of sodium caseinate, 0.1 g of L-Asparagine, 4.0 g of Sodium propionate, 0.5 g of Dipotassium phosphate, 0.1 g of Magnesium sulphate, 0.001 g of Ferrous sulphate, 15 g of agar 15.000 and 1000 ml of both RO and artificial sea water. The media was sterilized by autoclaving at 121^0C for 15 minutes

at 15 mmHg. All media were supplemented with various concentrations of antibacterial and antifungal agents comprising of nalidixic acid (25 to 50µg/ml) from Acros organic, USA; cycloheximide (0.1 % of 50 to 100mg/ml) and Nystatin (25µg/ml) (Alfa Aesar, China). The reagents were sterile filtered and added to the cooled (40-50^{0}C) freshly prepared sterile selective media. Agar plates consisting of various selective media were inoculated with 100 µl aliquot of the serially diluted samples. The plates were then incubated at 28^{0}C for three weeks.

2.2.3. Selection and purification of colonies

Single and distinctive colonies were selected across the various dilution plates and re-streaked on to new media. The colonies were further purified by re-streaking them onto a fresh media three times (three stages re-streaking purification process), and each time, the plates were incubated at 28^{0}C for 48hours. Purified colonies were Gram-stained and Gram-positive isolates with vivid (distinctive) actinobacterial morphology were stored in 20% glycerol at -80^{0}C for further analysis.

2.2.4 Genomics DNA Extraction

DNA was extracted by using Qiagen DNeasy Blood and Tissue extraction kit, Germany, with minor modifications (Qiagen, 2016). A 1.5 ml of the cell culture grown in tryptic soy broth (TSB) was centrifuged at 14000 RPM for 10 minutes, and the pellets were re-suspended in 200 µl of phosphate-buffered saline (PBS). The lysis step was modified by adding a 20 µl of 50mg/ml lysozyme to the cell and was incubated for 30 minutes at 37^{o}C, after which 20 µl proteinase K was added. This was followed by adding 200 µl Buffer AL (Lysis buffer), and the mixture was vortexed thoroughly, after which it was incubated at 56^{o}C for 10 minutes. After the incubation, 200 µl

of 100% ethanol was added and was again vortexed entirely. Next, 700 µl of the mixture was pipette into a DNeasy mini spin column placed in a 2 ml collection tube. This was centrifuged at 8000 RPM for 1 minute. The flow-through and the collection tube were discarded, and the spin column was placed in a new 2 ml collection tube. Next, 500 µl Buffer AW1 (Wash buffer 1) was added and was centrifuged for 1 minute at 8000 RPM. The flow-through and the collection tube were again discarded as before, and the spin column was placed in a new 2 ml collection tube. Next, 500 µl Buffer AW2 (Wash buffer 2) was added and was centrifuged for 3 minutes at 14000 RPM. The flow-through and the collection tube were discarded again, and the spin column was transferred to a new 1.5 µl micro-centrifuge tube. The DNA was eluted by adding 200 µl Buffer AE (Elution buffer) to the centre of the spin column membrane and was incubated at room temperature (15-25^{o}C) for 1 minute. The mixture was then centrifuged at 8000 RPM for 1 minute, and the eluted DNA was placed on ice for further analysis. The DNA band was run on a 1% agarose gel containing 5µl ethidium bromide. Gels were electrophoresed at 100 V for 40 minutes in 1x TAE buffer. The gels were then viewed and scanned for DNA bands in a Gel Doc apparatus (Gel-Doc-it^{TM} 310 Imaging System), USA.

2.2.5 Amplification of 16S rRNA gene

The gene coding 16S rRNA was amplified by the polymerase chain reaction (Techne PCR machine 3 Prime G/02, USA) method using universal primers 27F (5'–AGAGTTTGATCMTGGCTCAG–3'), 1492R (5'–GTTACCTTGTTACGACTT–3'), 1525R (5'-AAGGAGGTGATCCAGCC-3') and 63F (5'-CAGGCCTAACACATGCAAGTC-3') (Downes et al., 2000; Frank *et*

al., 2008). Positive (of known actinobacteria) DNA template and negative (without any DNA sample) controls were also included in the PCR experiments. A 50 µl PCR reaction was set up (Bora et al., 2015). The reaction mixture had a 10 µl of 5X PCRBIO Buffer, 1.5 µl of 15mM $MgCl_2$, 1 µl of 10 mM dNTP, 2 µl each of 20 mM of both primers, 2 µl of DNA template, 31.25 µl of nuclease-free water and 0.25 µl of *Taq* DNA polymerase. The PCR conditions followed with an initial denaturation at 95^{o}C for 10 minutes, 30 cycles at 95^{o}C for 1 minute, primer annealing at 50^{o}C for 1minute, elongation at 72^{o}C for 1 minute and final cycle of the DNA extension at 72^{o}C for 10 minutes and a final cooling step at 4°C. PCR amplicons were run on 1% agarose gel consisting of 5 µl ethidium bromide. Gels were electrophoresed at 100 V for 40 minutes in 1x TAE buffer. The gels were then view and scanned for DNA bands in a Gel Doc apparatus (Gel-Doc-it^{TM} 310 Imaging System), USA.

2.2.6 Repetitive Extragenic Palindromic (REP) PCR DNA Finger Typing

To de-replicate similar clones of the same culture, repetitive extragenic palindromic (REP) PCR was carried out using REP primers RepIRI (forward): IIIICGIGCICATCIGGC and Rep2I (reverse): ICGICGTATCIGGCCTAC (Versalovic *et al*., 1994). A 50 µl PCR reaction was set up (Bora et al., 2015) consisting of 10 µl of 5X PCRBIO Buffer, 1.5 µl of 15mM $MgCl_2$, 1 µl of 10 mM dNTP, 2 µl each of 20 mM for both primers, 1 µl of DNA template, 32.25 µl of nuclease-free water and 0.25 µl of *Taq* DNA polymerase. The PCR conditions followed with an initial denaturation cycle at 95^{o}C for 10 minutes, 28 cycles of denaturation at 95^{o}C for 1 minute, primer annealing at 40^{o}C for 1 minute, extension at 65^{o}C for 8

minutes and final extension at 65°C for 16 minutes and was then cooled and held at 4°C. The 0.25 µl of *Taq* DNA polymerase was added after the completion of the initial denaturation step as a hot start PCR. The amplified rep-PCR genomic fingerprint fragments were run on a 1% agarose gel containing 5µl ethidium bromide. Gels were electrophoresed at 120 V for 2 h in a 1x TAE buffer electrophoresis tank. The gels were viewed and scanned for DNA bands in a Gel Doc apparatus (Gel-Doc-it™ 310 Imaging System), USA.

2.2.7 Detection and Identification of Actinobacteria

The isolates were subjected to PCR screening with Actinobacteria specific primers S-c-Act-0235-a-S-20 (forward): 5'-CCGTACTCCCCAGGCGGGG-3' and S-c-Act-0878-A-19 (reverse): 5'-CGCGGCCTATCAGCTTGTTG-3') for the amplification of the V3 to V5 regions of the 16S rDNA gene from Actinobacteria (Stach *et al*., 2003). A 50 µl PCR reaction was set up. The reaction contained a 10 µl of 5X PCRBIO Buffer, 1.5 µl of 15mM $MgCl_2$, 1 µl of 10 mM dNTP, 2 µl each of 20 mM of both primers, 2 µl of DNA template, 31.25 µl of nuclease-free water and 0.25 µl of *Taq* DNA polymerase. The PCR conditions followed with an initial denaturation at 95°C for 5 minutes, 30 cycles for denaturation at 95°C for 1 minute, primer annealing at 55°C for 1minute, extension at 72°C for 1.5 minutes and final extension at 72°C for 10 minutes and was then cooled and held at 4°C. Amplified PCR product was also separated on a 1% agarose gel electrophoresis containing 5µl ethidium bromide. Gels were electrophoresed at 100 V in 1x TAE buffer for 40 min. The gels were viewed and scanned for DNA bands in a Gel Doc apparatus (Gel-Doc-it™ 310 Imaging System), USA.

2.2.8 DNA Gel Extraction

To have pure PCR products especially in samples (isolates) where there were multiple DNA bands on the gel electrophoresis, there was need to physically extract the biggest DNA band based on the expected DNA size from the gel. The procedure involved first running the whole and unpurified DNA samples on gel electrophoresis. The gel containing the DNA band was excised with a clean razor blade under Ultra-violet light. The gel slice with the DNA was placed in a pre-weighed 1.5 ml centrifuge tube and the weight of the gel slice was recorded. Three times the volume of the weight (3:1; volume: weight) of the DNA of Qiagen buffer PB was added to the gel slice. The mixture was incubated in a water bath preheated to 65^{o}C for about 8 minutes or till the gel slice was completely dissolved. The centrifuge tube was inverted intermittently during the incubation period. At the end of the incubation, the mixture was poured into a fresh tube with an adsorption column, and it was centrifuged at 13,000 RPM for 1 minute, and the flow-through was discarded. The column was put back on the collection tube, and 750 µl buffer PE containing 100% alcohol was added and this was centrifuged for 1 minute at 13000 RPM. The flow-through was discarded, and the column was again placed on the same collection tube and was further centrifuged for 1 minute at 13000 RPM to remove any residual buffer. A 50 µl buffer EB was added to the centre of the membrane, and this was centrifuged for 1 minute at 13000 RPM. The purified samples were electrophoresed on 1% gel for 40 minutes.

2.2.9 Purification of DNA

The DNA samples were purified using the Qiagen QIAquick PCR Purification Kit (Qiagen, 2008). A 200 µl of Buffer PB was added to 40 µl of the PCR

product, and this was mixed thoroughly. The mixture was then added to the purification kit column and placed on a 2 ml collection tube. The mixture was centrifuged for 1 minute at 13000 RPM, and the flow-through was discarded. The column was put back on the collection tube, and 750 µl buffer PE was added and was centrifuged for 1 minute at 13000 RPM. The flow-through was discarded, and the column was again placed on the same collection tube and was further centrifuged for 1 minute at 13000 RPM to remove any residual buffer. A 50 µl buffer EB was added to the centre of the membrane, and this was centrifuged for 1 minute at 13000 RPM. The bands of the purified DNA were seen on the gel after 1% gel electrophoresis was run.

2.2.10 Agarose Gel electrophoresis

A 1.0% agarose gel containing 5 µl ethidium bromide was prepared in the gel room by weighing and dissolving 1.5 g of the agarose powder in 150 ml of 1X TAE (Tris-acetate-EDTA) Buffer. The mixture was heated in an oven for 1-2 minutes to ensure complete dissolution. This was allowed to cool down for a few minutes, and 5 µl (10 mgs/ml) of ethidium bromide was added. The semi-cooled mixture was poured into a pre-assembled casting tray with relevant combs. The gel agarose was allowed to solidify for 25-30 minutes. After solidifying, some 1X TAE buffer was poured on the gel, and the comb was removed. The cast containing the gel was transferred to the electrophoresis unit filled with the 1X TAE buffer. The samples and the ladder were loaded onto the gel wells. The electrophoresis unit was connected to the electricity supply, and the gels were electrophoresed at 100 V for 40 minutes. The gels were viewed and

scanned for DNA bands in a Gel Doc apparatus (Gel-Doc-it™ 310 Imaging System), USA.

2.2.11. Sequencing and phylogenetic analysis

A 20 µl aliquot of purified amplicons of the samples (isolates), as well as 50 µl of 3.2mM primers (27F, 1492R and 1535R), were prepared and used for sequencing (Bora et al., 2015). The samples in labelled tubes were sent to Source Bioscience, Nottingham, the United Kingdom, for sequencing (Sanger). The chromatogram of the sequenced amplicons was viewed with Snap Genes viewer version 6.0.2. For the 16S rDNA sequencing, the forward and reverse (complements) sequences were assembled and aligned with ClustalX to form a long and continuous strand of the DNA (Thompson *et al*., 1997). The partial 16S rRNA gene sequences (averaging 1,400 nucleotides) were used to search the NCBI GenBank database with Blastn (nucleotide BLAST against highly similar sequences) algorithm to determine the relative phylogenetic positions (Zhang *et al*., 2000; Morgulis *et al*., 2008). Selected type strains of the blast results were aligned with the nucleotide of the isolates by the Clustal omega online alignment tools (https://www.ebi.ac.uk/Tools/msa/clustalo/). Unreliable parts and gaps of the aligned sequence were trimmed off. Phylogenetic trees of the evolutionary relationship between the isolates and sequences with close similarity from NCBI blast result based on 16 rRNA gene clusters were determined by the neighbour-joining method (Saitou & Nei, 1987). Percentage values of ≥99% identity of the blast query sequence to the closest relative database sequence were considered as species identification, whereas percentage identity values between ≥97% and

≤99% were considered as identification to a genus level and percentage identity value of ≥95% were considered potential new genus.

2.3 Results

2.3.1 Selective isolation of novel marine actinomycetes

We used selective media with varying concentrations of inhibitory agents (nystatin, nalidixic acid and cycloheximide) prepared with both RO water and ASW to stimulate and cultivate marine Actinobacteria from the samples collected from Newcastle and Liverpool seas. Antibiotics were used to inhibit non-target bacteria and fungi. Nine different selective media (ISP 2, ISP 4, ISP 5, AGS, Marine agar 2216, NaST21Cx, R2A (Reasoners 2A Agar), SCA and AIA) were tried and used for the isolation. A total number of nine (9) Actinobacterial species belonging to six genera: *Brachybacterium, Kocuria, Micrococcus, Micromonospora, Streptomyces* and *Salinibacterium* were isolated (Table 2.1). The highest number of Actinobacteria were isolated with ISP2-AWS, followed by AGS-ASW, AIA-ASW and SCA-ASW media (figure 2.1A). Six actinobacteria species (66.7%) were isolated from the Newcastle Sea and three (33.3%) from the Liverpool Sea (figure 2.2B). The percentage of the total number of rare actinobacterial species isolated was seven (77.8%). There was no single isolation with the media prepared with RO water. Also, Marine agar 2216, NaST21Cx, R2A, ISP 4, and ISP 5 did not support the isolation of any actinobacteria in the two forms of the media prepared. The isolates were characterized morphologically (Gram staining and microscopy) as well as by biochemical assays, and they were selected for further analysis and downstream processes. Several non-Actinobacteria such as *Bacillus*, *Pseudomonas*, *Halomonas*, *Marinobacter, Limmaricola* and *Roseobacter* were also isolated with these media. The phylogenetic trees of these non-target groups are shown in appendix 7.1 (figures 7.1a-7.1d)

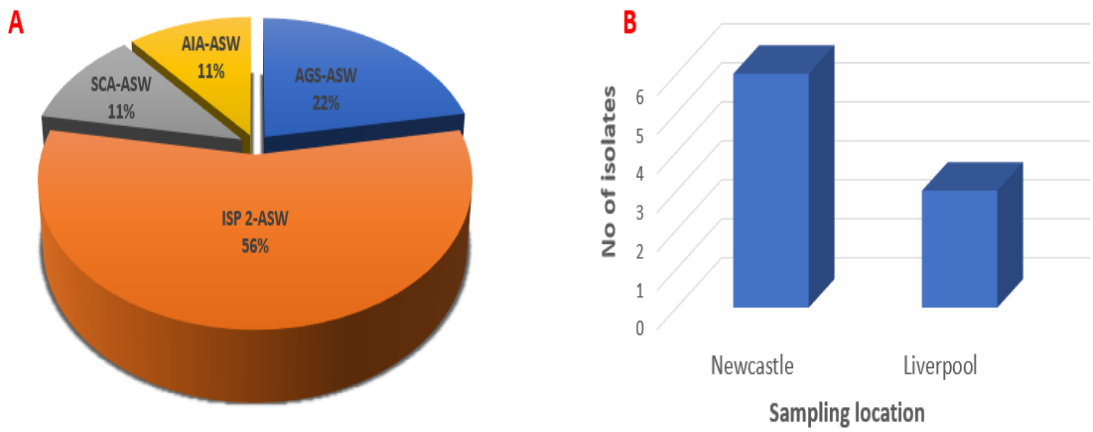

Figure 2. 2: Diagrammatic representation of isolates from the marine habitat. (A). Pie chart showing the total number of isolates based on the selective media used. ISP 2-ASW supported the highest number of isolates followed by AGS-ASW, SCA-ASW and AIA-ASW. (B). Histogram showing the number of isolates in the sample area.

2.3.2 Detection and Identification of Actinobacteria

To identify and assign the isolate to a particular taxonomic group, genomic DNA of the pure culture isolates was first extracted (Figure 2.2A). Bacterial 16S rRNA universal primers 27F, 63F, 1525R and 1492R (Table 2.1) were used to amplify the 16S rDNA gene by PCR reaction (Figure 2.2B). Figure 1C shows the amplification of the isolates with Actinobacterial primers (S-c-Act-0235-a-S-20-forward and S-c-Act-0878-A-19-reverse). Rep-PCR DNA finger-typing techniques was used to dereplicate the isolates belonging to the same genus or species (Figure 2.2D). Table 2.1 shows the primers used for the selective isolation of marine actinobacteria in this study.

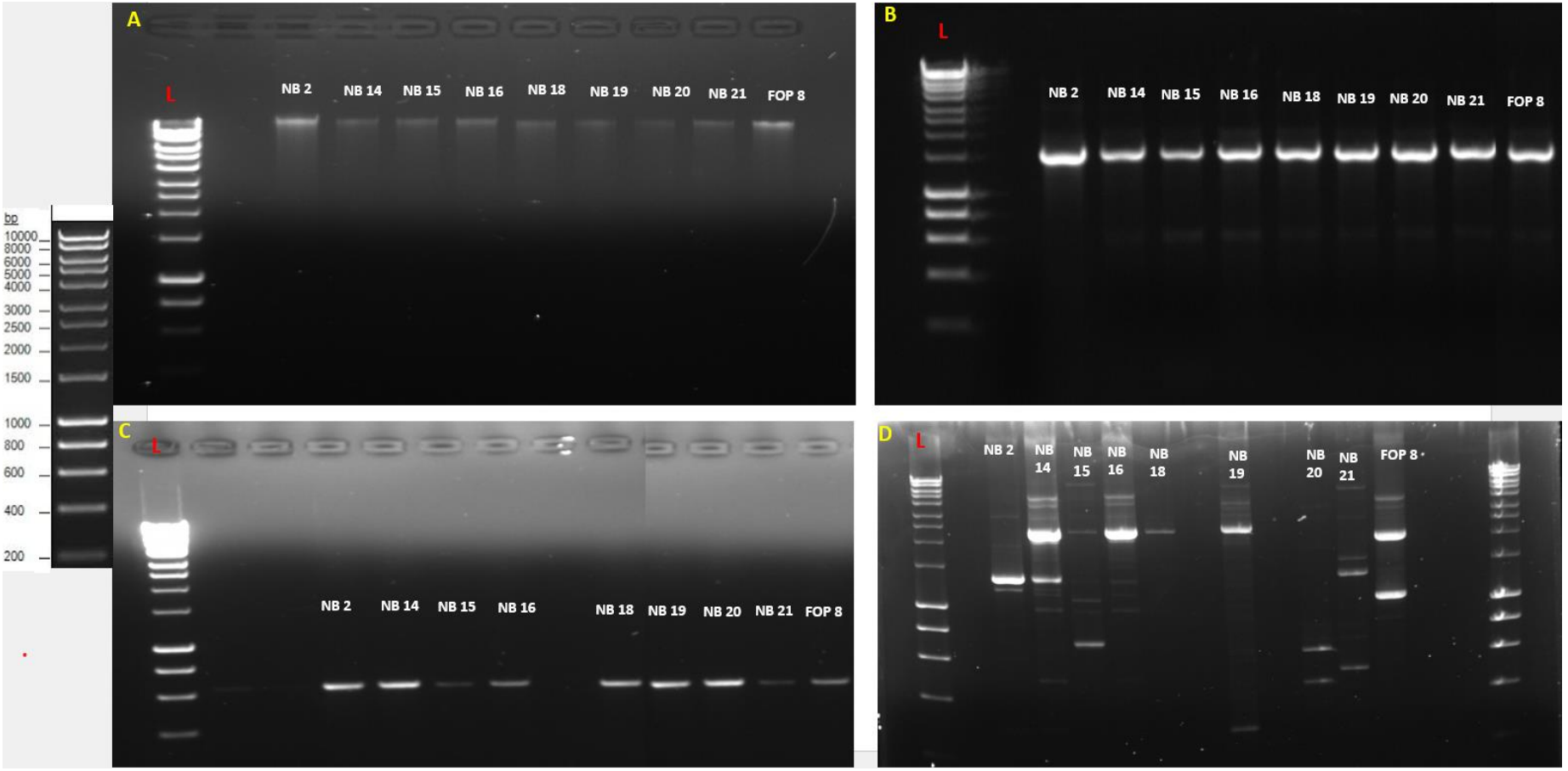

Figure 2. 3: A representation of DNA bands on 1.5% gel electrophoresis. (A). Genomic DNA extraction. The expected size of the total (genomic) DNA of the isolates was obtained as shown (B). PCR amplification of the 16S rDNA gene with universal primers. Various universal primers pairs (27F/1492R and 27F/1525R) were used to amplify the rDNA gene of the isolates with an approximate size of 1500 bp. (C). PCR amplification of V3 to V5 region of 16S rDNA genes with actinobacteria specific primers. (D). Rep-PCR DNA finger typing of the isolates.

2.3.3 16S rRNA sequencing and phylogenetic analysis

16S rRNA gene was sequenced for phylogenetic analysis of selected actinobacterial isolates. The aligned 16S rDNA gene sequences of the isolates and those of the type strain of the actinobacterial genera obtained from NCBI database were analysed and compared phylogenetically by constructing an evolutionary tree as shown by a dendrogram in figures 2.3 to 2.7. The sequence similarities of selected actinobacterial type strains with related species are shown in table 2.2. The nucleotide sequences of the isolates were deposited in NCBI GenBank database, and a unique accession number was assigned (Table 2.2)

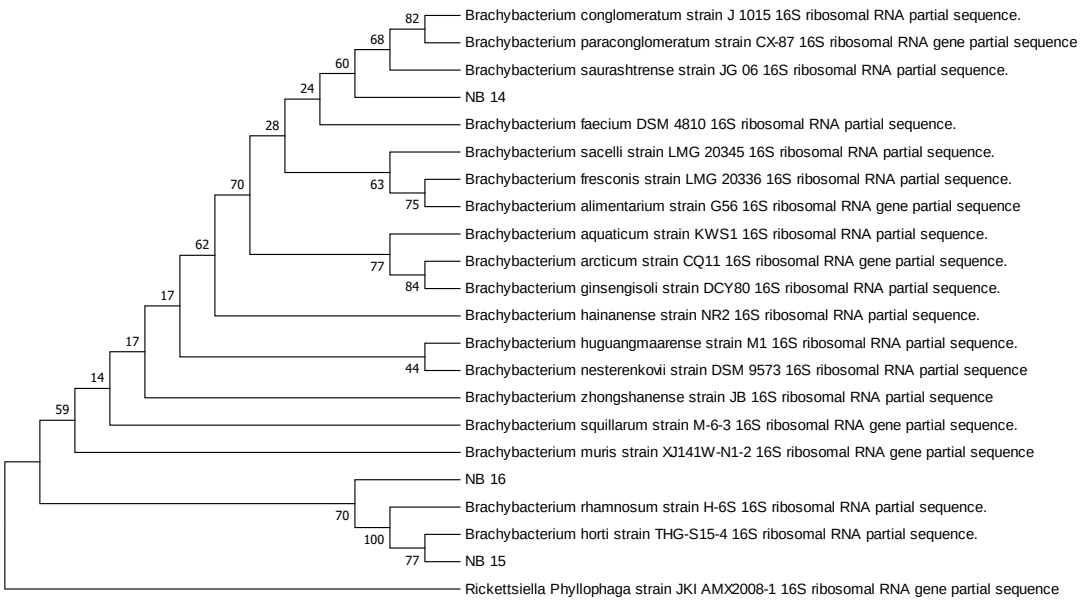

Figure 2. 4: Phylogenetic tree showing evolutionary relationship amongst isolates NB 14, NB 15, and NB 16 and *Brachybacterium* type strain. This was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree was inferred from 1000 replicates (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Jukes-Cantor method (Jukes et al., 1969) and are in the units of the number of base substitutions per site. The analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1262 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). *Rickettsiella Phyllophaga* strain JKI AMX2008-1 16S ribosomal RNA gene was used as the outgroup

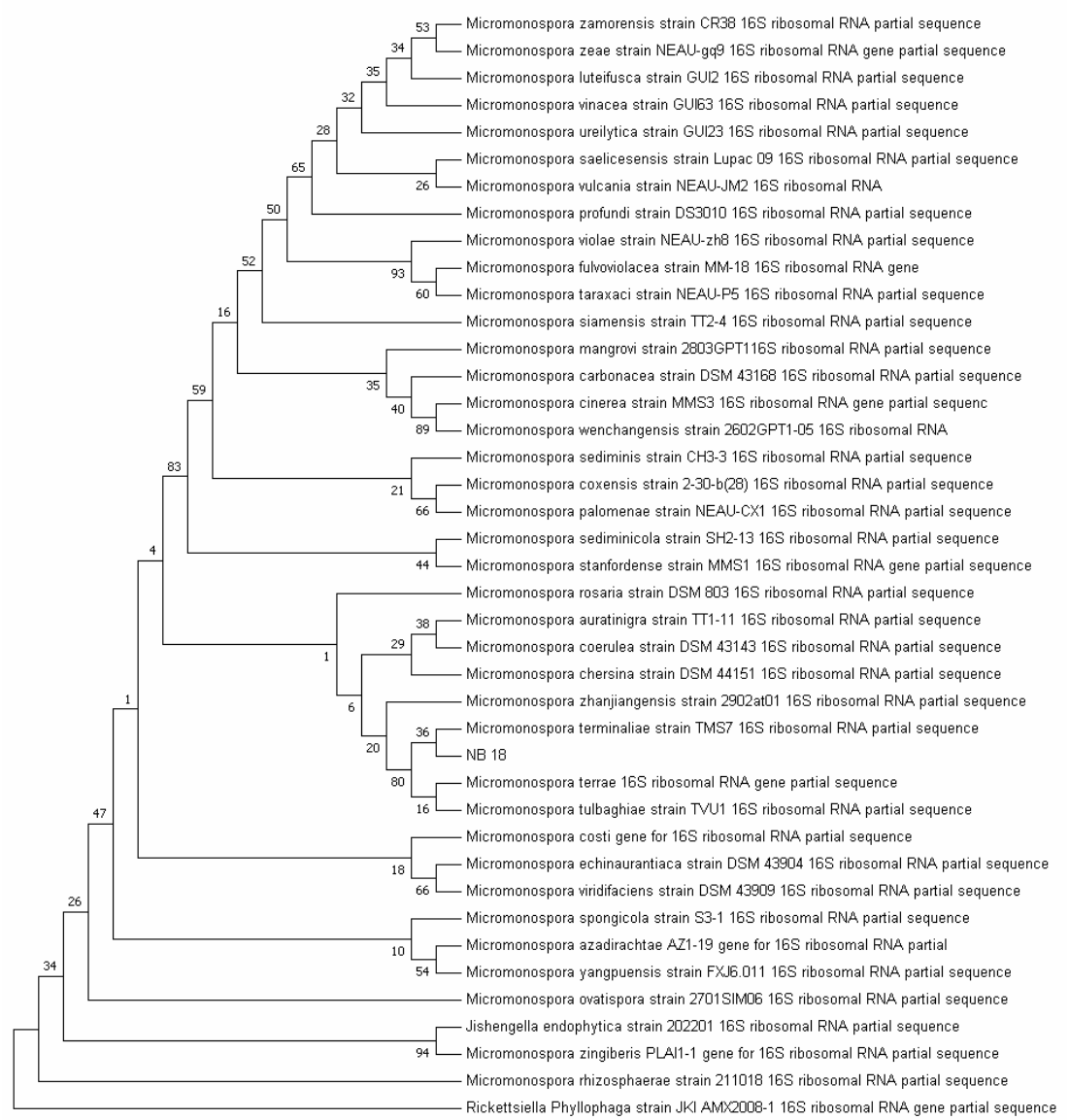

Figure 2. 5: Phylogenetic tree showing evolutionary relationship amongst isolates NB 18 and *Micromonospora* type strain. This was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree was inferred from 1000 replicates (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Jukes-Cantor method (Jukes et al., 1969) and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 767 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). *Rickettsiella Phyllophaga* strain JKI AMX2008-1 16S ribosomal RNA gene was used as the outgroup.

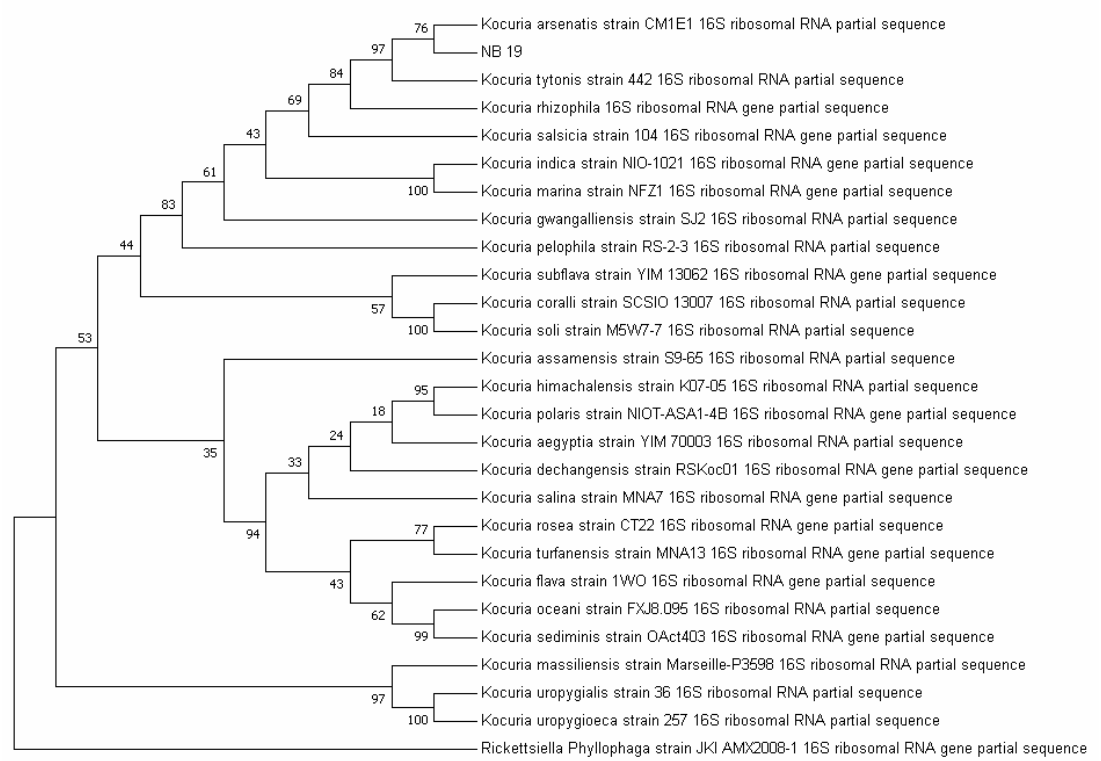

Figure 2. 6: Phylogenetic tree showing evolutionary relationship amongst isolates NB 19 and *Kocuria* type strain. This was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree was inferred from 1000 replicates (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Jukes-Cantor method (Jukes et al., 1969) and are in the units of the number of base substitutions per site. The analysis involved 26 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 826 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). *Rickettsiella Phyllophaga* strain JKI AMX2008-1 16S ribosomal RNA gene was used as the outgroup.

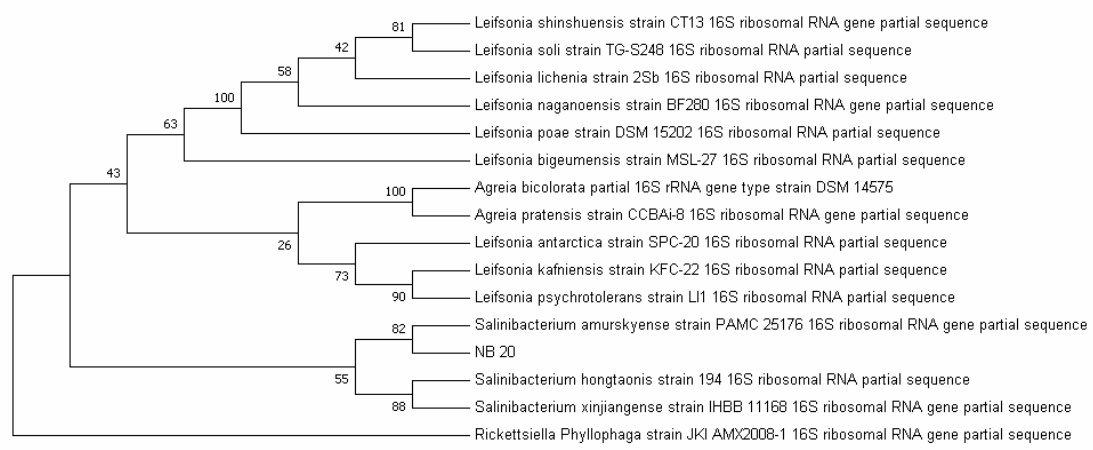

Figure 2. 7: Phylogenetic tree showing evolutionary relationship amongst isolates NB 20 and *Salinibacterium* type strain. This was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree was inferred from 1000 replicates (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Jukes-Cantor method (Jukes et al., 1969) and are in the units of the number of base substitutions per site. The analysis involved 40 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 832 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). *Rickettsiella Phyllophaga* strain JKI AMX2008-1 16S ribosomal RNA gene was used as the outgroup.

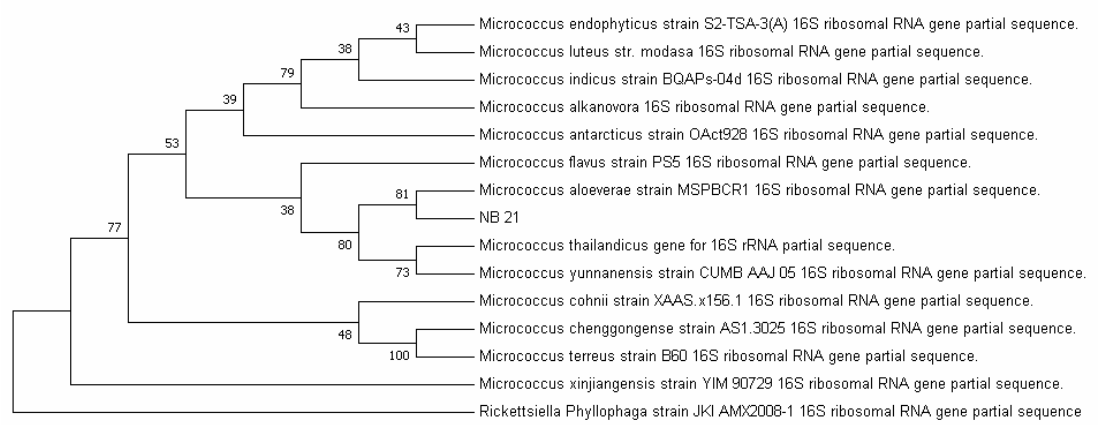

Figure 2. 8: Phylogenetic tree showing evolutionary relationship amongst isolates NB 21 and *Micrococcus* type strain. This was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree was inferred from 1000 replicates (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Jukes-Cantor method (Jukes et al., 1969) and are in the units of the number of base substitutions per site. The analysis involved 14 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 1228 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). *Rickettsiella Phyllophaga* strain JKI AMX2008-1 16S ribosomal RNA gene was used as the outgroup.

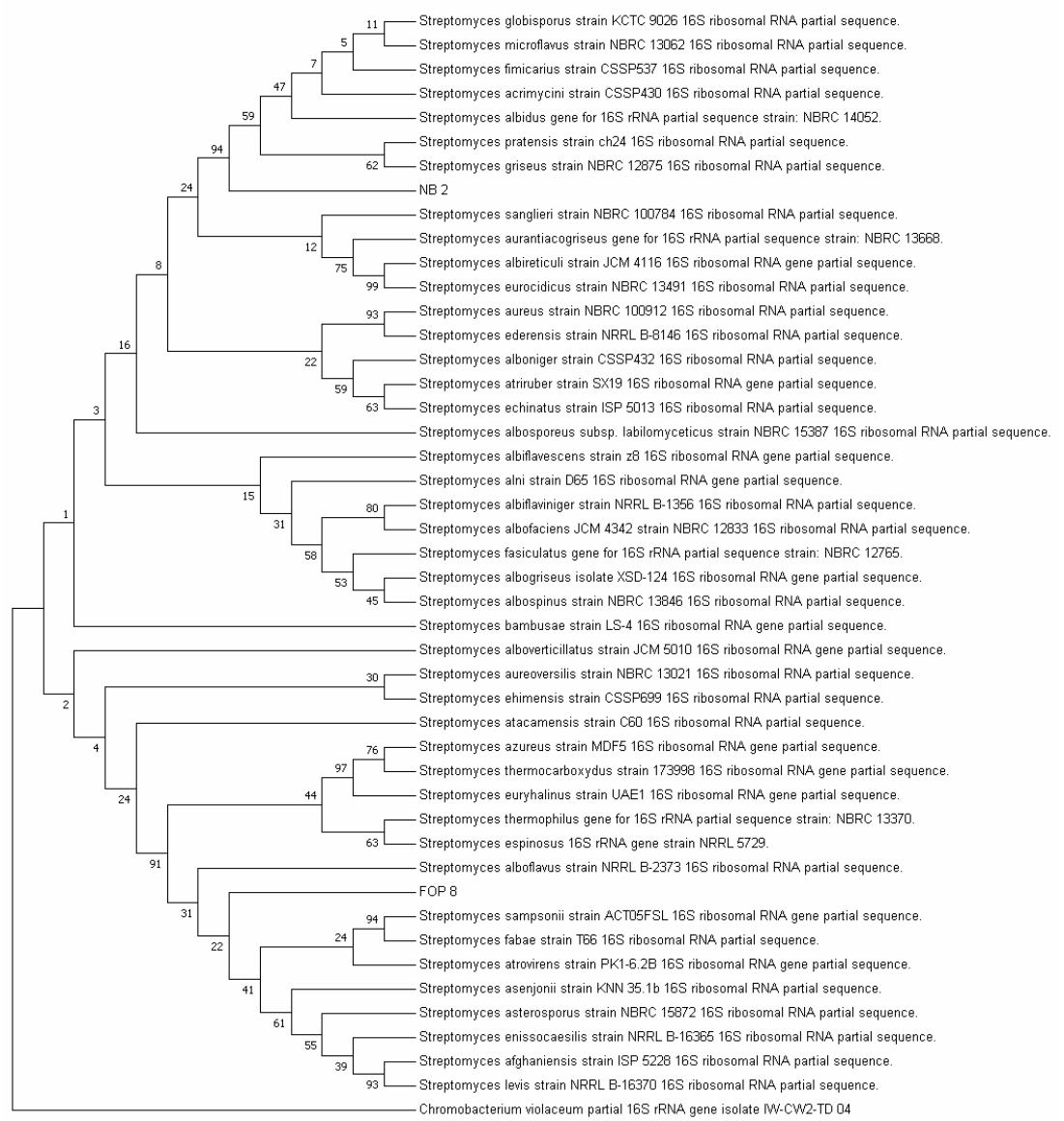

Figure 2. 9: Phylogenetic tree showing evolutionary relationship amongst isolates NB 2 and FOP 8 and *Streptomyces* type strain. This was inferred using the Neighbour-Joining method (Saitou & Nei, 1987). The bootstrap consensus tree was inferred from 1000 replicates (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Jukes-Cantor method (Jukes et al., 1969) and are in the units of the number of base substitutions per site. The analysis involved 161 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 445 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016). *Chromobacterium violaceum* partial 16S ribosomal RNA gene was used as the outgroup

Table 2. 1: List of actinobacterial isolates based on 16S rRNA gene analysis

S/N	Isolates/ Accession number	Genus	% Identity	Closest species (16S rRNA Sequencing)	Origin of isolates	Comment
1	NB 14 (ON023828)	*Brachybacterium*	91.61	*Brachybacterium conglomeratum* strain J 1015 *Brachybacterium paraconglomeratum* strain CX-87 *Brachybacterium saurashtrense* strain JG 06	Marine sediment	This study
2	NB 15 (ON023829)	*Brachybacterium*	99.05	*Brachybacterium horti* strain THG-S15-4 *Brachybacterium rhamnosum* strain H-6S	Marine sediment	This study
3	NB 16 (ON023830)	*Brachybacterium*	97.79	*Brachybacterium horti* strain THG-S15-4 *Brachybacterium rhamnosum* strain H-6S *Brachybacterium muris* strain XJ141W-N1-2	Marine sediment	This study
4	NB 18 (ON514127)	*Micromonospora*	99.37	Micromonospora terrae *Micromonospora soli* SL3-70	Marine sediment	This study
5	NB 21 (ON023827)	*Micrococcus*	97.26	Micrococcus aloeverae strain MSPBCR1,	Marine sediment	This study
6	NB 19 (ON023825)	*Kocuria*	98.03	*Kocuria arsenatis* strain CM1E1 *Kocuria tytonis* strain 442	Marine water	Previous study
7	NB 20 (ON023826)	*Salinibacterium*	98.33	*Salinibacterium* amurskyense strain PMAC 25176	Marine water	Previous study
8	NB 2 (ON023824)	*Streptomyces*	97.86	*Streptomyces albidus* strain NBRC 14052 *Streptomyces acrimycini* strain CSSP430 *Streptomyces microflavus* strain NBRC 13062 *Streptomyces fimicarius* strain CSSP537	Soil (Sea bank)	Previous study
9	FOP 8 (ON023831)	*Streptomyces*	99.93	*Streptomyces thermocarboxydus* strain 173998	Soil (Sea bank)	Previous study

2.4 Discussion

Marine rare actinobacteria have proven to be a source of wide ranging applications in several industries (Azman et al., 2015; Bull & Stach, 2007; Subramani & Aalbersberg, 2013). This has led to search and discovery strategies to hunt for novel strains in recent years. Continuous exploration of the terrestrial environment and repeated isolation of the same genera and species of actinobacteria have resulted in a reduction in the discovery of new metabolites, including novel bioactive compounds (Debbab et al., 2010). The marine environment represents an underexplored niche for isolating new actinobacterial taxa and novel secondary metabolites (Lilja, 2013). The marine environment is also a valuable source for isolating novel actinomycetes with metabolic potential (Blunt et al., 2017). This chapter was aimed at using different selective media to isolate novel actinobacterial species from samples collected from different marine environments in UK. Samples were pre-treated by preheating at 56^{o}C (section 2.2.1). Sample preparation and pre-treatment techniques could affect the kind of actinobacteria that can be isolated. It has been reported in previous studies that preheating stimulates the isolation of actinobacteria while reducing the rate of isolating unwanted microorganisms (Baskaran et al., 2011; M. Hayakawa et al., 1991; Niyomvong et al., 2012; Seong et al., 2001; Zainal Abidin et al., 2016). Pre-treatment of samples also promotes the isolation of rare genera of actinobacteria (Bredholt et al., 2008). The culture-dependent approach with nine media (ISP 2, ISP 4, ISP 5, AGS, Marine agar 2216, NaST21Cx, R2A, SCA and AIA) targeting actinobacteria were used to isolate actinobacteria from the marine samples (figure 2.1). Non-target and unwanted bacteria were inhibited by nalidixic acid, 0.1%

cycloheximide and nystatin at varying concentrations for fungi. Moreover, care was taken with the concentration used as too high a concentration may affect the germination and isolation of target actinobacteria while a low concentration could promote the isolation of unwanted and non-target groups. This result supports the prediction that diverse groups of actinomycetes require different media components such as carbon, nitrogen, trace metals, vitamins for their growth and differentiation. Suitable culture media promotes the isolation of actinomycetes from different samples and nutrient availability is one of the main factors determining the growth and isolation of actinobacteria. Most actinobacteria can use a wide variety of compounds such as glucose, starch, proteins, and amino acids as their energy source, unlike other bacterial groups that favour simple carbon and nitrogen sources (Gil et al., 2009). Several types of selective isolation media were also used to increase the number of non-Streptomyces actinomycetes (Bredholt et al., 2008). In this study, nine actinobacterial species were isolated from marine samples taken from Newcastle and Liverpool Sea with the maximum isolates coming from Newcastle Sea. The number of rare actinobacteria isolated from the sampling area as evident in table 2.2 was low. Some factors such as the sampling methods, the amount of the aliquot used in the inoculation and even representative amount (about 1 g and 1 ml) of the sample used in preparing the dilution could not have been a good representation of the whole samples and non-target bacteria in the sample could have competed with the target rare actinobacteria for nutrients. Actinobacteria also represent a small portion of the total bacteria population in the marine environment (Goodfellow & Williams, 1983). Our results indicated that ISP

2-ASW was the best medium for the selective isolation as it supports the isolation of the highest number of rare actinobacterial species. This agreed with the study of Suthindhiran and colleagues where they used ISP 2 media to isolate the highest number of actinobacteria from marine sediment in the South Coast of India (Suthindhiran et al., 2014). There was no single actinobacteria detected with media prepared with RO water. It has been reported that media prepared with seawater supports the isolation and maintenance of marine microorganisms (Ramesh & Mathivanan, 2009). The media used in this isolation were prepared within pH a range of 7.2 – 7.4. This agreed with the report of (Taber 1960) that actinomycetes prefer neutral or slightly alkaline environment for their growth. The distribution of actinobacteria is influenced by the pH of the respective environment (Ramesh & Mathivanan, 2009). The total number of actinobacteria isolated in this study was low as compared to previous studies (Janssen et al., 2002; Magarvey et al., 2004; Pathom-Aree et al., 2006; Undabarrena et al., 2016). This is not surprising as actinobacteria usually make up only a small fraction of bacterial communities (Bull et al., 2005; Pathom-Aree et al., 2006). *Brachybacterium* was the most dominant rare actinobacterial genus isolate followed by *Micrococcus*, *Micromonospora*, *Salinibacterium* and *Kocuria* in the marine environment sampled. To the best of our knowledge, these rare actinobacteria have not yet been reported from the UK's marine sediment and have been isolated for the first time in the present study. The isolates were delineated by PCR reaction with specific actinobacterial primers and REP-PCR DNA finger-typing (table 2.1; figure 2.1 C and D). The PCR amplification with actinobacterial specific primers was used to eliminate numerous non-actinobacteria from the pool of our

isolates. The actinobacterial specific primers only amplifies the V3 to V5 region of the 16S rDNA gene of actinobacteria (Stach *et al*., 2003). The Rep-PCR DNA finger-typing technique was used to delineate isolates based on their DNA fragmentation pattern (figure 2.1D). This assay was carried out to avoid the tendency of isolating and then sequencing and characterizing the same species of the genera (isolates). Sample NB 14, NB 15 and NB 16 had remarkably similar DNA fragmentation and typing patterns consisting of a distinct band of about 2000 bp confirming that they are indeed from the same genus (*Brachybacterium*). The DNA fragmentation of other isolates were also quite different inferring that they are from a different genus. One would have also expected the fragmentation of NB 2 and FOP 8 to be similar since they are from the same genus (*Streptomyces*). However, they both have similar bands around the 3000 bp region. The isolates (strains) identification and classification were made primarily by 16S rRNA. The 16S rDNA gene identification is one gold standard in identification of bacteria (Valli et al., 2012). This gene gives unique and important phylogenetic information about the isolates (Bora et al., 2015). It is highly conserved in prokaryotes thereby allowing the use of universal primers in its amplification (Bora et al., 2015; Lane, 1991; Marchesi, et al., 1998). It also helps to place a new organism in the correct classification (genus) and close to related strains with the highest percentage identity. It has been revealed from previous studies that the 16S rDNA of organism with a percentage identity similarity of above 97 % correlates with at least 70 % DNA: DNA similarity for species' delineation (Wayne et al., 1987; Stackebrandt and Goebel 1994; Stach et al., 2002). Also, 16S rDNA of isolates (organism) with a

percentage identity similarity of less than 95 % correlates to a new genus (Bora et al., 2015). Isolate NB 14 had a percentage identity of 91.61% (table 2.2). Though blast analysis of this isolate indicated that it was in the genus *Brachybracterium*, further study and DNA: DNA analysis may be required to make a valid conclusion whether they are indeed a new member of a new genera or not.

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Chapter Three

3.0 Genomic Sequencing

3.1 Introduction

Actinobacteria are group of bacteria with diverse metabolic potential (Monciardini et al., 2014) considered to be economically and biotechnologically important as they can produce natural products with pharmaceutical and industrial applications (Zhao et al., 2017). They are renowned for their ability to produce secondary metabolites such as antibiotics, enzymes, biosurfactants, pigments, VOCs, antivirals, antiparasitics and anticancer compounds (Schorn et al., 2016). Many of the old genera (*Streptomyces* and *Mycobacterium*) of Actinobacteria have had hundreds of their genomes sequenced (Doroghazi & Metcalf, 2013; Nett et al., 2009). Traditionally, the discovery of secondary metabolites depended heavily on the ability of cultured strains of microorganisms to synthesize and produce metabolites in media at detectable levels by varying cultural conditions (Schorn et al., 2016). Secondary metabolite biosynthetic gene clusters (SMBGCs) harbour genes involved in the synthesis and regulation of secondary metabolites (Weber et al., 2015). Most bacterial secondary metabolite biosynthesis pathways are organized in SMBGCs (Weber et al., 2015). Often, conventional method of discovering secondary metabolites result in a high chance of re-discovering a known compound. Some Cryptic (silent) BGCs of actinomycetes could hide a vast array of potentially important metabolites in their genomes (Choi et al., 2015). It is exceedingly difficult to characterize and access these cryptic gene clusters from the genomes of organisms using traditional culturing methods (Choi et al., 2015). The new era of sequencing and bioinformatics

have transformed the discovery of metabolites by revealing such metabolites gene clusters harboured in bacterial genomes (Bentley et al., 2002; Ikeda et al., 2003; Udwary et al., 2007). The sequences of bacterial genomes contain the blueprint of the potential metabolites that they (bacterium) can produce (Schorn et al., 2016). Genomic mining in bacteria, including actinobacteria, has revealed that their potential for producing secondary metabolites is much higher than evident solely through culture-dependent methods (Bachmann et al., 2014). Genomic analysis has shown that actinobacteria could have BGCs that are not expressed in laboratory media but could still have biological and industrial potential (Genilloud, 2014). Genomic mining has also enabled the de-replication of known compounds, structural predictions, and identification (Jensen et al., 2014; Tang & Li, 2015). Genome sequencing has become an indispensable technique for unmasking the metabolic potential of Actinobacteria by identifying BGCs for the secondary metabolites (Weber et al., 2015). Also, advances in heterologous expression and regulation manipulation have increased access to cryptic clusters in the biosynthetic pathways (Tang & Li, 2015; Yamanaka et al., 2014). Next generation sequencing technologies such as Illumina®, Ion Torrent®, Pacific Biosciences® and Oxford Nanopore Technologies® provides high-throughput sequencing and data analysis with low-cost sequencing (Slatko et al., 2018). Illumina genome sequencing technology for example provides an effective method for genomics studies and sequence analysis of individual genes, BGCs or entire genomes of any organism (Bentley, 2006; Castro et al., 2018). The technology is based on bridge amplification technique wherein DNA with a size 500 bp and appropriate adapters ligated

on each end are used as substrates for repeat amplification reactions on a solid support that contains oligonucleotide sequences complementary to a ligated adapter (Slatko et al., 2018). The need to explore the genome of actinobacteria for secondary metabolites comes from the outcome of previous genome sequencing of *Streptomyces* genus such as *Streptomyces coelicolor* (Bentley et al., 2002) and *Streptomyces avermitilis* (Ikeda et al., 2003). The analysis revealed many biosynthetic gene clusters in the genome of the species thus implying their potential to produce more metabolites (Udwary et al., 2007). The complete genome sequencing of *S. coelicolor* and *S. avermitilis* and many other *Streptomyces* species have revealed silent BGCs for secondary metabolites (Bentley et al. 2002; Choi et al. 2015). For example, in *S. coelicolor* A3(2), only four secondary metabolites was produced in 40 years prior to genomic sequencing even though it was the main genetic power house of Actinobacteria (Bentley et al., 2002). With the advent of genome sequencing, an additional 18 BGCs for metabolites (Prodiginines, Actinorhodin, Coelibactin, Geosmin, calcium-dependent antiobiotic, desferrioxamines, TW95a, Hopanoids, Butyrolactones etc.) have been revealed (Bentley et al., 2002). In recent years, other metabolites that genomic sequencing have revealed in *Streptomyces* include scleric acid (Alberti et al., 2019), streptosermycin (Zhang et al., 2018), Actinoallolides (Inahashi et al., 2018), cosmomycins (Larson et al., 2017), Thaxtomins (Jiang et al., 2018) and syringolin (Huang et al., 2018). Rare Actinobacteria (non-*Streptomyces*) have attracted attention in the past decade for discovering of secondary metabolites (Azman et al., 2015). Some species of genus *Brachybracterium* for example contain unique polyketide synthase (PKS)

genes (Selvin et al., 2016), and have shown antimicrobial activities against some pathogens (Undabarrena et al., 2016). However, the genus has not been fully studied because as from 2009, only one species *B. faecium* DSM 4810 have had its genome completely sequenced (Lapidus et al., 2009). Also, there were only about 8 draft genomes sequences that are available in GenBank database as in 2017 (Zhao et al., 2017). Genomic-based information have been used to forecast the chemical structures of earlier undetected metabolites and optimize fermentation methods that enhance their production (Bok et al., 2006; Gross et al., 2007; Song et al., 2006). Bioinformatics-based approaches to natural product discovery at industries has led to the discovery and commercialization of significant novel secondary metabolites (McAlpine et al., 2005; Zazopoulos et al., 2003). These approaches have helped to eliminate the rigorous laboratory methods of selective isolation of previously described compounds while allowing detailed expression studies and molecular cloning experiments to be focused on strains that possess a high probability of producing secondary metabolites (Udwary et al., 2007). This chapter aims to carry out next-generation (genomic) sequencing (NGS) of the actinobacteria genera of our novel isolates (selected genera) to understand and analyse their genome for the

potential metabolites they can produce.

3.2 Material and methods

3.2.1 Sample preparation

Selected genera of our isolates (NB 2, NB 16, NB 18, NB 20, NB 19, NB 21, and FOP 8) earlier identified and characterized by 16s rDNA sequencing (chapter two) were used for genomic sequencing.

3.2.1 Extraction of Genomic DNA

DNA was extracted by using Qiagen DNeasy Blood and Tissue extraction kit, Germany, with minor modifications (Qiagen, 2016). A 1.5 ml of the cell culture grown in TSB was centrifuged at 14000 RPM (Revolutions Per Minute) for 10 minutes, and the pellets were re-suspended in 200 µl of phosphate-buffered saline (PBS). The lysis step was modified by adding a 20 µl of 50mg/ml lysozyme to the cell and was incubated for 30 minutes at 37^{o}C, after which 20 µl proteinase K was added. This was followed by 200 µl Buffer AL (Lysis buffer), and the mixture was vortexed thoroughly, after which it was incubated at 56^{0}C for 10 minutes. After the incubation, 200 µl of 100% ethanol was added and was again vortexed thoroughly. Next, 700 µl of the mixture was pipette into a DNeasy mini spin column placed in a 2 ml collection tube. This was centrifuged at 8000 RPM for 1 minute. The flow-through and the collection tube were discarded, and the spin column was placed in a new 2 ml collection tube. Next, 500 µl Buffer AW1 (Wash buffer 1) was added and was centrifuged for 1 minute at 8000 RPM. The flow-through and the collection tube were again discarded as before, and the spin column was placed in a new 2 ml collection tube. Next, 500 µl Buffer AW2 (Wash buffer 2) was added and was centrifuged for 3 minutes at 14000 RPM. The flow-through and the collection tube were again discarded as before, and the spin column was transferred to a new

1.5 µl micro-centrifuge tube. The DNA was eluted by adding 200 µl Buffer AE (Elution buffer) to the centre of the spin column membrane and was incubated at room temperature (15-25^{o}C) for 1 minute. The mixture was then centrifuged at 8000 RPM for 1 minute, and the eluted DNA was placed on ice for further analysis. The DNA band was run on a 1% agarose gel containing 5µl ethidium bromide. Gels were electrophoresed at 100V for 40 min in 1x TAE buffer. The gels were then viewed and scanned for DNA bands in a Gel Doc apparatus (Gel-Doc-it^{TM} 310 Imaging System), USA.

3.2.2 Purification and Quantification DNA samples

The genomic DNA was purified using the Qiagen QIAquick DNA Purification Kit (Qiagen, 2008). A 200 µl of Buffer PB was added to 40 µl of the DNA, and this was thoroughly mixed. The mixture was then added to the purification kit column placed on a 2ml collection tube. The mixture was centrifuged for 1 minute at 13000 RPM, and the flow-through was discarded. The column was put back on the collection tube, and 750 µl buffer PE was added and was centrifuged for 1 minute at 13000 RPM. The flow-through was discarded, and the column was again placed on the same collection tube was further centrifuged for 1 minute at 13000 RPM to remove any residual buffer. A 50 µl buffer EB was added to the centre of the membrane, and this was centrifuged for further 1 minute at 13000 RPM. The bands of the purified DNA were seen on the gel after 1% gel electrophoresis was run. The purified DNA samples were quantified by Nanodrop spectrophotometer (IMPLEN nanophotometers $^{(TM)}$ N60).

3.2.3 Agarose Gel electrophoresis

A 1.0% agarose gel containing 5 µl ethidium bromide (10 mg/ml) was prepared in the gel room by weighing and dissolving 1.5 g of the agarose

powder in 150 ml of 1X TAE (Tris-acetate-EDTA) Buffer. The mixture was heated in an oven for 1-2 minutes to ensure complete dissolution. This was allowed to cool down for a few minutes, and 5 µl was added. The semi-cooled mixture was poured into the casting tray that was earlier set up, and the comb was inserted. The agarose was allowed to solidify for 25-30 minutes. After solidification, some 1X TAE buffer was poured on the gel, and the comb was removed. The cast containing the gel was transferred to the electrophoresis unit filled with the 1X TAE buffer. The samples and the ladder were loaded onto the gel comb. The electrophoresis unit was connected to the electricity supply, and the gels were electrophoresed at 100V for 40 minutes. The gels were viewed and scanned for DNA bands in a Gel Doc apparatus (Gel-Doc-it™ 310 Imaging System), USA.

3.2.4 Whole genome sequencing

Purified and quantified samples were sequenced using Illumina NovaSeq PE150 at the Beijing Novogene Bioinformatics Technology Co., Ltd for whole-genome sequencing.

3.2.4.1 Library construction

A total amount of 1 µg of DNA per sample was used as input material for DNA sample preparations. Sequencing libraries were generated using NEBNext® Ultra™ DNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations and index codes were added to attribute sequences to each sample (https://www.neb.uk.com/products/neb-catalogue/ngs-sample-prep/nebnext-lt;sup-gt;-amp;reg;-lt;-sup-gt;-ultra-amp;trade;-dna-library-prep-kit-for-illumina-lt;sup-gt;-amp;reg;-lt;-sup-gt). Briefly, as shown in figure 3.1 below, the DNA sample was fragmented by sonication to a size of 350bp, and the DNA fragments were

end-polished, A-tailed and ligated with the full-length adaptor for Illumina sequencing with further PCR amplification. Lastly, PCR products were purified (AMPure XP system) and libraries were analysed for size distribution by Agilent2100 Bioanalyzer and quantified using real-time PCR.

Table 3. 1: Sequences of adapter

5’ Adapter	5'-AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGTAGATCTCGGTGGTCGCCGTATCATT-3'
3’ Adapter	5'-GATCGGAAGAGCACACGTCTGAACTCCAGTCACGGATGACTATCTCGTATGCCGTCTTCTGCTTG-3'

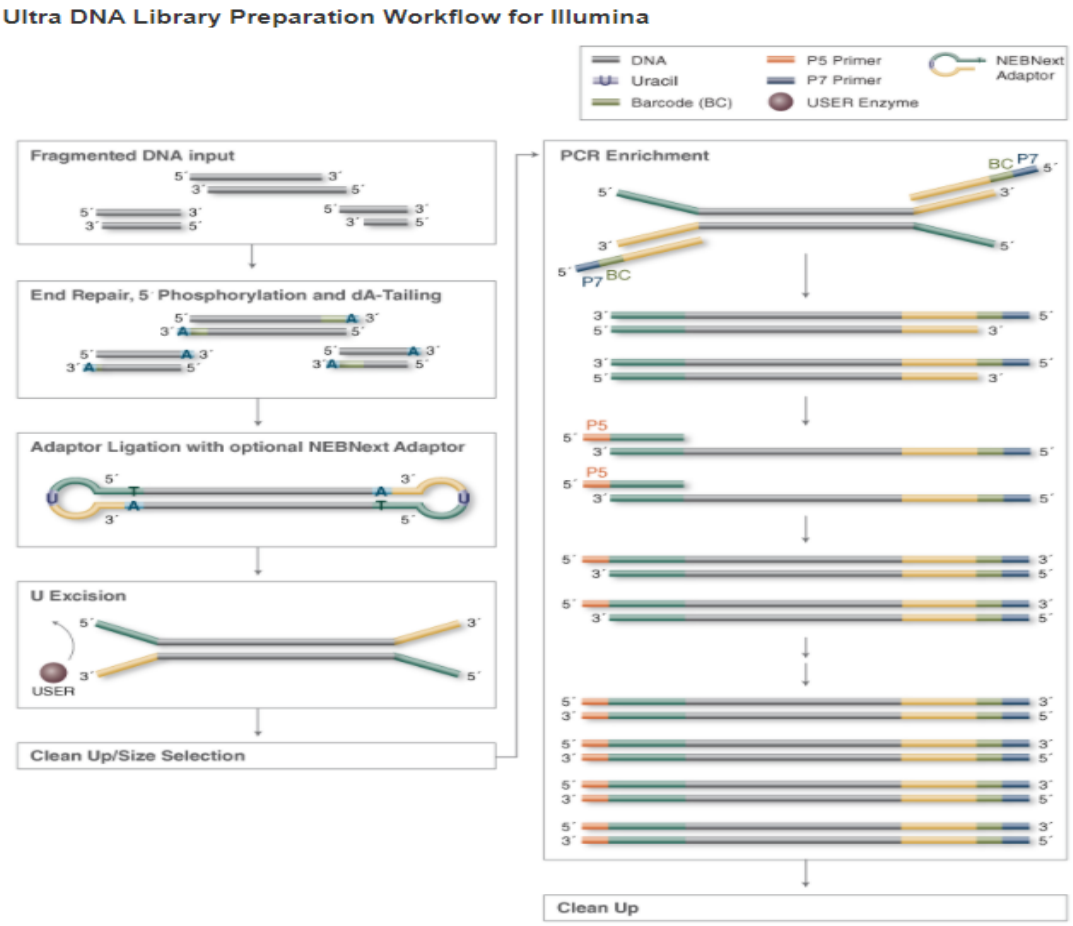


Figure 3. 1: Schematic diagram for library preparation.

Source: https://www.neb.uk.com/products/neb-catalogue/ngs-sample-prep/nebnext-lt;sup-gt;-amp;reg;-lt;-sup-gt;-ultra-amp;trade;-dna-library-prep-kit-for-illumina-lt;sup-gt;-amp;reg;-lt;-sup-gt;?returnurl=/SearchResults%3Fs%3DNEBNext%AE%20Ultra%E2%84%A2%20DNA%20Library%20Prep%20Kit%20for%20Illumina&pn=1&ps=12&b=true#jump. (Accessed on 25th February 2022)

3.2.4.2 Data Processing

To ensure the accuracy and reliability of the analysed data, the original data were filtered (cleaned) to obtain valid data since the raw data contains certain element of low-quality reads. The specific processing steps involved the removal of reads containing low-quality bases (mass value ≤20) over a certain percentage (40% default), the removal of 'N' reads beyond a certain proportion (10% default), removal of overlapping reads between the adapter which exceeded a certain threshold (15 bp default) and removal of three mismatches bases between them.

3.2.4.3 Genome assembly and annotation

The sequenced reads generated from the NGS by Illumina technology were assembled by strategic k-mer extension for scrupulous assemblies (SKESA) de-novo assembler (Souvorov et al., 2018). Gene calling was annotated using the Prokaryotic Genome Annotation Pipeline (PGAP) at NCBI (Tatusova et al., 2016). Annotated features include genes, coding sequences (CDSs), ribosomal ribonucleic acid (rRNA), transfer ribonucleic acid (tRNA), non-coding ribonucleic acid (ncRNA) and repeat region. The read assembly and annotation pipeline tool (RAPT) was performed at NCBI as shown figure 3.2 (https://www.ncbi.nlm.nih.gov/rapt/documentation/)

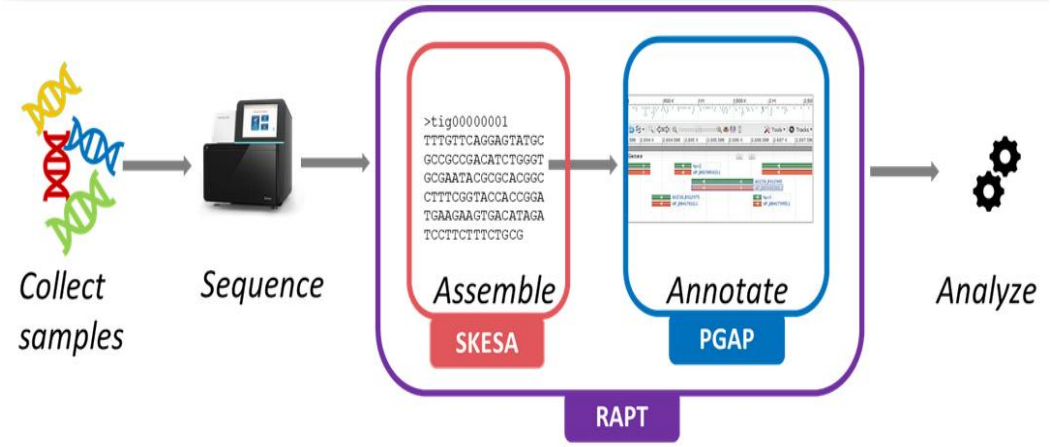


Figure 3. 2: Genomic reads assembly and annotation pipeline. Purified and quantified DNA samples were collected and sequenced (Illumina). The sequenced were assembled by SKESA and long (good) sequence contigs were annotated and then analysed

Source: https://www.ncbi.nlm.nih.gov/rapt/documentation/. (Accessed on 28th February 2022)

3.2.5 Phylogenetic analysis of 16S rRNA gene

The 16S rRNA sequences were extracted from the whole-genome assemblies for all strains. The 16S rRNA gene sequences of approximately 1500 bp of the type strains within the same genera from marine sources were selected as collected from NCBI blast. All sequences were aligned with NCBI BLAST pairwise alignment. The 16S phylogenetic tree from the top 100 hits in NCBI blast (blast tree view) was constructed using the neighbour joining methods with 1000 bootstrap replicates.

3.2.6 Biosynthesis gene clusters (BGCs) analysis

Potential secondary metabolites of our isolates were analysed by the antibiotics and Secondary Metabolite Analysis Shell (antiSMASH) version 5.1.2, online platform to predict the presence of diverse types of BGCs that encode potential secondary metabolites (Medema et al., 2011).

3.3 Results

3.3.1 Extraction, purification, and quantification of genomic DNA.

The genomic DNA for each of the isolates was extracted, purified, and quantified according to Illumina quality control standard. Digital images of agarose gel captured by Gel Doc apparatus (Gel-Doc-it™ 310 Imaging System), USA, confirmed the high quality extracted and purified DNA from the isolates. The result of the quantified genomic DNA was obtained by nanodrop spectrophotometer as shown in table 3.2

Table 3. 2: Quantity and quality of samples by Nanodrop

Samples	**ug/ul**	**260/280**
NB 2	96.15	2.00
NB 16	23.50	1.98
NB 18	51.50	2.03
NB 19	52.55	1.96
NB 20	124.70	1.99
NB 21	146.05	2.00
FOP 8	49.00	1.95

3.3.2 Whole-genome sequencing, assembly, and annotation.

To get a better understanding of the metabolic potential of the isolates, their whole genomes were sequenced by Illumina sequencing technology and then assembled and annotated. The de novo genome assembly shows that the graft genome of the isolates consisted of assembled genome size which ranged from 3,994,542 bp for NB 16 to 9,849,154 bp for NB 20. The total percentage content of G+C ranged from 62.1% for NB 20 to 72.8% for NB 21. The total number of genes ranged from 2,416 for NB 19 to 9,567 for NB 18. Full details of the genome annotation features of the isolates are shown in table 3.3. A summary of the PGAP genome annotation process is provided in the supplementary section (Appendix 7.3)

3.3.3 Construction of 16S phylogenetic trees

The isolates were characterised and grouped into different actinobacterial genera based on their 16S rRNA gene sequences. The phylogenetic tree analysis shows that the selected isolates were a true representation of the actinobacterial genera (Table 3.5). The phylogenetic tree analysis is shown in appendix 7.4.1 to 7.4.6.

Table 3. 3: 16S rRNA gene analysis from the genome of the isolates

Isolates	**Genera**	**Closest species (16 rRNA BlastN sequences)**	**% Identity**
NB 2	*Streptomyces*	*Streptomyces flavogriseus* strain USC061	97.70
NB 16	*Brachybacterium*	*Brachybacterium rhamnosum* strain S2-11	99.79
NB 18	*Micromonospora*	*Micromonospora tulbaghiae* strain UAE1	99.96
NB 19	*Kocuria*	*Kocuria rhizophila* strain 3333	99.87
NB 20	*Salinibacterium*	*Salinibacterium amurskyense* strain y182	99.12
NB 21	*Micrococcus*	*Micrococcus luteus* strain NSM12	99.89
FOP 8	*Streptomyces*	*Streptomyces thermocarboxydus* strain K155	99.80

3.3.4 Analysis of biosynthetic gene clusters (BGCs)

To understand and know the kind, types, and functions of biosynthetic gene clusters as well as the possible potential secondary metabolites and antibiotics (bioactive compounds) that our isolates can produce, we used the antiSMASH v5.1.2 platform to predict the presence of several types of BGCs that code for potential secondary metabolites present on different regions of the genomes of the isolates. The genomic data prediction by antiSMASH for BGCs revealed a total of 26 BGCs coding for different secondary metabolites including nonribosomal peptides synthetases (NRPS), polyketides (PKs), ribosomally synthesized and post-translationally modified peptides (RiPPs), RiPP-like, terpene, beta-lactone, NRPS-likes, PKS-likes, NAPAA (non-alpha poly-amino group acids), siderophores, NAGGN (N-acetylglutaminylglutamine amide), amglyccycl, LAP (Linear azol(in)e-containing peptide), melanin, ectoine, butyrolactone, lantipeptide class I, redox-cofactor, siderophore, t3pks (type III PKS), t2pks (type II PKS), t1pks (type I PKS), blactam, hgIE-ks (heterocyst glycolipid synthase like PKS), thiopeptide, and RRE (RiPP recognition element)-containing. Comparative analysis among the isolates shows that isolate NB 2 has the highest of 30 (35%) of BGCs and potential to produce secondary metabolites while the least was in isolates NB 16 and NB 20 with 3 (4%) BGCs each (figure 3.5). Terpene BGCs were common in the genome of all the isolates. Siderophores were also found in all the isolates except in NB 20. BGCs NAGGN, amglyccycl and LAP were unique to NB 18 as these BGCs were only found in the genome of this isolate. The BGCs of the isolates are shown in tables 3.4.1 to 3.4.7.

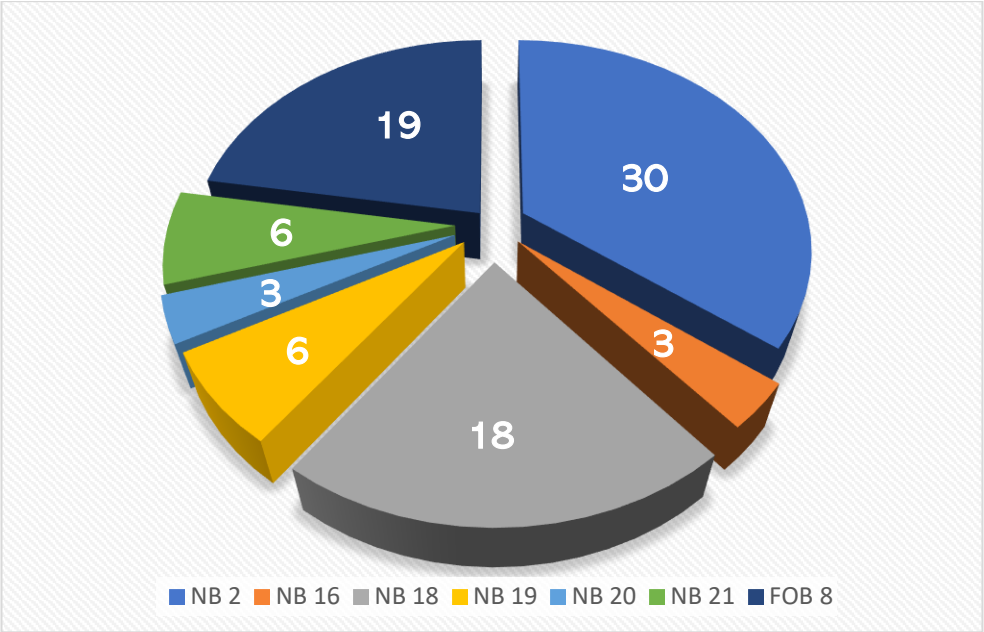


Figure 3. 3: Pie chart showing the number of BGCs for each isolate. The highest number of BGCs were found in NB 2 followed by FOP 8, NB 18, NB 19, NB 21, NB 16, and NB 20

.

Table 3.4 1: Potential gene clusters that code for secondary metabolites in NB 2 as predicted by antiSMASH

Region	Type	From	To	Most similar known cluster		Similarity
Region 2.1	ectoine	64,465	74,863	ectoine	Other	100%
Region 15.1	RiPP-like	27,311	38,109	tetronasin	Polyketide	3%
Region 21.1	melanin	124,437	134,964	melanin	Other	100%
Region 27.1	butyrolactone	1	6,672	prejadomycin / rabelomycin / gaudimycin C / gaudimycin D / UWM6 / gaudimycin A	Polyketide:Type II + Saccharide:Hybrid/tailoring	12%
Region 34.1	RiPP-like	54,882	65,085			
Region 39.1	terpene	59,746	75,233	geosmin	Terpene	100%
Region 40.1	terpene	134,128	155,180	steffimycin D	Polyketide:Type II + Saccharide:Hybrid/tailoring	16%
Region 44.1	RiPP-like	19,674	30,966			
Region 51.1	hglE-KS	1	46,280	meilingmycin	Polyketide	2%
Region 52.1	siderophore	1	12,607	ficellomycin	NRP	3%
Region 59.1	terpene	9,288	30,358	A-500359 A / A-500359 B	NRP	5%
Region 63.1	NRPS-like , NRPS , PKS-like	19,169	82,165	BD-12	NRP	17%
Region 65.1	terpene	13,330	38,825	isorenieratene	Terpene	100%
Region 70.1	NRPS	1	33,298	coelichelin	NRP	90%
Region 71.1	terpene	1	11,656	hopene	Terpene	53%
Region 76.1	T3PKS	10,404	51,462	alkylresorcinol	Polyketide	100%
Region 79.1	terpene	25,051	46,082			
Region 82.1	NAPAA	1	30,509	stenothricin	NRP:Cyclic depsipeptide	13%
Region 90.1	T2PKS	1	5,233	jadomycin	Polyketide	100%
Region 95.1	butyrolactone	114,996	125,907	lactonamycin	Polyketide	3%
Region 96.1	NRPS , T3PKS	1	30,542	feglymycin	NRP	42%
Region 99.1	NRPS	1	34,108	thiocoraline	NRP:Cyclic depsipeptide	7%
Region 105.1	T2PKS	1	16,920	spore pigment	Polyketide	66%
Region 109.1	hglE-KS , T1PKS	93,539	148,160	cremeomycin	Other	21%
Region 112.1	NRPS	65,259	108,914	streptobactin	NRP	47%
Region 112.2	blactam	145,453	165,030	clavulanic acid	Other:Non-NRP beta-lactam	20%
Region 117.1	RiPP-like	49,422	59,649			
Region 120.1	siderophore	10,917	22,698	desferrioxamin B	Other	100%
Region 128.1	NRPS	3,791	53,771	mirubactin	NRP	50%
Region 129.1	terpene	102,205	116,670	hopene	Terpene	30%

A total number of 30 regions within the genome were identified for the biosynthesis of potential secondary metabolites. Specific colour represents the type of individual BGCs within the genome. In the table, the colour green for instance represents NRPS BGCs in region 70.1 of the genome. This gene cluster occupies a start position of 1 bp to end position of 33,298 bp within the genome. The known potential secondary metabolite is a non-ribosomal peptide (NRP).

Table 3.4 2: Potential gene clusters that code for secondary metabolites in NB 16 as predicted by antiSMASH

Region	Type	From	To	Most similar known cluster		Similarity
Region 5.1	siderophore	27,526	39,874	desferrioxamine	Other	50%
Region 7.1	NAPAA	1	20,306			
Region 9.1	terpene	217,282	238,142	vazabitide A	NRP	8%

A total number of 3 regions within the genome were identified for the biosynthesis of potential secondary metabolites. Specific colour represents the type of individual BGCs within the genome. In the table, the colour red for instance represents siderophore BGCs in region 5.1 of the genome. This gene cluster occupies a start position of 27,526 bp to end position of 39,874 bp within the genome. The most similarly known cluster to the siderophore is desferrioxamine with 50% similarity.

Table 3.4 3: Potential gene clusters that code for secondary metabolites in NB 18 as predicted by antiSMASH

P024087.1 (Micromonospora tulbaghiae)

Region	Type	From	To	Most similar known cluster		Similarity
Region 1	terpene	500,735	519,836			
Region 2	RiPP-like, terpene	659,950	683,906	lymphostin / neolymphostinol B / lymphostinol / neolymphostin B	NRP + Polyketide	33%
Region 3	lanthipeptide-class-i	941,680	966,471	tylactone	Polyketide	6%
Region 4	NRPS, T1PKS	1,074,979	1,124,053	collismycin A	NRP + Polyketide:Modular type I	62%
Region 5	amglyccycl, lanthipeptide-class-iii	1,263,143	1,316,585	neomycin	Saccharide	47%
Region 6	transAT-PKS, NRPS, NRPS-like, PKS-like	1,366,847	1,462,180	leinamycin	NRP + Polyketide:Modular type I + Polyketide:Trans-AT type I	15%
Region 7	T2PKS	1,702,266	1,774,840	paramagnetoquinone 1 / paramagnetoquinone 2	Polyketide	25%
Region 8	terpene	1,959,656	1,979,857	nocathiacin	RiPP:Thiopeptide	4%
Region 9	lanthipeptide-class-iii	2,056,216	2,078,939	hedamycin	Polyketide	9%
Region 10	NRPS, T1PKS, NRPS-like	2,462,437	2,530,071	crochelin A	NRP + Polyketide	11%
Region 11	siderophore	2,689,074	2,700,858	desferrioxamine E	Other	100%
Region 12	NAGGN	4,362,139	4,376,951			
Region 13	lanthipeptide-class-i	4,649,081	4,673,687			
Region 14	LAP	4,688,051	4,720,078	arenimycin A	Polyketide:Type II + Saccharide:Hybrid/tailoring	9%
Region 15	terpene	5,093,747	5,114,078	phosphonoglycans	Saccharide	3%
Region 16	terpene	5,285,557	5,306,483	isorenieratene	Terpene	25%
Region 17	T3PKS	6,016,583	6,057,635	alkyl-O-dihydrogeranyl-methoxyhydroquinones	Terpene + Polyketide	71%
Region 18	T1PKS	6,765,983	6,921,668	JBIR-100	Polyketide:Modular type I	72%

A total number of 18 regions within the genome were identified for the biosynthesis of potential secondary metabolites. Specific colour represents the type of individual BGCs within the genome. In the table, the colour blue for instance represents amglyccycl and ianthipeptide class III BGCs in region 5 of the genome. This gene cluster occupies a start position of 1,262,143 bp to end position of 1,316,585 bp within the genome. The known potential secondary metabolite in this BGC is a saccharide

Table 3.4 4: Potential gene clusters that code for secondary metabolites in NB 19 as predicted by antiSMASH

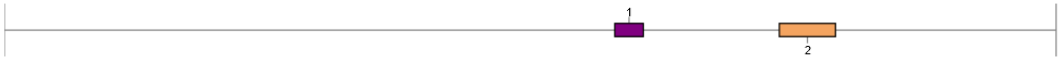
Region	Type	From	To	Most similar known cluster		Similarity
Region 2.1	betalactone	37,046	63,879	microansamycin	Polyketide	7%
Region 11.1	terpene	69,094	89,993	carotenoid	Terpene	25%
Region 18.1	T3PKS	1	37,947			
Region 20.1	NAPAA	72,559	106,683			
Region 20.2	siderophore	142,245	154,164			
Region 28.1	RiPP-like	46,929	57,729			

A total number of 6 regions within the genome were identified for the biosynthesis of potential secondary metabolites. Specific colour represents the type of individual BGCs within the genome. In the table, the colour purple for instance represents terpene BGCs in region 11.1 of the genome. This gene cluster occupies a start position of 69,094 bp to end position of 89,993 bp within the genome. The known potential secondary metabolite in this BGC is a terpene.

Table 3.4 5: Potential gene clusters that code for secondary metabolites in NB 20 as predicted by antiSMASH

Region	Type	From	To	Most similar known cluster		Similarity
Region 2.1	betalactone	146,960	174,214	microansamycin	Polyketide	7%

Contig_4_470.131 (Salinibacterium amurskyense)



Region	Type	From	To	Most similar known cluster		Similarity
Region 4.1	terpene	446,271	467,137	carotenoid	Terpene	33%
Region 4.2	T3PKS	566,561	607,622	alkylresorcinol	Polyketide	100%

A total number of 3 regions within the genome were identified for the biosynthesis of potential secondary metabolites. Specific colour represents the type of individual BGCs within the genome. In the table, the colour orange for instance represents T3PKS BGCs in region 4.2 of the genome. This gene cluster occupies a start position of 566,561 bp to end position of 607,622 bp within the genome. The known potential secondary metabolite in this BGC is polyketide.

Table 3.4 6: Potential gene clusters that code for secondary metabolites in NB 21 as predicted by antiSMASH

Region	Type	From	To	Most similar known cluster		Similarity
Region 8.1	terpene	8,409	22,710	carotenoid	Terpene	66%
Region 60.1	NAPAA	1,511	31,347	stenothricin	NRP:Cyclic depsipeptide	31%
Region 65.1	ectoine	13,761	24,132			
Region 83.1	siderophore	11,427	23,262			
Region 93.1	betalactone	36,694	56,278	microansamycin	Polyketide	7%
Region 120.1	RRE-containing	47,157	67,489			

A total number of 6 regions within the genome were identified for the biosynthesis of potential secondary metabolites. Specific colour represents the type of individual BGCs within the genome. In the table, the purple colour for instance represents betalactone BGCs in region 93.1 of the genome. This gene cluster occupies a start position of 36,694 bp to end position of 56,278 bp within the genome. The known potential secondary metabolite in this BGC is polyketide.

Table 3.4 7: Potential gene clusters that code for secondary metabolites in FOP 8 as predicted by antiSMASH

Identified secondary metabolite regions using strictness 'relaxed'

Region	Type	From	To	Most similar known cluster		Similarity
Region 20.1	lanthipeptide-class-i	40,571	65,087			
Region 27.1	lanthipeptide-class-i	31,719	46,937			
Region 28.1	terpene	1	18,600	hopene	Terpene	61%
Region 47.1	siderophore	31,039	44,221	grincamycin	Polyketide:Type II + Saccharide:Hybrid/tailoring	8%
Region 49.1	NRPS-like	1	15,735	arsono-polyketide	Polyketide	25%
Region 53.1	terpene	62,763	79,285	carotenoid	Terpene	54%
Region 58.1	thiopeptide	20,564	71,139	granaticin	Polyketide:Type II	16%
Region 85.1	NRPS-like	135,049	176,878	toyocamycin	Other	20%
Region 87.1	siderophore	17,579	29,351	desferrioxamin B / desferrioxamine E	Other	83%
Region 95.1	RRE-containing	1	19,823			
Region 121.1	ectoine	7,716	18,114	ectoine	Other	100%
Region 132.1	terpene	57,878	78,963	albaflavenone	Terpene	100%
Region 134.1	T3PKS	36,371	77,447	alkylresorcinol	Polyketide	100%
Region 136.1	terpene	5,907	26,908	cyslabdan	Terpene	81%
Region 146.1	RiPP-like	72,353	82,568	informatipeptin	RiPP:Lanthipeptide	57%
Region 154.1	T2PKS	18,439	90,948	spore pigment	Polyketide	83%
Region 169.1	redox-cofactor	37,465	51,696	lankacidin C	NRP + Polyketide	26%
Region 175.1	RiPP-like	1,018	12,328			
Region 175.2	terpene	37,170	59,326	geosmin	Terpene	100%

A total number of 19 regions within the genome were identified for the biosynthesis of potential secondary metabolites. Specific colour represents the type of individual BGCs within the genome. In this table, the blue colour for instance represents redox-cofactor BGCs in region 169.1 of the genome. This gene cluster occupies a start position of 37,465 bp to end position of 51,696 bp within the genome. The known potential secondary metabolites in this BGC are NRP and polyketide.

Table 3. 4: Genome annotation features of the isolates

Isolates	Assembled Genome size (bp)	Genome topology	G+C (%)	No of BGCs	Genes (total)	CDSs (total)	CDSs (with protein)	CDSs (without protein)	Contigs	rRNAs	tRNA
NB 2	7,513,185	Linear	70.6	30	7191	6873	6632	241	106	6(5S, 16S, 23S)	68
NB 16	3,994,542	Linear	70.2	3	3477	3359	3297	62	25	6(5S, 16S, 23S)	50
NB 18	7,234,650	Linear	72.6	18	9567	9446	9305	141	43	6(5S, 16S, 23S)	101
NB 19	7,607,128	Circular	70.6	6	2416	2360	2341	19	55	5(5S, 16S, 23S)	46
NB 20	9,813,922	Linear	62.1	3	2644	2592	2580	12	7	6(5S, 16S, 23S)	44
NB 21	6,715,068	Circular	72.8	6	2433	2378	2326	52	133	6(5S, 16S, 23S)	48
FOP 8	7,362,642	Linear	72.0	19	7087	6840	6668	172	150	6(5S, 16S, 23S)	66

3.4 Discussion

Actinobacteria are a group of microbes with many BGCs and high potential to produce different secondary metabolites (Monciardini et al., 2014) that have found end applications in the environment, medical, and biotechnological industries (Zhao et al., 2017). Exploring the metabolic potential of Actinobacteria especially the rare ones from underexplored niches such as the marine environment have been hindered by conventional and laboratory isolation and screening techniques. Conventional methods of secondary metabolites discoveries often result in the re-isolation and re-discovering of already discovered metabolites. This is because novel metabolites are present in cryptic BGCs of actinobacteria and accessing these BGCs by traditional culturing methods is quite difficult (Choi et al., 2015). This chapter was aimed at carrying out genomic sequencing of novel actinobacteria obtained from marine environment in the UK. Linking the ability to produce metabolites to the genes that code for them through analysis of genomic sequenced data has helped in the discovery of new metabolites (Zerouki et al., 2021). High-quality genomic DNA (gDNA) was extracted from our characterized and verified isolates (chapter two). The DNA products of these isolates were purified and quantified according to the standard (requirement) and quality control measures specified by the commercial sequencing (genome) firms (Novogen). Prior to the extraction of the gDNA, extra care was taken to avoid contamination of the pure isolate by ensuring that the agar media used were freshly prepared and autoclaved at the correct parameters. The inoculation and addition of inhibitory agents were done inside a class II safety cabinet. Whole-genome sequencing of short reads was carried out

by Illumina sequencing technology. The reads were assembled into different contigs and the various contigs were annotated by NCBI PGAP. Housekeeping genes *rpoB* was used to delineate species which had close and overlapping phylogenetic classification. During the genomic data analysis, isolate NB 18 (*Micromonospora*) was initially coming under the *Brachybacterium* genus (NB 16) until rpoB was used to delineate these two genera. The *rpoB* gene has been assessed as potential alternative for universal phylogenetic marker metabarcoding analysis (Ogier et al., 2019). Their result reveal that *rpoB* gene for taxonomic classification and assignation was more accurate than that of 16S rRNA gene (Jean-Claude Ogier et al., 2019). Previous studies have also reported that *rpoB* gene marker could be suitable for phylogenetic analysis as it gives a better resolution than 16S rRNA gene at species level (Adékambi et al., 2008, 2009; Drancourt & Raoult, 2002; Mollet et al., 1997). The isolates were characterized based on the 16S rRNA gene sequences obtained from the assembled genome sequences. The result of the phylogenetic analysis correlates with the 16S rDNA characterization of the isolates in chapter two. This genomic analysis further confirms and validates our result in chapter two that the isolates are indeed true genera of Actinobacteria.

The potential of our isolates to produce secondary metabolites was determined by searching for the presence of BGCs using antiSMASH. AntiSMASH is an online tool or platform for mining the genomes of microbes for their secondary metabolites BGCs (Blin et al., 2021). BGCs are groups of genes responsible for producing secondary metabolites and are they usually located together in clusters within the genome of an organism (Doroghazi & Metcalf, 2013). BGCs analysis indicated that our

isolates have enormous potential for secondary metabolites biosynthesis. Our result reveals that the potential for secondary metabolites was highest in isolate NB 2 because it had the highest number of BGCs as determined by antiSMASH analysis. The antiSMASH tool has been used for predicting BGCs in Actinobacteria (Amin et al., 2019; Gosse et al., 2019; Guerrero-Garzón et al., 2020; Herdini et al., 2017; Najah et al., 2017). This agrees with the previous report that the genus *Streptomyces* contains more BGCs that code for secondary metabolites than the currently discovered ones (Belknap et al., 2020). Also, some actinobacteria can produce up to 50 diverse types of secondary metabolites as per the sequenced genomic data (Katz & Baltz, 2016). Similarly, the result also shows that isolates NB 16 and NB 20 host the least number of secondary metabolites gene clusters. Terpene BGCs were found in the genomes of all the isolates. This is not so surprising because of the ubiquity of terpene pathways in the bacterial genome (Schorn et al., 2016). Terpenes are natural metabolites mostly made by plants and fungi, though some (odoriferous terpenes) have been obtained from bacterial origin in recent years (Cane & Ikeda, 2012; Yamada et al., 2015). Genes coding for terpene synthases are distributed in bacteria thus making them a good reservoir for the discovery of secondary metabolites (Yamada et al., 2015). Terpenes are the main biosynthetic building blocks of steroids, a component of squalene (triterpene) (Omar et al., 2016). Terpene and its derivatives (terpenoids) are the main constituents of essential oils (Omar et al., 2016). Terpenes are used as a precursor in the biosynthesis of polymers such as polyisoprene (Silvestre & Gandini, 2008). They are also used as fragrances and flavours in perfumes, cosmetics, cleaning products, and food and

drinks (Steenackers et al., 2015). For example, hops used in the brewing industries for better quality (aroma and flavour) come partly from sesquiterpenes such as alpha-humulene and beta-caryophyllenen (Steenackers et al., 2015). The ubiquity of terpene BGCs in all our isolates could imply that these isolates could be a huge reservoir for the biosynthesis of terpenes from marine rare actinobacteria. The second most pervasive and abundant class of BGCs found in the genomes of our isolates was siderophores. This BGC was found in all the isolates except isolate NB 20. Siderophores are BGCs for the biosynthesis of chemically diverse secondary metabolites with a strong affinity for ferric iron (Hider & Kong, 2010; Kramer et al., 2020). These secondary metabolites have biological effects on microbial community development as well as therapeutic potential (Kramer et al., 2020; Wilson et al., 2016). There has been a renewed interest in identifying and characterizing new siderophores because of their areas of applications (Kreutzer et al., 2012). Actinomycetes have become a promising source for the biosynthesis of siderophores with marine rare actinomycetes as an important emerging isolating niche (Wang et al., 2014). Siderophores are normally biosynthesized by microorganisms to survive in an iron-depleted environment or conditions (Shen et al., 2021). The biosynthesis is regulated by the levels of iron within their cells which are also affected by their habitats (Hider & Kong, 2010; Sandy & Butler, 2009). In contrast to other habitats, iron levels in the marine environment are low (Shen et al., 2021). Also, the dilute nature of the marine environment promotes the diffusive losses of siderophores, and this makes the efficiency for the normal uptake of siderophore-based iron an issue for marine organisms

(Shen et al., 2021). In such conditions, the siderophores produced by marine bacteria show different properties compared to their terrestrial counterparts (Chen et al., 2019; Hider & Kong, 2010; Kümmerli et al., 2014; Sandy & Butler, 2009; Zane et al., 2014). The non-ribosomal peptides synthetases (NRPS) and polyketides (PKs) BGCs and their derivatives (NRPS-likes, PKS-likes, RiPPs, RiPPs-likes, RRE, t1pks, t2pks, t3pks and hgIE-ks) were primarily found in isolates NB 2 and FOP 8 and a few in NB 18. This is not surprising because *Streptomyces* accounts for over 70% of known bioactive secondary produced by microorganisms (Bérdy, 2005; Valli et al., 2012). Gene studies in actinomycetes revealed that their genome codes for different BGCs for secondary metabolites and many of these clusters are associated with NRPS and PKS pathways (Komaki et al., 2016). The non-ribosomal peptides and polyketide compounds are the main secondary metabolites of actinomycetes (Nett et al., 2009). Most of them have shown useful bioactivities which have been developed into various drugs such as antibiotics, anticancer agents and immunosuppressants (Berdy, 2005; Komaki et al., 2016; Watve et al., 2001). The presence of NRPS and PKS BGCs in in the genome of actinobacterial strains has been used to evaluate the strain's potential to produce secondary bioactive compounds (Komaki et al., 2012, 2014, 2015). None of our rare marine actinobacteria isolates (NB 16, NB19, NB 20 and NB 21) had either NRPS or PKS BGCs in their genome based on our antiSMASH analysis. This could imply that these isolates might not be producing secondary metabolites with bioactivity (pharmaceutical and antimicrobial activities). However, further analysis is necessary to fully ascertain this assumption because it could be that the antiSMASH analysis

did not predict all the potential BGCs especially for NRPS and PKS for these rare marine actinobacteria. Contrary, terpene, siderophores and betalactone BGCs were mostly common among these isolates. The presence of these BGCs could be explored and investigated further to produce novel secondary metabolites. Some of the BGCs for example siderophores have shown in previous reports to have some therapeutic usefulness (Kramer et al., 2020; Wilson et al., 2016). In addition to common BGCs that the isolates shared, NB 18 had other three BGCs (NAGGN, LAP and amglyccycl) which were only indigenous to isolate NB 18. LAB and amglyccycl BGCs are also known to have most similar cluster of neomycin and arenimycin A (Table 3.4.3). The presence of these BGCs in isolate NB 18 could imply that this isolate could biosynthesized secondary metabolites with antibacterial activity.

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Chapter Four

4.0 Bioprospecting and identification of biosurfactants from novel isolates

4.1 Introduction

Recently, the demand and search for novel biosurfactants has increased due to their numerous applications (Sachdev & Cameotra, 2013). Biosurfactants are amphiphilic compounds with emulsification properties and the ability to reduce surface tension across polar compounds (Smith et al., 2020). Biosurfactants and their synthetic (chemical) counterpart are types of surface-active compounds with emulsifying properties. Surfactants are substances that are used in cleaning and removal of undesirable substances or dirty by a process called emulsification (Santos et al., 2018). They have unique characteristic such as detergency, solubilisation, lubrication, foaming, phase dispersion, and stabilizing (Sobrinho et al., 2013). Surfactants have a huge global market estimated to be about $39.86 billion by 2021 (Markets and Markets 2016). Globally, biosurfactant production was 344.06 kilotons in 2013 and was expected to increase to 462 kilotons by 2020 (Grand View Research 2015). As amphiphilic compounds, surfactants have both hydrophobic and hydrophilic ends (Elkhawaga, 2018). These two ends enable it to lower the surface or interfacial tension between two liquid phases such as oil/water interface (Gudiña et al., 2013; Luna et al., 2013). The hydrophobic part could be a long chain of fatty acids, hydroxy fatty acids, or α-alkyl-α-hydroxyl fatty acids (Elkhawaga, 2018). The water-soluble end (hydrophilic) could be a chain of carbohydrate, amino acid, cyclic peptide, phosphate, carboxylic acid, or alcohol (Elkhawaga, 2018; Mao et al.,

2015). Most of the surfactants available in the market are chemically synthesised from petroleum (Banat et al., 2000). In the last decade, several scientific research and investigations have been conducted to isolate microorganisms that can produce biosurfactants with surface-active features that includes low critical micelle concentration (CMC), low toxicity and high emulsifying activity (Shekhar et al., 2015; Silva et al., 2014). This research became essential due to the need to replace synthetic surfactants with microbial produced ones for many applications in the industry, medicine, and environment (Banat et al., 2000). Biosurfactants stand out from synthetic surfactants mainly because of their biological and, therefore, eco-friendly and renewable origins, when sourced from microorganisms and plants (Smith et al., 2020). They also have more excellent emulsification activities, suitable across a different range of temperature conditions, and have been proven to exhibit a low degree of cytotoxicity (Abdel-Mawgoud et al., 2010). An exciting feature of biosurfactants is forming micellar structures around their CMC. However, the value of CMC depends on the kind of biosurfactant (Smith et al., 2020). These characteristic features make biosurfactants better alternatives to chemically synthesised surfactants (Banat et al., 2010; El-Sheshtawy et al., 2016).

Biosurfactants can be grouped based on their ionisation state in aqueous solution as anionic (negatively charged), cationic (positively charged), non-ionic (neutral) and amphoteric (both negatively and positively charged) (Ana Paula Pereira dos Santos et al., 2016). Biosurfactants can also be grouped based on their chemical composition into glycolipids (rhamnolipids, sophorolipids, trehalolipids, mannosylerithritol lipids),

lipopeptides (surfactin, lichenysin, iturin, fengycin, serrwettin), fatty acids/phospholipids/neutral lipids (phosphatidylethanolamine, spiculisporic acid), polymeric biosurfactants (emulsan, alasan, biodispesan, liposan), and particulate biosurfactants (Muthusamy et al., 2008; Pacwa-Płociniczak et al., 2014; Rani et al., 2020; Stancu, 2015). They can also be grouped based on their molecular weight as low or high molecular weight biosurfactants (Cameotra et al., 2010; Fenibo et al., 2019). Lipopeptides, glycolipids, and phospholipids are low molecular weight biosurfactants, while polymeric and particulate biosurfactants are high molecular weight biosurfactants (Cameotra et al., 2010; Shekhar et al., 2015). Glycolipids biosurfactants are made up of sugars with long chains of aliphatic or hydroxy-aliphatic acids (Arpita, 2017). Rhamnolipids contains rhamnose sugar moiety linked to β-hydroxylated fatty acid chains (Chong & Li, 2017). Sophorolipids on the other hand are made up of hydrophobic fatty acid and a hydrophilic sophorose (glucose disaccharide) sugar moiety (Delbeke et al., 2018; Kulakovskaya & Kulakovskaya, 2014). Rhamnolipids for instance can be classified into various kinds depending on the number of rhamnose moieties attached to the lipid part (Behrens et al., 2016). Mono-rhamnolipids contain one rhamnose moiety, and di-rhamnolipids contain two (Behrens et al., 2016).

Biosurfactants have found application in the cosmetics, chemicals, food, pharmaceutics, medical, agriculture, cleaning, and environmental industries (El-Sheshtawy et al., 2016; Joshi et al., 2008). Biosurfactants also have many applications in the environmental sector, such as in bioremediation, soil washing and soil flushing (Elkhawaga, 2018). Surfactants are the single most essential ingredients in laundry and

household cleaning products because they account for about 40% of the composition (Yangxin et al., 2008). Glycolipids (sophorolipids, rhamnolipids, and mannosylerythritol lipids) are the most commercialised biosurfactants in the cleaning industries (Smith et al., 2020). Companies such as Saraya, Ecover, Henkel, BASF, Evonik, TeeGene and Unilever are applying biosurfactants in their cleaning and commercialising rhamnolipids and lipopeptide biosurfactants based products (Klosowska-Chomiczewska et al., 2011; Kosaric & Sukan, 2014; Sekhon Randhawa & Rahman, 2014; Singh et al., 2019). Biosurfactants have been employed to enhance oil production, especially in microbial enhanced oil recovery (MEOR), due to their low toxicity, high biodegradability, and ecological acceptability (El-Sheshtawy et al., 2016; Fang et al., 2007; Nitschke & Pastore, 2006). Several biosurfactants also exhibit antibacterial, antifungal, antiviral, or anti-tumour activities, making them potential alternatives to conventional therapeutic agents in many biomedical applications (Marchant & Banat, 2012; Müller et al., 2012). Recently, biosurfactants have found applications in the medical sectors as antimicrobial, antiadhesive, antitumor and immunomodulation (Smith et al., 2020). Studies involving mammalian cells indicates that biosurfactants participate in many intercellular molecular recognition steps such as signal transduction, cell differentiation and cellular immune response, acting as antitumor agents by interfering with cancer progression processes (Fracchia et al., 2015; Gudiña et al., 2013; Sajid et al., 2020). Lipopeptides and glycolipids are the most effective antimicrobial and could represent essential sources for discovering new antibiotics (Smith et al., 2020). Different lipopeptides have reached a commercial antibiotic status, such as echinocandins,

micafungin, anidulafungin, and daptomycin (Fracchia et al., 2015). Biosurfactants could also be used as a drug delivery system. For example, the micellar nature of biosurfactants makes them an ideal candidate for drug delivery systems by allowing them to form a stable liposome that can encase the drug, thus protecting them from damage (Sosnowski & Gradon, 2009). This chapter aims to investigate the potential of our isolated Actinobacteria to produce biosurfactants that could be of industrial, environmental, and medical importance.

4.2 Material and methods

4.2.1 Bioprospecting and detection of biosurfactants.

Our isolates (both actinobacteria and non-actinobacteria) were screened for their ability to produce biosurfactants by the hemolytic assay, drop collapsing test, and oil displacement test. Isolated actinobacteria (NB 2, NB 14, NB 15, NB 16, NB 18, NB 19, NB 20, NB 21, and FOP 8) were the target in this screening exercise. However, other non-actinobacteria isolates were also screened in order to have a better understanding of the biodiversity of biosurfactants production in the marine habitat under focus.

4.2.1.1 Hemolytic assay

This was used as the primary screening assay for biosurfactant production (Kiran et al., 2009). The isolates were streaked onto Blood agar (5.0 g of peptone, 3.0 g of yeast extract, 5.0 g of sodium chloride, 50 ml of horse blood, and RO (Reverse Osmosis) water 1000ml) in a multi-well plate. The plates were incubated at 37^0C for 48 hrs. At the end of the incubation period, the plates were observed for a zone of clearance around the isolates. The presence of a zone around the test isolates indicated positive results for producing biosurfactants. The assay was carried out in duplicate, and blood agar without test isolates was used as the negative control.

4.2.1.2 Drop collapse test

This test was done according to (Kiran et al., 2009) with some modifications. A 2 µl aliquot of mineral oil (paraffin oil) was added to 96-well microlitre plates. The plate was left at room temperature (25^0C) to equilibrate for 1 hr. A 5 µl of supernatant from active culture grown overnight (24 hrs at 28^0C) and centrifuged with Eppendorf centrifuge 5810 R at 4000 RPM/2755 g, 4^oC for 20 minutes was added to the surface of the

oil. The shape of the drop on the oil surface was observed after 2 minutes and recorded. Wells without any isolates were used as control. The culture supernatant that made the drop collapse was indicated as a positive result, and the drops that remained beaded were scored as negative.

4.2.1.3. Oil displacement test

This was determined according to Kiran et al., 2009 with some modification. A 15 µl of crude oil were placed on the surface of 40 µl distilled water in a Petri dish, and 10 µl of the supernatant from an active culture grown overnight (24 hrs at 28^0C) and centrifuged with Eppendorf centrifuge 5810 R at 4000 RPM/2755 g, 4^oC for 20 minutes was gently added onto the surface of the oil film. Crude and distilled water without isolates were used as control. The diameter and area of clear halo visualised under visible light were measured and recorded as positive after 1 minute.

4.2.2 Small scale expression and extraction of crude biosurfactant

4.2.2.1 Small scale expression and production of biosurfactants

Isolates with positive results from the combined phenotypic assay (hemolytic, oil collapse and oil displacement test) were selected for the fermentation and production of biosurfactants. Mineral salt medium (MSM), according to (Adebajo et al., 2020), was prepared with some modification. The medium composed of the following, 2.5 g of $NaNO_3$, 0.1 g of KCL, 3.0 g of KH_2PO_4, 7.0 g of K_2HPO_4, 0.01 g of $CaCl_2$, 0.5 g of $MgSO_4.7H_2O$) and 5 ml of trace element solution prepared in 1000ml of RO water (0.116 g of $FeSO_4.7H_2O$, 0.232 g of H_3BO_3, 0.41 g of $CoCl_2.6H_2O$, 0.008 g of

$CuSO_4.5H_2O$, 0.008 g of $MnSO_4.H_2O$, 0.022 g of $[NH4]_6Mo_7O_{24}$ and 0.174 g of ZnSO4). 50ml (5%) of vegetable oil was used as the carbon source. The pH was adjusted to 7.0 by 1 M of NaOH. The medium was autoclaved at 121°C with 15 mmHg for 15 min. A 100 ml of the medium was then inoculated in a 250 ml shake flask with the cell pellets. The cell pellets were obtained after the isolates earlier grown in 10 ml of TSB were centrifuged (Eppendorf centrifuge 5810 R) at 4000 RPM/2755 g, 4^oC for 20 minutes. The flasks were incubated at 28°C at 200 RPM (Revolutions Per Minute) for seven days.

4.2.2.2 Extraction of biosurfactants

The extraction techniques involved the use of acid precipitation and solvent extraction methods. The fermentation broth sample was centrifuged (Eppendorf centrifuge 5810 R) at 4000 RPM/2755 g, 4^oC for 30 minutes. The obtained supernatant served as a crude biosurfactant.

4.2.2.2.1 Acid precipitation

Crude biosurfactant was treated by acidification to a pH of 2.0 using 6 M of HCl. The acidified supernatant was left overnight at 4°C for complete precipitation of the biosurfactant. The next day, precipitated samples were centrifuged with Eppendorf centrifuge 5810 R at 4000 RPM/2755 g, 4^oC for 30 minutes, and the pellets obtained served as crude biosurfactant from acid precipitated samples.

4.2.2.2.*2* Ethyl acetate extraction

Crude biosurfactant samples with average pH of 7.2 were extracted with ethyl acetate using three times the volume of supernatant. This concoction was left to stand for 30 minutes, and the upper organic phase containing the extract was transferred to a new Durham bottle. The process was

repeated three times to increase the quantity of the extracts. The organic solvent was evaporated using a rotary evaporator, and the residue obtained served as the crude biosurfactant (Saravanan & Vijayakumar, 2012)

4.2.2.3 Analysis of extracted biosurfactants

4.2.2.3.1 Thin-layer chromatography (TLC)

Silica aluminium gel plates (matrix) labelled with fluorescent dye was obtained from Sigma-Aldrich, Germany. The plates were cut according to a pre-determined size. A starting line at about 1 cm from the bottom was made. One microliter of the (samples) crude extracts from ethyl acetate was spotted on the gel plates about 1 cm apart. A lipid standard (rhamnolipids) was also spotted on the gel plate as a control. A solvent system of chloroform: methanol: water (65:15:2 v/v) was prepared in a final volume of 82 ml. About 25 ml of this solvent system was placed into an air-tight container. A filter paper was placed inside the air-tight container to help in the saturation, and this was removed after the filter paper is fully saturated (Bora et al., 2015). The silica gel plate with the spotted samples and the standard was placed inside the air-tight container so that the solvent system was below the starting line of the gel plate. The solvent system was allowed to migrate for about 15-20 minutes, and the solvent front was marked at the finished line. The container was then opened inside the fume-hood, and the gel plate was removed and placed on a dried blue roll paper and was allowed to dry for 3 minutes. The dried plate was observed under UV light. Observed spots were marked as a positive area for the presence of biosurfactants. To visualise the diverse types of biosurfactants present in our samples, the plate was sprayed with

0.2% ninhydrin in ethanol (Bora et al., 2015). The sprayed plate was allowed to be air-dried inside the fume-hood for about 4 minutes, and the plate was charred in an oven at 120^{o}C for 5 minutes. The presence of purple colour indicates the presence of biosurfactants (lipopeptide/proteins). The plate was also saturated with iodine by placing the plate inside an air-tight container containing iodine crystals (Bora et al., 2015). The presence of dark brown spots indicates the presence of biosurfactants (lipids). The retention factor (Rf) of the biosurfactants was calculated by dividing the distance (cm) migrated by the samples by the distance (cm) migrated by the solvent front. The distance was measured from the starting line.

4.2.2.3.2 High-performance liquid chromatography-mass spectrometer (HPLC-MS)

Crude extracts were analysed by HPLC-MS All samples were made up to 1ml in Acetonitrile (ACN) in an HPLC vial. All samples were then sonicated for 2 minutes for a full dissolution. A 10 ul of the sample was injected into the system with mobile phase A of 0.1 % Formic acid in water and mobile phase B of 0.1 % Formic acid in ACN. Mobile phase gradient A: B (50: 50) to (5: 95) over 7.5 minutes, A: B (0: 100) for 1 minute and A: B (95: 0) for 1.5 minutes were set up, and column Xbridge C18 3.5 um x 2.1 x 30 mm, PN: 186003020 was used. Mass spectrometry was performed using Agilent 6120 using Electro-Spray Ionisation (ESI) and quadrupole mass spectrometer and UV detector. m/z range: 100 – 1500, run in Positive mode only. Rhamnolipids and sophorolipids were used as standard.

4.2.2.4 Amplification of surfactin genes by conventional and gradient PCR (Polymerase Chain Reaction)

The genomic DNA from the isolates with positive hemolytic assay was screened to detect surfactin genes by both conventional and gradient PCR. Primers for surfactin genes, sfp: 5'-ATGAAGATTTACGGAATTTA-3' (forward) and 5'-TTATAAAAGCTCTTCGTACG- 3' (reverse); srfAA: 5'-TCGGGACAGGAAGACATCAT-3' (forward) and 5'-CCACTCAAACGGATAATCCTGA-3' (reverse) were used for the amplification (Plaza et al., 2015). 50 µl PCR reaction containing 10 µl of 5X PCRBIO Buffer, 1.5 µl 15mM $MgCl_2$, 1 µl of 10 mM dNTP, 2 µl each of 20 mM of both primers, 1 µl of DNA template, 32.25 µl of nuclease-free water and 0.25 µl of *Taq* DNA polymerase. The PCR conditions followed with an initial denaturation at 95^{o}C for 4 minutes, 30 cycles for denaturation at 94^{o}C for 1 minute, an annealing temperature of 48^{o}C for 30 seconds for the conventional PCR and a temperature range of 48^{o}C to 65^{o}C for the gradient PCR for 30 seconds, extension at 72^{o}C for 1 minute. The final cycle involved extension at 72^{o}C for 5 minutes. Amplified PCR products were separated on a 1% agarose gel electrophoresis containing 5µl ethidium bromide. Gels were electrophoresed at 100 V in 1x TAE buffer for 40 min. The gels were viewed and scanned for DNA bands in a Gel Doc apparatus (Gel-Doc-it^{TM} 310 Imaging System), USA

4.3 Results

4.3.1 Bioprospecting and detection of biosurfactants

Isolates were screened phenotypically by hemolytic assay on blood agar, the drop collapse test, and the oil displacement test to determine if they (both actinobacteria and non-actinobacteria) could produce biosurfactants.

Figure 4.1 below shows the hemolytic assay by blood agar on multi well-plates with a clearing zone (hemolysis) around the isolates, indicating a positive result for biosurfactants production Drop collapse and oil displacement tests were also used to screen the isolates to know if they could produce biosurfactants. The results are shown in figure 7.5.1 (appendix 7.5). The presence of hemolysis on duplicate samples (isolates) was taken as positive, and these were selected for further analysis. Samples without hemolysis (dark spots) were recorded as negative. In the oil drop collapse test, a decrease in the interface tension on the supernatants of the isolates and the paraffin oil led to spreading of the oil on the surface of the supernatants. In the oil displacement assay, a clear and concave zone formed on crude oil by the supernatants was observed and measured on the positive results. The diameter of the displacement is shown in table 7.5.1 (appendix 7.5), with isolate NB 20 having the largest diameter of 8.50 mm (figure 7.5.1). A total of five (5) isolates out of our 9 isolates representing 55.55% were positive in all the three (hemolysis, drop collapse test, and oil displacement test) screening assays carried out (Table 4.1).

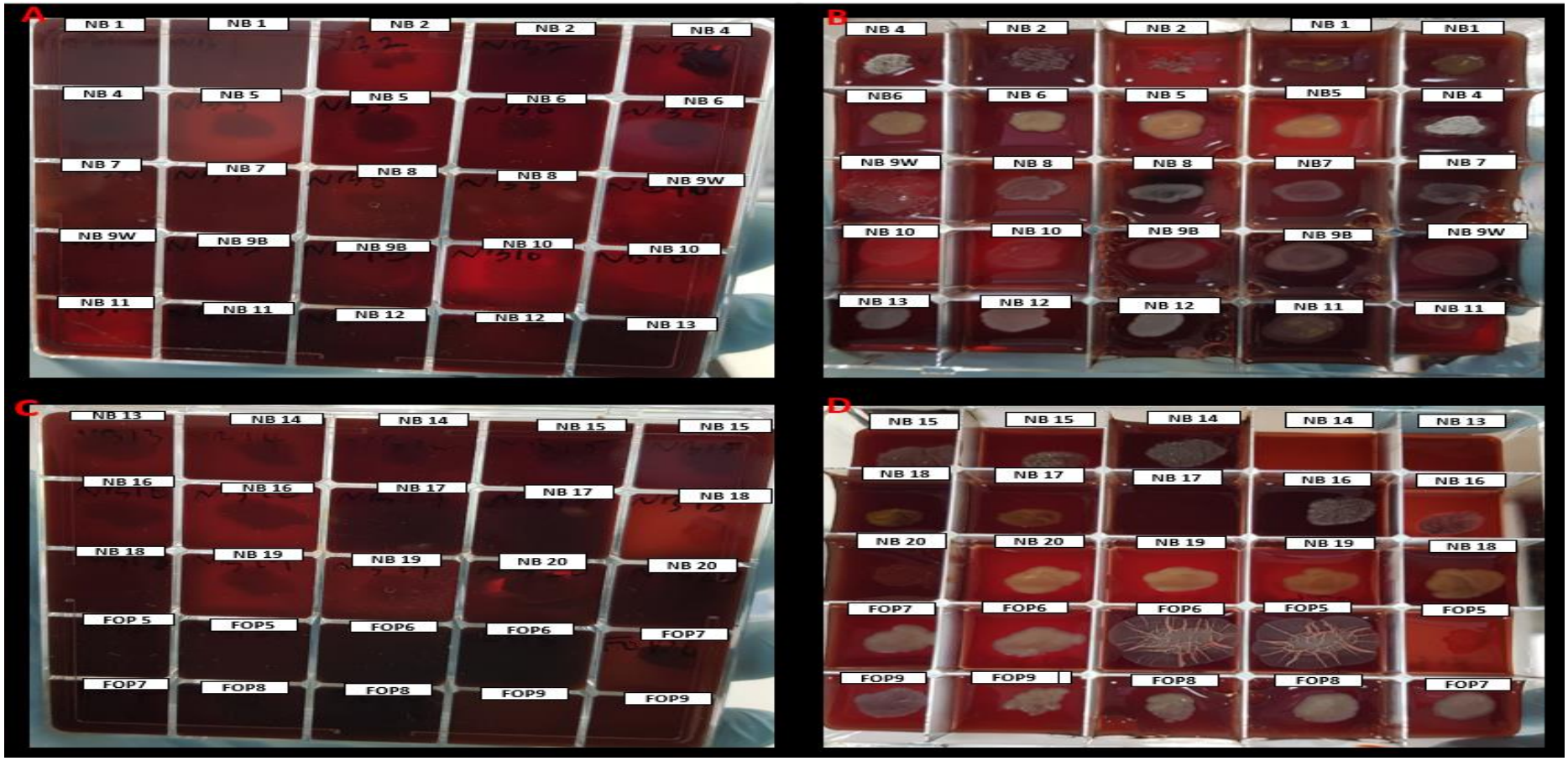

Figure 4. 1: Bioprospecting for biosurfactants production by the isolates through hemolysis of blood agar on multi-well plates. A and C are rear views of the multi-well plate. B and D are front views of the multi-well plate. Positive results were inferred from duplicate assays with complete hemolysis on blood agar. Isolates NB 2, NB 14, NB 15, NB 16, NB 18, NB 19, NB 20, NB 21, and FOP 8 which are marine actinobacteria (chapter two) were the target of this assay.

Table 4. 1: Screening result of biosurfactants-producing isolates

Isolates	**Hemolysis test**	**Oil spreading test**	**Drop collapse test**
NB2	-	-	-
NB14*	+	+	+
NB15*	+	+	+
NB16*	+	+	+
NB18	-	-	+
NB19*	+	+	+
NB20*	+	+	+
NB21	-	-	+
FOP 8	-	-	+

Key: + = Positive, - = Negative, * = Isolates with positive results in the three screening assays.

4.3.2 Small scale expression and extraction of biosurfactants

Small scale expression in 50 ml of MSM was carried out with the isolates that gave a combined positive result for the phenotypic (haemolytic assay, drop collapse test and oil displacement test) assay in the screening and prospecting of biosurfactants production. The supernatants from the fermented broth were extracted with both acid precipitation and ethyl acetate methods. Figure 4.2A shows some representatives of the crude extracts of biosurfactants by acid (6M HCl) precipitation, and figure 4.2B shows the crude and evaporated extracts by ethyl acetate of the extracts. From the nine (9) isolates used in screening for biosurfactants production (Figure 4.1 and Table 4.1), a total of five (5) extracts representing 55.6% was obtained based on ethyl acetate extraction protocol and six (6) crude extract respecting 66.7 % based on acid precipitation protocol. There were no noticeable crude extracts from samples NB 15 with ethyl acetate extraction and NB 15 and NB 14 with acid precipitation protocol.

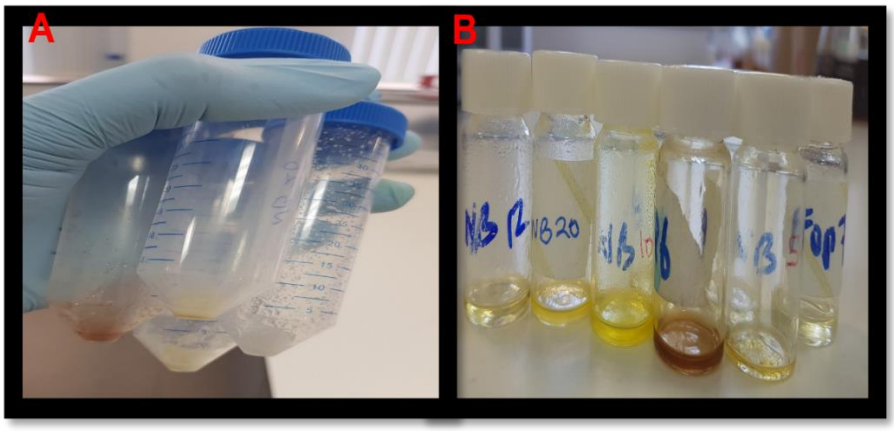

Figure 4. 2: Representative of biosurfactants crude extracts from 50 ml MSM broth culture. (A). crude extracts by acid precipitation. (B). Crude extracts by ethyl acetate.

4.3.3 Analysis of extracted biosurfactants

The crude and evaporated biosurfactant extracts from the isolates were analysed by both TLC and HPLC-MS.

4.3.3.1 Thin-layer chromatography (TLC)

TLC was used to determine the presence of any types of biosurfactants in the ethyl acetate extracts of our isolates. A total of five extracts (NB 14, NB 15, NB 16, NB 19, and NB 20) were chosen and analysed by TLC (Figure 4.3) based on the phenotypic assay result. The result shows that four (80%) of our isolated actinobacteria could produce biosurfactants based on the observed spots on the TLC gel plates (figure 4.3). Firstly, the spots on the TLC plates were observed under ultra-violet light (Figure 4.3A). Since the plates were fluorescent, bright spots indicated the presence of biosurfactants. Various degrees of white migrating fluorescent pattern against dark background could be seen on the plates, confirming biosurfactants' presence in our extracts. Secondly, on spraying the plates with 0.2% ninhydrin in ethanol and charring it for 5 minutes at 120^{o}C, the appearance of purple spots on the plates indicated the presence of lipopeptides, type of biosurfactants in our samples (Figure 4.3B). However, there were no noticeable purple spots on the lanes corresponding to the standards (std). This was expected as our standard (rhamnolipid), a lipid, will not be observed when sprayed with 0.2% ninhydrin (protein indicator). Thirdly, the plates were saturated with iodine vapour when placed inside an air-tight container containing iodine crystals. The presence of brown spots on the TLC plates confirms the presence of lipid biosurfactants in our extracts (Figure 4.3C). The various brown spot corresponds to different lipids biosurfactant compounds in the extracts. As expected, there were

noticeable spots for the standards. The retention factor (Rf) values of the migrated spots were calculated (Table 4.2). The result of the TLC analysis further confirms the earlier result of the phenotypic assay, especially the hemolysis on blood agar, the primary screening test for biosurfactant production.

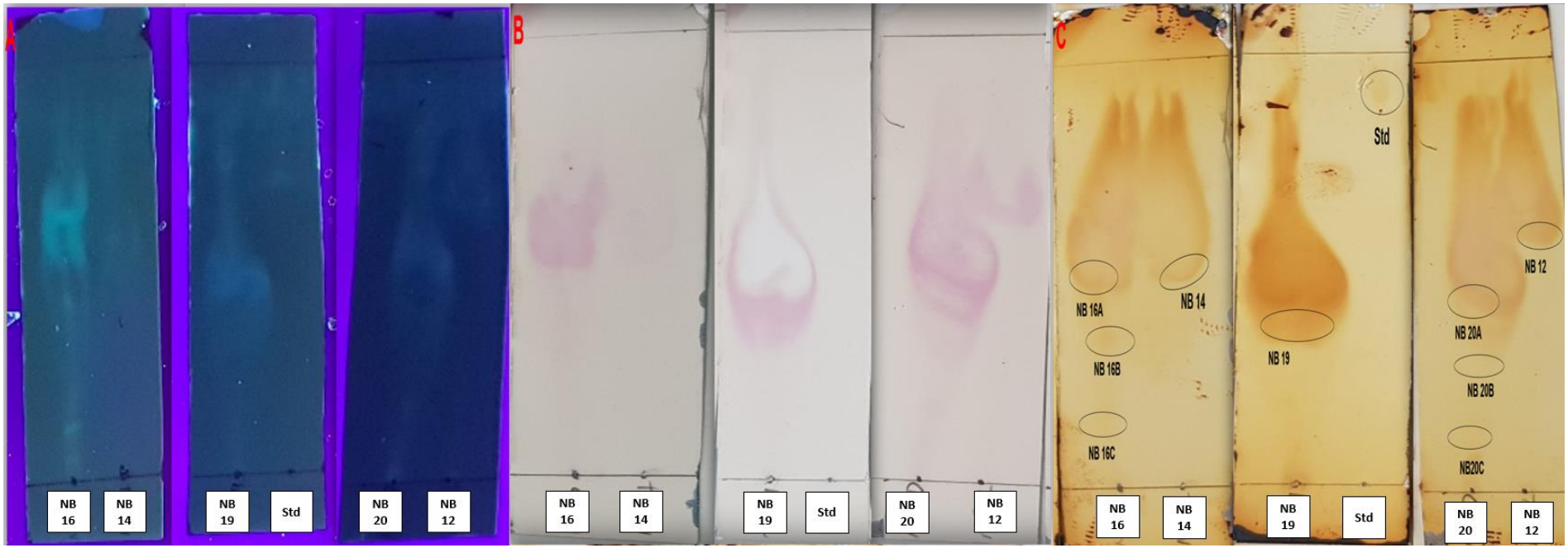

Figure 4. 3: TLC analysis of ethyl acetate extracts. A silica aluminium gel plates impregnated with fluorescent dye in chloroform: methanol: water (65:15:2 v/v) solvent system. Spotted samples were std (rhamnolipid) and rare marine actinobacteria isolates (NB 14, NB 16, NB 19, and NB 20). (A). Migration of separated biosurfactants as seen with UV light. (B). Migration of separated biosurfactants after spraying with 0.2% ninhydrin in ethanol and charring for 5 minutes at 120^{o}C. (C). Migration of separated biosurfactants in crystal iodine.

Table 4. 2: Retention factor (Rf) of migrated biosurfactants

Migrated samples	Rf values
Std (Rhamnolipid)	0.88 ± 0.02
NB 19	0.28 ± 0.00
NB 16A	0.28 ± 0.00
NB 16B	0.48 ± 0.00
NB 16C	0.51 ± 0.00
NB 14	0.52 ± 0.02
NB 20A	0.13 ± 0.01
NB 20B	0.17 ± 0.01
NB 20C	0.29 ± 0.00

Rf value measurement based on the spots as observed with iodine vapour (Figure 4.3C)

4.3.3.2 High-performance liquid chromatography mass spectrometry (HPLC-MS)

HPLC-MS analysis of the ethyl acetate crude extract from the isolates as well as the two standards was carried out. The result shows that our isolates produce biosurfactants. The result of the HPLC-MS is shown in Figures 4.4 to 4.9 and Table 4.3 to 4.8. The HPLC profiles and the total ion chromatogram (TIC) of our isolates were compared with the standards and published data (Table 4.9). The result revealed that our isolates could produce glycolipids (rhamnolipids and sophorolipids) at varying degree and with different molecular weights. Only NB 14 (*Brachybacterium*) produced diacylglycerol at a RT of 8.71 minutes with mass to charge ratio (m/z) of 607.4 and 663.4. The least RT at which glycolipid biosurfactants were observed was 2.379 minutes for sophorolipids (sample NB 16) while the highest RT at which noticeable glycolipids biosurfactants was observed was 9.635 minutes for rhamnolipids (sample NB 14).

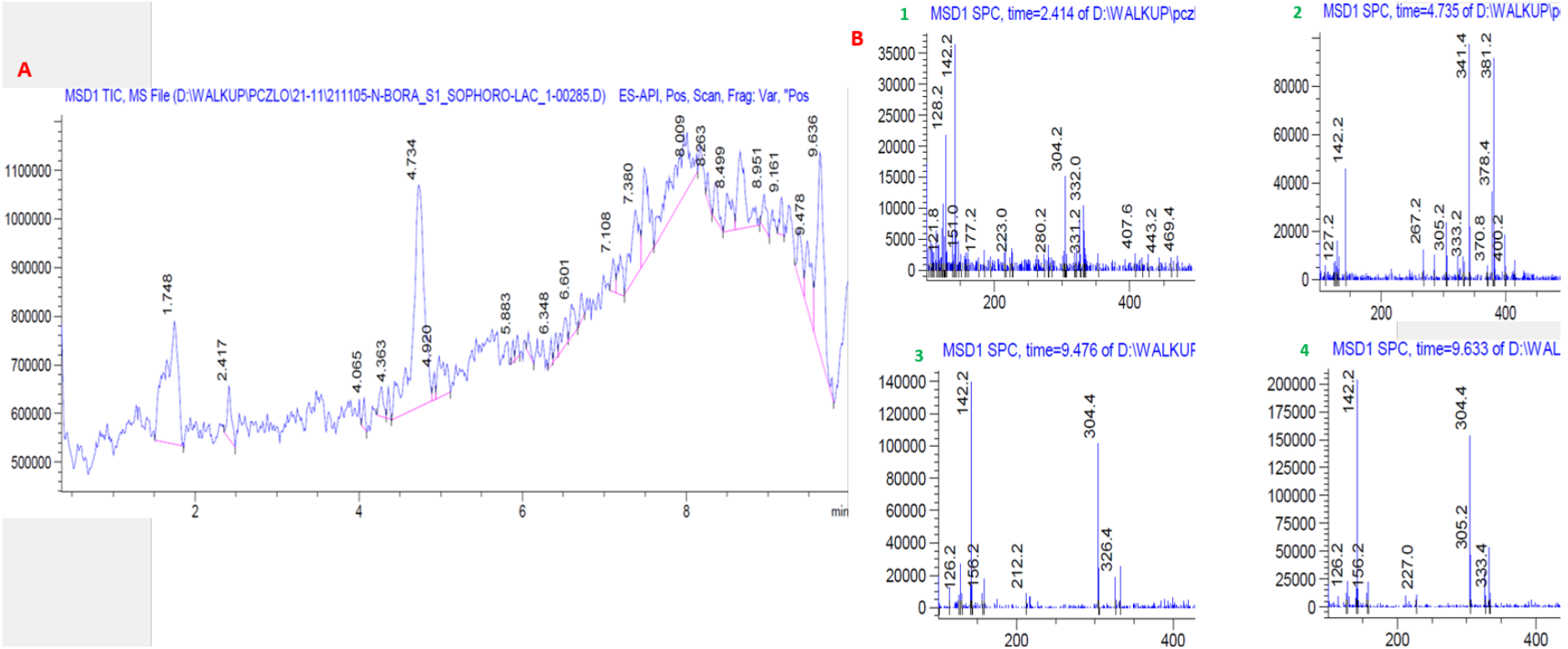

Figure 4. 4: HPLC profile of Sophorolipid standard. (A). Total ion chromatogram (TIC). (B). Mass to charge ratio (m/z) at various retention times.

Table 4. 3: Mass to charge (m/z) ratio at various retention times of Sophorolipid standard

Retention time (RT)	Mass to charge ratio (m/z)
2.417	128.2
	142.2
	304.2
	332.0
4.734	142.2
	341.4
	378.4
	381.2
9.478	142.2
	304.4
9.636	142.2
	304.4

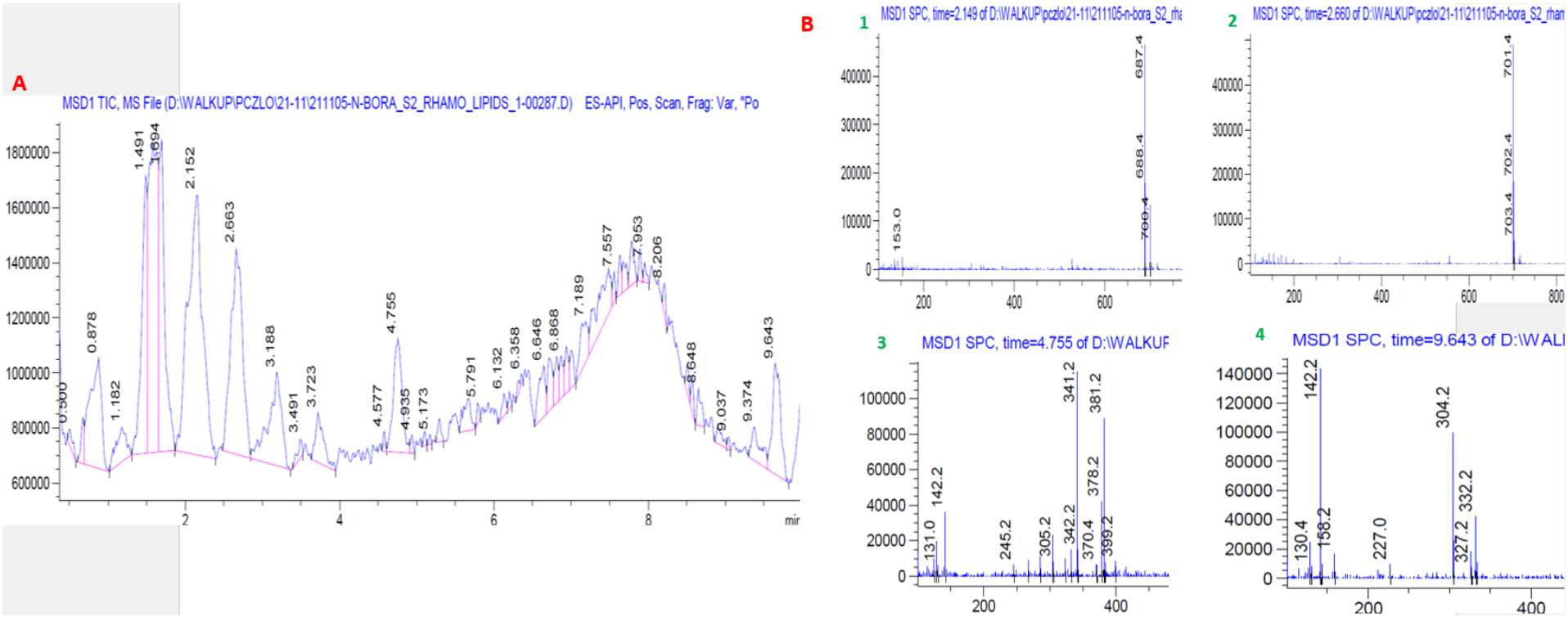

Figure 4. 5. HPLC profile of Rhamnolipids standard. (A). Total ion chromatogram (TIC). (B). Mass to charge ratio (m/z) at various retention times.

Table 4. 4: Mass to charge (m/z) ratio at various retention times of Rhamnolipids standard

Retention time (RT)	m/z
2.152	687.4
	688.4
2.663	701.4
	702.4
4.755	142.2
	341.2
	378.2
	381.2
9.643	142.2
	304.2
	332.2

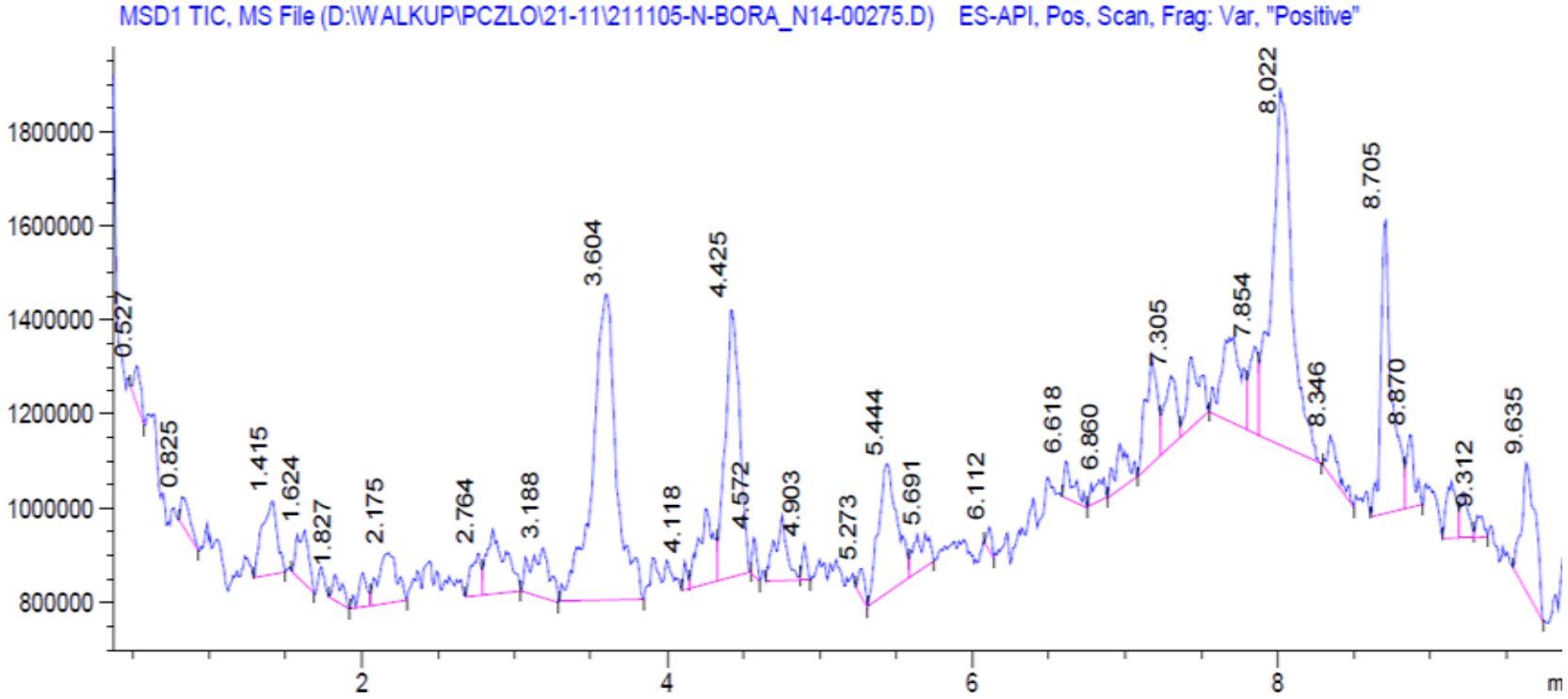

Figure 4. 6A: HPLC profile of total ion chromatogram (TIC). of biosurfactant extracted from sample NB 14.

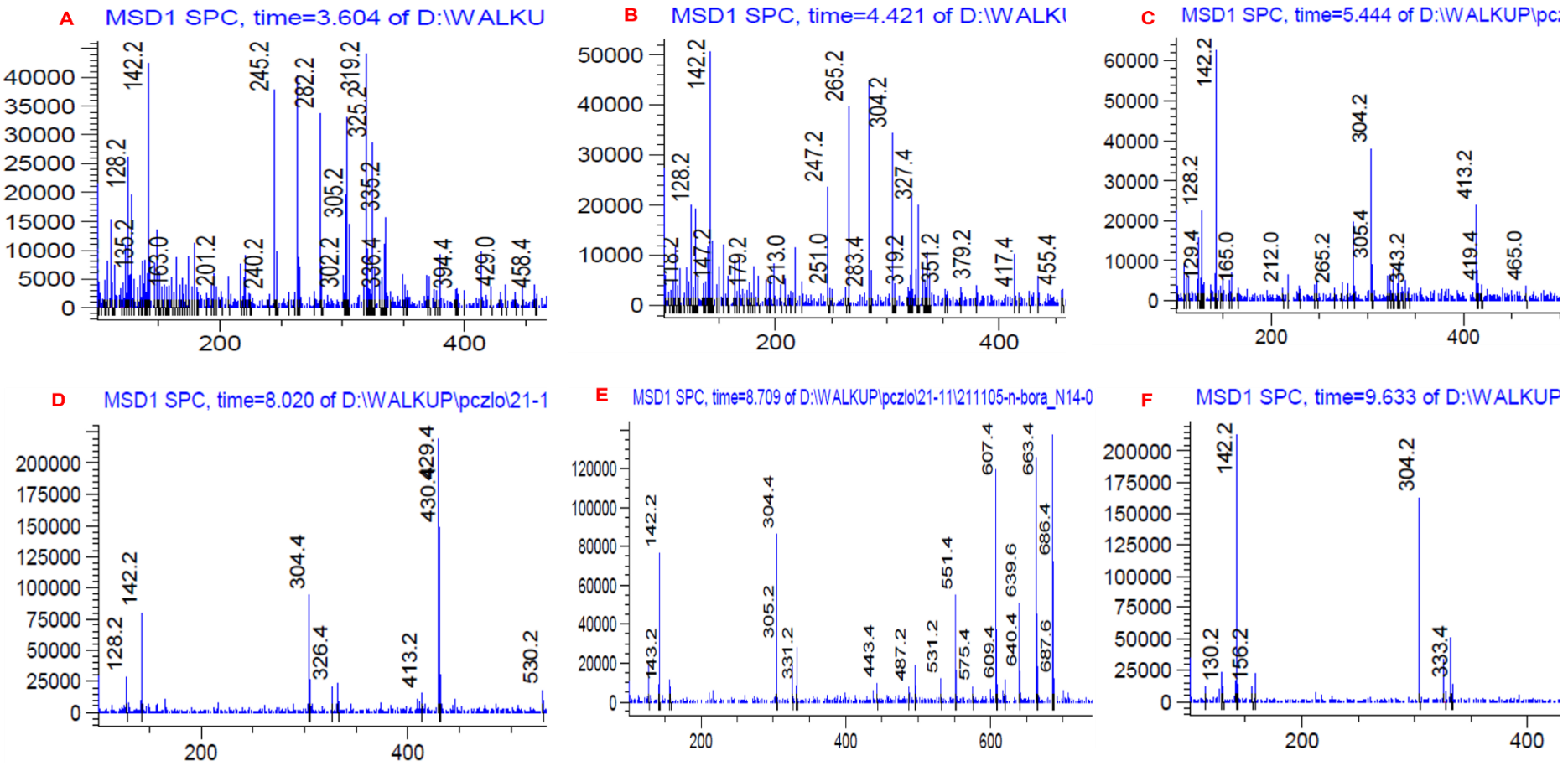

Figure 4. 7B: HPLC profile of the mass to charge ratio (m/z) at various retention times of TIC of biosurfactant extracted from sample NB 14.

Table 4. 5: Mass to charge (m/z) ratio at various retention times of Sample NB 14

Retention time (RT)	m/z
3.604	142.2
	245.2
	282.2
	319.2
	325.2
4.425	142.2
	247.2
	265.2
	304.2
	327.4
5.444	142.2
	304.2
	413.2
8.022	142.2
	304.4
	429.4
	430.1
8.705	142.2
	304.4
	607.4
	663.4
9.635	142.2
	304.2

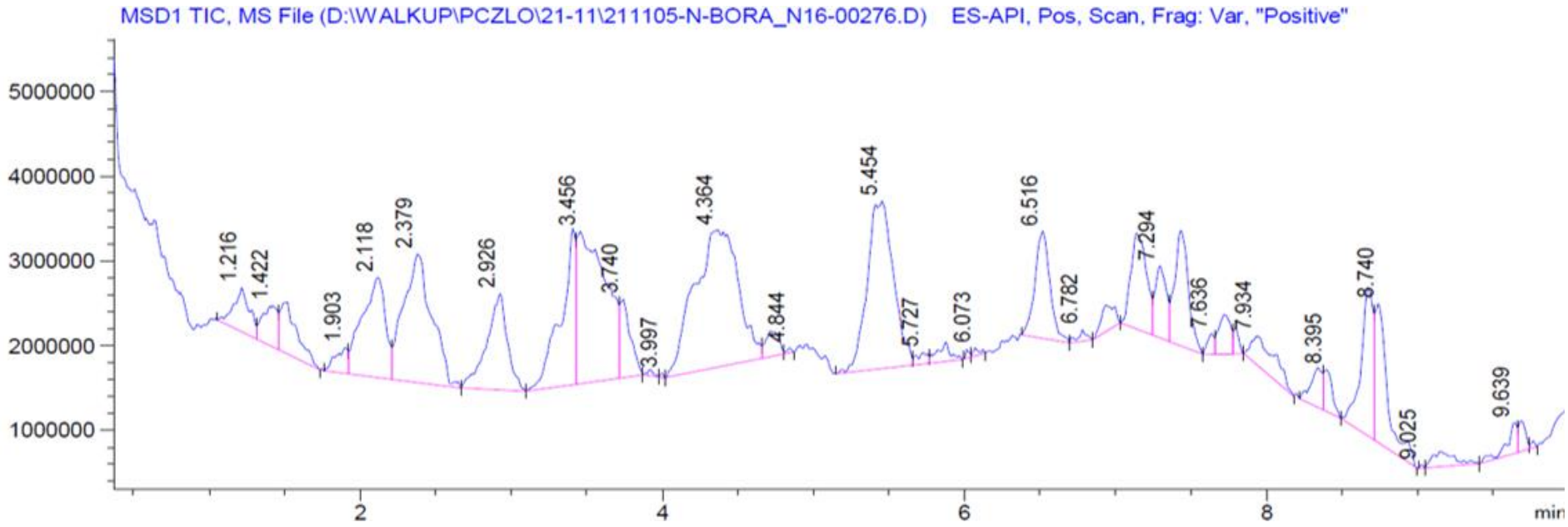

Figure 4. 7A: HPLC profile of total ion chromatogram (TIC). of biosurfactant extracted from sample NB 16.

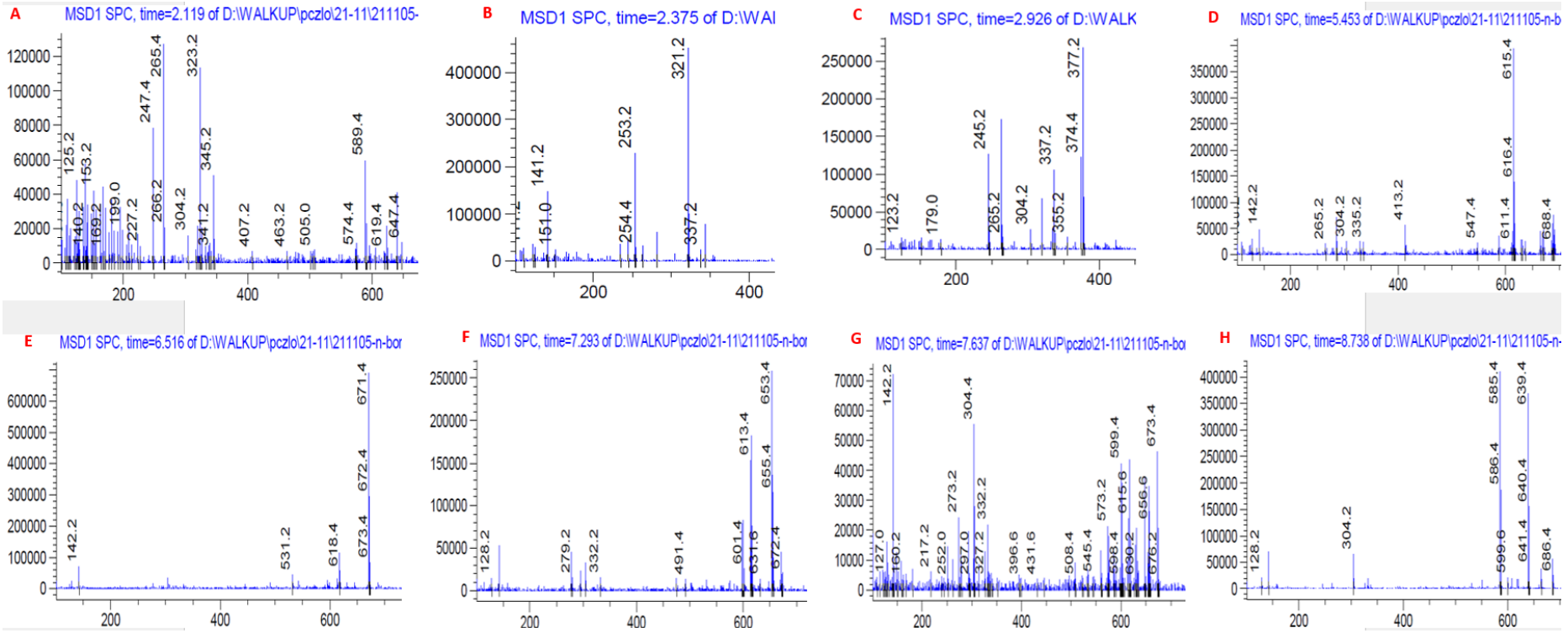

Figure 4. 7B: HPLC profile of the mass to charge ratio (m/z) at various retention times of TIC of biosurfactant extracted from sample NB 16.

Table 4. 6: Mass to charge (m/z) ratio at various retention times of Sample NB 16

Retention time (RT)	Mass/charge ratio (m/z)
2.118	247.4
	265.4
	323.2
	345.2
	589.4
2.379	253.2
	321.2
2.926	245.2
	265.2
	337.2
	374.4
	377.2
5.454	615.4
	616.4
6.516	671.4
7.294	613.4
	653.4
7.636	142.2
	304.4
	599.4
	673.4
8.740	585.4
	639.4

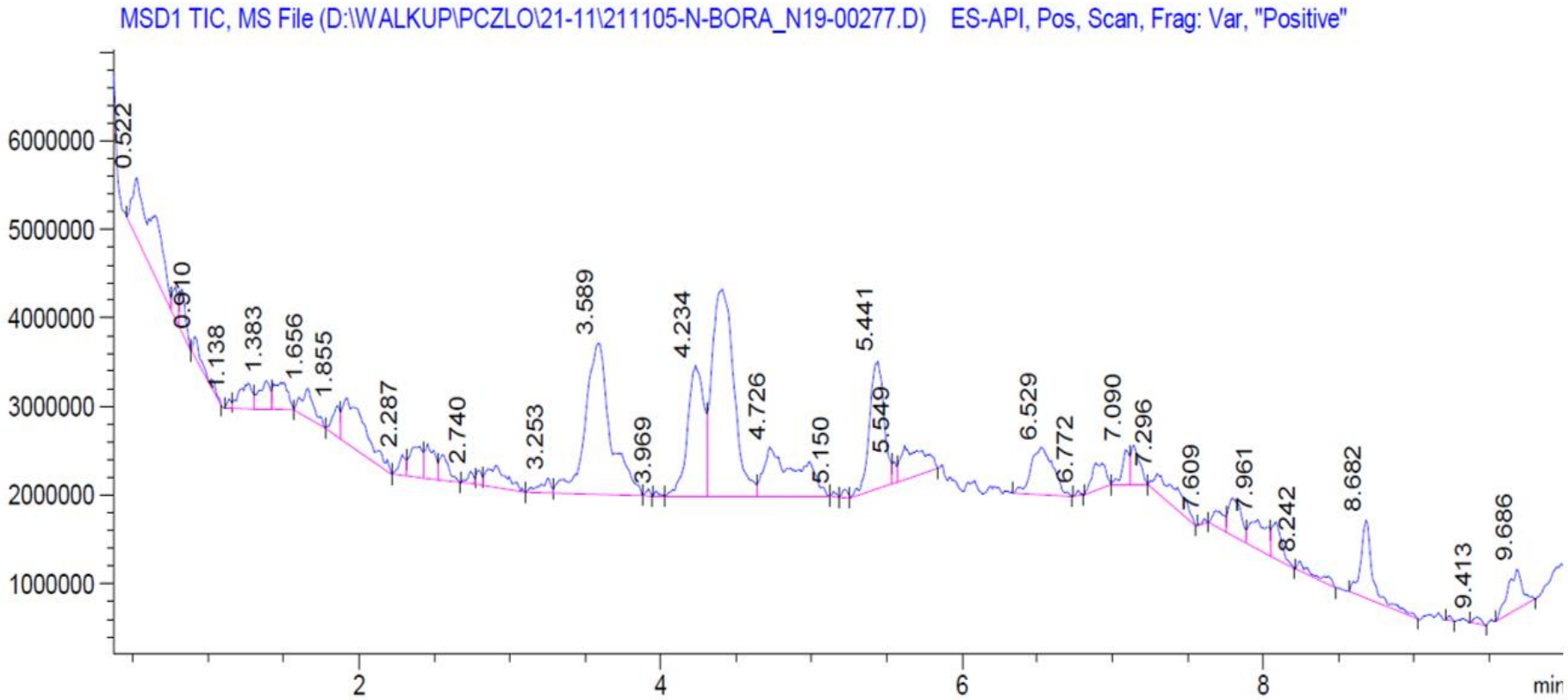

Figure 4. 8A: HPLC profile of total ion chromatogram (TIC). of biosurfactant extracted from sample NB 19.

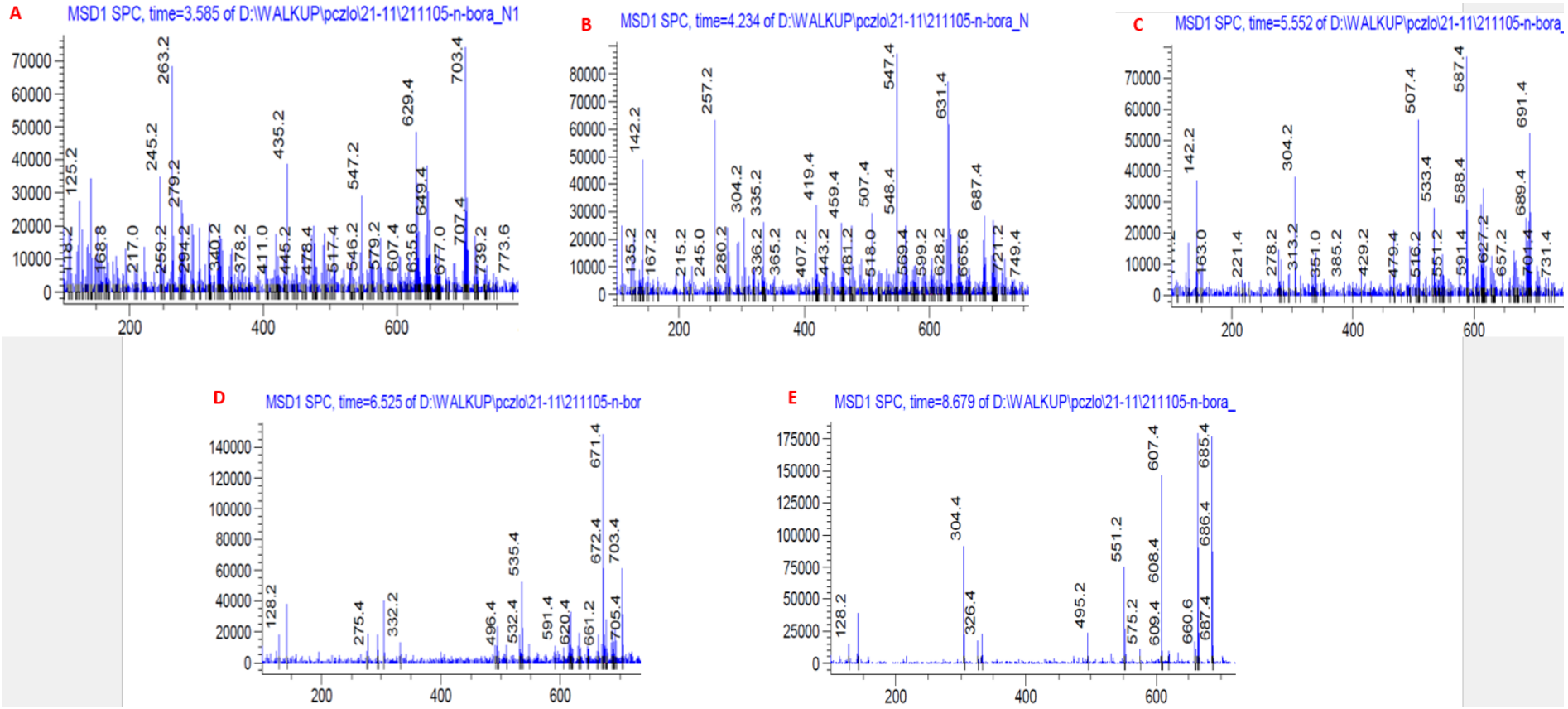

Figure 4. 8B: HPLC profile of the mass to charge ratio (m/z) at various retention times of TIC of biosurfactant extracted from sample NB 19.

Table 4. 7: Mass to charge (m/z) ratio at various retention times of Sample NB 19

Retention time (RT)	Mass to charge ratio (m/z)
3.589	263.2
	435.2
	629.4
	703.4
4.234	142.2
	257.2
	547.4
	631.4
5.549	142.2
	304.2
	507.4
	587.4
	691.4
6.529	671.4
	672.4
8.682	304.4
	607.4
	685.4
	686.5

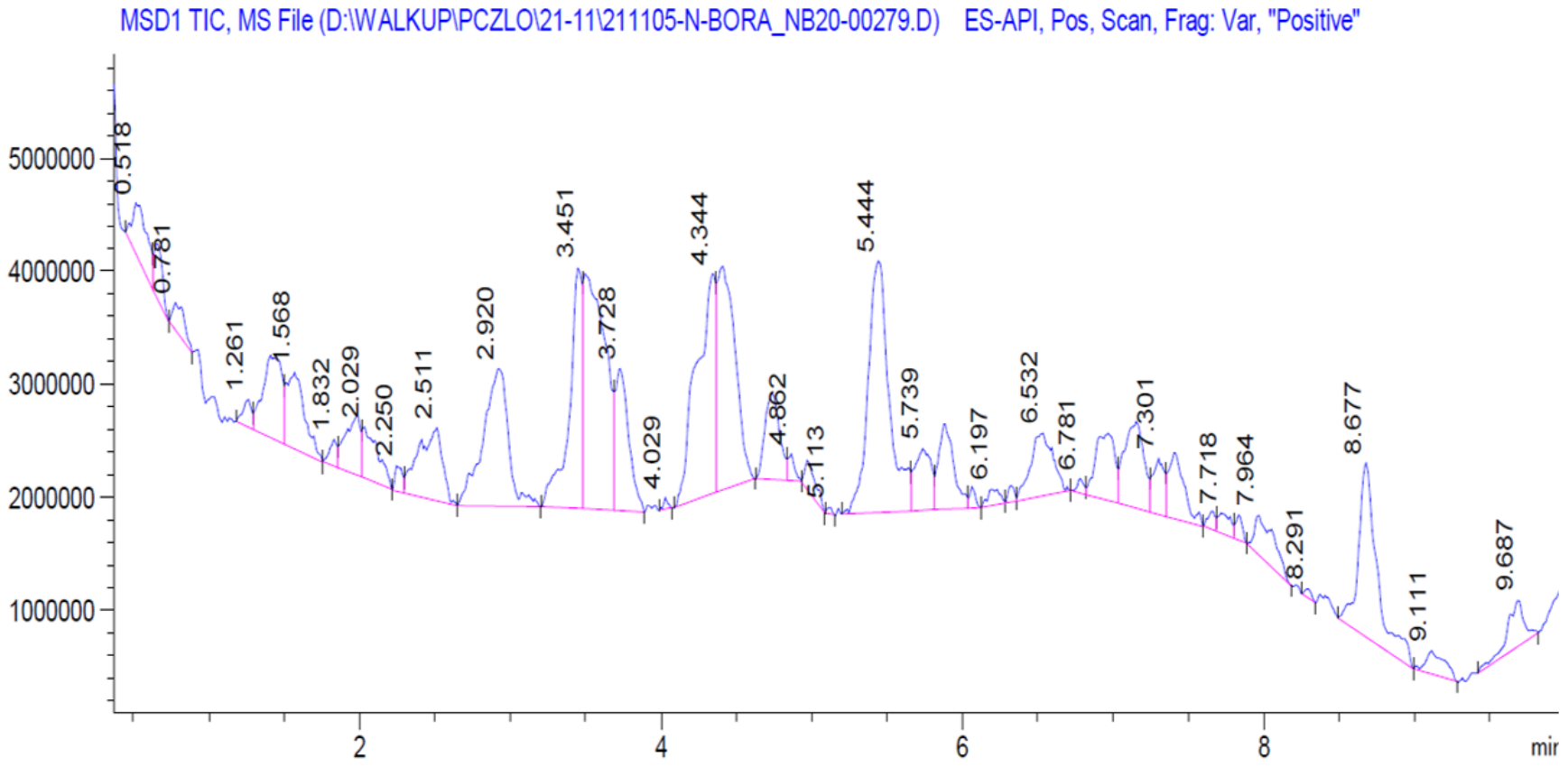

Figure 4. 9A: HPLC profile of total ion chromatogram (TIC). of biosurfactant extracted from sample NB 20.

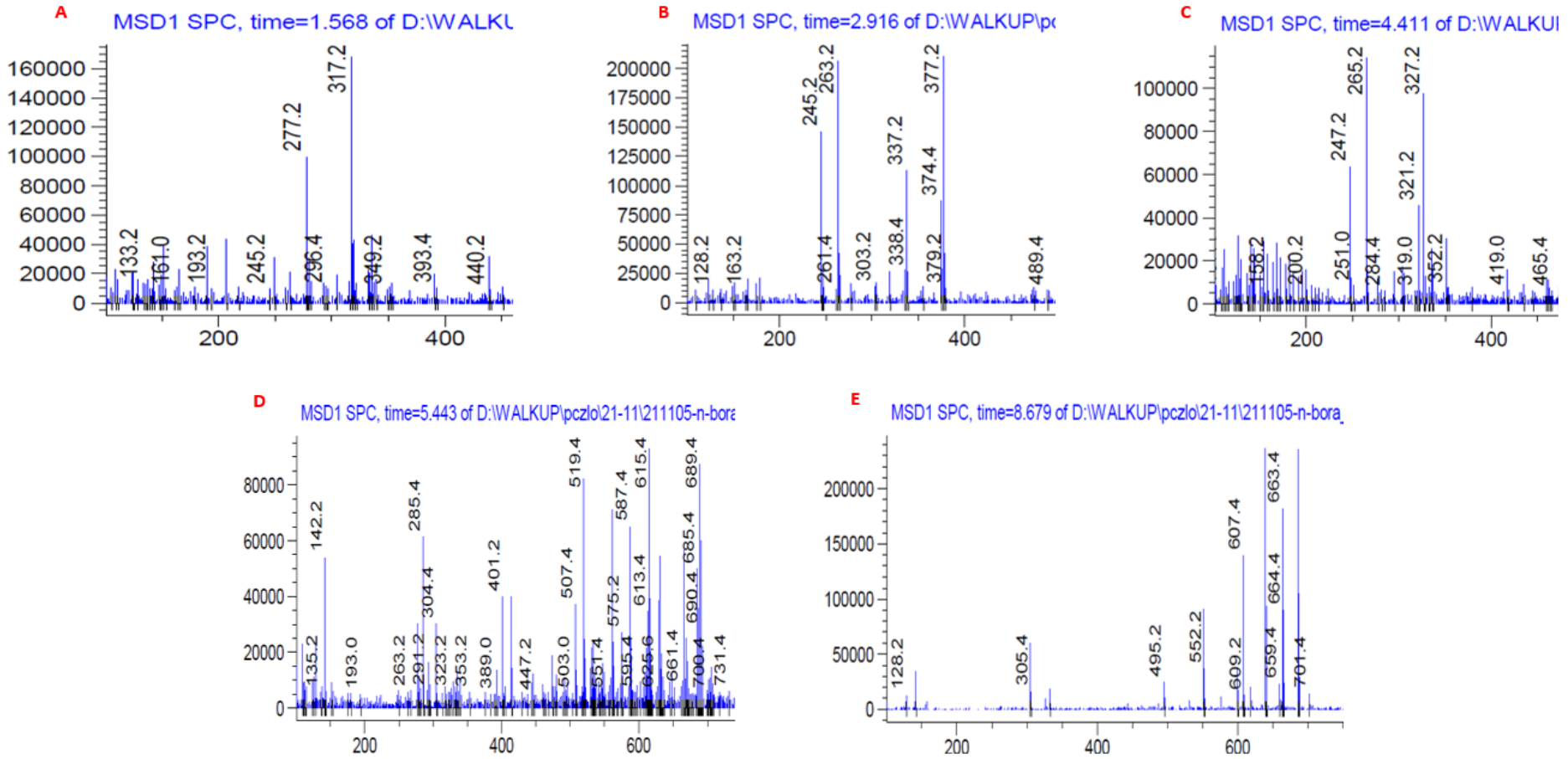

Figure 4. 9B: HPLC profile of the mass to charge ratio (m/z) at various retention times of TIC of biosurfactant extracted from sample NB 20

Table 4. 8: Mass to charge (m/z) ratio at various retention times of Sample NB 20

Retention time (RT)	Mass to charge ratio (m/z)
1.568	277.2
	317.2
2.920	245.2
	263.2
	337.2
	374.4
	377.2
4.344	247.2
	265.2
	327.2
	631.4
5.444	142.2
	285.4
	519.4
	587.4
	615.4
	685.4
	689.4
8.677	607.4
	663.4
	664.4

Table 4. 9: Comparison of HPLC-MS profiles of our samples with the standard and published data.

Samples			Comparison					
NB 14	**RT**	**m/z**	**compounds**	**RT**	**m/z**	**MW**	**Symbol**	**Reference**
	3.604	142.2 245.2 282.2 319.2 325.2						
	4.425	142.2 247.2 265.2 304.2 327.4	Rhamnolipids Sorphorolipid Rhamnolipids	4.755 4.734	142.2 142.2 304.2	 306.35 334.41	 Rha-C8 Rha-C10	Standard Standard Standard
	5.444	142.2 304.2 413.2	Rhamnolipids		304.2	306.35	Rha-C_8	Standard
	8.022	142.2 304.4 429.4 430.1	Rhamnolipids		304.2	306.35	Rha-C_8	Standard
	8.705	142.2 304.4 607.4 663.4	Diacylglycerol Diacylglycerol Rha-Rha-C_8-C_8	8.0 8.5 13.13	614.571 642.603 593	 306.35 664.82	 Rha-C_8 Rha-Rha-C_{10}-C_{10}-C_{H3}	Kiran et al., 2014 Camilios-Neto et al., 2011
	9.635	142.2 304.2	Rhamnolipids Sophorolipid Rhamnolipids	9.643 9.636	142.2 1422.2,304.4 304.2	 306.35	 Rha-C_8	Standard Standard Standard
NB 16	2.118	247.4 265.4 323.2 345.2 589.4				334.41 594.69	Rha-C_{10} Rha-Rha-C_8-C_8	
	2.379	253.2 321.2	Sophorolipid	2.417	357, 332.0	334.41	Rha-C_{10}	Standard

Samples			Comparison					
	RT	**m/z**	**compounds**	**RT**	**m/z**	**MW**	**Symbol**	**Reference**
	2.926	245.2 265.2 337.2 374.4 377.2				 386.48 386.48	 Rha-$C_{14:2}$ Rha-$C_{14:2}$	
	5.454	615.4 616.4	Rha-Rha-C_{10}-C_8 Rha-Rha-C_{10}-C_8	15.87 15.87	621 621	616.87 616.87	Rha-C_{14}-C_{14} Rha-C_{14}-C_{14}	Camilios-Neto *et al.*, 2011
	6.516	671.4	Rha-Rha-C_{10}-$C_{12:1}$	19.75	675	672.97	Rha-C_{16}-C_{16}	Haba et al., 2003
	7.294	613.4 653.4	 Rha-Rha-C_{10}-C_{10}		 650	616.87 656.89	Rha-C_{14}-C_{14} Decenoyl-Rha-C_{10}-C_{10}	 Camilios-Neto *et al.*, 2011
	7.636	142.2 304.4 599.4 673.4	 Rhamnolipids Rha-Rha-C_8-C_8 Rha-Rha-C_{10}-$C_{12:1}$	 13.13 	 304.2 593 676	 306.35 616.87 	 Rha-C_8 Rha-C_{14}-C_{14} 	 Standard Camilios-Neto et al., 2011 Haba et al., 2003
	8.740	585.4 639.4	Rha-Rha-C_8-C_8 Rha-Rha-C_{10}-$C_{10:1}$	13.13 17.51	593 647	588.81 644.92	Rha-C_{12}-C_{14} Rha-C_{14}-C_{16}	Camilios-Neto et al., 2011
NB 19	3.589	263.2 435.2 629.4 703.4	 Rha-Rha-C_8-C_8 Rha-Rha-C_8-C_{10} Rha-Rha-C_{12}-$C_{12:1}$	 13.7 15.87 21.99	 447 621 703	 644.92 704.89	 Rha-C_{14}-C_{16} Rha-Rha-C_{12}-$C_{12:1}$	 Camilios-Neto et al., 2011 Camilios-Neto et al., 2011 Camilios-Neto et al., 2011
	4.234	142.2 257.2 547.4 631.4	Rhamnolipids Sophorolipids Rha-Rha-C_{10}-C_8	4.755 4.734 15.87	142.2 142.2 621	 558.74 644.92	 Rha-C_{12}-$C_{12:1}$ Rha-C_{14}-C_{16}	Standard Standard Camilios-Neto et al., 2011
	5.549	142.2 304.2 507.4 587.4 691.4	 Rhamnolipids Rha-C_{10}-C_{10} Rha-Rha-C_8-C_8 	 19.05 13.13 	 304.2 503 593 	 306.35 588.81 704.89	 Rha-C_8 Rha-C_{12}-C_{14} Rha-Rha-C_{12}-$C_{12:1}$	 Standard Camilios-Neto *et al.*, 2011
	6.529	671.4 672.4	Rha-Rha-C_{10}-$C_{12:1}$ Rha-Rha-C_{10}-$C_{12:1}$		676 676	672.97 672.97	Rha-C_{16}-C_{16} Rha-C_{16}-C_{16}	Haba *et al.*, 2003 Haba *et al.*, 2003

	Samples		**Comparison**					
	RT	**m/z**	**compounds**	**RT**	**m/z**	**MW**	**Symbol**	**Reference**
	8.682	304.4				306.35	Rha-C_8	
		607.4				616.87	Rha-C_{14}-C_{14}	
		685.4	Rha-Rha-C_{10}-C_{12}		678	691.06	Rha-Rha-C_{14}-C_{16}	Haba *et al.*, 2003
		686.5	Rha-Rha-C_{12}-C_{10}		678	691.06	Rha-Rha-C_{14}-C_{16}	Haba *et al.*, 2003
NB 20	1.568	277.2						
		317.2						
	2.920	245.2						
		263.2						
		337.2						
		374.4				386.48	Rha-$C_{14:2}$	
		377.2				386.48	Rha-$C_{14:2}$	
	4.344	247.2						
		265.2						
		327.2	Sophorolipids	4.734	341.4			Standard
		631.4	Rha-Rha-C_{10}-C_{10}	4.27	649			Filloux & Ramos, 2014
	5.444	142.2						
		285.4						
		519.4				518.68	Rha-C_{10}-C_{10}-CH_3	
		587.4				588.81	Rha-C_{12}-C_{14}	
		615.4	Rha-Rha-C_{10}-C_8	15.87	621	616.87	Rha-C_{14}-C_{14}	Camilios-Neto *et al.*,
		685.4	Rha-Rha-C_{10}-C_{12}	20.78	677	691.06	Rha-Rha-C_{14}-C_{16}	2011
		689.4				691.06	Rha-Rha-C_{14}-C_{16}	
	8.677	607.4				616.87	Rha-C_{14}-C_{14}	
		663.4				664.82	Rha-Rha-C_{10}-C_{10}-CH_3	
		664.4	Rha-Rha-C_{10}-C_{12-1}	19.76	675	664.82	Rha-Rha-C_{10}-C_{10}-CH_3	Camilios-Neto *et al.*, 2011

4.3.4 Detection of surfactin genes by conventional and gradient PCR

Primers specific for surfactin genes in *Bacillus* species were tried for the amplification of any potential surfactin genes in our isolates by both conventional and gradient PCR. Our result shows that there was no noticeable amplification with any of the PCR technique employed as evidenced by absence of any band in the agarose gel electrophoresis.

4.4 Discussion

Recently, there has been an increase in the research for the microbial production of biosurfactants. Many research projects are being carried out worldwide to isolate and identify biosurfactant producing bacteria (Ben Ayed et al., 2014; Chittepu, 2019). This chapter was aimed at screening our isolates for their metabolic potential to biosynthesize biosurfactants. In this study, we used blood hemolysis, drop collapse, and oil displacement tests to screen the isolates for the ability to produce biosurfactants. Various screening methods were required to understand the ability of our isolates to make biosurfactants, mainly because there are different classes of biosurfactants, and a single screening method will not be sufficient. However, hemolysis on blood agar is the primary screening assay for biosurfactant activities (Seghal Kiran et al., 2009; Shubhrasekhar et al., 2013). There is also an association between hemolytic activity and biosurfactant production, so blood agar was recommended for lysis activity (Carrillo et al., 1996). The hemolytic assay was carried out in duplicates to avoid a false-positive result and isolates with the same results in the duplicates assays were considered positives and were selected for further analysis Biosurfactant activity of the cell-free supernatant of the isolates was also determined by both the oil displacement and oil collapse test. In this study, there were many positive results for both the drop collapse and oil displacement test. This disagreed with the study of Adebajo and colleagues (Adebajo et al., 2020) but agreed with the study of (Ndibe et al., 2018; Kiran et al., 2009; Youssef et al., 2004) where there was a high positive result for both oil displacement and collapse test. Since phenotypic assay for elucidating the ability of microorganisms, including actinobacteria

(isolates), is not enough and not entirely reliable to conclude the ability of microbes to produce biosurfactants or any other secondary metabolites, we decided to carry out broth culture fermentation in MSM and extraction of the cell-free supernatants. Vegetable oil was used as the carbon source for the screening process. This was in agreement with previous studies of Hamed and colleagues where olive oil was used as carbon source in the production of biosurfactants from Actinomycetes (Hamed et al., 2021). The broth culture was extracted by acid precipitation and by solvent (ethyl acetate). The ethyl acetate crude extracts from the combined positive results of the phenotypic assays were analysed by TLC. TLC is one of the most frequently used chromatographic techniques for identifying biosurfactants. Since biosurfactants are heterogeneous compounds, it was interesting to analyse the separation of the chromatogram with different indicators to have a better idea of the class of biosurfactants in our extract. A 0.2% ninhydrin was used to detect the presence of lipopeptide/peptide/amino groups in our extract, while iodine vapour was used for the presence of lipids component in the biosurfactant extract. The analysis of the TLC assay further confirms that the extracts from our isolates were producing biosurfactants, especially of the class of lipopeptide (amino group) and lipids. This was in agreement with previous studies of Joy et al., 2017; Phulpoto et al., 2020 on biosurfactants production and characterization. In the study of Phulpoto and colleagues, they used 0.2% ninhydrin to indicate lipopeptide biosurfactants spots on TLC plate with RF values of 0.25, 0.35 and 0.75. Similarly, the study of Joy and colleagues on lipopeptide biosurfactants production by Bacillus sp. (SB2) also used 0.2% ninhydrin to indicate lipopeptide spots on TLC plates.

During the TLC assay, precaution was taken when placing the gel plates with the spotted samples inside the air-tight container to avoid splashing and sub-merging the spotted samples (starting line) inside the solvent system (chloroform: methanol: water). All the sample extracts were stored at -20^{o}C prior to analysis to prevent solvent loss due to evaporation of the samples at room temperatures. The freshly prepared mobile phase was used to develop each plate as the solvent system's re-use might change the solvent's polarity and the nature of its migration. These preventive measures ensure good migration and a good formation of bands.

HPLC-MS was used to reconfirm the biosurfactants produced by the phenotypic assay and confirmed by TLC by analysing the ethyl acetate crude extracts of the samples. Production of biosurfactants have been confirmed by HPLC-MS analytical method in previous study (Abdel-Mawgoud et al., 2010). The HPLC-MS profile (TIC and m/z) of the isolates was compared with the standards (rhamnolipids and sophorolipids) and those from literature (Table 4.9). The result revealed that our isolates could be producing biosurfactants at varying degree (figure 4.6 to 4.9 and table 4.5 to 4.8). Our result for the production of biosurfactants was in agreement with previous studies for biosurfactants production (Gudiña et al., 2016; Joy et al., 2019; Ramírez et al., 2015). Different species of actinobacteria such as *Renibacterium salmoninarum* (Christova et al., 2004), *Cellulomonas cellulans* (Arino et al., 1998), *Nocardioides* sp. (Vasileva-Tonkova & Gesheva, 2005), and *Brachybacterium paraconglomeratum* MSA21 (Kiran et al., 2014) have been reported to produce various kind of biosurfactants . Production of biosurfactants has been reported to be more when the source of carbon is from oil (Mata-

Sandoval et al., 2001). The use of vegetable oil as source of carbon in this study could have contributed to the extremely high number of biosurfactants produced. The result observed in isolate NB 14 was in agreement with the study of Kiran et al for the production of glycolipid biosurfactants from sponge associated marine *Brachybacterium paraconglomeratum* MSA21 (Kiran et al., 2014). In their study, the strain produced diacylglycerol (DAG) with m/z 614.571 and 642.603 at RT of 8.0 minutes and 8.5 minutes respectively. DAG is a glyceride that consist of two fatty acids linked to a glycerol by an ester linkage (Wood & Woltjer, 2020). DAG has been investigated as a fat substitute because of its ability to suppress the accumulation of body fat (Lo et al., 2008; Phuah et al., 2015).

Both conventional and gradient PCR were tried for the amplification of any potential surfactin gene that might be present in our isolates. Specific primers for surfactin genes (sfp and srfAA) that have been designed from *Bacillus subtilis* gene fragment to amplify about 675 bases (sfp) and 202 bases (srfAA) from the CDSs region (Plaza et al., 2015) were used for the PCR amplification. These specific primers for amplifying the surfactin gene in *Bacillus* species were chosen and tried because of the evolutionary lineage relationship between *Bacillus* and Actinobacteria. No amplified PCR product was observed on gel electrophoresis in all the isolates with the primers' set of surfactin genes. Several reasons could be responsible for the unamplified PCR products from the isolates. The specific primers could be solely particular to the *Bacillus* genus and not the Actinobacteria genera from this study. To date, there are no specific primers in the literature for the amplification of any genes responsible to produce biosurfactants in

Actinobacteria. Also, there are no universal primers for amplifying the heterogenous biosurfactants in microorganisms, including Bacillus, the primary main of biosurfactants. This could be an exciting research topic or area for the design and optimisation of universal primers to amplify genes in the biosynthesis of the heterogenous biosurfactants in microbes. Primer choices are essential when screening for specific gene fragments by PCR reaction (Plaza et al., 2015). Since the biosurfactants produced by our isolates are heterogeneous, the PCR reaction and PCR condition might need different primers and optimization for each class of biosurfactants for the amplification to work. The biosurfactants might also need a mixture of different homologous for the amplification to succeed. Surfactin, for example, is synthesised as a mixture of different homologous forms. Pecci and colleagues reported a combination of three homologous (C13, C14 and C15) surfactin in *B. licheniformis* (Pecci et al., 2010). In the study of Bacon and colleagues, seven surfactin homologues (C11 to C17) were identified in the synthesis of surfactin (Bacon et al., 2012).

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Chapter five

5.0 Extracellular enzymes from Actinobacteria

5.1 Introduction

Enzymes are protein biomolecules that speed up the rate of a chemical reaction, and they have found wide applications in several industries (Markel et al., 2020). They are also organic biocatalysts produced by living organisms, and most industrial processes and reactions are simplified using enzymes (Kulkarni and Maurya, 2017). They have application in many industries such as biofuel, paper, animal feed, biomedicine, and food (Kulkarni and Maurya, 2017). Microbial extracellular enzymes are important biocatalysts with application in textile, bio-refineries, food, pulp and paper, agriculture, detergent, and pharmaceuticals industries (Janaki, 2017). Soil microbes also use extracellular enzymes to break down complex molecules into valuable and essential nutrients for easy absorption (Janaki, 2017). They play an essential role in the marine environment by recycling organic carbon and nitrogenous compounds (Vijayan et al., 2012). Microbial extracellular production is enhanced by the high yields, scalability, cost-efficiency, and susceptibility to genetic manipulation of the producing microbes (Vaijayanthi et al., 2016). The enzymes are used in food processing, detergent manufacturing, the textile and pharmaceutical industries, medical therapy, bioorganic chemistry as well as in research (molecular biology) (Ivanova et al., 2016). Enzymes produced by microorganisms are potential biocatalysts for many reactions, and their wide use reflects their distinct specificity of action as biocatalysts (Mukhtar et al., 2017). Enzymes derived from the microbial source are generally regarded as safe (GRAS). They function well at a wide range of

temperature, pH, salinity, or other extreme conditions (Mukhtar et al., 2017). Microbially sourced enzymes specifically have gained global attention in recent years and have replaced the chemical catalyst used in pharmaceuticals, textiles, paper, and food industries (Kumar et al., 2018). Nowadays, enterprises utilise microbes as sustainable and alternative sources of extracellular enzymes. Microbial enzymes are also more stable than their counterparts such as those from plant and animal and their production is more convenient and safer (Hasan et al., 2006). This has led to the massive exploration of new niches, such as marine habitat for their production (Kumar et al., 2020).

Actinobacteria are among the most diverse groups of bacteria that are well characterised and recognised for their metabolic versatility (Mukhtar et al., 2017) and for their rich source of enzymes, antibiotics, biosurfactants and other secondary metabolites (Vaijayanthi et al., 2016). It has been reported that actinobacteria possess the capacity to secrete and produce various extracellular enzymes (Janaki, 2017; Saadoun et al., 2007; Sathya & Ushadevi, 2014; Tan et al., 2009). Several genera of Actinobacteria produce a wide array of extracellular enzymes which have found application in biotechnological, medical, and pharmaceutical sectors (Nawani et al., 2013). With the advent of the genome and protein sequencing and bioinformatic, actinobacteria have been continuously screened to produce industrial enzymes (Vaijayanthi et al., 2016). Different commercial enzymes such as L-glutaminase, α galactosidase, amylase, cellulase, chitinase, xylanases, lipase, protease, and L-asparaginase, were obtained from the marine actinobacteria

(Lakshmanaperumalsamy, 1978; Lekshmi, Jayadev, and Navami, 2014; Vaijayanthi, Vijayakumar and Dhanasekaran, 2016).

Amylases (EC: 3.2.1.1) are starch degrading enzymes with a wide range of biotechnological applications in the food industry, fermentation, textile, and paper industries (Mantiri et al., 2019; Rengasamy & Thangaprakasam, 2018). They accounts for above 25% of demand in the global industrial enzyme market (Rajagopalan & Krishnan, 2008; Reddy et al., 2003; R. Singh et al., 2016). The ability of amylase to convert starch into simple sugars (saccharides) makes them as essential components in the feed industry to improve the digestibility of fibre (John, 2017). Amylases are three types, such as α-amylase which hydrolyses α-1,4 bonds and bypasses branched linkages, β-amylase which breaks down α-1,4 and cannot bypass α-1,6 branch linkages and produces maltose as a product, and γ-amylase that hydrolyses α-1,4 and α-1,6 linkages and breaks down the substrate from the non-reducing end, and in turn releasing monosaccharides as the product (Mantiri et al., 2019). Cellulases (endo-1, 4-β-glucanase, EC 3.2.1.4) are a group of hydrolytic enzymes that hydrolyse the glycosidic bonds of cellulose and related cello-disaccharide derivatives (Lekshmi et al., 2014). They are employed in colour extraction from juices, detergents causing colour brightening and softening, bio-stoning of jeans, pre-treatment of biomass that contains cellulose to improve the nutritional quality of forage, and pre-treatment of industrial wastes (Ito, 1997; Lekshmi et al., 2014). They are also compounds produced by microorganisms during their growth phase on cellulosic materials (Lee & Koo, 2001; Rajagopal & Kannan, 2017). Lipase (triacylglycerol acyl hydrolase, EC 3.1.1.3) is an enzyme that catalyse the

breakdown of triglycerides into fatty acids and glycerol (Pirahanchi, & Sharma, 2021). They have broad applications in the food, oleochemical, pharmaceutical and detergent industries as well as in medicines (diagnostic) (Lekshmi et al., 2014). They are also used in biotechnological industries to synthesize biopolymers and biodiesel (Jaeger & Eggert, 2002). They have gained special industrial attention due to their stability, selectivity, and broad substrate specificity (Griebeler et al., 2011). Lipase could be produced from a variety of bacteria, fungi and actinomycetes (Kulkarni & Gadre, 2002). Proteases are enzymes that break the peptide bond in protein molecules and they represent important enzymes produced commercially for industrial purposes (Lekshmi et al., 2014). Microbial protease represents about 60% of the world's market for industrial enzymes. They have commercial applications in toothpaste as antiplaque and anti-tartar, cosmetics, and in recovery of silver from used X-ray films (Lekshmi, Jayadev, and Navami, 2014; Ishikawa *et al.*, 1993). In addition to their potential industrial applications, they also regulate algal blooms in coastal waters and recycle organic matter (Lee et al., 2000). This chapter aims to screen our isolates for the presence of such robust enzymes and their ability to produce extracellular enzymes (amylase, cellulase, protease and lipase) for end use industrial applications.

5.2 Material and methods

Our isolates (both actinobacteria and non-actinobacteria) were screened for their potential to produce extracellular enzymes (amylase, cellulase, protease and lipase). Isolates NB 2, NB 14, NB 15, NB 16, NB 18, NB 19, NB 20, NB 21, and FOP 8 were the target of this screening exercise, but other non-actinobacteria were also screened in other to understand the biodiversity of extracellular enzymes production from the marine environment.

5.2.1 Screening and detection of amylase production

The ability of our isolates to produce amylase was analysed by streaking them on starch agar (Rengasamy & Thangaprakasam, 2018; Vijayan et al., 2012) consisting of 3 g of beef extract, 0.2% (20.0 g) of soluble starch, 12 g of agar and 1000 ml of RO water (Rengasamy & Thangaprakasam, 2018). Half strength of starch casein agar (5.0 g soluble starch, 0.15 g casein, 1.0 g potassium nitrate, 0.025 g magnesium sulphate heptahydrate, 1.0 g dipotassium hydrogen phosphate (K_2HPO_4), 1.0 g sodium chloride, 0.01 g calcium carbonate, iron II sulphate heptahydrate, 9.0 g agar and 1000 ml water) containing 0.5% of starch (soluble) according to Vijayan *et al.*, 2012 was also prepared to detect the ability to produce amylase. The isolates were streaked onto the agar plates and were incubated at 28°C for 7 days. Plates without isolates were used as negative control. At the end of incubation period, plates were flooded with Gram's iodine. The development of a bright and transparent zone around the colonies on Gram's iodine background indicated the production of amylase.

5.2.1.1 PCR amplification of the amylase gene

The amylase gene within the genomic DNA of the isolates that were positive in the screening assay of agar plate techniques were subjected to PCR for detection of the relevant gene. DNA samples of respective isolates were amplified using specific primers amy3-F (5'-ACGAACGGCGAGGGTGCAGC -3') and ldh2-R (5'-GCCGCTGCCGATGACGCG -3') (Mantiri et al., 2019). The PCR reaction mixture consisted of 50 µl containing a 10 µl of 5X PCRBIO Buffer, 1.5 µl of 15mM $MgCl_2$, 1 µl of 10 mM dNTP, 2 µl each of 20 mM of both primers, 1 µl of DNA template, 32.25 µl of nuclease-free water and 0.25 µl of Taq DNA polymerase. The PCR conditions followed with an initial denaturation at 94^{o}C for 5 minutes, 30 cycles at 94^{o}C for 45 seconds, primer annealing at 54^{o}C for 45 seconds, elongation at 72^{o}C for 1.5 minutes and final cycle of DNA extension at 72^{o}C for 5 minutes and then cooled to 4°C. PCR amplicons were run on a 1% agarose gel containing 5µl ethidium bromide. Gels were electrophoresed at 100V for 40 min in 1x TAE buffer. The gels were then viewed and scanned for DNA bands in a Gel Doc apparatus (Gel-Doc-it^{TM} 310 Imaging System), USA.

5.2.2 Protease detection

The ability of isolates to produce protease enzyme was detected by streaking them onto skim milk agar (SMA) [skimmed milk powder 28.0 g, tryptone 5.0 g, yeast extract 2.5 g, glucose 1.0 g, agar 15.0 g and water 1000 ml] according to the method of Jeyadharshan, 2013. The isolates were streaked onto the SMA at the centre of the agar plate. Plates without isolates were used as a negative control. The plates were incubated at 28°C for 7 days. At the end of the incubation period, the plates were

observed for the development of a zone of clearance around the colony for proteolytic activity (Menasria et al., 2018).

5.2.2.1 PCR amplification of the protease/peptidase gene

The protease gene within the genomic DNA of the isolates that gave a good positive result by the screening assay of the agar plate techniques was amplified using specific primers FP: 5'-TAYGGBTTCAAYTCCAAYAC-3' and RF:5'-VGCGATSGAMACRTTRCC-3'); for apr gene (Bach et al., 2001). The PCR reaction mixture that consisted of 50 µl had a 10 µl of 5X PCRBIO Buffer, 1.5 µl of 15mM $MgCl_2$, 1 µl of 10 mM dNTP, 2 µl each of 20 mM of both primers, 1 µl of DNA template, 32.25 µl of nuclease-free water and 0.25 µl of Taq DNA polymerase. The PCR program was set at with a hot start having an initial denaturation of 95^{o}C for 5 minutes. *Taq* polymerase was added after this step. The second step of 30 cycles consisted of denaturation at 94^{o}C for 30 seconds, primer annealing at 53^{o}C for 30 seconds, extension at 72^{o}C for 20 seconds and final extension at 72^{o}C for 10 minutes and then cooled to 4°C. PCR amplicons were run on a 1% agarose gel containing 5µl ethidium bromide. Gels were electrophoresed at 100 V for 40 minutes in 1x TAE buffer. The gels were then viewed and scanned for DNA bands in a Gel Doc apparatus (Gel-Doc-it^{TM} 310 Imaging System), USA.

5.2.2.2 Gradient PCR amplification of protease gene

A gradient PCR was performed to amplify the protease gene of the isolates using specific primers FP: 5'-TAYGGBTTCAAYTCCAAYAC-3' and RF:5'-VGCGATSGAMACRTTRCC-3'); for apr gene (Bach et al., 2001). The PCR reaction mixture of 50 µl had a 10 µl of 5X PCRBIO Buffer, 1.5 µl of 15mM $MgCl_2$, 1 µl of 10 mM dNTP, 2 µl each of 20 mM of both primers, 1 µl of

DNA template, 32.25 µl of nuclease-free water and 0.25 µl of Taq DNA polymerase. The PCR program consisted of a hot start with initial denaturation of 95°C for 5 minutes. *Taq* polymerase was added after this step. The second step of 30 cycles consisted of denaturation at 94°C for 30 seconds, a gradient temperature of annealing step of 45°C, 48°C, 53°C, 55°C, 57.5°C and 60°C for 30 seconds and an extension at 72°C for 20 seconds. A final extension step at 72°C for 10 minutes was carried. PCR amplicons were run on a 1% agarose gel containing 5µl ethidium bromide. Gels were electrophoresed at 100 V for 40 min in 1x TAE buffer. The gels were then viewed and scanned for DNA bands in a Gel Doc apparatus (Gel-Doc-it™ 310 Imaging System), USA.

5.2.3 Lipase detection

The ability of the isolates to produce lipase enzyme was detected by streaking the test isolates onto half strength of starch casein agar (5.0 g soluble starch, 0.15 g casein, 1.0 g potassium nitrate, 0.025 g magnesium sulphate heptahydrate, 1.0 g dipotassium hydrogen phosphate, 1.0 g sodium chloride, 0.01 g calcium carbonate, iron II sulphate heptahydrate, 9.0 g agar and 1000 ml water) containing 0.5% of tween-80 according to Vijayan *et al.*, 2012. The isolates were streaked onto the centre of the agar plate. Plates without isolates were used as a negative control. The plates were incubated at 28°C for seven days. At the end of the incubation period, the plates were observed for the ability to form an opaque zone around the isolates for positive results.

5.2.3.1 PCR amplification of lipase A gene and lipase gene

Since lipase enzymes are encoded by lipase A gene and lipase gene family, there was need to amplify the genomic DNA of our isolates that gave a

positive result by the phenotypic assay for possible presence of the lipase genes. Specific primers (FP: 5′-ATGGTTCACGGTATTGGAGG-3′ and RP: 5′-CTGCTGTAAATGGATGTGTA-3′) for lipase A gene (Mir Mohammad Sadeghi et al., 2010) and FP: 5'- CATATGATGAAAKGCTGYCGGGT-3' and RP: 5'-GGATCCTTAAGGCCGCAARCTCGCCA-3') for lipase gene (Sifour et al., 2010) were used for the PCR amplification reaction. The PCR reaction mixture consisted of 50 µl with 10 µl of 5X PCRBIO Buffer, 1.5 µl of 15mM $mgCl_2$), 1 µl of 10 mM dNTP, 2 µl each of 20 mM of both primers, 1 µl of DNA template, 32.25 µl of nuclease-free water and 0.25 µl of *Taq* DNA polymerase. The PCR conditions followed with an initial denaturation at 94^{o}C for 5 minutes, 30 cycles at 94^{o}C for 45 seconds, primer annealing at 58.3^{o}C for 30 seconds (lipase A gene) and 54^{o}C for 45 seconds (lipase gene), extension at 72^{o}C for 30 seconds minutes and final cycle of the DNA extension at 72^{o}C for 5 minutes and then cooled to 4°C. PCR amplicons were run on a 1% agarose gel containing 5µl ethidium bromide. Gels were electrophoresed at 100 V for 40 min in 1x TAE buffer. The gels were then viewed and scanned for DNA bands in a Gel Doc apparatus (Gel-Doc-it™ 310 Imaging System), USA.

5.2.3.2 Gradient PCR amplification of lipase gene

A gradient PCR was performed to amplify the lipase gene of our isolates which gave a good positive phenotypic result. Specific primers (FP: 5′-ATGGTTCACGGTATTGGAGG-3′ and RP: 5′-CTGCTGTAAATGGATGTGTA-3') for lipase A gene (Mir Mohammad Sadeghi et al., 2010) and FP: 5'-CATATGATGAAAKGCTGYCGGGT-3' and RP: 5'-GGATCCTTAAGGCCGCAARCTCGCCA-3') for lipase gene (Sifour et al., 2010) were used. The PCR reaction mixture consisted of 50 µl with 10 µl

of 5X PCRBIO Buffer, 1.5 µl of 15mM $MgCl_2$, 1 µl of 10 mM dNTP, 2 µl each of 20 mM of both primers, 1 µl of DNA template, 32.25 µl of nuclease-free water and 0.25 µl of *Taq* DNA polymerase. The PCR conditions followed with an initial denaturation at 94^{o}C for 5 minutes, 30 cycles at 94^{o}C for 45 seconds, a gradient temperature for the annealing step of 48^{o}C, 50^{o}C, 52^{o}C, 54^{o}C, 57^{o}C, 59.5^{o}C and 61^{o}C at 30 seconds for lipase A gene and 54^{o}C, 56.5^{o}C, 58.3^{o}C, 60^{o}C, 62.5^{o}C and 65^{o}C for lipase gene at 45 seconds and an extension at 72^{o}C for 30 seconds. A final extension step at 72^{o}C for 5 minutes was carried out. PCR amplicons were run on a 1% agarose gel containing 5µl ethidium bromide. Gels were electrophoresed at 100 V for 40 min in 1x TAE buffer. The gels were then viewed and scanned for DNA bands in a Gel Doc apparatus (Gel-Doc-it^{TM} 310 Imaging System), USA.

5.2.4 Cellulase detection

The ability of the isolates to produce cellulase enzyme was determined according to the study of Kasana et al., 2008. This was carried out by streaking the test isolates on carboxymethyl cellulase (CMC) agar containing peptone 0.2 g, potassium chloride 0.5 g, magnesium sulphate 0.5 g, dipotassium hydrogen phosphate 1.0 g, CMC 5.0 g, sodium nitrate 2.0 g, agar 17.0 g and water 1000 ml. The isolates were streaked onto CMC agar plates. Plates without isolates were used as a negative control. The plates were incubated at 28°C for 7 days. At the end of the incubation period, the plates were flooded with Gram's iodine. The formation of a clear zone around the isolates indicates a positive result for cellulase production.

5.2.4.1 PCR amplification of the cellulase gene

The cellulase gene of the isolates that gave a positive result for the screening of the cellulase gene by agar plate techniques were amplified

using specific primers CelF (5'-ATGAAACG GTCAATCTC-3') and CelR (5'-CTAATTTGGTTCTGTTC CC-3') (Thakkar & Saraf, 2014). The PCR reaction mixture of 50 µl had a 10 µl of 5X PCRBIO Buffer, 1.5 µl of 15mM $MgCl_2$, 1 µl of 10 mM dNTP, 2 µl each of 20 mM of both primers, 1 µl of DNA template, 32.25 µl of nuclease-free water and 0.25 µl of Taq DNA polymerase. The PCR conditions followed with an initial denaturation at 95°C for 2 minutes, 30 cycles at 94°C for 30 seconds, primer annealing at 58°C for 30 seconds, extension at 72°C for 1.5 minutes and final extension at 72°C for 10 minutes and then cooled to 4°C. PCR amplicons were run on a 1% agarose gel containing 5µl ethidium bromide. Gels were electrophoresed at 100 V for 40 min in 1x TAE buffer. The gels were then viewed and scanned for DNA bands in a Gel Doc apparatus (Gel-Doc-it™ 310 Imaging System), USA.

5.2.4.2 Gradient PCR amplification of cellulase gene

A gradient PCR was performed to amplify the possible presence of cellulase gene in our isolates which gave a good positive phenotypic result. Specific primers CelF (5'-ATGAAACG GTCAATCTC-3') and CelR (5'-CTAATTTGGTTCTGTTC CC-3') (Thakkar & Saraf, 2014) were used. The PCR reaction mixture of 50 µl had a 10 µl of 5X PCRBIO Buffer, 1.5 µl of 15mM $MgCl_2$), 1 µl of 10 mM dNTP, 2 µl each of 20 mM of both primers, 1 µl of DNA template, 32.25 µl of nuclease-free water and 0.25 µl of *Taq* DNA polymerase. The PCR conditions followed with an initial denaturation at 95°C for 2 minutes, 30 cycles at 94°C for 30 seconds, a gradient temperature for the annealing step of 54°C, 56°C, 58°C, 60.5°C, 62°C and 64°C at 30 and an extension at 72°C for 1.5 minutes. A final extension step at 72°C for 10 minutes was carried. PCR amplicons were run on a 1%

agarose gel containing 5µl ethidium bromide. Gels were electrophoresed at 100 V for 40 min in 1x TAE buffer. The gels were then viewed and scanned for DNA bands in a Gel Doc apparatus (Gel-Doc-it™ 310 Imaging System), USA.

5.2.4.3 Confirmation of presence of cellulase gene

The possible presence of cellulase gene in the genome of our sequenced (genomic) isolates (NB 2, NB 16, NB 18, NB 19, NB 20, NB 21, and FOP 8) was analysed by the web-based enzyme function initiative-enzyme similarity tool (EFI-EST) (Zallot et al., 2019). Earlier sequenced and annotated genomes in chapter three was used for the analysis. Different contigs of the annotated genome was searched for the presence of the enzyme. Protein sequence was extracted from the genome and NCBI blastP was carried out. The nucleotide sequence of the most hit from the blastP was used to construct a phylogenetic tree.

5.3 Results

5.3.1 Screening and detection of amylase production

The ability of our isolates (both actinobacteria and non-actinobacteria) to produce amylase were tested both by analysing the phenotype and genotype. Figure 5.1 shows the result of the phenotypic assay to produce amylase on both starch agar and starch casein agar. The assay was carried out in triplicate, and the result was inferred from a continuous positive (clearing zone around the isolate) of at least two from the triplicates. Inference from the phenotypic effect is shown in table 5.1. The result shows that 33.33% of the isolates (NB 2, NB 15, and NB 16) produces amylase, while 66.67% of the isolates (NB 14, NB 18, NB 19, NB 20, NB 21, and FOP 8) did not produce any amylase based on the phenotypic assay (figure 5.1 and table 5.1).

5.3.1.1 PCR amplification of the amylase gene

The amylase genes of the isolates (NB 2, NB 15, and NB 16) which gave a positive result of the phenotypic assay on starch agar for amylase production were amplified by PCR reaction. The result is shown in figure 5.2. The result indicates that the three isolates can produce amylase.

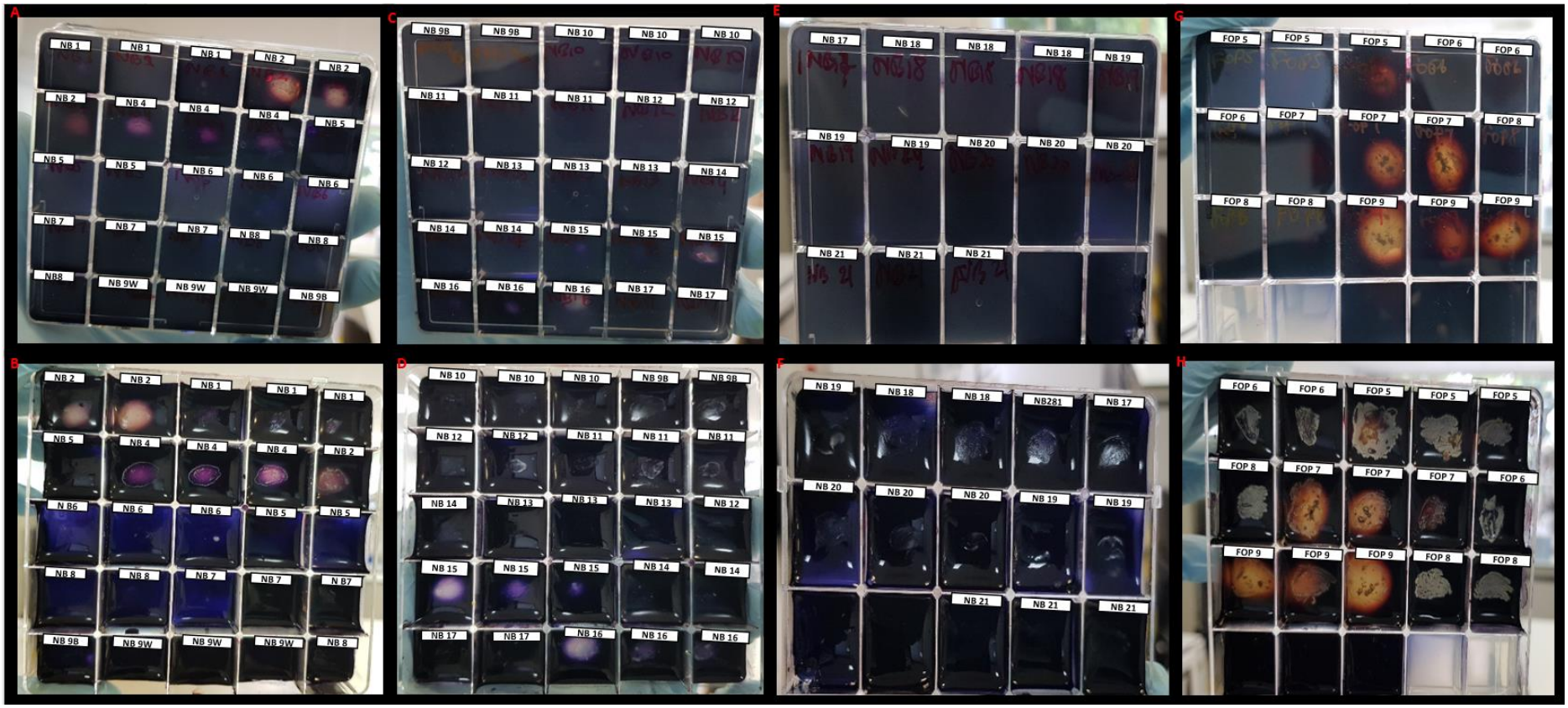

Figure 5. 1: Bioprospecting to produce amylase by isolates on starch agar on multi-well plates. Plates A, C, E and G are rear views of the multi-well plate while B, D, F and H are front views of the multi-well plate. Positive results were inferred with at least two positive results from the triplicate assays with a clearing zone around the isolates. Isolates NB 2, NB 14, NB 15, NB 16, NB 18, NB 19, NB 20, NB 21, and FOP 8 which are marine actinobacteria (chapter two) were the target of this assay.

Table 5. 1: Inference from the screening assay for amylase production by the isolates

Isolates	**Replicate 1**	**Replicate 2**	**Replicate 3**
NB2	+	+	+
NB14	-	-	-
NB15	+	+	+
NB16	+	+	+
NB18	-	-	-
NB19	-	-	-
NB20	-	-	-
NB21	-	-	-
FOP 8	-	-	-

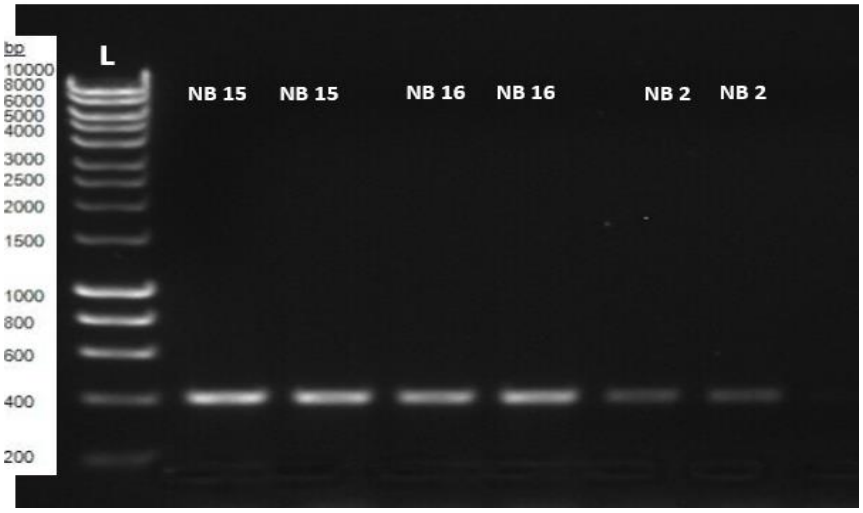

Figure 5. 2: A 1.5% gel electrophoresis of PCR amplification of amylase gene with an expected size of 405 bp

5.3.2 Screening and detection of protease production

The ability of our isolates was screened for their ability to produce protease. Figure 5.3 shows the result of the phenotypic assay for protease production on skimmed milk agar. The assay was carried out in triplicate, and the result was inferred from a continuous positive (clearing zone around the isolate) of at least two from the triplicates. Inference from the phenotypic effect is shown in table 5.2. The result shows that 88.89% of the isolates (NB 2, NB 14, NB 15, NB 16, NB 18 NB 19, NB 20, and FOP 8) produces protease, while only 11.11% of the isolates (NB 21) did not produce any protease based on the phenotypic assay (figure 5.3 and table 5.2).

5.3.2.1 PCR amplification of the protease gene

The possible presence of protease gene in our isolates (NB 2, NB 14, NB 15, NB 16, NB 18 NB 19, NB 20, and FOP 8) that gave a positive result as determined by the phenotypic assay were amplified by PCR reaction. The result indicates that there was no amplification of the protease gene in any of the isolates by both the convectional and gradient PCR reactions used.

Figure 5. 3: Bioprospecting of isolates to produce protease on skimmed milk agar in multi-well plates. Plates A, C and E are rear views of the multi-well plate while B, D and F are front views of the multi-well plate. Positive results were inferred from at least two positive results from the triplicate assays with a clearing zone around the isolates. Isolates NB 2, NB 14, NB 15, NB 16, NB 18, NB 19, NB 20, NB 21 and FOP 8 which are marine actinobacteria (chapter two) were the target of this assay.

Table 5. 2: Inference from the screening assay for protease production by the isolates

Isolates	**Replicate 1**	**Replicate 2**	**Replicate 3**
NB2	+	+	+
NB14	+	+	+
NB15	+	+	+
NB16	+	+	+
NB18	+	+	+
NB19	+	+	+
NB20	+	+	+
NB21	-	-	-
FOP 8	+	+	+

5.3.3 Screening and detection of lipase production

The potential of our isolates to produce lipase was screened. Figure 5.4 shows the result of the phenotypic assay for lipase production on half strength starch casein agar. The assay was carried out in triplicate, and the result was inferred from a continuous positive (opaque zone around the isolate) of at least two from the triplicates. Inference from the phenotypic effect is shown in table 5.3. The result shows that 11% of the isolates (NB 2) produces lipase while 89% of the isolates (NB 14, NB 15, NB 16, NB 18 NB 19, NB 20, NB 21, and FOP 8) did not produce any lipase based on the phenotypic assay (figure 5.4 and table 5.3).

5.3.3.1 PCR amplification of lipase A gene and lipase gene

The possible presence of lipase A gene and lipase gene in the isolate (NB 2) that gave a positive result by the phenotypic assay was amplified by PCR reaction. The result indicates that there was no amplification of either of the gene (lipase A gene and lipase gene) in the isolate by both conventional and gradient PCR reactions used.

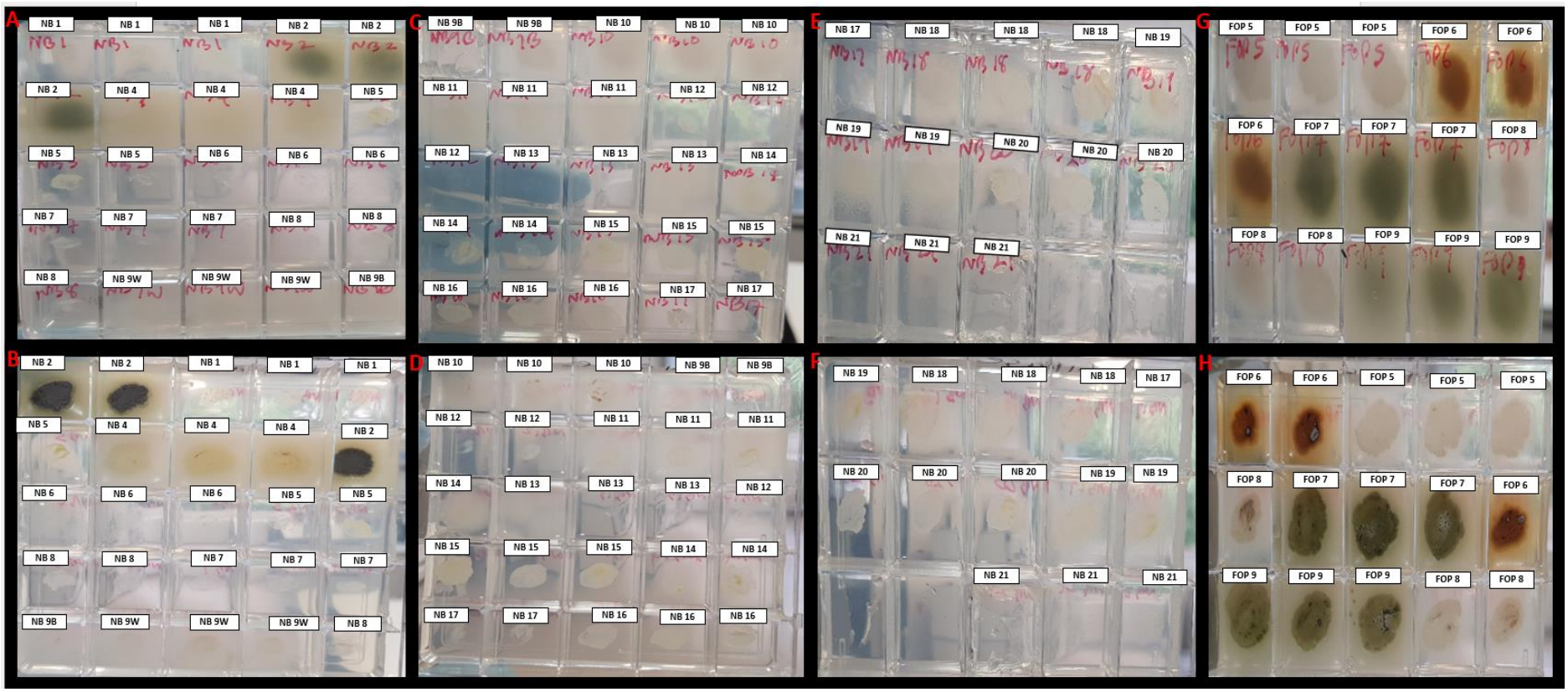

Figure 5. 4: Screening for lipase production by the isolates on half strength of starch casein agar with multi-well plates. Plates A, C, E and G are rear views of the multi-well plate, while B, D, F and H are front views of the multi-well plate. Positive results were inferred with at least two positive results from the triplicate assays by the ability to form an opaque zone around the isolates. Isolates NB 2, NB 14, NB 15, NB 16, NB 18, NB 19, NB 20, NB 21 and FOP 8 which are marine actinobacteria (chapter two) were the target of this assay.

Table 5. 3: Inference from the screening assay for lipase production by the isolates

Isolates	Replicate 1	Replicate 2	Replicate 3
NB 2	+	+	+
NB 14	-	-	-
NB 15	-	-	-
NB 16	-	-	-
NB 18	-	-	-
NB 19	-	-	-
NB 20	-	-	-
NB 21	-	-	-
FOP 8	-	-	-

5.3.4 Screening and detection of cellulase production

The potential of our isolates was screened for their ability to produce amylase. Figure 5.7 shows the result of the phenotypic assay for cellulase production on carboxymethyl cellulase (CMC) agar. The assay was carried out in triplicate, and the result was inferred from a continuous positive (clearing zone around the isolate) of at least two from the triplicates. Inference from the phenotypic result is shown in table 5.4. The result shows that 89% of the isolates (NB 2, NB 14, NB 15, NB 16, NB 18 NB 19, NB 20, and FOP 8) produced cellulase, while only 11% of the isolates (NB 21) did not produce any cellulase based on the phenotypic assay (figure 5.5 and table 5.4).

5.3.4.1 PCR amplification of the cellulase gene

The possible presence of cellulase genes in the isolates (NB 2, NB 14, NB 15, NB 16, NB 18 NB 19, NB 20, and FOP 8) which gave a positive result by the phenotypic assay were subjected to amplification by PCR reaction. The result indicates that there was no amplification of the cellulase gene in any of the isolates by both conventional and gradient PCR used.

5.3.4.2 Confirmation of presence of cellulase gene

The result of our phenotypic assay shows that majority of the isolates could produce cellulase gene. There was a need to confirm the presence of this gene in the genome of the isolates. The result of the analysis shows that some of the isolates have cellulase and cellulose-related genes in specific contig of the annotated genome. A blastp analysis shows that *Streptomyces* was the genus with the most hits for the enzyme. Figure 5.7 shows the phylogenetic tree for the evolutionary relationship of cellulase

gene with the nucleotide sequence obtained from the protein sequence of our isolates. The annotated genome of the isolates was searched for the presence of cellulase gene. The genome of the isolates NB 2, FOP 8 and NB 18 had good hits for different cellulase gene and other cellulose-related gene in different contigs as shown in Table 7.2 (appendix 7.6). There was no single hit for any cellulase enzyme in isolates NB 16, NB 19, NB 20, and NB 21.

Figure 5. 5: Bioprospecting to produce cellulase by isolates on carboxymethyl cellulase (CMC) agar in multi-well plates. Plates A, C and E are rear views of the multi-well plate, while B, D and F are front views. Positive results were inferred with at least two positive results from the triplicate assays by forming a clearing zone around the isolates. Isolates NB 2, NB 14, NB 15, NB 16, NB 18, NB 19, NB 20, NB 21 and FOP 8 which are marine actinobacteria (chapter two) were the target of this assay.

Table 5. 4: Inference from the screening assay for cellulase production by the isolates

Isolates	Replicate 1	Replicate 2	Replicate 3
NB2	+	+	+
NB14	+	+	+
NB15	+	+	+
NB16	+	+	+
NB18	+	+	+
NB19	+	+	+
NB20	+	+	-
NB21	-	-	-
FOP 8	+	+	+

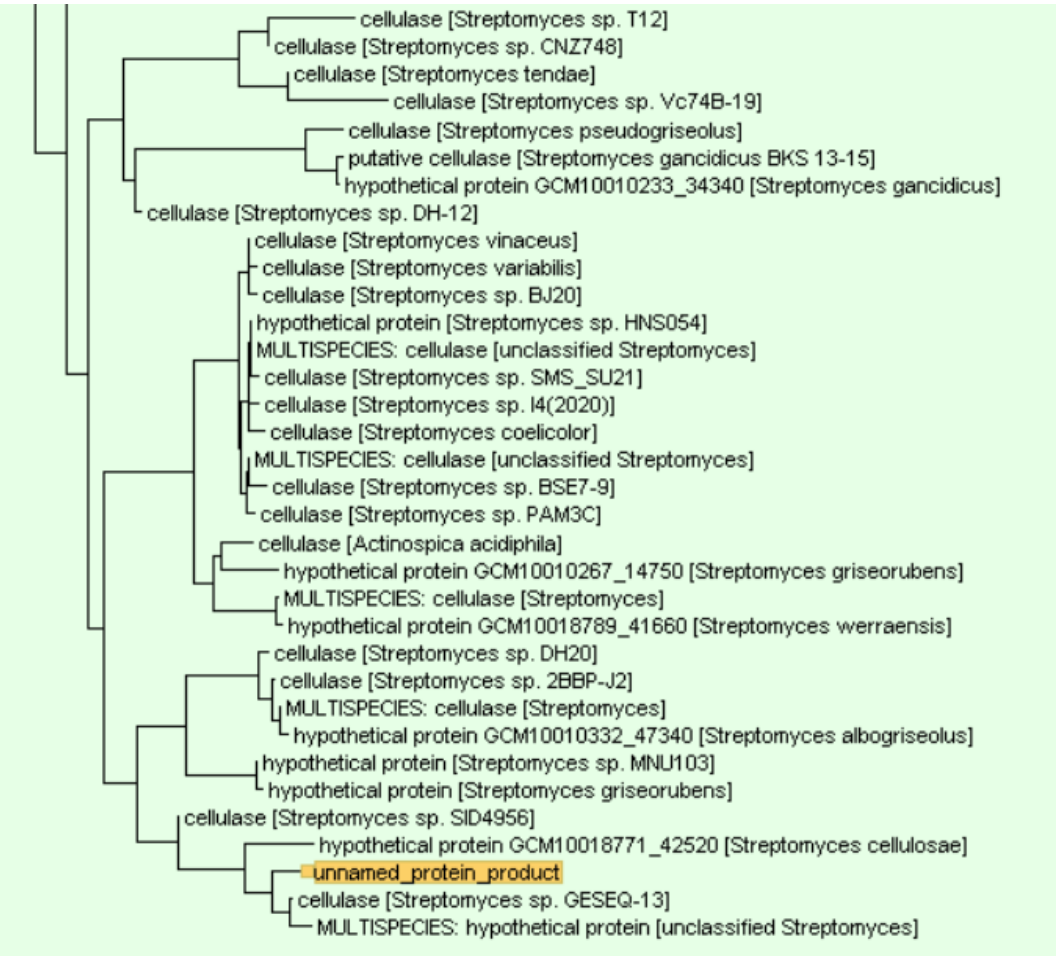

Figure 5.6 phylogenetic tree of cellulase gene. Nucleotide sequence extracted from genomic sequencing data was used for NCBI blast. Tree was prepared from top 100 hits in NCBI blastp using Blast tree view with neighbour-joining method.

5.4 Discussion

Actinobacteria are known as rich source of enzymes and other secondary metabolites (Vaijayanthi et al., 2016). This chapter was aimed at screening our isolates for their metabolic potential to produce various extracellular enzymes such as amylase, protease, cellulase and lipase. The majority of the actinobacterial isolates produce protease, cellulase, followed by amylase and lipase. This agreed with the study of Kumar and colleagues, where they screen for extracellular enzymes production from actinomycetes isolated from earthworm castings (Kumar et al., 2012). The Actinobacterial isolates used in this study produced at least one extracellular enzyme under the screening conditions employed, showing an enormous enzymatic diversity and biotechnological potential.

Phenotypic assay was carried out in triplicate and positive results were recorded from plates that had consistent two positive results. This was done to avoid false-positive results. For the amylase enzyme screening assay, Gram's iodine was used as an indicator because iodine reacts with starch to form a dark bluish colour and starch hydrolysis in the presence of amylase will create a clear zone around the isolates. Our result agreed with the findings of Rengasamy and colleague on the isolation, screening, and determination of alpha-amylase activity from marine *Streptomyces* species (Rengasamy and Thangaprakasam, 2018) Gopinath and colleagues also used agar plates to detect amylase production and determined its activity in *Penicillium* sp and *Aspergillus versicolor* (Gopinath et al., 2017). Though phenotypic assay for screening for the microbial production of amylase in Actinobacteria and other bacteria is simple and easy to achieve, the result is not exceptionally reliable as there could be false positives and

biases in the media composition and preparation. To avoid this and have an exceptionally reliable screening assay for the ability of our isolates to produce amylase, we carried out PCR amplification of the amylase gene. We used the PCR conditions and reaction mixture (primers and buffers) as previously reported (Mantiri et al., 2019). Our result (figure 5.2) revealed that the isolates (positive for amylase) could have an amylase gene. The result further confirms our phenotypic assay for the screening of amylase enzyme. It will be interesting to carry out a systematic molecular study to characterize and group the amylase into the different kind of amylase (alpha, beta and gamma amylase) while also optimising the phenotypic culture. media.

All our isolates except NB 21 produced cellulase according to the phenotypic assay carried out on agar multi-well plates. Cellulase is an inducible enzyme, and its nature is affected by the kind of substrate used in its production (Huang and Monk, 2004). The cellulose degradation zone was high in all our isolates, and this could be because cellulase is easily assimilated by microbes during its production process (Huang & Monk, 2004; Sadhu et al., 2013). Our result was in agreement with previous studies on screening of isolates for cellulases from marine sediment where over 50% of isolates shows zones of hydrolysis on CMC (Gobalakrishnan et al., 2016; Rajagopal & Kannan, 2017; Veiga et al., 1983). Our result revealed that majority of our isolates could produce both protease and cellulase enzymes. Cellulase producing marine actinobacteria have been isolated from marine sediments in La Corufia Bay, Spain (Veiga et al., 1983), Vellar estuary, Triuchendhur coastal area of Tamil Nadu, Bay of Bengal, and Andaman and Nicobar island in India (Meena et al., 2013;

Murugan et al., 2007; Sirisha et al., 2013; Stalin et al., 2012). Since phenotypic assay by agar method for bioprospecting for cellulase enzymes is not very reliable, and no cellulase gene was amplified by the PCR techniques employed, there was need to search for the presence of this gene in the annotated genome of the genomic sequenced data of the isolates. The result revealed that isolates NB 2, FOP 8 and NB 18 have cellulase and cellulose-related gene in different contigs of the annotated genome (Table 7.2). This result further re-confirmed the result of the phenotypic assay for isolates NB 2, FOP 8, and NB 18 which were positive for cellulase gene and NB 21 which was negative for cellulase gene. However, the result was different when compared with the result of the phenotypic assay in isolates NB 16, NB 19, and NB 20 with the genome sequence analysis. It will be interesting to also carry out genome sequencing, annotation and analysis for isolates NB 14 and NB 15 to arrive at a valid conclusion in determining if their genome contains the enzyme.

For the bioprospecting of protease by the isolates, the result of our phenotypic assay on skimmed milk revealed that all the isolates except NB 21 produced protease. Clearing zones around the isolates indicated that the isolates secrete proteolytic enzymes into the media by degrading the casein in the skimmed milk. It was observed that the longer the incubation time (up to 10 days) the clearer and wider the clearing zones were around the isolates. Our finding agreed with previous report of screening for protease enzyme production from actinobacteria isolated from marine environment (González et al., 2020; Ramesh & Mathivanan, 2009; Suthindhiran et al., 2014b; Vonothini et al., 2008).

Among the isolates, only NB 2 was able to produce lipases according to our phenotypic assay. This was in agreement with previous report where lipolytic activities derived from marine microbes was low (Lan et al., 2016; D. Yuan et al., 2014). Since the phenotypic assay for screening the ability of our isolates to produce lipase cannot distinguish between the kind of lipase the isolates could produce, specific primers for lipase A gene and lipase gene were used for the PCR amplification reaction. Lipase A (*LIPA*) gene for example is an enzyme that code for lysosomal acid lipase and it catalyzes the hydrolysis of cholesteryl esters and triglycerides (Zeljko et al., 2014) while lipase gene on the other hand comprises of three mammalian lipases (pancreatic lipase (LP), lipoprotein lipase and hepatic lipase) based on their amino acid configuration (Wong and Schotz, 2002). The production of lipase by marine microbes including actinobacteria is large because the ocean contains significant amounts of polymers which are degraded by lipase producing microbes (Ramesh & Mathivanan, 2009).

Due to the biases associated with phenotypic method of screening for extracellular production, both conventional and gradient PCR were tried for the amplification of the presence of cellulase gene, protease gene and lipase gene in the genomic DNA of our isolates which gave a good positive phenotypic result. Our result revealed that none of the genes were amplified by the PCR techniques used. The most probably reason for this result could be that the primers pair used for the amplification were not specific to our isolates as the primers were universal in nature which have been used to successfully amplify these genes in Bacillus species. It will be interesting to confirm the presence of the genes that code for the various

enzymes in this study by searching the genome (sequenced) and annotated data of the isolates to arrive at a good conclusion.

5.5 References

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Chapter six

6.0 General discussion, conclusion, and future work

6.1 General discussion

Actinobacteria are an exceptional group of bacteria with vast metabolic potential. Over the years, special attention has been paid to them by researchers and pharmaceutical companies as members of this group, especially the *Streptomyces* are known to harbour many BGCs for different secondary metabolites. They serve as an important source for the discovery of secondary metabolites that have found applications in different sectors (Ranjani Anandan & Manogaran, 2016; Zotchev, 2012; Hazarika & Thakur, 2020). They also account for more than half of the currently known bioactive compounds (Bérdy, 2005). Exploration of the same environment for their isolation has resulted in the isolation of the same species and re-discovery of same metabolites and compounds. This research study was aimed at exploring the novel diversity and metabolic potential of isolates obtained from the marine environment in the UK.

We used culture-dependent approach to isolate marine actinobacteria with a focus on rare actinobacteria. The marine environment represents one of the underexplored environments for the isolation of actinobacteria (Ma et al., 2017; Lilja, 2013). It was important to explore this habitat because the biodiversity of the marine environment is quite different from terrestrial, where much has been reported (Donia & Hamann, 2003). It is hypothesized that the marine environment did not receive much attention in the past for the isolation of actinobacteria and exploration of their metabolites because of the false impression that the marine environment contains very few microbes and that its condition of high salinity,

unfavourable temperature and pH does not support the growth and proliferation of many microorganisms (Flora et al., 2015). In this study, different selective media prepared in both RO water and ASW were tried and used for the isolation of marine actinobacteria. Nine isolates comprising of six genera *Brachybacterium, Micrococcus*, *Micromonospora*, *Salinibacterium*, *Kocuria* and *Streptomyces* were isolated from the marine environment. The presence of these genera supports the biodiversity of actinobacteria in the marine environment. Though the isolation of rare actinobacteria was the focus of this study, the isolation of *Streptomyces* was also included because it helps us in understanding the other isolates (rare actinobacteria) better since *Streptomyces* account for more than 50% of isolated actinobacteria. Our utmost focus in the selective isolation procedure was to obtain pure culture for other downstream processing. Obtaining pure culture isolates which is only possible by culture-dependent based approaches, was embarked on in this study because it enables us to know the different diversity of actinobacteria in the sampled environment in real time. Culture-independent (metagenomic) approach was not embarked on because this technique will not provide us real time pure culture for the actual practical application of the isolates even though it will give the total diversity of actinobacteria in the sample. The analysis of the 16S rRNA gene of our isolates revealed that they are indeed actinobacteria. This was further reconfirmed by the genome sequencing analysis of the isolates.

We carried genomic sequencing by using Illumina technology of selected genera of the isolates. It was important to do genomic sequencing because it has been reported that conventional cultural methods for the exploration

of secondary metabolites often result in the re-isolation of already isolated actinobacteria and re-discovery of already discovered metabolites (Choi et al., 2015). Often, novel metabolites are present in cryptic clusters and accessing the BGCs of microbes by traditional culturing methods is quite difficult (Choi et al., 2015). The draft genome annotation features of the isolates agreed with previous draft genomic sequencing results (Braun et al., 2018; Goh et al., 2021; Loong et al., 2017; Shin et al., 2012; Souak et al., 2020; Wang et al., 2014). The ability of the isolates to produce secondary metabolites was predicted by antiSMASH by searching different aligned contigs read within the annotated genome for potential BGCs. The results revealed that our isolates had different BGCs in their genome for potential metabolites. For example terpenes BGCs were found in the genomes of all the isolates. The presence of terpenes in these isolates could imply that the isolates could be important source for the producing of terpenes. Terpenes generally are constitutes of essential oils and precursors for the biosynthesis of polymers (Omar et al., 2016; Silvestre & Gandini, 2008). The BGCs NRPS and PKS and their derivatives for the biosynthesis of bioactive compounds was predicted in the genome of two isolates (NB 2 and FOP 8). This was highly expected as Streptomyces accounts for over 70 % of known bioactive compounds (Berdy, 2005). The genomic analysis has also revealed the different diversity of BGCs for the biosynthesis of different potential metabolites. The result suggests that the isolates could be potential reservoirs for the exploration of many diverse secondary metabolites.

It is good to discover and isolate novel actinobacterial species from different environmental niches including the marine environment which is

currently underexplored for actinobacteria. However, the exploitation or screening of these isolates in laboratory conditions for the metabolites they can produce with direct applications in different sectors is more important. Our isolates were therefore screened phenotypically by agar assay for their ability to biosynthesise biosurfactants and extracellular enzymes. The potential of the isolates to produce biosurfactants was investigated by both culture-based and analytical methods. The phenotypic assay on blood agar and TLC analysis of biosurfactants extract indicated that some of the isolates were producing biosurfactants. HPLC-MS analysis which is one of the most precision analytical methods also reconfirmed the result of the phenotypic assay and TLC analysis. The HPLC-MS result revealed that the isolates produced glycolipids (rhamnolipids, sophorolipids and diacylglycerol) biosurfactants. The majority of glycolipids produced by the isolates were mainly mono-rhamnolipids though small number of di-rhamnolipids were also observed in isolate NB 19 and NB 20. Our findings was in agreement with previous study on the bioproduction of rhamnolipids using olive oil as sole carbon source (Ramírez et al., 2015). The high number of rhamnolipids produced by our isolates would be attributed to the use of vegetable oil as carbon source in our MSM media. The production of DAG by NB 14 (*Brachybacterium*) was in agreement with previous study of Kiran and colleagues (Kiran et al., 2014). DAG has useful application in the food industry as it has been found to be a good substitute for body fat. The result suggests that these isolates could be explored and use as sources for the production of biosurfactants. The production and application of biosurfactants have been extensively reviewed (El-Sheshtawy et al., 2016; Fenibo et al., 2019).

The result of the screening assay by agar-based techniques for the potential of the isolates to produce extracellular enzymes (amylase, cellulase, protease and lipase) revealed that the isolates could produce the enzymes at varying degrees. Extracellular enzymes were screened because previous studies have reported that actinobacteria possess the capacity to produce several types of extracellular enzymes (Janaki, 2017; Saadoun et al., 2007; Sathya & Ushadevi, 2014; Tan et al., 2009). In recent years, extracellular enzymes have gained attention as they could replace their synthetic counterpart used in pharmaceuticals, textiles, paper, and food industries (Adrio & Demain, 2014). Though the phenotypic agar-based assay for bioprospecting for the potential of our isolates to produce extracellular enzymes shows positive results, more studies such as molecular studies involving the designing of specific primers and PCR amplification of the genes as well as genomic sequencing analysis for the presence of the gene sequences for these enzymes in the genome of the isolates should be carried out. Due to biases in the choices and preparation of screening media, the result for the phenotypic assay in bioprospecting for enzymes production by the isolates is not so reliable. Though the amplification of the gene by PCR reaction did not detect any cellulase gene, the annotated genomic sequenced data was analysed for possible presence of cellulase gene in the genome of the isolates. The result re-confirmed the result of the phenotypic assay which have earlier revealed that most of the isolates could produce cellulase. The analysis of the annotated genomic sequences of the isolates for cellulase gene further validate and support our earlier hypothesis that the absence of amplified cellulase gene by the PCR techniques could be due to the fact that the primers used for the

amplification were not specific to the different actinobacteria genera in our isolates. It will also be interesting to also carry out genomic sequencing analysis for the presence of other enzymes (amylase, protease, and lipase) in the genome of the isolates.

6.2 Conclusion

This research study has revealed that there is different diversity of actinobacteria in the marine environment (Liverpool and Newcastle Sea) sampled based on the selective isolation result using a culture-dependent approach. Nine species of Actinobacteria were isolated in five genera which include Brachybacterium*, Micromonospora, Micrococcus, Salinibacterium, Kocuria and Streptomyces*. Our result revealed that some of these isolates could produce glycolipids biosurfactants (rhamnolipids sophorolipids and DAG) and several extracellular enzymes (amylase, protease, cellulase and lipase). The isolates could also serve as a potential reservoir for the screening and isolation of secondary metabolites such as NRP, polyketide, terpene, and antibiotics because the analysis of our genomic sequencing data revealed that they possess several BGCs. The Liverpool and Newcastle Seas could be a rich environment for the isolation of marine actinobacteria and bioprospecting of secondary metabolites.

6.3 Future work

Our isolates from the marine environment in both Liverpool and Newcastle Seas in the United Kingdom have given insight into some of their metabolic potential (production of biosurfactants and extracellular industrial enzymes) as well as the numerous BGCs for secondary metabolites in their genome (genomic sequencing). There is therefore a need to carry out more

laboratory studies on these isolates for other secondary metabolites that they can produce. Area for possible further research includes.

1. Screening for bioactive compounds or antimicrobial activities in our isolates. Actinobacteria especially *Streptomyces* are known for their bioactivity against antagonistic (pathogens) as they have been the major sources of bioactive compounds such as antibacterial, anti-viral, anti-helminths, anti-parasites, antifungal etc. These compounds could be analysed in the laboratory either by mono or co-cultivation techniques. The co-cultivation could either be with another actinobacterium (actinobacteria with actinobacteria co-cultivation technique) or co-cultivation with another organism (actinobacteria with non-actinobacteria co-cultivation technique).
2. Detection of biosynthetic gene sequences (PKS and NRPS). The isolates could be tested for their antagonistic ability by the amplification of the biosynthetic genes cluster for the KS domains of Polyketide synthase (PKS) and the adenylation domains of non-ribosomal peptide synthetase (NRPS) by PCR reaction (Zothanpuia *et al*., 2016).
3. Metagenomic studies. It would be interesting in future studies to carry out culture-independent approaches in the isolation of marine actinobacteria to understand the general diversity of actinobacteria in the marine environment by isolating the community DNA and carrying out metagenomic analysis on them. This is pertinent since most microbes are not culturable and knowing the different diversity of actinobacteria in the marine environment could give better

explanation of their potential BGCs diversity and the kind of natural products that they could produce.

4. Molecular studies and primer design. Actinobacteria are extremely broad and highly diverse and so it might it difficult to use a particular primer pair for the amplification of genes of interest. PCR amplification with specific primers for the amplification of protease, lipase and cellulase could be designed specifically for each genus. It will also be interesting to carry out the analysis of the annotated genomic sequenced data for the presence of amylase, protease, and lipase genes in the genome of the isolates.
5. Structural characterization of the biosurfactants produced. It will be interesting to structurally characterize the biosurfactants produced by the isolates by using either tandem MS or MS/MS or nuclear magnetic resonance (NMR)
6. Optimization of cultural conditions for enhanced biosurfactant production. The effect of diverse cultural conditions such as incubation time, pH, temperature, nitrogen source, inoculum concentration, and carbon source on the growth of the isolates, and the ability of the strain to produce biosurfactant could be determined to fully ascertain the optimal conditions to produce biosurfactants from the isolates. Other studies that could be carryout on the biosurfactant includes

(i) Antimicrobial assay. The antimicrobial activity of produced and characterized biosurfactants can be carryout against common pathogens such as *Vibrio alginolyticus, Escherichia coli, Pseudomonas aeruginosa,* and *Staphylococcus aureus.*

This assay can be carryout in agar well plates and zones of inhibition around the test pathogens could confirm the antimicrobial activities of the biosurfactants.

(ii) Bioremediation of oil spill or polluted environment. The isolates will be screened for their ability to bioremediate oil spills or polluted environments. This could be done by taking contaminated samples from the polluted environment and screening.

(iii) Genomic sequencing analysis of the BGCs for presence of biosurfactants in the genome of the isolates

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7.0 Appendices

Appendix 7.1: Phylogenetic tree of non-actinobacteria

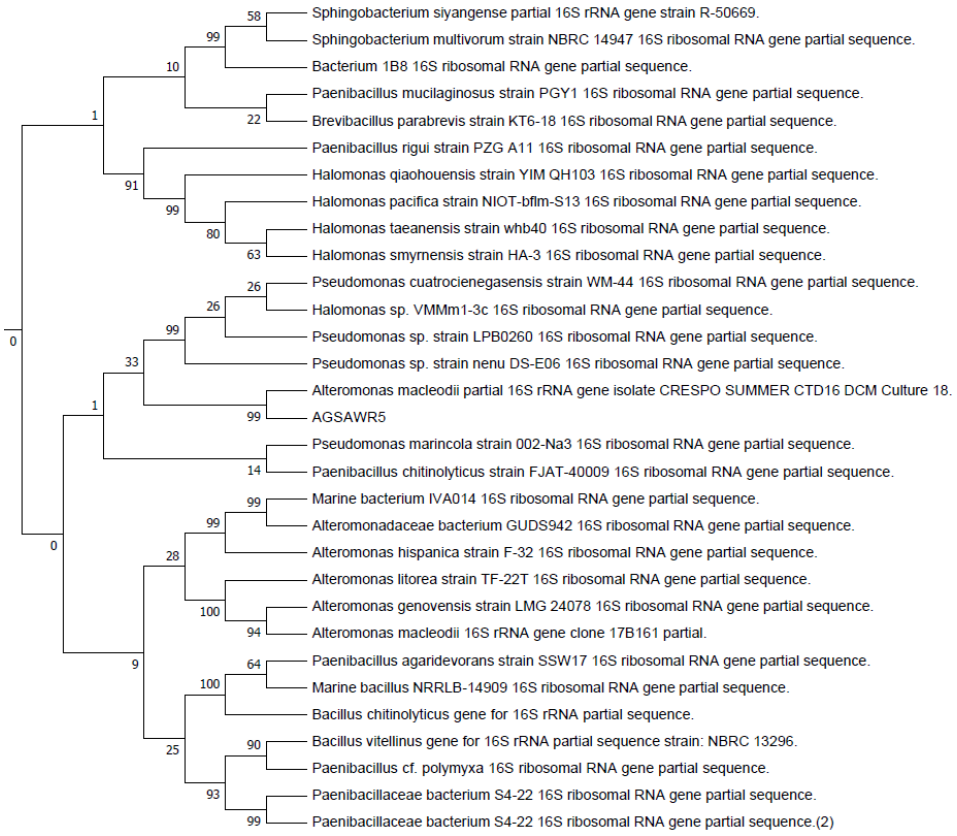

Figure 7. 1a: Phylogenetic tree showing evolutionary relationship between non-actinobacteria isolates and closely related published type strains as inferred using the Neighbour-Joining method (Saitou & Nei, 1987).

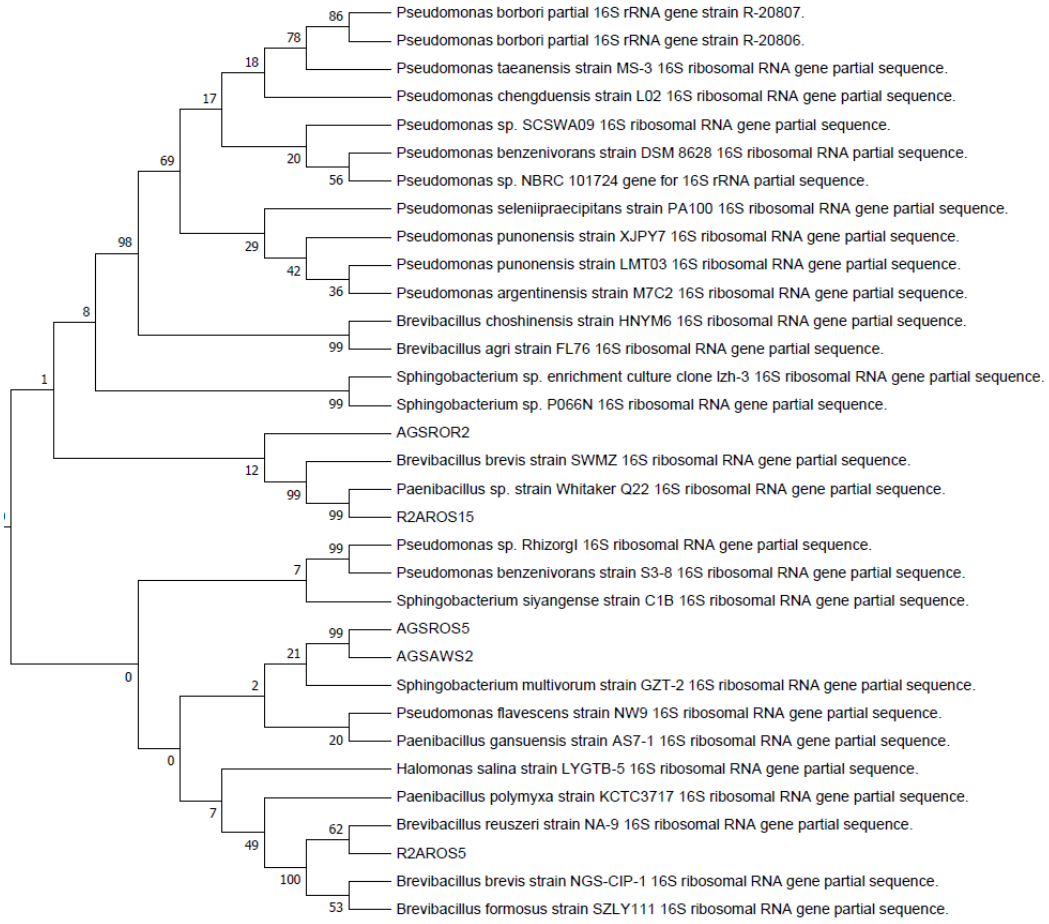

Figure 7. 1b Phylogenetic tree showing evolutionary relationship between non-actinobacteria isolates and closely related published type strains as inferred using the Neighbour-Joining method (Saitou & Nei, 1987).

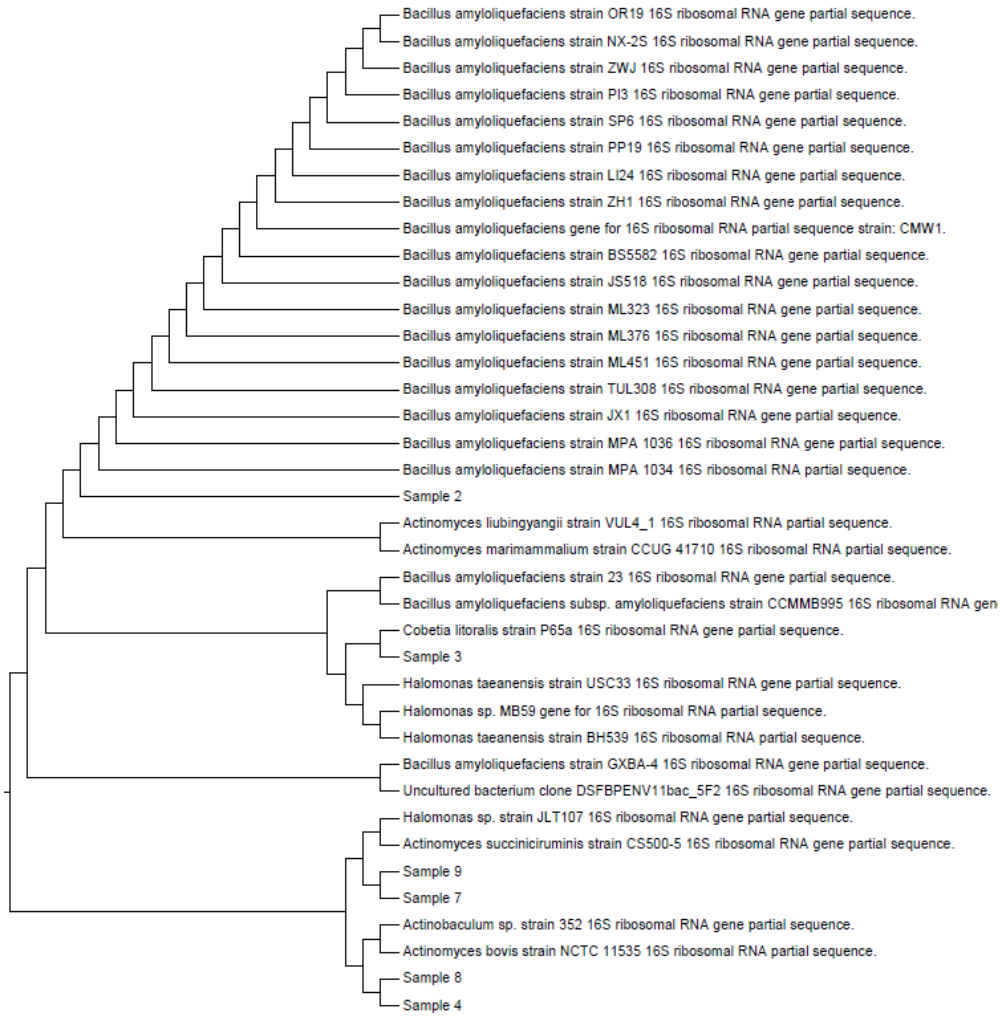

Figure 7. 1c : Phylogenetic tree showing evolutionary relationship between non-actinobacteria isolates and closely related published type strains as inferred using the Neighbour-Joining method (Saitou & Nei, 1987).

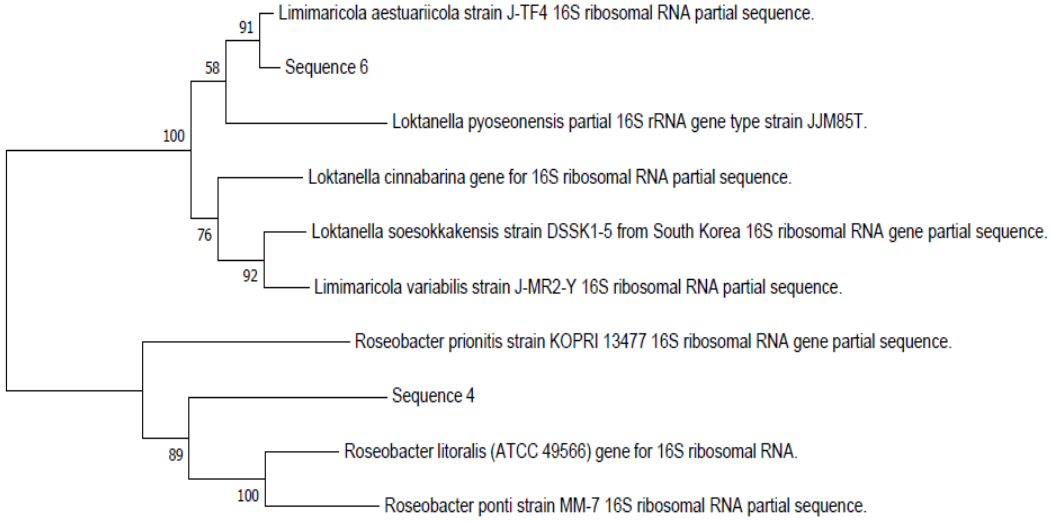

Figure 7. 1d: Phylogenetic tree showing evolutionary relationship between non-actinobacteria isolates and closely related published type strains as inferred using the Neighbour-Joining method (Saitou & Nei, 1987).

Appendix 7.2: 16S rRNA sequences of our isolates

NB 2: *Streptomyces* (ON023824)

TGCAGTCGCGATGAACCCTTCGTGGATTAGTGGCGAACGGGTGAGTAACACGA
AGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACCG
GATAACACTCTGTCCCGCATGGGACGGGGTTAAAAGCTCCGGCCCGGGGGGT
AAAATCCCGGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCT
ACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCAC
AATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACCGGGCCTTC
GGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGA
AGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAA
GCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCG
GATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCTAGCTA
GAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGAT
ATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCT
GAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACG
CCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCA
GCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGAC
GCAACGCGAAGAACCTTACCAAGGCTTGACATATACCGGAAAGCATCAGAGAT
GGTGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGT
GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTT
GCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACTGCCGGGGTCAA
CTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGC
TGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATGCCGCGAGGCGGA
GCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCC
GAAGTCGGAATTGCTAGTAATCGAGAACAACATTGCTGGGAAACCCGGCCCGC
GCCAAATAACGGGGCCCCCCTTGGCAAGGGGGGGAGAAAAAAGAAGAAAA

NB 19: *Kocuria* (ON023825)

GCAGTCGACGCTGGCTTGTGATAGCTTGCACTGGGTGGATGAGGTGGAGTGG
CGAACGGAATACGTGAGTAACCTGCCCTTGACTCTGGGATAAGCCTGGGAAAC
TGGGTCTAATACTGGATACGACATGTCACCGCATGGTGGTGTGTGGAAAGGGT
TTTACTGGTTTTGGATGGGCTCACGGCCTATCAGCTTGTTGGTGGGGTAATGG
CTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGC
ACAATGGGCGGAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTC
GGGTTGTAAACCTCTTTCAGCACGGAAGAAGCGAAAGTGACGGTACGTGCAGA
AGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAA
GCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCT
GCTGTGAAAGCCCGGGGCTTAACCCCGGGTGTGCAGTGGGTACGGGCAGACT
TGAGTGCAGTAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGA
TATCAGGAGGAACACCGATGGCGAAGGCAGGTCTCTGGGCTGTTACTGACGCT
GAGGAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATG
CCGTAAACGTTGGGCACTAGGTGTGGGGAACATTCCACGTTTTCCGCGCCGTA
GCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCA
AAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCGGATTAATTCGAT
GCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGACCGGGCCAGAGAT
GGTCTTTCCCCCTTGTGGGGCTGGTGTACAGGTGGTGCATGGTTGTCGTCAGC
TCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCTA
TGTTGCCAGCACGTGATGGTGGGGACTCATAGGAGACTGCCGGGGTCAACTC
GGAGGAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTC
ACGCATGCTACAATGGCCAGTACAATGGGTTGCGATGCCGCGAGGTGGAGCT
AATCCCAAAAAGCTGGTCTCAGTTCGGATCGTGGTCTGCAACTCGACCACGTG
AAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCC
CGGGCCTTGTACACCGTCAAGTCACGAAAGTTGGTCACCCGATCGTGGCCTAG
GAG

NB 20: *Salinibacterium* (ON023826)

TGCAGTCGCGATGACCGGAGCTTGCTCTGGTGGATTAGTGGCGAACGGGTGA
CGAGTAACCTGCCCTTGACTCTGGAATAAGCGTTGGAAACGACGTCTAATACC
GGATACGAGCTTCAGCCGCATGGCTAGGAGCTGGAAAGAATTTCGGTCAAGGA
TGGACTCGCGGCCTATCAGGTAGTTGGTGAGGTAATGGCTCACCAAGCCTACG
ACGGGTAGCCGGCCTGAGAGGGTGACCGGCCACACTGGAACTGAGACACGGT
CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGC
CTGATGCAGCAACGCCGCGTGAGGGACGACGGCCTTCGGGTTGTAAACCTCTT
TTAGTAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAAAAGCACCGGCTAA
CTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTTATCCGGAATTA
TTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCTGCTGTGAAAACTGGAG
GCTCAACCTCCAGCCTGCAGTGGGTACGGGCAGACTAGAGTGCGGTAGGGGA
GATTGGAATTCCTGGTGTAGCGGTGGAATGCGCAGATATCAGGAGGAACACC
GATGGCGAAGGCAGATCTCTGGGCCGTAACTGACGCTGAGGAGCGAAAGCAT
GGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTGGGAA
CTAGATGTAGGGACCATTCCACGGTTTCTGTGTCGCAGCTAACGCATTAAGTTC
CCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCGGCGGAGCATGCGGATTAATTCGATGCAACGCGAAGAACCTT
ACCAAGACTTGACATATACGAGAACGGGCCAGAAATGGTTCACTCTTTGGACA
CTCGTAAACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGG
TTAAGTCCCGCAACGAGCGCAACCCTCGTTCTTTGTTGCCAGCACGTAATGGT
GGGAACTCAAAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGAC
GTCAAATCATCATGCCCCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGA
TACAAAGGGCTGCAATACCGCGAGGTGGAGCGAATCCCAAAAAGTCGGTCTCA
GTTCGGATTGAGGTCTGCAACTCGACCTCATGAAGTCGGAGTCGCTAGTAATC
GCAGATCAACAACCTTCCGGTGAATACTTCCCGGGCCTTCCCGCCCCAAGGAA
GGACCCACCCCCCAAGGGGTAAGTGGGGAAAATAAAAAATAAAAAAAACTG

NB 21: *Micrococcus* (ON023827)

TGCAGTCGCGATGAACCAGATGTGGGTGGATAGTGGCGAACGGGTGAGTAAC
CTGCCCTTAACTCTGGGATAAGCCTGAACTGGGTCTAATACCGGATAGGAGCG
CCCACCGCATGGTGGTGAAAGATTTATCTTTTGGATGGACTCGCGGCCTATCA
GCTTGTTGGTGAGGTAATGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGA
GAGGGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAG
GCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCG
CGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGTAGGGAAGAAGCG
AAAGTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCG
CGGTAATACGTAGGGTGCGAGCGTTATCCGGAATTATTGGGCGTAAAGAGCTC
GTAGGCGGTTTGTCGCGTCTGTCGTGAAAGTCCGGGGCTTAACCCCGGATCTG
CGGTGGGTACGGGCAGACTAGAGTGCAGTAGGGGAGACTGGAATTCCTGGTG
TAGCGGTGGAATGCGCAGATATCAGGAGGAACACCGATGGCGAAGGCAGGTC
TCTGGGCTGTAACTGACGCTGAGGAGCGAAAGCATGGGGAGCGAACAGGATT
AGATACCCTGGTAGTCCATGCCGTAAACGTTGGGCACTAGGTGTGGGGACCAT
TCCACGGTTTCCGCGCCGCAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTAC
GGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGG
AGCATGCGGATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATG
TTCTCGATCGCCGTAGAGATACGGTTTCCCCTTTGGGGCGGGTTCACAGGTGG
TGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACG
AGCGCAACCCTCGTTCCATGTTGCCAGCACGTCGTGGTGGGGACTCATGGGAG
ACTGCCGGGGTCAACTCGGAGGAAGGTGAGGACGACGTCAAATCATCATGCC
CCTTATGTCTTGGGCTTCACGCATGCTACAATGGCCGGTACAATGGGTTGCGA
TACTGTGAGGTGGAGCTAATCCCAAAAAGCCGGTCTCGTTCGAGATTGGGGTC
TGCAACTCGACCCCATGAAGTCGGAGTTGCTAGTAATCCCAAGCTGCGGTTAA
TCTTCCCGGCCTTCCCCCCCCAAAAAGTACCCAACCGGAACCTGGGGGCAGGG
CCGATAATTAAA

NB 14: *Brachybacterium* (ON023828)

TGCACGCGTGGGTGCACGCGAACATGCGGCGACGGAAAACCGCGGCCTCTTG
ATTGGGAACGTGGTTCTATCTGTTTTGCACCTCGGATGGTTAGGCCAGATTTGG
TGAGGGATGGACTCGCGGCCTATCAGTTTGTTGGTGAGGTGATGGCTCACCAA
GACGATGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGA
GACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATAATGCACAATGG
GCGAAAGCCTGATGCAGCGACGCCGCGTGGGGATGACGGCCTTCGGTGTAAA
CCCCTTTCAGTAGGGAAGAAGCGAGAGTGACGGTACCTGCTGAAGAAGCGCC
GGCTAACACGTGCCAGCAGCCCGGTAATACGTAGGGCGCAAGCGTTGTCCGG
AATTATTGAGCGTAAAGAGCTTGTAGGTGGCTTGTCGCGTCTGCCGTGAAAAC
CCGAGGCTCAACCTCGGGCGTGCGGTGGGTACGGGCAGGCTAGAGTGTGGTA
GGGGAGACTGGAACTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAAGA
ACACCGATGGCGAAGGCAGGTCTCTGGGCCATTACTGACACTGAGAAGCGAAA
GCATGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTG
GGCACTAGGTGTGGGGGACATTCCACGTTTTCCGCGCCGTAGCTAACGCATTA
AGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGAC
GGGGGCCCGCACAAGCGGCGGAGCATGCTGATTAATTCGATGCAACGCGAAG
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TGGATGGAGTACGAGGACATCTAATGATGTCGCTCCCTCAGTTTCGATTCGTCA
ATGGGAATCTGCGTTGCCATCCGCGTCTTACGGATATTGCCCCCTTTCAGCGCT
ACACCGGGATTTCTGGGGACTCATAGGAGACTGCCGGGGTCAACTCGGAGGA
AGGTGGGGACGACGTCAAATCATCATGCCCCTTATGTCTTGGGCTTCAAGCAT
GCTACAATGGTCGGTACAATGGGTTGCGAAACTGTGAGGTGGAGCGAATATCC
CAAAAAGCCGGCCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTC
GGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACTCGGCAGGG
TACCGTAATTTCCCTTTCTTCGCTGAAAGAAGTTTTACAACCCCGAAGGGAGTG
GCCCATCCTCGTGAGGGAGCTGTCGAAGGTGGGATCGGTGATTGGACTAAGT
CG

NB 15: *Brachybacterium* (ON023829)

GAGATGCACCACCGTATTGCTCGGCCTGATTAGTGGCGAACGGGTGAGTAACA
CGTGAGCAACCTGCCCTTCACTCTGGGATAACCTCGGGAAATCGGGGCTAATA
CCGGATATGAGCTCCTGTCGCATGGCGGGTGTTGGAAAGTTTTTCGGTGAAGG
ATGGGCTCGCGGCCTATCAGTTTGTTGGTGAGGTAGTGGCTCACCAAGGCGAT
GACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACG
GCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAA
GCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCT
CTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGC
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ATTATTGGGCGTAAAGAGCTTGTAGGTGGCTTGTCGCGTCTGCCGTGAAAACC
CGAGGCTCAACCTCGGGCGTGCGGTGGGTACGGGCAGGCTAGAGTGTGGTAG
GGGAGACTGGAACTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAAGAA
CACCGATGGCGAAGGCAGGTCTCTGGGCCATTACTGACACTGAGAAGCGAAA
GCATGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGTTG
GGCACTAGATGTGGGGAACATTCCACGTTTTCCGCGTCGTAGCTAACGCATTA
AGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGAC
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AACCTTACCAAGGCTTGACATGCACCGGACGACTCCAGAGATGGGGTTTTCTT
CGGACTGGTGCACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGANATGT
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TGGCGGGGAACTCATGGGAAGACTGCCGGGGTCAACTCGGAGGAAGGTGGG
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GGCCGGTACAAAGGGTTGCGAAACTGTGAGGTGGAGCGAATCCCAAAAAGCC
GGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGTC
TAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACA
CACCGCCCGTCAAGTCACGAAAGTCGGTAACACCCAGTGGCCCATCCTCGTTA
GGGAGCTGTCGT

NB 16: *Brachybacterium* (ON023830)

GTGTTCGCGTGACGGCCGACTGTTGATCGGGATGGGTGATAACACGTGCCGC
CCTTACTCTGGGATAACTCGGAAATCGGGGCTAATACCGGATGAGCTCCGTCG
CATGGCGGGGAAAGTTTTTCGTAAGATGGGCTCGCGGCCTATCAGTTTGTTGG
TGAGGTAGTGGCTCACCAAGGCGATGACGGGTAGCCGGCCTGAGAGGGCGAC
CGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGA
TGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACG
GTACCTGCAGAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATAC
GTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTTGTAGGTGG
CTTGTCGCGTCTGCCGTGAAAACCCGAGGCTCAACCTCGGGCGTGCGGTGGG
TACGGGCAGGCTAGAGTGTGGTAGGGGAGACTGGAACTCCTGGTGTAGCGGT
GAAATGCGCAGATATCAGGAAGAACACCGATGGCGAAGGCAGGTCTCTGGGC
CATTACTGACACTGAGAAGCGAAAGCATGGGGAGCGAACAGGATTAGATACCC
TGGTAGTCCATGCCGTAAACGTTGGGCACTAGATGTGGGGAACATTCCACGTT
TTCCGCGTCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAA
GGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGCG
GATTAATTCGATGCAACGCGAAGAACCTTACCAAGGCTTGACATGCACCGGAC
GACTCCAGAGATGGGGTTTTCTTCGGACTGGTGCACAGGTGGTGCATGGTTGT
CGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT
CGTTCCATGTTGCCAGCGCGTAATGGCGGGGACTCATGGGAAGACTGCCGGG
GTCAACTCGNAGGAAGGTGGGGACGACGTCAAATCATCATGCCCCTTATGTTT
GGGGCTTCACGCATGCTACAATGGCCCGGTACAAGGGTTGCGAAACTGTGAG
GTGGAGCGAATCCCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCG
ACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAA
TACGTTCCCGGGCCTTGTACACACCGCCCGTCAAGTCACGAAAGTCGGTAACA
CCCGAAGCCAGTGGCCCATCCTCGTGAGGGAGCTGTCGAAGGTGGGATCGGT
GAAG

NB 18: *Micromonospora* (ON514127)

CTCGCGGCCTATCAGCTTGTTGGTGGGGTGATGGCCTACCAAGGCGACGACG
GGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCA
GACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGGAAGCCTG
ATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCA
GCAGGGACGAAGCGTAAGTGACGGTACCTGCAGAAGAAGCGCCGGCCAACTA
CGTGCCAGCAGCCGCGGTAAGACGTAGGGCGCGAGCGTTGTCCGGATTTATT
GGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGACTGTGAAAACCCGCAG
CTCAACTGCGGGCCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAG
ACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGG
TGGCGAAGGCGGGTCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGG
GGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGTTGGGCGCT
AGGTGTGGGGGGCCTCTCCGGTTCCCTGTGCCGCAGCTAACGCATTAAGCGC
CCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGG
CCCGCACAAGCGGCGGNAGCATGCGGATTAATTCGATGCAACGCGAAAGAAC
CTTACCTGGGTTTGACATGGCCGCAAAAACTGTCAGAGATGGCAGGTCCTTCG
GGGGNCGTCACAGGTGNTGCATGGCTGTCGTCAGCTCGTGTNGTGANGATGT
GGGTTAAGTCCCGCAACGAGCGCAACCCTCGTTCGATGTTGCCAGCGCGTTAT
GGCGGGGACTCATCGAAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGAT
GACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACGCATGCTACAATGG
CCGGTACAATGGGCTGCGATACCGTGAGGTGGAGCGAATCCCAAAAAGCCGG
TCTCAGTTCGGATCGGGGTCTGCAACTCGACCCCGTGAAGTCGGAGTCGCTAG
TAATCGCCTGCGGTGAATACGTTCCCGGGCCTTGTACGTCACGAAAGTCGGNA
CACCCTTG

FOP 8: *Streptomyces* (ON023831)

GAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCA
AGTCGAACGATGAAGCCCTTCGGGGTGGATTAGTGGCGAACGGGTGAGTAAC
ACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGGGTCTAAT
ACCGGATACTGATCGCCTTGGGCATCCTTGGTGATCGAAAGCTCCGGCGGTGC
AGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAATGGCTCACCAAGGC
GACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCG
AAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAA
CCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCC
GGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCGAGCGTTGTCC
GGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCGCGTCGGTTGTGAAA
GCCCGGGGCTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGG
TAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAG
GAACACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCG
AAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GGTGGGCACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACG
CATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAAT
TGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGC
GAAGAACCTTACCAAGGCTTGACATACACCGGAAACGTCCAGAGATGGGCGCC
CCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGA
GATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTGTTGCCAGCA
GGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGG
AAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCACACG
TGCTACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTC
AAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCG
GAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCC
TTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGG
CCCAACCCCCTTGTGGGGAGGGAGCTGTCGAAGGTGGGACTGGCGATTGGGA
CGAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCTCCT

Appendix 7.3: Genome annotation data

##Genome-Annotation-Data-START##: **NB 2**

Annotation Provider	:: National Library of Medicine
Annotation Date	:: 01/13/2022 20:33:51
Annotation Pipeline	:: NCBI Prokaryotic Genome Annotation Pipeline (PGAP)
Annotation Method	:: Best-placed reference protein set; GeneMarkS-2+
Annotation Software revision	:: 2021-11-29. build5742
Features Annotated	:: Gene; CDS; rRNA; tRNA; ncRNA. repeat region
Genes (total)	:: 7,191
CDSs (total)	:: 6,873
Genes (coding)	:: 6,632
CDSs (with protein)	:: 6,632
Genes (RNA)	:: 52
rRNAs	:: 3, 2, 1 (5S, 16S, 23S)
complete rRNAs	:: 3, 2, 1 (5S, 16S, 23S)
tRNAs	:: 68
ncRNAs	:: 3
Pseudo Genes (total)	:: 241
CDSs (without protein)	:: 241
Pseudo Genes (ambiguous residues)	:: 0 of 12
Pseudo Genes (frameshifted)	:: 2 of 12
Pseudo Genes (incomplete)	:: 10 of 12
Pseudo Genes (internal stop)	:: 4 of 12
Pseudo Genes (multiple problems)	:: 3 of 12

##Genome-Annotation-Data-END##

##Genome-Annotation-Data-START##: **NB 16**

Annotation Provider	:: National Library of Medicine
Annotation Date	:: 01/13/2022 20:33:51
Annotation Pipeline	:: NCBI Prokaryotic Genome Annotation Pipeline (PGAP)
Annotation Method	:: Best-placed reference protein set; GeneMarkS-2+
Annotation Software revision	:: 2021-11-29. build5742
Features Annotated	:: Gene; CDS; rRNA; tRNA; ncRNA. repeat region
Genes (total)	:: 3,477
CDSs (total)	:: 3,359
Genes (coding)	:: 3,297
CDSs (with protein)	:: 3,297
Genes (RNA)	:: 52
rRNAs	:: 3, 2, 1 (5S, 16S, 23S)
complete rRNAs	:: 3, 2, 1 (5S, 16S, 23S)
tRNAs	:: 50
ncRNAs	:: 3
Pseudo Genes (total)	:: 62
CDSs (without protein)	:: 62
Pseudo Genes (ambiguous residues)	:: 0 of 12
Pseudo Genes (frameshifted)	:: 2 of 12
Pseudo Genes (incomplete)	:: 10 of 12
Pseudo Genes (internal stop)	:: 4 of 12
Pseudo Genes (multiple problems)	:: 3 of 12

##Genome-Annotation-Data-END##

##Genome-Annotation-Data-START##: **NB 18**

Annotation Provider	:: National Library of Medicine
Annotation Date	:: 01/13/2022 16:50:14
Annotation Pipeline	:: NCBI Prokaryotic Genome Annotation Pipeline (PGAP)
Annotation Method	:: Best-placed reference protein set; GeneMarkS-2+
Annotation Software revision	:: 2021-11-29.build5742
Features Annotated	:: Gene; CDS; rRNA; tRNA; ncRNA; Repeat region
Genes (total)	:: 9,567
CDSs (total)	:: 9,446
Genes (coding)	:: 9,305
CDSs (with protein)	:: 9,305
Genes (RNA)	:: 121
rRNAs	:: 5, 2, 7 (5S, 16S, 23S)
complete rRNAs	:: 5 (5S)
partial rRNAs	:: 2, 7 (16S, 23S)
tRNAs	:: 101
ncRNAs	:: 6
Pseudo Genes (total)	:: 141
CDSs (without protein)	:: 141
Pseudo Genes (ambiguous residues)	:: 0 of 141
Pseudo Genes (frameshifted)	:: 23 of 141
Pseudo Genes (incomplete)	:: 127 of 141
Pseudo Genes (internal stop)	:: 10 of 141
Pseudo Genes (multiple problems)	:: 17 of 141

##Genome-Annotation-Data-END##:

##Genome-Annotation-Data-START##: **NB 19**

Annotation Provider	:: National Library of Medicine
Annotation Date	:: 01/13/2022 18:44:44
Annotation Pipeline	:: NCBI Prokaryotic Genome Annotation Pipeline (PGAP)
Annotation Method	:: Best-placed reference protein set; GeneMarkS-2+
Annotation Software revision	:: 2021-11-29. build5742
Features Annotated	:: Gene; CDS; rRNA; tRNA; ncRNA. repeat region
Genes (total)	:: 2,416
CDSs (total)	:: 2,360
Genes (coding)	:: 2,341
CDSs (with protein)	:: 2,341
Genes (RNA)	:: 56
rRNAs	:: 3, 1, 3 (5S, 16S, 23S)
complete rRNAs	:: 3 (5S)
partial rRNAs	:: 1, 3 (16S, 23S)
tRNAs	:: 46
ncRNAs	:: 3
Pseudo Genes (total)	:: 19
CDSs (without protein)	:: 19
Pseudo Genes (ambiguous residues)	:: 0 of 19
Pseudo Genes (frameshifted)	:: 5 of 19
Pseudo Genes (incomplete)	:: 15 of 19
Pseudo Genes (internal stop)	:: 3 of 19
Pseudo Genes (multiple problems)	:: 3 of 19

##Genome-Annotation-Data-END##

##Genome-Annotation-Data-START##: **NB 20**

Annotation Provider	:: National Library of Medicine
Annotation Date	:: 01/13/2022 20:33:51
Annotation Pipeline	:: NCBI Prokaryotic Genome Annotation Pipeline (PGAP)
Annotation Method	:: Best-placed reference protein set; GeneMarkS-2+
Annotation Software revision	:: 2021-11-29. build5742
Features Annotated	:: Gene; CDS; rRNA; tRNA; ncRNA. repeat region
Genes (total)	:: 2,644
CDSs (total)	:: 2,592
Genes (coding)	:: 2,580
CDSs (with protein)	:: 2,580
Genes (RNA)	:: 52
rRNAs	:: 3, 1, 1 (5S, 16S, 23S)
complete rRNAs	:: 3, 1, 1 (5S, 16S, 23S)
tRNAs	:: 44
ncRNAs	:: 3
Pseudo Genes (total)	:: 12
CDSs (without protein)	:: 12
Pseudo Genes (ambiguous residues)	:: 0 of 12
Pseudo Genes (frameshifted)	:: 2 of 12
Pseudo Genes (incomplete)	:: 10 of 12
Pseudo Genes (internal stop)	:: 4 of 12
Pseudo Genes (multiple problems)	:: 3 of 12

##Genome-Annotation-Data-END##

##Genome-Annotation-Data-START##: **NB 21**

Annotation Provider	:: National Library of Medicine
Annotation Date	:: 01/13/2022 18:23:37
Annotation Pipeline	:: NCBI Prokaryotic Genome Annotation Pipeline (PGAP)
Annotation Method	:: Best-placed reference protein set; GeneMarkS-2+
Annotation Software revision	:: 2021-11-29. build5742
Features Annotated	:: Gene; CDS; rRNA; tRNA; ncRNA; Repeat region
Genes (total)	:: 2,433
CDSs (total)	:: 2,378
Genes (coding)	:: 2,326
CDSs (with protein)	:: 2,326
Genes (RNA)	:: 55
rRNAs	:: 1, 2, 1 (5S, 16S, 23S)
complete rRNAs	:: 1, 1, 1 (5S, 16S, 23S)
partial rRNAs	:: 1 (16S)
tRNAs	:: 48
ncRNAs	:: 3
Pseudo Genes (total)	:: 52
CDSs (without protein)	:: 52
Pseudo Genes (ambiguous residues)	:: 0 of 52
Pseudo Genes (frameshifted)	:: 13 of 52
Pseudo Genes (incomplete)	:: 40 of 52
Pseudo Genes (internal stop)	:: 4 of 52
Pseudo Genes (multiple problems)	:: 5 of 52
CRISPR Arrays	:: 3

##Genome-Annotation-Data-END##

##Genome-Annotation-Data-START##: **FOP 8**

Annotation Provider	:: National Library of Medicine
Annotation Date	:: 01/13/2022 20:33:51
Annotation Pipeline	:: NCBI Prokaryotic Genome Annotation Pipeline (PGAP)
Annotation Method	:: Best-placed reference protein set; GeneMarkS-2+
Annotation Software revision	:: 2021-11-29. build5742
Features Annotated	:: Gene; CDS; rRNA; tRNA; ncRNA. repeat region
Genes (total)	:: 7,087
CDSs (total)	:: 6,840
Genes (coding)	:: 6,668
CDSs (with protein)	:: 6,668
Genes (RNA)	:: 54
rRNAs	:: 3, 2, 1 (5S, 16S, 23S)
complete rRNAs	:: 3, 2, 1 (5S, 16S, 23S)
tRNAs	:: 68
ncRNAs	:: 3
Pseudo Genes (total)	:: 172
CDSs (without protein)	:: 172
Pseudo Genes (ambiguous residues)	:: 0 of 12
Pseudo Genes (frameshifted)	:: 2 of 12
Pseudo Genes (incomplete)	:: 10 of 12
Pseudo Genes (internal stop)	:: 4 of 12
Pseudo Genes (multiple problems)	:: 3 of 12

##Genome-Annotation-Data-END##

Appendix 7.4: Phylogenetic trees based on 16S rDNA data from genomic sequencing of the isolates.

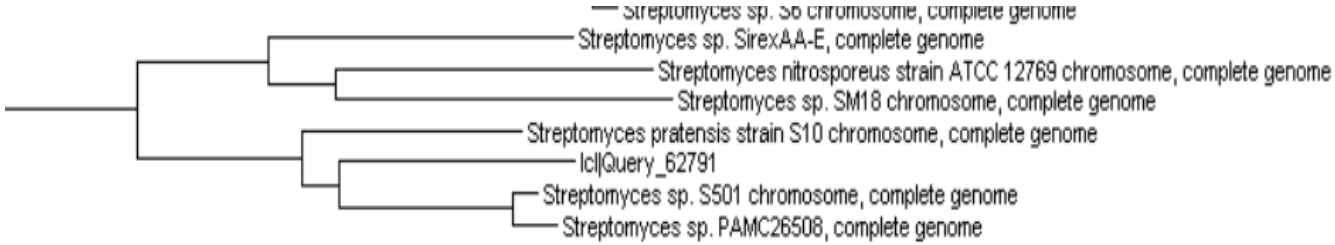

Figure 7.4 1: 16S rDNA phylogenetic tree of isolate NB 2 from genomic sequencing data. Tree was prepared from top 100 hits in NCBI blastN using Blast tree view

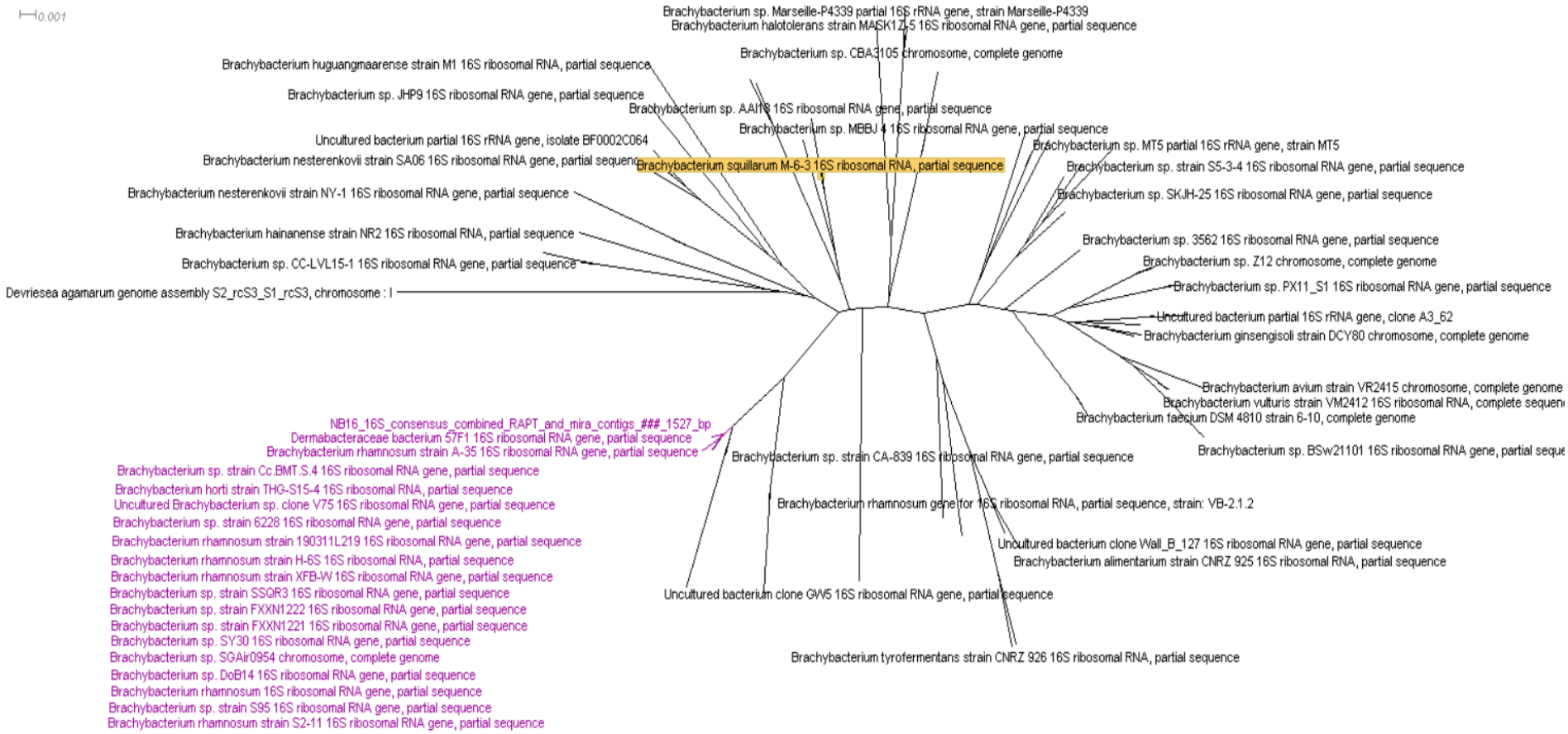

Figure 7.4 2: A 16S rDNA phylogenetic tree of isolate NB 16 from genomic sequencing data. Tree was prepared from top 100 hits in NCBI blastN using Blast tree view

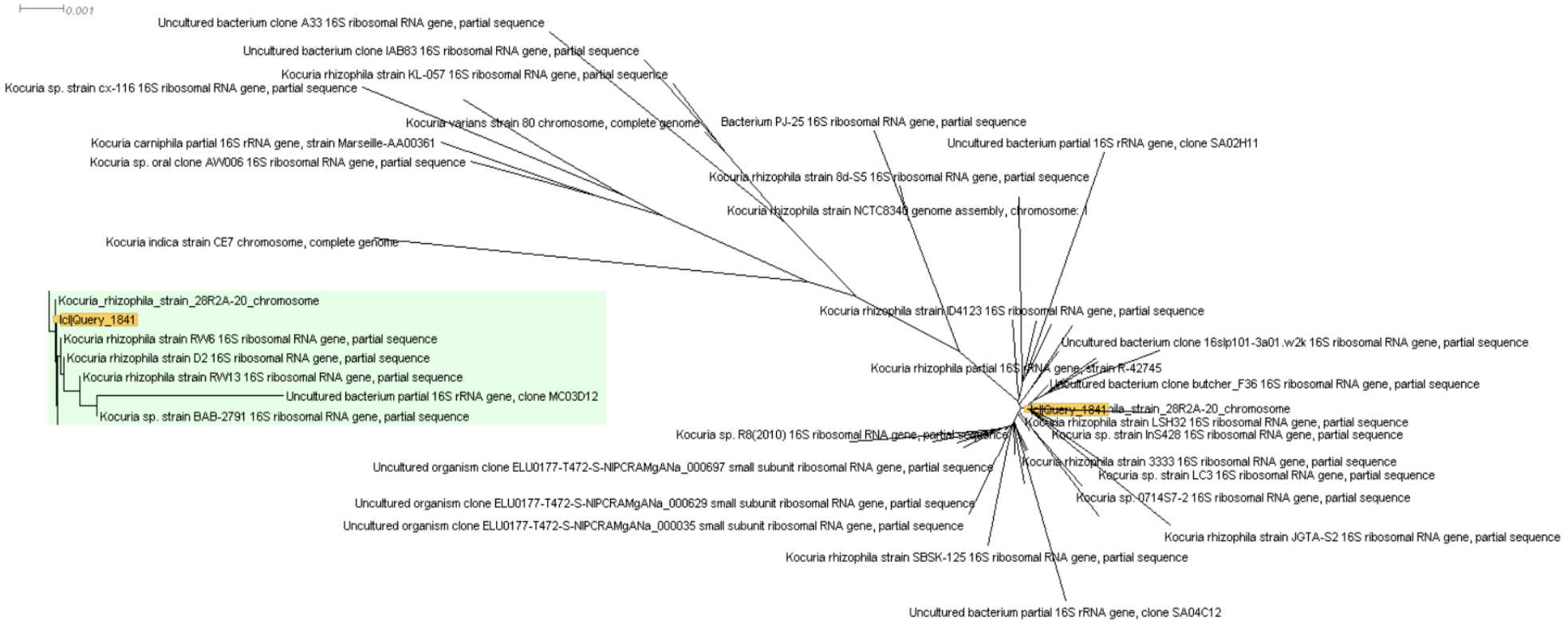

Figure 7.4 3: A 16S rDNA phylogenetic tree of isolate NB 19 from genomic sequencing data. Tree was prepared from top 100 hits in NCBI blastN using Blast tree view.

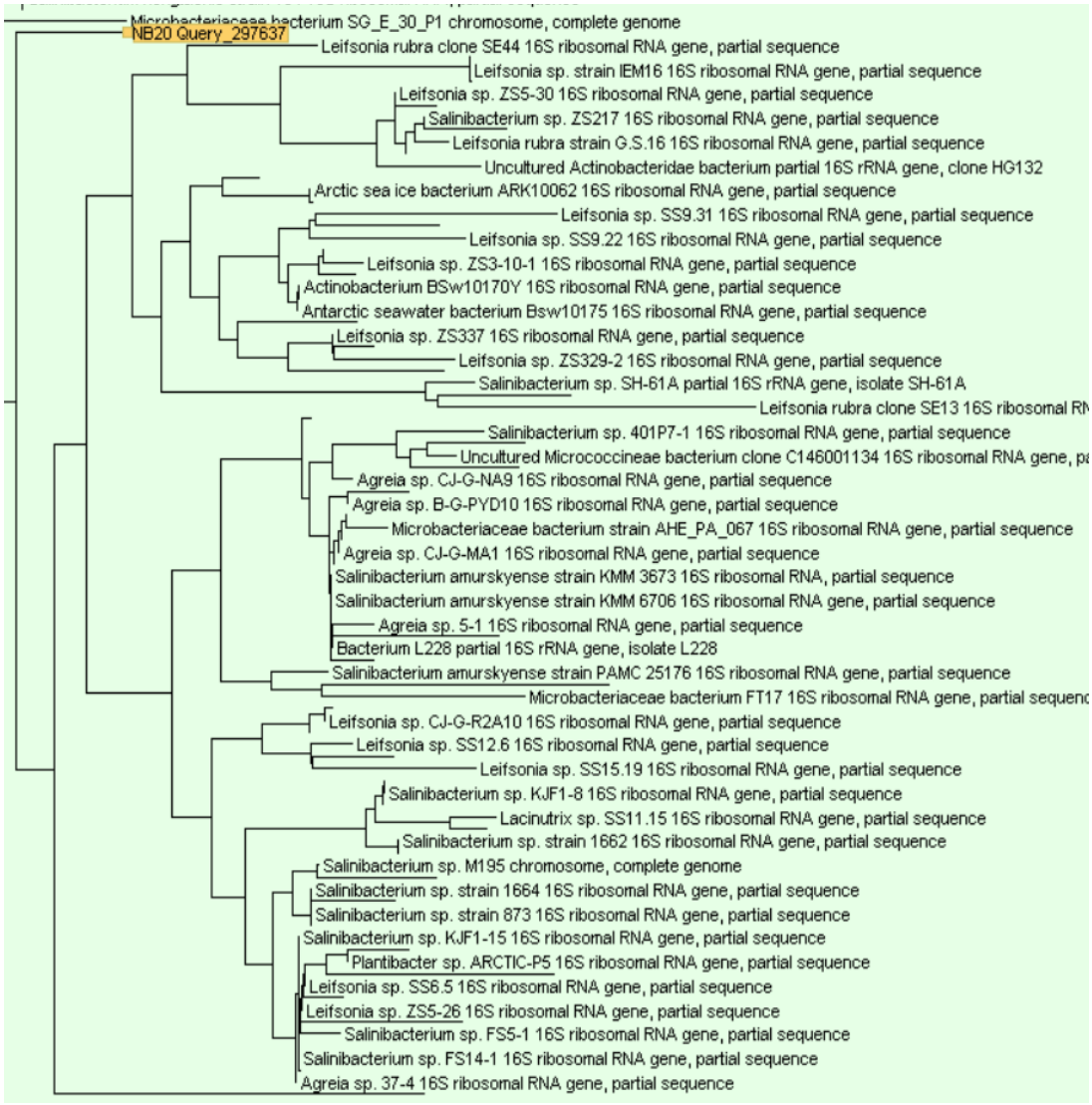

Figure 7.4 4: A 16S rDNA phylogenetic tree of isolate NB 20 from genomic sequencing data. Tree was prepared from top 100 hits in NCBI blastN using Blast tree view.

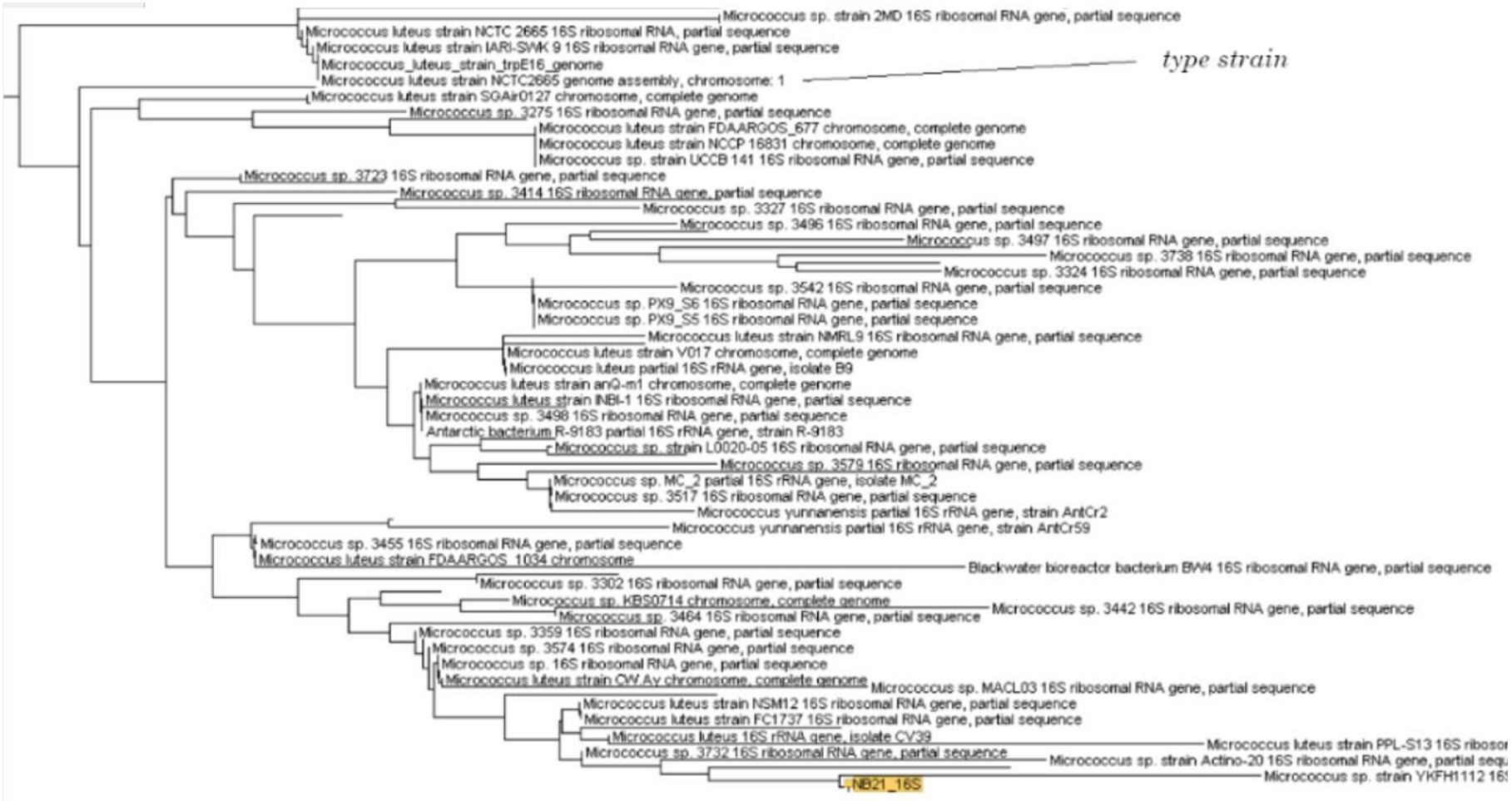

Figure 7.4 5: A 16S rDNA phylogenetic tree of isolate NB 21 from genomic sequencing data. Tree was prepared from top 100 hits in NCBI blastN using Blast tree view

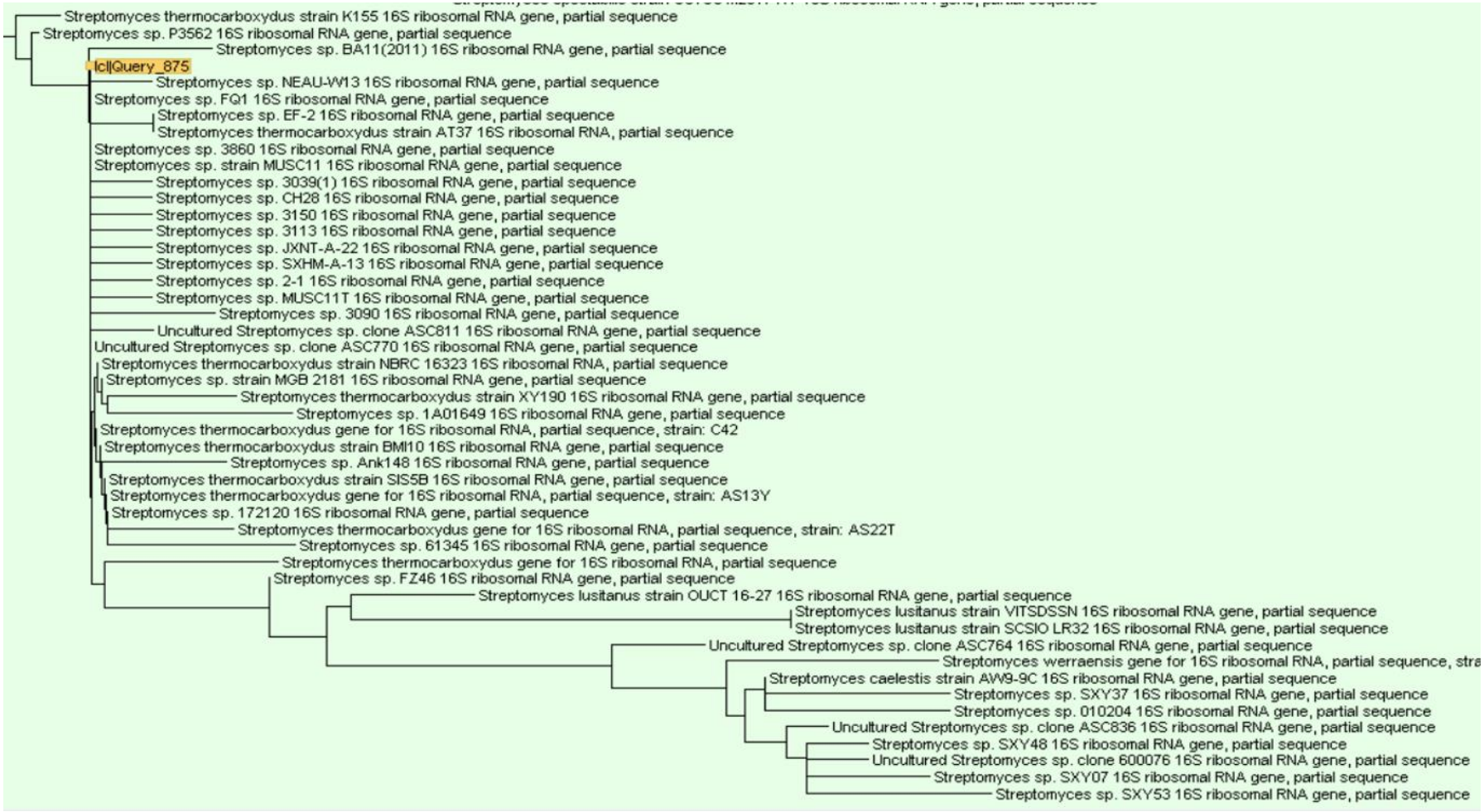

Figure 7.4 6. 16S rDNA phylogenetic tree of isolate FOP 8 from genomic sequencing data. Tree was prepared from top 100 hits in NCBI blastN using Blast tree view.

Appendix 7.5: Result of drop collapse and oil displace test

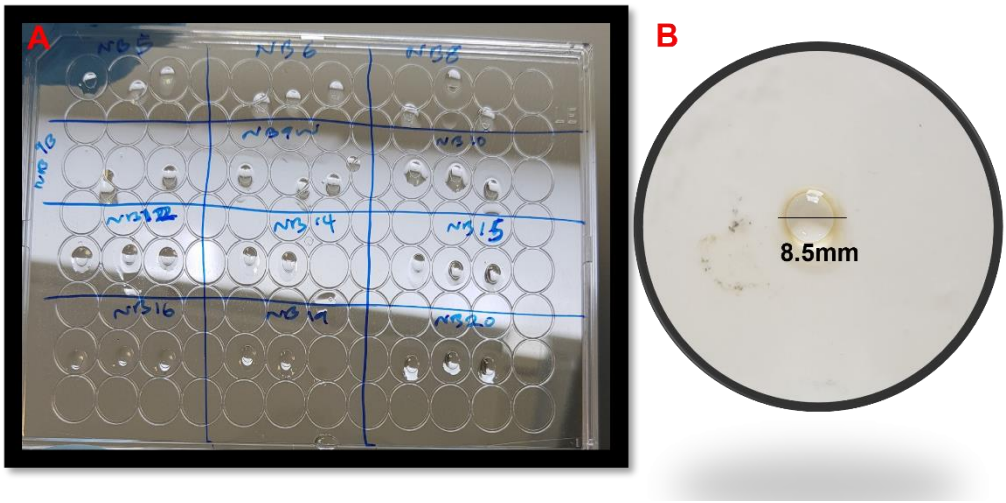

Figure 7.5 1: Representative of the screening for biosurfactant production by the isolates. (A) Drop collapse test. (B) Oil displacement test

Table 7. 1**:** Measurement of the diameter (mm) of the oil collapse test

Isolates	**Oil collapse test (mm)**
NB2	0.00 ± 00
NB14	7.40 ± 00
NB15	7.60 ± 02
NB16	7.20 ± 01
NB18	6.70 ± 00
NB19	7.00 ± 02
NB20	8.50 ± 02
NB21	8.00 ± 00

Appendix 7.6: Cellulose-related genes in genome of the isolates

Table 7.2: Cellulose-related genes in genome of the isolates

Isolates	Contig	Cellulase annotation	Reference strain	GenBank protein ID
FOP 8	23	Cellulase	*Streptomyces sp.* GESEQ_13	WP_210637842 mannosidase/endoglucanase
	23	Cellulose binding	*Streptomyces sp.* GESEQ_13	WP_210637843
	41	Cellulase	*Streptomyces sp.* GESEQ_13	WP_210637934
	47	Cellulose binding	*Streptomyces sp.* GESEQ_13	WP_210637761
	102	Cellulose binding	*Streptomyces sp.* GESEQ_13	WP_210636528
	102	Cellulose binding	*Streptomyces sp.* GESEQ_13	WP_246887781 xyloglucanase
	109	Cellulose binding	*Streptomyces sp.* GESEQ_13	WP_210635093
	139	Cellulose binding	*Streptomyces sp.* GESEQ_13	WP_210635767
	210	Cellulose binding	*S. cellulosae*	GHE68903
NB 2	22	Cellulose synthase	*Streptomyces sylvae*	WP_202076305 CelA-like
	39	Cellulose binding	*Streptomyces silvae*	WP_202077176
	40	Cellulose binding	*Streptomyces silvae*	WP_202074818
	41	Cellulose binding	*Streptomyces silvae*	WP_236058318 exoglucanase B precursor
	68	Glycosyl hydrolase	*Streptomyces silvae*	WP_202077786 endoglucanase CelA
	68	Cellulose binding	*Streptomyces sp.* ADI93-02	RPK50176
	88	Cellulase	*Streptomyces silvae*	WP_236058050
	104	Cellulose binding	*Streptomyces silvae*	WP_124274716
	129	Cellulose binding	*Streptomyces silvae*	WP_236057926 xyloglucanase
	132	Cellulase	*Streptomyces silvae*	WP_202077141 beta-mannosidase
NB 18	7	Cellulose binding	*Micromonospora tulbaghiae*	WP_120570040
	16	Cellulose binding	*Micromonospora tulbaghiae*	WP_208577583 lytic polysaccharide monooxygenase
	35	Cellulose binding	*Micromonospora tulbaghiae*	WP_120573898 glycoside hydrolase family 9 protein
	84	Cellulose binding	*Micromonospora tulbaghiae*	WP_244255611
	85	Cellulose binding	*Micromonospora tulbaghiae*	WP_120573835
	90	glycosylhydrolase	*Micromonospora tulbaghiae*	WP_091419873
	105	Cellulose binding	*Micromonospora tulbaghiae*	WP_120569461 PHB (polybetahydroxybutyrate) depolymerase

	111	Cellulose binding	*Micromonospora tulbaghiae*	WP_091427105 arabinofuranosidase
	118	Cellulase	*Micromonospora tulbaghiae*	WP_120571390 expansin peptidoglycan binding
	201	Cellulose binding	*Micromonospora tulbaghiae*	WP_205776079 cellulose 1,4 beta-cellobiosidase
	211	Cellulase	*Micromonospora tulbaghiae*	WP_120571736 endoglucanase
	212	Cellulose binding	*Micromonospora tulbaghiae*	WP_120571221
	246	Cellulose binding		WP_120570033
	255	Cellulase	*Micromonospora tulbaghiae*	WP_091428138 endoglucanase CelA
	303	Cellulose-binding	*Micromonospora tulbaghiae*	WP_120573312 endoglucanase
	371	Cellulose binding	*Micromonospora tulbaghiae*	WP_091416657 cellulase/cellobiose CelA
	413	Cellulose binding	*Micromonospora tulbaghiae*	WP_120571417 glycosyl htdrolase famly 5
	418	Cellulose binding	*Micromonospora tulbaghiae*	WP_120570846
	418	Cellulose binding	*Micromonospora tulbaghiae*	WP_120570852 glucanase
	418	Cellulose binding	*Micromonospora tulbaghiae*	WP_120570835
	470	Cellulase family	*Micromonospora tulbaghiae*	WP_120570924/WP_091427714 glycosyl hydrolase family 5
	505	Cellulose-binding	*Micromonospora tulbaghiae*	WP_120568972
	562	Cellulose binding	*Micromonospora tulbaghiae*	WP_120570835
	572	Cellulose binding	*Micromonospora tulbaghiae*	WP_120573318 chitinase