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Assessment of uncultivable soil microorganisms as a source of novel antibiotic

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Abstract

Multi drug resistance microorganisms are an increasing problem and more than 70% of clinically significant pathogens possesses resistance to currently existing antibiotics. Most antibiotics in clinical use were discovered by screening cultivable soil microorganisms and of these only 1% are cultivable using laboratory media and rest are uncultivable. Soil still offers a great potential for antibiotic discovery. In this study, iChip *in situ* environmental cultivation technology was used to cultivate previously uncultivated microorganisms and screened for antagonistic organisms. Soil samples were collected from various geographic locations in UK based on soil characteristics. Two to three samples were collected from each site and screened for antagonistic microorganisms using iChip-based technology along with soil supplemented nutrient agar which enabled the growth of previously uncultivated microorganisms. This study found an antagonistic microorganism and identified as *Bacillus pumilus*. Cell free supernatant of *B. pumilus* fermented broth showed 12 mm zone of inhibition against *Staphylococcus aureus* ATCC 25922 and butanol extract and compound-3 showed 9 mm and 8 mm zone of inhibition respectively. *Bacillus pumilus* has been previously described to produce antibacterial and antifungal compounds. Mueller Hinton broth with addition of 5% glucose was used for *B. pumilus* fermentation to assess secondary metabolites. Fermented broth supernatant and extracts produced zone of inhibition (ZI) ranging from 8-12mm against *S. aureus* but could not determine minimum inhibitory concentration. Fermented broth was extracted sequentially with ethyl acetate, n-butanol and methanol. n-Butanol extract in thin layer chromatography showed three compounds, and of these only Compound 3 showed inhibitory effect on *S. aureus*. Nuclear magnetic resonance (both 1D and 2D) analysis revealed compound 3 as a mixture of possible 3 compounds or their fragments but could not identify these compounds. However, compounds fragments had chemical structures containing peptide bonds and aliphatic chains which are also found in known peptide antibiotic, for example, vancomycin. Further studies are needed to fully characterise this compound-3.

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Abbreviations

AcOH	Acetic acid
ATCC	American type culture collection
API	Analytical profile index
β	Beta
BA	Blood agar
^{13}C	13 Carbon
CO_2	Carbon dioxide
cm	Centimetre
cfu	Colony forming unit
ºC	Degree centigrade
δ	Delta
EtOAc	Ethyl acetate
FSSNA	Filtrated soil supplemented nutrient agar
g/L	Gram per litre
H_z	Hertz
HPLC	High-Performance Liquid Chromatography
^{1}H	Hydrogen (Protons)
H_2O_2	Hydrogen peroxide
iChip	Isolation chip
MALDI-TOF	Matrix Assisted Laser Desorption Ionisation, Time of Flight
MH_z	Megahertz
MeOH	Methanol
MRSA	Methicillin resistant *Staphylococcus aureus*
µg	Microgram
µl	Microlitre
ml	Millilitre
µm	Micrometre
mm	Millimetre
MDCT	Morphologically different colonies types
MHA	Mueller Hinton agar
MHB	Mueller Hinton broth
MIC	Minimum inhibitory concentration
ηm	Nanometre
NG	No growth
NMR	Nuclear Magnetic Resonance
NA	Nutrient agar
OG	overgrowth
O_2	Oxygen
ppm	Parts per million
%	Percent
PBS	phosphate buffer saline
PTLC	Preparative thin layer chromatography
R_f	Retardation Factor
SSNA	soil supplemented nutrient agar
TLC	Thin layer chromatography
TSA	Tryptone soya agar
UV	Ultraviolet
UK	United Kingdom

USA	United State of America
UEL	University of East London
VRE	Vancomycin resistant *Enterococcus*

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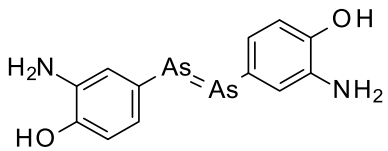
Chapter 1: Introduction

1.1 Pre-antibiotic era and discovery of first natural antibiotic

In the pre-antibiotic era, natural remedies such as herbs, honey, mouldy breads and animal faeces were used in different communities and countries (Gould, 2016). A study showed that a 1000 years old Anglo-Saxon remedy can kill *Staphylococcus aureus* biofilms in soft tissue infection in an *in vitro* model and can kill methicillin-resistant *S. aureus* (MRSA) in a mouse chronic wound model (Harrison et al., 2015). However, Paul Ehrlich who is regarded as the pioneer in antibiotic research showed scientific approach in screening antibiotics that led the development of Salvarsan, an arsenic based compound for the treatment of syphilis and trypanosomiasis (Aminov, 2010) in 1909 . Later a sulfa drug called prontosil (**2**) discovered by Gerhard Domagk (Gradmann, 2008) was a breakthrough to treat the infections until the availability of penicillin in early 1940s. The fortuitous discovery of penicillin (**3**) in 1928 (Fleming, 1929) is regarded as a new era of modern antibiotics from microbes that formed the foundation of current antibiotic researches (Gould, 2016).



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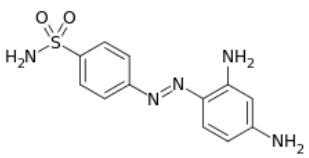

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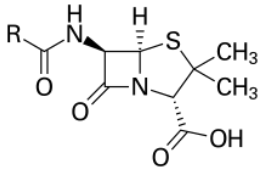

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Figure 1.1: Structures of Salvarsan (**1**), prontosil (**2**) and penicillin (**3**)

1.2 Antibiotics challenges and rise of multi-drug resistance (MDR)

Soon after introduction of Penicillin 1941 and its wide uses, resistance to Penicillin had reported in *Staphylococcus aureus* in 1942 (Rammelkamp and Maxon, 1942) and now resistance to many other pathogens (Coates and Hu, 2007). Although antibiotic resistance is a current problem due to rise of multi drug resistance (MDR) and lack of discovery of new broad-spectrum antibiotics, but this phenomenon is an ancient by far several millenniums. Beringian permafrost sediments DNA from 30,000-year-old showed genes encoded resistance to beta-lactam, tetracycline and glycopeptide antibiotics in

metagenomic analyses. Study also confirmed VanA element of vancomycin resistance from this ancient DNA sample was similar to modern VanA variant by structure and functions (D'Costa et al., 2011). Multidrug resistance is a natural phenomenon and occurs when an organism is exposed to same antibiotic over and over or abuse or misuse of antibiotics. Microorganisms can produce various enzymes and become resistance to current antibiotics. Globally over 2 million people die each year due to bacterial infections (Bérdy, 2012). Approximately 25,000 patients die each year from MDR in the EU and about 1.5 billion Euro worth of extra healthcare costs and productivity losses due to infections by MDR organisms in the EU. More than 70% of the clinically significant or pathogenic bacteria possesses resistance to currently existing antibiotics (Kitchel et al., 2009). Methicillin resistance *Staphylococcus aureus* (MRSA), vancomycin resistance *Enterococcus* (VRE), Extended spectrum beta lactamase (ESBL) producing Enterobacteriaceae, Carbapenemase resistance Enterobacteriaceae (CRE), Multidrug resistance *Pseudomonas* species are the key MDR mostly seen in the patients (van Duin and Paterson, 2016). According to The UK Government-commissioned O'Neill report 10 million people a year will die from MDR infections by 2050 if no urgent action is taken and recommended to boost the development of antibiotics (Antimicrobial resistance ,2019) . Hence this study was undertaken in search of novel antibiotic.

1.3 Approaches for antibiotic discovery

Varied approaches have been used in the past 40 years to discover and develop antibiotics from various sources. Technological advancement over the last few decades helped our understanding of the microbial resistance mechanisms. However, developing new antibiotics remains as a big challenge although various approaches have taken in antibiotic discovery.

1.3.1 Genomics Approaches

The whole genome sequence of *Haemophilus influenzae* in 1995 marked as genetic revolution and regarded as a potential new era for antibiotic discovery (Payne et al., 2007). Since then, there had been significant development in molecular microbiology and whole genome sequencing of 1000s of pathogenic and non-pathogenic microorganisms were done to understand their characteristics and find suitable drug target(s) (Livermore, 2011). On the other hand, metagenomic analysis of soil bacteria enabled to find secondary

metabolites producing biosynthetic gene clusters (BGCs), such as nonribosomal peptide synthetases (NRPSs) and polyketide synthases (PKSs) and these are the two largest classes of BGCs, encoded for most of the known antibiotics and antifungals metabolites (Sharrar et al., 2020). Whole genome sequences of *Streptomyces coelicolor* and *Streptomyces avermitilis* and their secondary metabolites analysis revealed more secondary metabolites initially scientists thought of (Bentley et al., 2002; Ikeda et al., 2003). AntiSMASH-3.0 tool can detect 44 different classes of BGCs present in a microbe (Weber et al., 2015). Most of the known antibiotics came from cultivable *Actinobacteria*, *Proteobacteria*, and *Firmicutes* member of bacterial family and yet the diverse uncultivable soil microbial communities are wealthy potential sources for secondary metabolites (Sharrar et al., 2020). Genomics revolution contributed significantly in diagnosis rather the antibiotic development.

1.3.2 Plant sources (phytochemical compounds)

Plant based remedies (bark, roots, leaf) had been in use for centuries and herbal medicines are still important in many communities and counties (Gould, 2016; Khameneh et al., 2019). Plant based compounds reported to be active against bacteria (Rahman et al., 2008a, 2008b; Wan et al., 1998), fungi (Hufford et al., 1993; Rana et al., 1997) including MDR strains (Dwivedi et al., 2019; Mun et al., 2014) and *Mycobacterium tuberculosis* (Hochfellner et al., 2015) and sweet wormwood (*Artemisia annua*) derived drug, Artemisinins, is currently in use for the treatment of malaria, caused by parasites, *Plasmodium* species, including drug-resistant strains (Krishna et al., 2008). Hence it remains as potential resources for antimicrobial compounds.

1.4 Main Source of Antibiotics

Most of the antibiotics currently in clinical uses come from soil microorganisms; for example, Streptomycin from *Streptomyces griseus,* Cephalosporins from *S. clavuligerus*, Bacitracin from *Bacillus licheniformis*, Polymyxin from *Bacillus polymyxa*, Chloramphenicol from *S. venezuelae*, Tetracycline from *S. aureofaciens*, Erythromycin *Saccharopolyspora erythraea*, Gentamicin from *Micromonospora purpurea*, Mupirocin from *Pseudomonas fluorescens* and more (de Lima Procópio et al., 2012). However, last antibiotics class Carbapenem was discovered in 1976 and came in clinical use in 1985

(Hutchings et al., 2019). There is a huge gap in the new class of antibiotic discovery, and it is an urgent need to find the new class of antibiotic.

Millions of microbial species exists in soil (Bollmann et al., 2007) but 1% can grow on laboratory media and rest are uncultivable (Ling et al., 2015). One-gram soil possibly can have millions of bacteria and fungi and a clear majority of these natural resources are not yet explored for their biodiversity and their bioactivity is not clearly known (Bérdy, 2012). Soil still offers a great potential for antibiotic discovery. Organisms in soil could produces multiple secondary metabolites with possible various functions, for example, to suppress the growth of competitors or as a predator, or even as signalling molecules to interact with eukaryotic hosts and this phenomenon is backed by the evidence of evolution of Streptomyces species and other filamentous actinomycetes and the plant species colonised earth circa 440 million years ago simultaneously (Hutchings et al., 2019).

1.4.1 Uncultivable Microorganisms

Metagenomic analysis of soil samples revealed that only 1% can grow on laboratory media and rest are uncultivable (Bollmann et al., 2007; Sharrar et al., 2020). The term un-cultivable microorganisms mean, organisms those cannot grow in standard laboratory-based culture medium. Natural environments or simulated natural environments using a diffusion growth chamber can enable growth of some previously uncultivated microorganisms and this concept was proposed by Kaeberlein *et al*. in 2002 (Kaeberlein, 2002). Since then, several other techniques such as soil substrate membrane system (Ferrari et al., 2008), Hallow fibre membrane chamber (Aoi et al., 2009), iChip (Nichols et al., 2010), I-tip (Jung et al., 2014) were developed and employed to cultivate uncultivated microorganisms.

iChip is one of the simplest yet much efficient device that facilitate the growth of uncultivable environmental organisms in their natural habitats. The principle behind this approach is that the 100s of miniature diffusion chamber will take growth factors for the microbes from their natural environment and allow to grow *in situ* cultivation (Nichols et al., 2010). A novel broad spectrum antibiotic was discovered from previously uncultivated microorganism using iChip (Ling et al., 2015). For this study, concept of

iChip and environmental cultivation was used for screening antagonistic soil microorganisms along with soil supplemented nutrient agar (SSNA).

1.5 Aim of the study

Isolation of previously uncultivated soil microorganisms using iChip device and their potential as a source of novel antibiotic.

1.6 Objective of the study

1. Screening and isolation of new organism(s) with potential antimicrobial activities by using iChip device.
2. Subculturing and identifying the organisms those grow on iChip and screen for their secondary metabolites.
3. Testing for antimicrobial activities against common pathogenic Gram positive and Gram-negative organisms including their resistant strains.
4. Fermentation, extraction and purification of active metabolites.

Chapter 2: Optimisation of sample collections, preparations, cultures

2.1 Introduction

A gram of soil sample contains billions of microbes (Sánchez-Marañón et al., 2017) with mass of fungal hyphae (Paul, 2007). Microbial diversity and numbers varies from soil to soil depending composition, natural environments, plant species and other atmospheric conditions (Martiny and Walters, 2018). Whatever the soil type is, we can grow only 1% of that microbial population (Delgado-Baquerizo et al., 2018). Microbial communities may be affected through use of fertilisers, pesticides, crop cycles, plant species, aeration, natural climate and seasons and soil types (Zhou et al., 2017). Soil sample of a grassland, park, home garden and forest have different microbial flora (Griffiths et al., 2011). Surface, subsurface and deep soil have different microbial numbers and types (Paul, 2007). Hence, selection of a soil sample is very important to screen for antagonistic microorganisms.

Optimisation is an essential prerequisite prior to any research. Depending on the research and methodologies different optimisation criteria might apply. For the isolation of antagonistic soil microorganisms several key stages required optimisation such as isolation culture medium, temperature and atmospheric conditions. Culture medium alone can be the key factor for isolating antagonistic microorganisms. Various techniques and culture medium such as Tryptone Soy Agar (TSA), Casein-starch Thornton, M3 and Czapeck were tried for the maximum recovery of soil microbes (Acea and Carballas, 1990; Buyer, 1995; Martin, 1950; Sørheim et al., 1989) and 21 different medium were proposed for Streptomycetes isolation alone (Küster and Williams, 1964). It was also important to optimise sample collection, transportation, and inoculation volume for the semi-confluent growth of organisms for subsequent analysis.

Microorganisms require various atmospheric condition for their optimal growth along with appropriate nutrition. Some organisms are strict aerobic e.g. *Pseudomonas aeruginosa* whereas others are strict anaerobes e.g. *Clostridium perfringens*, some prefer microaerophilic condition e.g. *Campylobacter* species and some thrives in the presence of CO_2 e.g. *Haemophilus* species. Microorganisms on the surface soils are mostly aerophilic and microaerophilic whereas subsurface samples are most likely an anaerobic organisms (Marteinsson et al., 2015). Hence, it is important to cultivate the samples in different atmospheric conditions to isolate possible antagonistic microorganisms.

2.2 Soil sample collection

For this research, soil samples were collected from various geographic location in London, Hertfordshire and Southend, UK. Locations were chosen either based on the soil characteristics or natural environments. Permissions was requested in writing from national nature reserves sites such as Burnham Beech, Buckinghamshire, Aston Rowan, Oxfordshire, Ruislip Woods, Greater London and Epping forest, Essex to utilises their sites for this research project. Permission was granted from Ruislip Woods and Epping forest sites. Request letters were also sent to friends, family members and colleagues who lives various part of the UK and have different soil types for example soil from my supervisor's garden was chalky and soil from my nephew's garden in Southend on Sea was sandy. Soil samples collected for the initial optimisation were from Dagenham area of London from two residential back gardens. Samples were collected from the random places in the garden from the surface and sub-surface layers up to 20 centimetres depth from the surface. Soil samples were collected into sterile containers using a clean trowel and transported to the UEL laboratory within maximum of 2 hours. Written permission was obtained prior to collection of all test samples.

2.3 Sample preparation

Collected samples were brought to the laboratory for sorting, preparation, screening and optimisations of culture methods within 2 hours. Collected samples were placed onto a sterile tray for sorting. Leaves, tree roots, grasses, stones and other large debris were handpicked from the samples and removed. Samples were then placed onto a sterile stainless-steel mesh colander having a pore size 2 mm in diameter. With the help of a spatula soil, samples were passed through the colander to make fine and uniform soil samples and collected in a tray. It was then used for soil supplemented nutrient agar (SSNA) medium preparation and cultivation. Remaining samples were returned to their original containers labelling with the details of sample collection sites and dates. Samples were stored in the fridge at 4-7ºC for up to 7 days for further use such as microbial re-culture, chemical profiling, making SSNA medium and so on. Some soil microorganisms might have been attached to tree and grass roots which can be lost through sample sorting and preparation processes. Sample stored for later use might compromise the diversity of the organisms for example anaerobic and other fastidious organisms might not survive up to seven days storing periods, so potential antagonistic organisms might get lost. So far

antibiotics have come from aerobic and facultative anaerobic organisms. However, genetic studies showed secondary metabolites potential from strict anaerobic organisms, for example, *Clostridium* species (Pahalagedara et al., 2020). Hence, results might vary depending on processing time and incubation conditions and might lost potential metabolites producing strict anaerobic organisms.

2.4 Determination of dilution factors and inoculation volumes and culture medium

It is important to do serial dilution of the sample to determine quantitative and qualitative measurement of the soil as well as to fulfil the crowded plate criteria to isolate antagonistic microorganisms i.e. to have around 300-400 cfu or slightly above in one standard agar plate (Sambamurthy and Kar, 2006). Crowded plate technique is a simple, yet the best screening technique employed for the isolation of antagonistic microorganisms. Antagonistic organisms will grow with a clear zone around them and suppress the growth of other organisms which will then be further evaluated against pathogenic microorganisms (Sambamurthy and Kar, 2006).

One-gram soil was transferred onto a falcon tube containing 10 ml of sterile phosphate buffer saline (PBS) ($1:10^{-1}$), vortexed over a period of 10 minutes with regular intervals to get a uniform mixture of the sample. It was then diluted further using serial dilutions methods in PBS to achieve 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} dilutions. Fifty µl of sample from each dilution were inoculated onto nutrient agar plates in triplicate and spread the using L-shaped spreader (Fisher Scientific, UK). Plates were incubated aerobically at 37ºC for 72 hours and checked for microbial growth at regular 24 hours intervals.

For the optimisation purposes, four different types of agar were used. Nutrient agar (NA) and Tryptone soya agar (TSA) (both from Oxoid, UK) and two variants of formulated medium, soil supplemented nutrient agar (SSNA) and filtrated soil supplemented nutrient agar (FSSNA). TSA is the most used nonselective agar for isolation of soil microbes(Vieira and Nahas, 2005); hence it's used in this study to compare mainly with SSNA and FSSNA. Resulting colonies were compared with each other and counted colony forming unit (cfu) by Miles and Misra method (Miles et al., 1938).

2.4.1 Preparation of Soil Supplemented Nutrient Agar (SSNA)

To maximise the recovery of soil microorganisms, we hypothesised that addition of soil to nutrient agar might improve the isolation of soil microorganisms. It was believed that

supplementing with soil might provide yet undisclosed essential nutrients to promote microbial growth as a main nutritional source and would improve the isolation of microorganisms compared to other medium.

Fifty-five grams of pre-prepared soil sample (see 2.3 above) were added to 550 ml of distilled water to make 10% w/v soil supplementation in 1 litre Duran bottle. Bottle was shaken vigorously to mix the soil sample with water and then placed onto a magnetic stirrer plate to stir and mix for 10 minutes. It was then filtered using cotton wool and a final volume of 500 ml was collected in a separate Duran bottle and 14 grams nutrient agar powder was added. Agar was stirred and mixed and then autoclave at 121ºC for 15 minutes. Once autoclaved, medium was left to cool down around 50ºC and poured into sterile petri dishes and stored at 4ºC for later use. One plate was incubated at 37ºC for up to 48 hours without inoculating to check batch sterility.

2.4.2 Preparation of Filtrate Soil Supplemented Nutrient Agar (FSSNA)

FSSNA was prepared in two stages, prefiltration and filtration stage and medium preparation stage.

Soil samples were collected and prepared as described above (2.3). Sequential prefiltration was achieved with the samples being initially sieved using a clean kitchen strainer, then prefiltered using and cotton wool and thirdly by grade-1 filter paper with particle retention capacity >11µm under vacuum (Whatman, UK).

Filtration were done in three steps with the help of peristaltic pump (Millipore, UK). Initially, it was filtered using coarse membrane filter 0.8 µm pore size and 47mm in diameter, followed by 0.45 µm pore and finally using 0.2 µm pore filter (Whatman, UK), which is regarded as sterile filtration and collect them in a sterile jar.

In order to prepare 200 ml FSSNA, 5.6 gram of NA powder was dissolved into 100 ml of distilled water and autoclaved at 121ºC for 15 minutes. After autoclaving 100 ml of filtered soil supplement were added into medium and mixed them well by shaking the medium contents. Approximately 20 ml agar was poured onto per petri dish and allowed to solidify. Randomly selected one plate from the batch was incubated at 37º C for 48 hours to check their sterility.

2.5 Cultivation of soil samples

Soil samples were prepared in $1:10^{-2}$, $1:10^{-3}$, $1:10^{-4}$, $1:10^{-5}$, $1:10^{-6}$ dilutions and NA, TSA, SSNA and FSSNA plates were inoculated with 50 ul of sample from each dilution in triplicate. Plates were then spread using L-shaped spreader both clockwise and anticlockwise and incubated for 72 hours at 37º C. Plates were observed in 24 hours intervals and growth recorded at 72 hours and compared with other replicates. Additionally, parallel sets of plates were compared for growth following incubation under different atmospheric incubation conditions such as aerobic with and without CO_2, microaerophilic and anaerobic.

Three sets of plates were inoculated with different dilutions as per chapter 2.8 and incubated in aerobic condition at 37ºC without CO_2 for up to 72 hours. Plates were observed at 24 hours interval and growth were recorded and plates were photographed. Like 2.10.2a, 3 sets of plates were prepared and incubated at 37ºC with CO_2 for 72 hours. Microbial growths observed at regular interval of 24 hours, recorded and photographed for growth comparison and evidences.

Three sets of plates were prepared and incubated for 3 days at 37ºC using a 3.5 litre jar containing a CampyGen™ sachet (Fisher Scientific, UK) that create microaerophilic condition within the jar. A control plate with *Campylobacter jejuni* ATCC 3291 was placed in the jar to confirm the microaerophilic condition. Plates were checked after 72 hours incubations, growth recorded and photographed.

2.6 Optimisation Results

2.6.1 Comparison of Agar Medium

Growth on different medium were compared based on their nutritional supplements, atmospheric conditions, morphologically different colony type (MDCT), colonies counts to select the suitable medium for this study. GraphPad prism 7 statistical software was used to determine the significance or p-value. MDCT were determined based on their appearances on the agar plates and not by Gram staining or biochemical reactions and it was possible as I have more than twelve years experiences as specialist biomedical scientist (microbiology) in national health service, UK. Besides, aim of this study is to find new antagonistic microorganism but not new microbes, hence full identification of microorganisms were not carried out.

2.6.1a. SSNA versus NA

Soil sample were prepared as per the protocol and 50 µl of sample from each dilution were inoculated onto SSNA and NA plates in triplicates and colonies and their diversities were observed and recorded (Table 2.1).

Table 2.1: Comparison of triplicate tests of soil supplemented nutrient agar (SSNA) with nutrient agar (NA) (Oxoid). SSNA allowed significantly high number of Colony forming unit (cfu)/ml with p-value <0.0001 and significantly high number of MDCT with p-value 0.0139.

Culture medium	cfu/ml	MDCT
SSNA	4.44×10^6	19
	4.2×10^6	18
	4.02×10^6	14
NA	1.46×10^6	11
	1.36×10^6	9
	1.2×10^6	7

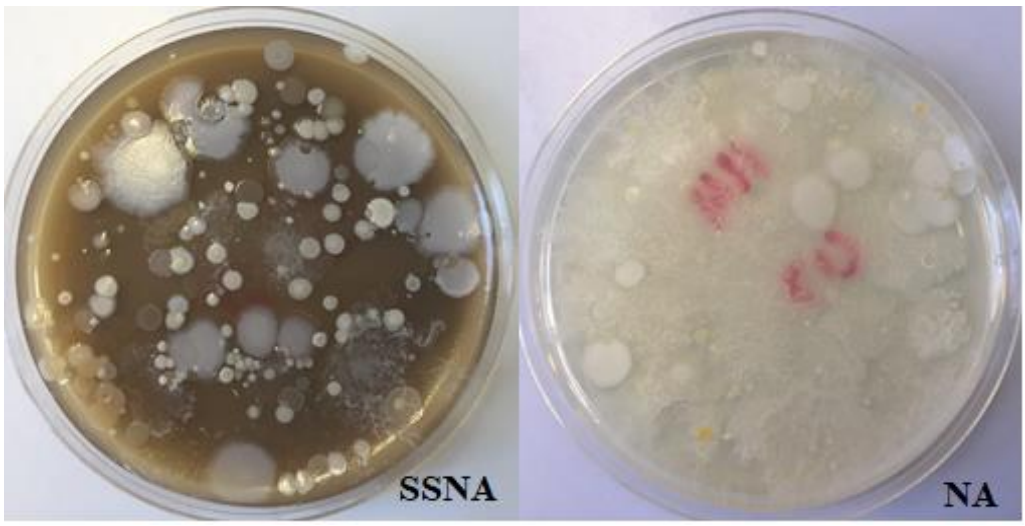

Figure 2.1: SSNA (on left) and NA (on the right) showing growth of different microorganisms and their colonies' morphologies.

2.6.1b. SSNA versus TSA

Microbial growth on SSNA and TSA plates at 37ºC after 72 hours aerobic incubation colonies counts (cfu/ml) and morphological different colonies types were compared given below.

Table 2.2: Colonies counts and morphologically different colonies types in SSNA and TSA. SSNA had significantly high number of cfu/ml with p-value 0.0021 and significantly higher number of MDCT with p-value 0.0132 compare to TSA.

Culture medium	cfu/ml	MDCT
SSNA	1.86×10^7	14
	1.72×10^7	11
	1.64×10^7	11
TSA	1.08×10^7	7
	9.6×10^6	7
	7.8×10^6	4

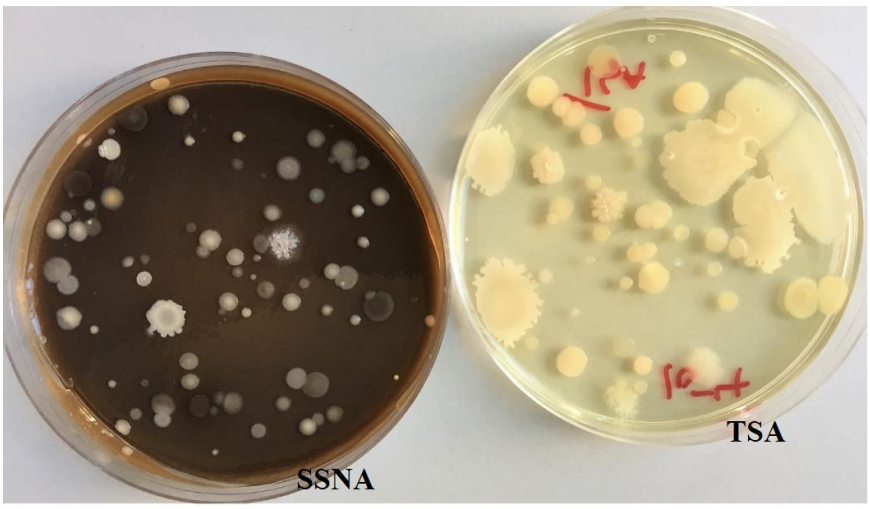

Figure 2.2: Growth of different microorganisms and their morphologies using SSNA (on left) and TSA (on the right)

2.6.1c. SSNA versus FSSNA

Microorganisms on SSNA and FSSNA plates showed following colonies counts and MDCT as shown in the table below.

Table 2.3: Colonies counts and morphologically different colonies types in SSNA and FSSNA. SSNA had significantly high number of cfu/ml with p-value 0.0065 when compared with FSSNA and had double number of MDCT in SSNA than FSSNA.

Culture medium	Cfu/ml	MDCT
SSNA	1.8×10^6	10
	1.72×10^6	10
	1.58×10^6	10
FSSNA	1.38×10^6	5
	1.32×10^6	5
	1.32×10^6	5

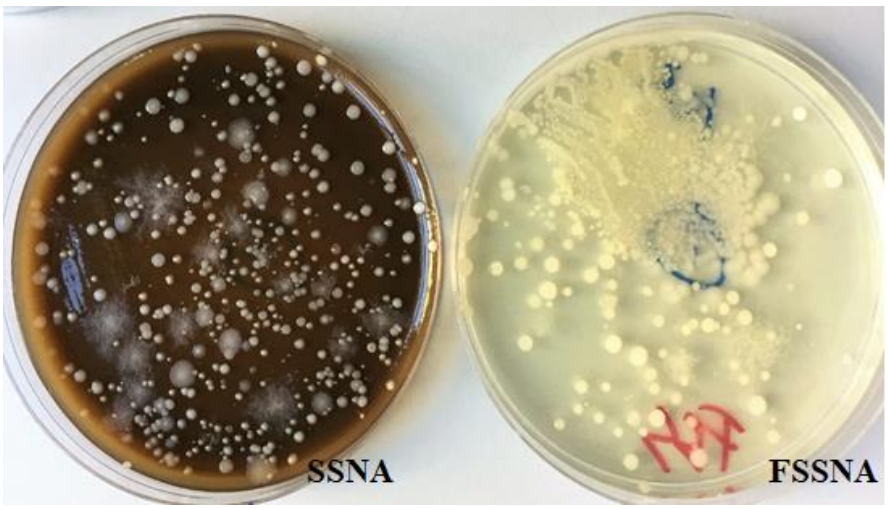

Figure 2.3: SSNA on left and FSSNA on the right showing growth of different microorganisms and their colony morphologies.

2.6.2 Atmospheric Conditions

The results of different atmospheric conditions for the growth and isolation of antagonistic microorganisms were studied. Plates were incubated in the aerobic incubator with 5% CO_2 and without CO_2 and in microaerophilic and anaerobic conditions. Triplicate plates were for all incubation conditions.

Table 2.4: Colony counts and MDCT on aerobic and microaerophilic conditions are given below in the table.

Culture medium	cfu/ml		MDCT	
	Aerobic	Microaerophilic	Aerobic	Microaerophilic
SSNA	4.44×10^6	8.4×10^4	19	4
	4.2×10^6	7.8×10^4	18	4
	4.02×10^6	7.4×10^4	14	4

Table 2.5: Colony counts and MDCT on aerobic and anaerobic conditions are given below in the table.

Culture medium	cfu/ml		MDCT	
	Aerobic	Anaerobic	Aerobic	Anaerobic
SSNA	4.44×10^6	4×10^4	19	1
	4.2×10^6	3.4×10^4	18	1
	4.02×10^6	3.2×10^4	14	1

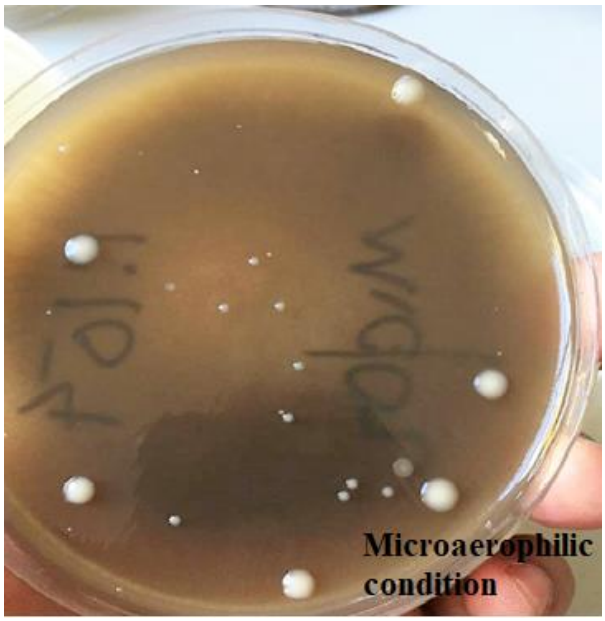

Figure 2.4: Microbial growth on SSNA plate in microaerophilic condition.

Figure 2.5: SSNA plate showing the growth of microorganisms in anaerobic conditions.

2.6.3 Combined results of all medium and culture conditions

Microbial yields varied from soil to soil including their MDCT. Overall performance of the culture medium can be summarised in the graph below.

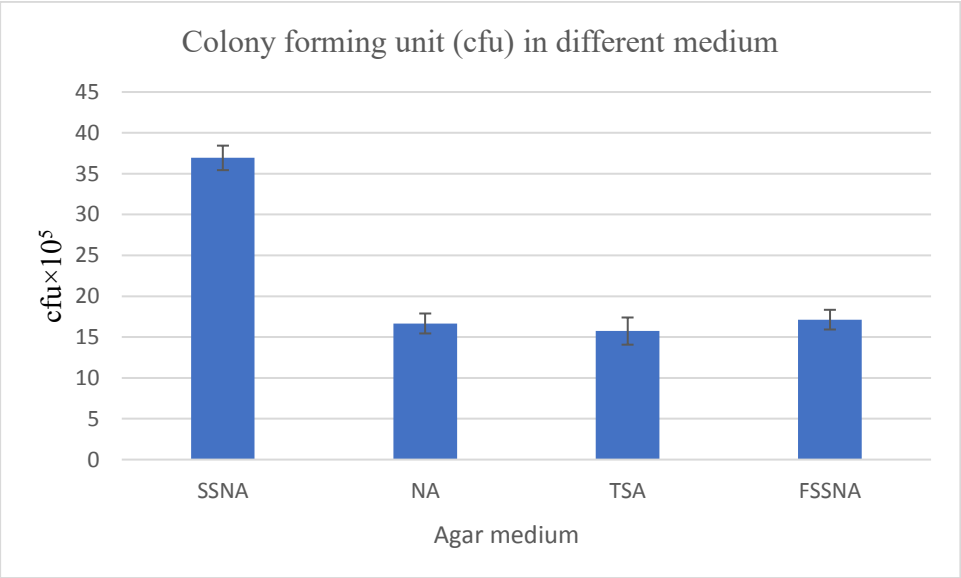

Figure 2.6: Effect of culture medium in colony forming unit per millilitre. Mean of triplicate plate sets data with error bars. SSNA had more than double the cfu/ml.

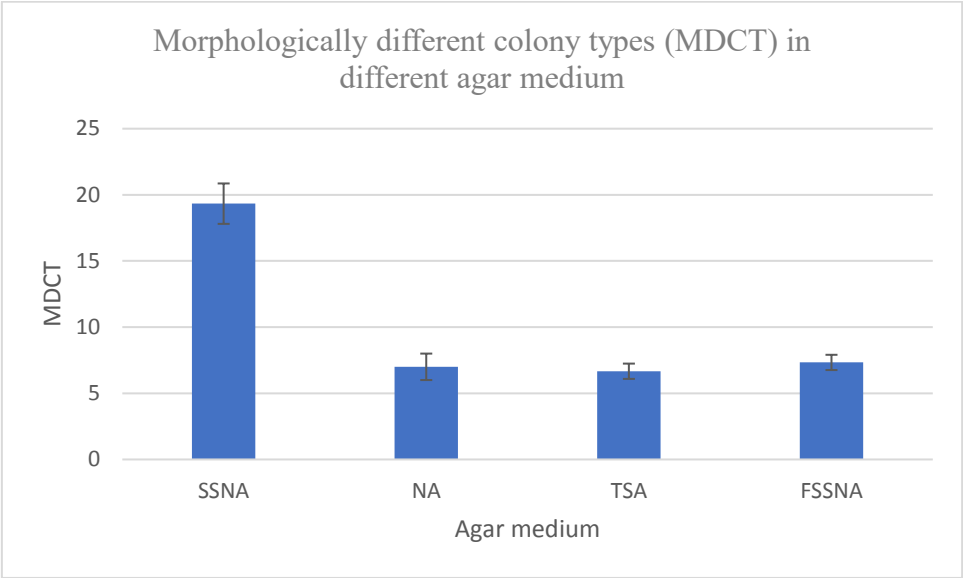

Figure 2.7: MDTC in different agar medium. SSNA had more than double the number of MDCT compared to NA, TSA and FSSNA.

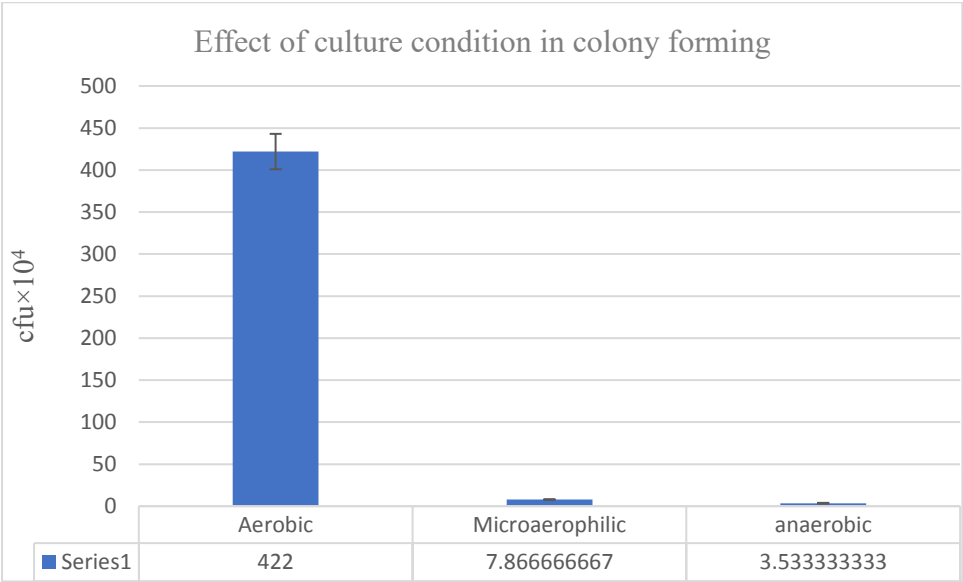

Figure 2.8: Different culture condition had different microbial yields. Aerobic condition had significantly high colonies counts with two log difference compare to microaerophilic and anaerobic conditions.

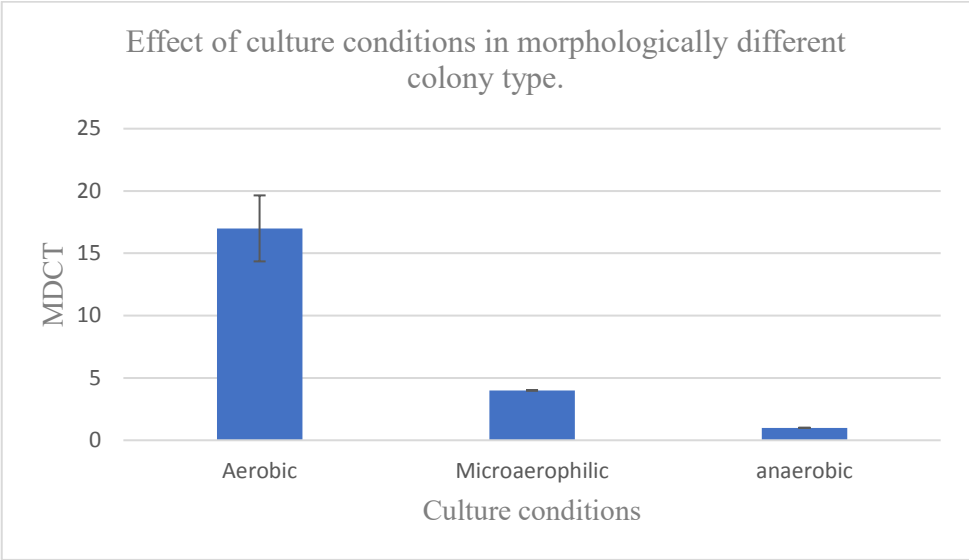

Figure 2.9: MDCT in different culture condition. Aerobic culture had higher MDCT compare to microaerophilic and anaerobic condition.

Table 2.6: Overview on effects of medium in cfu/ml and MDCT are given in the table below (means of triplicates).

Culture medium	Cfu/ml	MDCT
SSNA	36.93×10^5	19.33
NA	16.66×10^5	7
TSA	15.73×10^5	6.66
FSSNA	17.13×10^5	7.33

2.7 Conclusion of the optimisation procedure

All the medium were tested using soil samples from different sites and SSNA medium showed superiority in terms of cfu/ml and MDCT compared to NA, TSA and FSSNA in all cases. Agar medium were tested in pair and in combined with all medium. Data were analysed using GraphPad prism (version 7) statistical software. Study found that SSNA had significant high number of cfu/ml with p-value <0.0001 and significantly high number of MDCT with p-value 0.0139 compare to NA. When SSNA compared with TSA, high number of cfu/ml were seen with significant p-value 0.0021 and significantly higher number of MDCT with p-value 0.0132 were also seen in SSNA. SSNA had significantly high number of cfu/ml with p-value 0.0065 when compared with FSSNA and had double number of morphologically different colony type per plate in SSNA than FSSNA. Although cfu/ml were in same order that is no log difference, but p-value of cfu/ml were significant (<0.05) in SSNA compare to all medium. MDCT were not identified as individual species but categorised based on their colonial appearances on the agar medium. However, a Gram stain could have given basic microbial morphological identifications of those MDCT.

SSNA also produced differential colonies and when subcultured those colonies onto NA, FSSNA and TSA, they were unable to grow but grew only on SSNA subculture (figure-3.7). This means, SSNA supported growth of certain microorganisms which couldn't supported by NA, TSA and FSSNA. Beside it, FSSNA might have failed to support the growth as filtration might have retained certain macromolecule present in the soil sample which were essential for the growth of those organisms which formed colonies on SSNA or soil itself might play key role supporting the growth of those organisms. FSSNA showed no advantage over NA and preparation of FSSNA was time consuming and costly. Hence FSSNA was not used for further studies. Other studies showed soil extracts enabled to isolate previously uncultivated microorganisms (Hamaki et al., 2005; Nguyen et al., 2018). So, SSNA was selected as the main culture medium for this study.

Chapter 3: iChip Optimisation, Materials and methods for iChip Device

3.1: Introduction to iChip

iChip (isolation chip) was described as a technical device for cultivating previously uncultivated microorganisms within its natural environment (Nichols et al., 2010). To overcome the limitations of laboratory cultivation, the iChip utilises a diffusion chamber enabling access of components from their natural environment and thus provide essential growth factors for the microbes growing on the iChip. iChip showed growth of novel microbial species and helped to find new antibiotic (Ling et al., 2015).

Key advantages iChip are, a simple methodology to follow to build the device and their miniature chambers are designed to accommodate 1 cell per chamber to produce pure colony type and does not require sub-culturing for purity. 1 cell per well is determined by initial cells count and degree of dilution that gives 1 cell per 100 µl which is an approximate amount of agar for each well. Another advantage is using of 0.03µm membrane that prevent culture contamination from soil.

Disadvantages of iChip devices are, materials used in this methodology are not readily available from commercial sources and iChip was designed and developed by research team for their sole uses and 0.03µm membrane is not a common membrane type available from scientific manufacturers.

3.2 Concept of the Device

iChip is consist of a central plastic plate with 100s of miniature diffusion chambers and 2 metal plates to hold the central plate securely (Figure 3.1). Central plate is inoculated by dipping the plate into liquid agar medium and allowed to solidify. 0.03µm pore size membrane is then securely attached using glue on both top and bottom of iChip plate. Membrane inhibits microbial cell movement in or out of chambers but allowing only nutrients to pass though. The iChip is then left buried in the sampling site for microorganisms to grow on their own natural habitats for a prolonged period (Nichols et al., 2010).

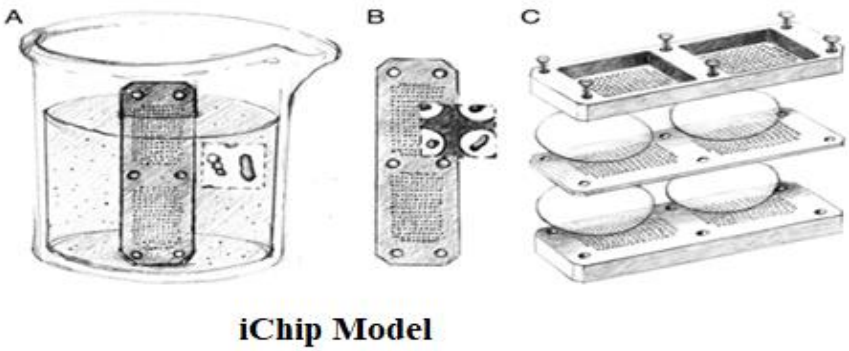

Figure 3.1: Original iChip model for *in situ* microbial cultivation (Nichols et al., 2010). (A) prepared iChip by dipping into unsolidified agar containing sample of interest, (B) iChip after inoculation that showed average 1 cell per chamber and (C) shows how iChip was assembled.

A protocol was published (Berdy et al., 2017) for alternative methods of constructing iChip devices and keeping original principle of *in-situ* cultivation. In this protocol, researchers used pipette tip rack for the construction of iChip devices. This variation provided the inspiration utilised during this current study.

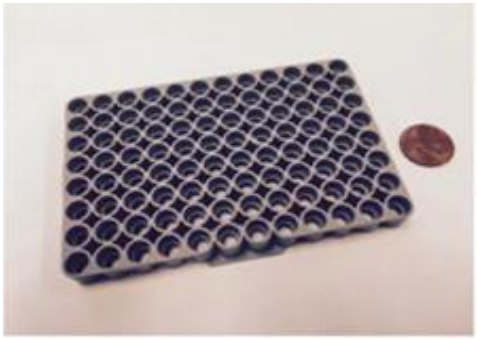

Figure 3.2: Example of pipette tip rack used to build iChip in the published protocol (Berdy et al., 2017).

3.3 Determination of Inoculation Volume for iChip for This Study

In original iChip method cell count was performed for every sample type to determine the inoculation volume. Unlike original study, to determine the inoculation volume for this study, every sample was cultured using a titration to determine the dilution factor

producing semiconfluent growth. This dilution was further diluted two-fold and 2 iChip devices were inoculated with 200 µl in each well.

3.4 iChip Devices Optimisation (Using Tip Rack)

Initial iChip devices were made according to the published protocol (Berdy et al., 2017). One pipette tip rack was taken and a polycarbonated membrane, pore size of 0.03 µm (Whatman, USA), was glued at the bottom of the pipette tip rack using silicon glue (Master Tradesman, USA) and allowed to dry for 30 minutes. Once polycarbonated membrane was dried and secure to tip rack, a 200 µl agar containing soil sample was added to all the wells in tip rack leaving the bordering wells empty which were inoculated with agar without soil sample as sterility control. Allowed the agar to solidify and second polycarbonated membrane was glued on the top of the tip rack. Three more iChip devices were made like wise. All four devices were then buried at the sample collection site around 15 to 20 cm below the surface. For this study optimisation purposes, two iChip devices were incubated for two weeks and other two devices for four weeks. Both microbial growth and devices stability were checked and recorded after appropriate incubation time.

A second type of iChip devices were made using 3 tip racks. In this method, 1 piece of polycarbonate membrane was glued at the bottom of the rack along with another tip rack using silicon glue to secure and support the membrane from any damage from rocks and other debris inside the ground. After inoculating the iChip device, a second polycarbonate membrane is place on the top of the tip rack and glued along with another rack on the top to protect the membrane and thus three more devices were made. Like first type, two iChip devices were incubated for 2 weeks and other two devices for 4 weeks. After incubation periods, devices were retrieved, checked for their stability, microbial growth and recorded findings for optimisations

3.5 Device Optimisation (Tip rack and 96 Well Plate)

Third type of iChip devices were made using tip racks and standard flat bottom 96 wells plates (Thermo Fisher Scientific, UK). Ninety-six well plates were inoculated like first device type. When agar was solidified in the wells, it was the covered with polycarbonate membrane and glued together with another tip rack on the top. Four devices were prepared for the optimisation studies. Two iChip devices were incubated for 2 weeks and other

two devices for 4 weeks. All the devices were buried at the sample collection site. Devices were retrieved after completing their incubation times.

3.6 Retrieval and Cleaning of the iChip

First sets of iChips (all three types) were retrieved after 2 weeks of incubation and brought back to UEL laboratory. iChips were placed in a clean tray inside the class-1 biological safety cabinet and mud and debris were removed carefully and cleaned using sterile distilled water. Tip racks were then removed slowly ensuring no damaged to the membrane. They were further washed with sterile water until all the mud was removed from iChips and membranes were then removed. Growth was checked in every well with naked eyes and using optical microscope with magnification of ×40. Any physical damage to the membranes or devices were visually checked and any contamination to the uninoculated agar wells were recorded (Figure 3.5).

3.7.1 Sub-Culturing iChip Growth in SSNA and NA

Agar from the wells were removed using sterile cocktail sticks and transferred into small bijou containing 3ml sterile phosphate buffer saline (PBS) and labelled with corresponding well number (A1, A2). Colonies from the agar were released into the PBS with cocktail sticks and by vortex. These suspensions were inoculated using sterile 1 µl plastic loop onto SSNA and NA plates labelled with corresponding well numbers. Plates were then incubated aerobically for 72 hours and checked them in every 24 hours interval.

3.7.2 Crowded plate technique

Crowded plate technique remains as a primary tool to screen soil microorganisms for their ability to produce antibiotic (Bavishi et al., 2017). This technique is both simple provides a rapid approach where organisms are allowed to grow confluently or semi-confluently on agar plates and organisms that produce inhibitory zones of inhibition around, termed as antagonistic microorganisms (Sambamurthy and Kar, 2006), are used for further analysis. Subcultures growth from the agar plugs removed from iChips were emulsified in PBS and cultured onto SSNA plated using crowded plate method to detect antagonistic microorganisms. Any organism that showed a clear round zone around it was sub-cultured again to make a pure culture of that specific organism for further antimicrobial testing.

3.8 Results

3.8.1 iChip using tip racks

iChip devices prepared using single pipette tip rack as per published protocol (Berdy et al., 2017), were all found to be damaged upon retrieval and were contaminated with mud and other debris (Figure 3.4).

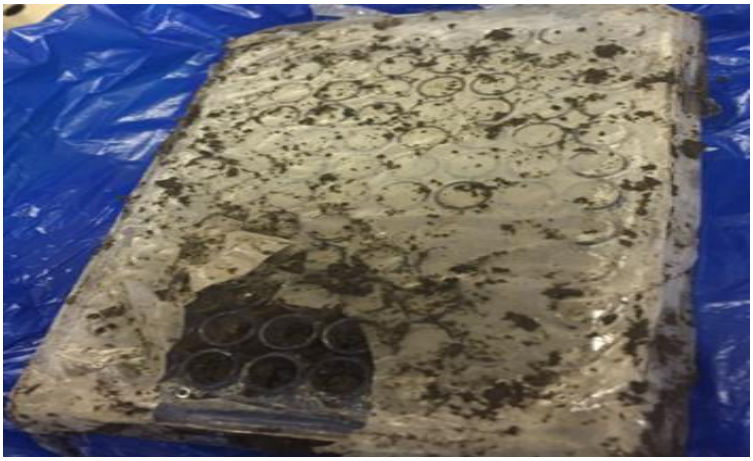

Figure 3.3: Single tip rack iChip. Damaged membrane and contaminated wells were seen in single tip rack iChip devices.

iChips having a support from top and the bottom using similar tip racks (triple) had no damage to the membranes or evidence of environmental contamination.

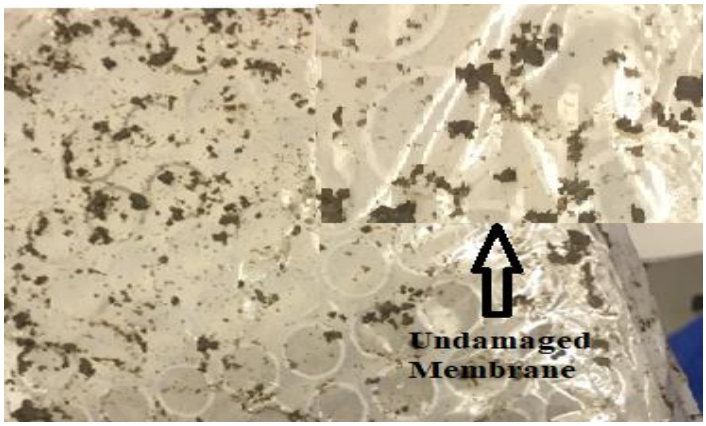

Figure 3.4: iChip with tip rack support. Undamaged membranes were seen in ichip devices using both triple racks and 96 well plate with tip rack support.

3.8.2 iChip using 96 well plate

Similarly, iChips using 96 well plates membranes were found firmly attached to the plate and had no contamination to the wells (Figures 3.4 and 3.4).

3.8.3 iChip sterility

Growth on the bordering wells of the 96 well plates checked both in naked eye and using optical microscopy with magnification ×40 which were loaded with agar and left uninoculated to check the iChip sterility. iChips made using 96 well plates and triple racks were found sterile in uninoculated wells and membranes were undamaged (Figure 3.5). Column C on Figure 3.6 illustrates the control column with no microbial growth and T columns were the test wells where there was microbial growth.

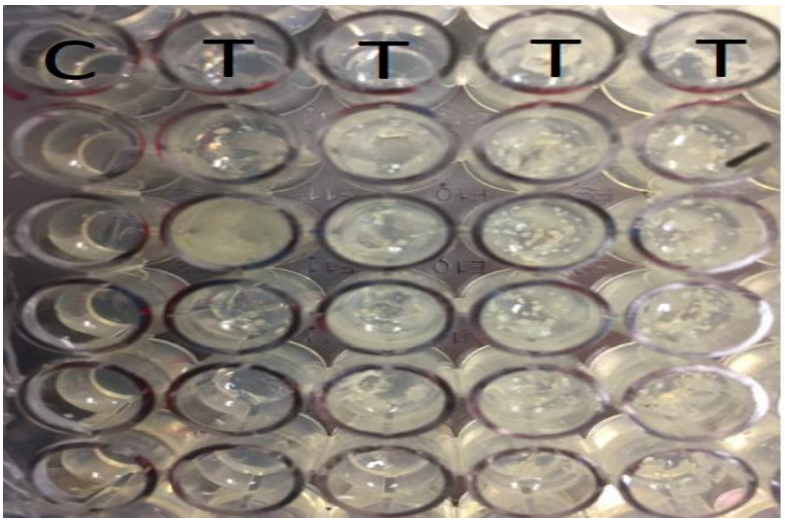

Figure 3.5: iChip sterility. Figure shows no growth on C (control) column and growth on T (test) column. Wells in the column C were inoculated with agar without sample.

3.8.4a Growth from iChip on Subcultured Medium

Growth from iChips were subculture onto NA and SSNA to compare their suitability as subculture medium. Certain organisms on NA suppressed the growth of other organisms and grew beyond their inoculation mark (Figure-3.6, A&B). On the other hand, certain organisms did not grow on their corresponding wells (marked as NG) on NA. Subculture on SSNA plates showed uniform growth and more consistent growth from the wells (Figure-3.6, C&D). Study believes that those organisms were unable to grow due to lack of essential nutrient on the NA medium which were present in SSNA from the soil source.

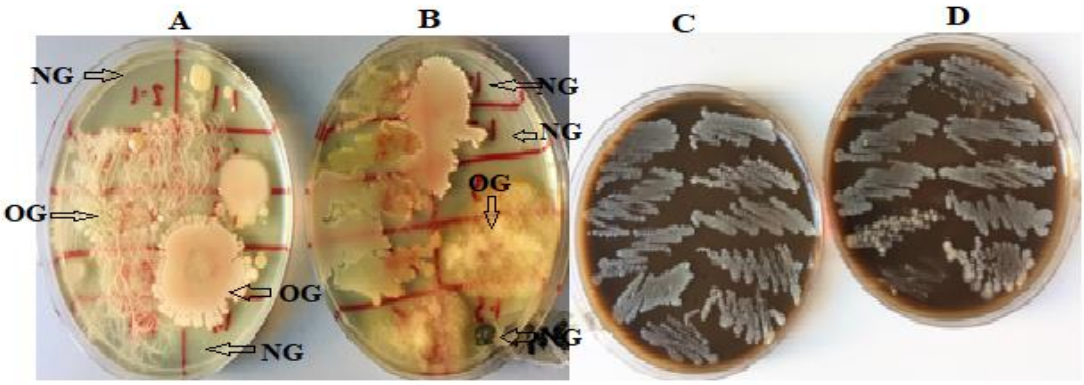

Figure 3.6: iChip subcultures growths comparison on NA (AB) and SSNA (CD). No growth (NG) and overgrowth (OG) (as marked in AB) of certain microorganisms were seen in NA. SSNA showed growth from all the wells.

3.8.4b Differential growth on the medium

When growth from the iChips wells were subcultured onto SSNA and NA medium, 90% on SSNA and 50-60% on NA formed colonies from iChips subcultures. In some cases, organism showed differential growth on SSNA and they were unable to on a medium without soil supplement. Organisms those grew only on SSNA were again subcultured onto SSNA and NA plates to recheck their growth and similar growth was seen (figure-3.7). This study believes that soil enriches the medium for certain organisms.

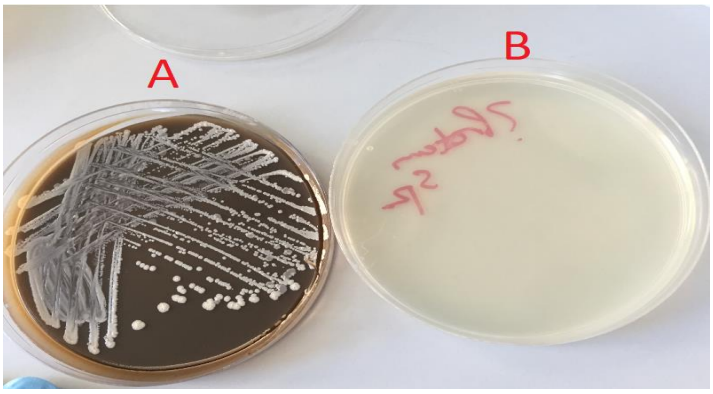

Figure 3.7: Differential growth. Organism showed differential growth, grew on SSNA (A) but not on NA (B).

3.8.4c Crowd plate for microbial antagonism

Microbial growth from subcultures were emulsified into bijou a containing 3 ml PBS and 50 µl PBS was inoculated and spread for crowd plate methods and incubated for 48 hours. Antagonistic microorganisms were seen growing with a small zone around them (circled in red, figure 3.8). Antagonistic microorganisms were then subcultured to get a pure culture for further investigations.

Figure 3.8: Microbial antagonisms. Microorganisms (circled red) showing small zone due to their antagonistic behaviour.

3.9 Summery of iChip Optimisation

During optimisation, iChip construction, subculturing and screening for antagonistic microorganisms were evaluated. Initial iChip construction suffered through issues of membrane integrity and contamination. Subsequently, iChip devices using triple racks and 96 well plate covered with rack, overcame these design difficulties. However, iChip devices using 96 well plates were easier to handle and economic. One membrane was needed to cover the top of the 96 well whereas 2 membranes were needed to cover on both top and the bottom in triple racks iChip devices.

"Domestication" of iChip growth was crucial for further investigation. Term domestication means any growth previously uncultivated requires maintaining for their cultures for further analysis. SSNA plates were used for subculturing the microorganisms for domestication purposes as SSNA supported growth from 90% wells (Figure 3.6). From this optimisation study it was concluded to use iChip devices constructed using 96 well plates and SSNA medium for subsequent investigations.

Chapter 4: Screening for Antagonistic Microorganisms

4.1 Introduction to the screening for antagonistic microorganisms

Most of the antibiotics currently in clinical uses come from soil microorganisms; for example, Streptomycin from *Streptomyces griseus,* Cephalosporins from *S. clavuligerus*, Bacitracin from *Bacillus licheniformis*, Polymyxin from *Bacillus polymyxa*, Chloramphenicol from *S. venezuelae*, Tetracycline from *S. aureofaciens*, Erythromycin *Saccharopolyspora erythraea*, Gentamicin from *Micromonospora purpurea*, Mupirocin from *Pseudomonas fluorescens* and more (de Lima Procópio et al., 2012).

4.2. Screening for antagonistic microorganisms

For this research, soil samples were collected from various geographic location in London, Hertfordshire and Southend, UK. Locations were chosen either based on the soil characteristics or natural environments. Permissions was requested in writing from national nature reserves sites such as Burnham Beech, Buckinghamshire, Aston Rowan, Oxfordshire, Ruislip Woods, Greater London and Epping forest, Essex to utilises their sites for this research project. Permission was granted from Ruislip Woods and Epping forest sites. Request letters were also sent to friends, family members and colleagues who lives various part of the UK and have different soil types for example soil from my supervisor's garden was chalky and soil from my nephew's garden in Southend on Sea was sandy. Two or more samples were collected from the random places within the permitted sites from the surface and sub-surface layers up to 20 cm depth from the surface for the screening of antagonistic microorganisms. Using a clean trowel approximately two to three hundred grams soil samples were collected in sterile containers to transport the samples to the laboratory.

4.2.1 Soil sample pH determination and cultivation

All the soil samples collected were checked for their pH level using a pH meter (HI 2210 pH meter, Hanna instruments, UK). Soil data for this study, especially, soil types and plant species around the soil were collected from published sites on UK soil types ("BBC - Gardening - How to be a gardener - Know your plot - Soil types," 2014; "Soilscapes soil types viewer - National Soil Resources Institute. Cranfield University," 2019). All

the samples were cultivated on both SSNA and iChip (Chapter 2; section 2.6 and Chapter 3; section 3.4).

4.2.2 Cultivation on Soil Supplemented Nutrient Agar (SSNA)

All samples were initially cultivated onto SSNA plate and incubated for 24 hours to determine dilution factor semi-confluent growth. Different soil types had a different colony forming units (cfu) per ml, hence it was important to determine dilution factor before inoculating onto iChip as iChip had higher cfu counts per ml compared to SSNA (Chapter 3; section 3.3).

4.2.3 Cultivation on iChip

Initially all soil samples were cultivated onto SSNA plates and incubated for 24 hours to determine semi-confluent growth for each soil samples types and their corresponding dilution factors for semi-confluent growth. Once dilution factor of a sample for semi confluent growth were determined, samples were then further diluted to next two-fold and inoculated the iChips. Thus 4 iChips per samples were made and 2 iChips were incubated for 2 weeks and other 2 iChips for 4 weeks. iChips were retrieved after completing their incubation periods and growths from iChips were assessed as described in section 3.7.1 and screened for antagonistic microorganisms as of section 3.7.2.

4.3 Isolation of antagonistic microorganism

Isolation of antagonistic microorganisms were achieved by crowded plate techniques (chapter-3.7.2). Any organism that showed any zone of inhibition was subcultured onto SSNA and incubated for 24 hours. Antagonistic microorganism was then tested against *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 for zones of inhibition. Two Mueller Hinton agar (MHA) plates were seeded with *S. aureus* ATCC 25923 or *E. coli* ATCC 25922 and test organism was applied by making a cross on the plates. Plates were incubated for 24 hours. One such organism was isolated using this method (figure 4.1).

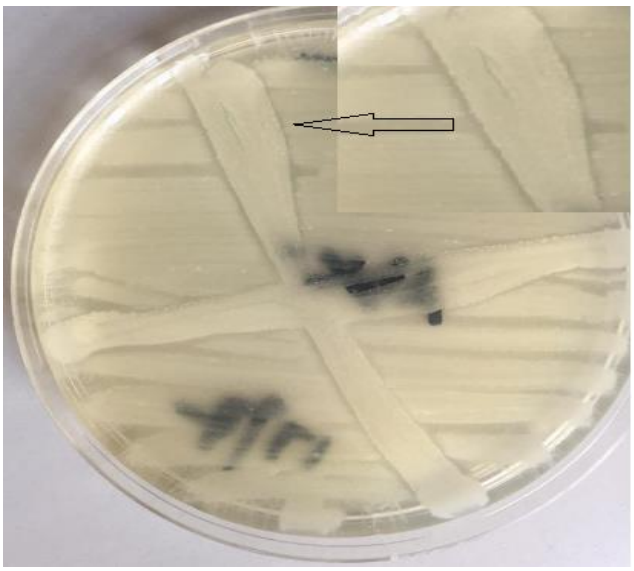

Figure 4.1: Test organism was seen producing clear zone along its growth on *S. aureus* plate.

4.4 Identification of the antagonistic organism

Once organism was found antagonistic, it was identified by morphology, cultural characteristics, biochemical reactions and MALDI ToF (Bruker Daltonic, Germany).

4.4.1 Identification based on morphology and cultural characteristics

Initial Gram stain was performed to identify the morphology of the organism and revealed Gram positive bacilli. To determine the cultural characteristics of the organism, it was sub-cultured onto nutrient agar (NA) and blood agar (BA) (Oxoid, UK) incubated aerobically and anaerobically for 24hours and colonies only formed in aerobic condition.

Morphology on NA (grey) and BA (off-white β-haemolytic) showed 2mm opaque round colonies that were dry looking having a rough margin. Catalase test on the colonies using H_2O_2 (3% H_2O_2, Sigma-Aldrich, UK) showed catalase positive reaction, oxidase test and DNase tests (Oxoid, UK) were negative. Spore staining showed endospores of *Bacillus* species in both Malachite green and Methylene blue spores staining (PHE, 2018).

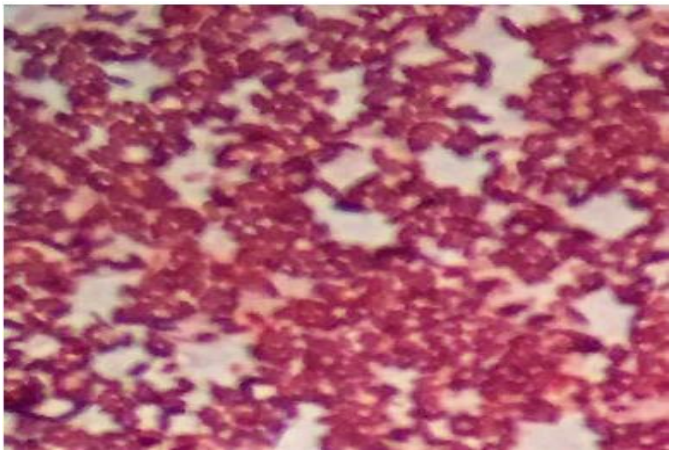

Figure 4.2: Gram stain showed short Gram-positive bacilli.

Analytical profile index (API) 20E (Biomerieux) was used to generate biochemical and sugar utilisation profile as describe in literature (Logan and Berkeley, 1984; Logan and Vos, 2015; O'Donnell et al., 1980). In API 20E organism had positive reactions on citrate, Voges-Proskauer, gelatine, glucose, mannitol, sucrose and arabinose and very weak positive reactions on ONPG (ortho-Nitrophenyl-ß-galactoside) and arginine which were considered as equivocal reactions and rest of them were negative and these biochemical results suggest the identity of *Bacillus* species.

4.4.2 Identification by Matrix Assisted Laser Desorption Ionisation Time of Flight, Mass Spectrometry (MALDI-TOF, MS)

MALDI-TOF MS is a very reliable and cost effective way to identify microorganism (Singhal et al., 2015). For this study, MALDI-TOF MS (BRUKER) was used as the main tool to identify the antagonistic microorganism. Two smears of thin film of freshly grown (24 hours) organism were made onto the MALDI target plate and allowed to dry. It was then covered with one µl HCCA (α-Cyano-4-hydroxycinnamic acid, Sigma-Aldrich) matrix solution within 1 hour and air dried. Two separate smears were done for *Escherichia coli* ATCC 25922 as a control as per standard protocol of MALDI quality control. Using Compass software data were input, and target plate was loaded into the MALDI analyser. Initial samples were processed by the direct method (Wunschel et al., 2005). Identification together with confidence scores were obtained (table-4.1) and interpreted as shown in table-4.4. Based on the initial results both extended direct transfer

and formic acid extraction methods (see appendix) were used for further identification and confirmation by MALDI (table 4.2 and 4.3).

Table 4.1: Direct method result of the antagonistic organism by MALDI-TOF.

Organism (best match)	Score Value	Organism (second-best match)	Score Value
Escherichia coli	2.23	Escherichia coli	2.19
Escherichia coli	2.24	Escherichia coli	2.17
Bacillus pumilus	1.8	No Organism Identification Possible	1.46
No Organism Identification Possible	1.53	No Organism Identification Possible	1.42

Table 4.2: Extended direct transfer method results of the antagonistic organism by MALDI-TOF.

Organism (best match)	Score Value	Organism (second-best match)	Score Value
Escherichia coli	2.311	Escherichia coli	2.298
Escherichia coli	2.299	Escherichia coli	2.291
Bacillus pumilus	2.093	Not reliable identification	1.65
Bacillus pumilus	2.15	Bacillus pumilus	1.891

Table 4.3: Formic acid extraction results of the antagonistic organism by MALDI-TOF.

Organism (best match)	Score Value	Organism (second-best match)	Score Value
Escherichia coli	2.411	Escherichia coli	2.369
Escherichia coli	2.407	Escherichia coli	2.395
Bacillus pumilus	2.391	Bacillus pumilus	2.379
Bacillus pumilus	2.372	Bacillus pumilus	2.365

Table 4.4: Meaning of score values and colour codes in MALDI identification system which are self-explanatory.

Range	Description	Symbols	Colour
2.300 ... 3.000	highly probable species identification	(+++)	Green
2.000 ... 2.299	secure genus identification, probable species identification	(++)	Green
1.700 ... 1.999	probable genus identification	(+)	Yellow
0.000 ... 1.699	not reliable identification	(-)	Red

4.5 Discussion on Organism's Species Identifications

MALDI-TOF identified the organism as *Bacillus pumilus* by all three methods. A duplicate smear was done in all three methods and *E. coli* ATCC 25923 strain was used as control. On initial direct method, score values for the identification was *B. pumilus* 1.8 having a low identification confidence. Improvements for identification score values from extended direct transfer methods and formic acid extractions for *B. pumilus* were 2.093 and 2.391 respectively giving high identification confidence by Bruker's manual (MALDI Biotyper CA System).

Bacillus pumilus is a spore forming Gram positive bacillus that produces beta haemolytic colonies on blood agar (Logan and Vos, 2015). It is catalase, oxidase and DNase negative, resistance to Penicillin (Liu and Jurtshuk, 1986; O'Donnell et al., 1980; Parvathi et al., 2009).

Cultural, morphological information from Gram stain and spore stain, physiological and biochemical characteristics and MALDI identifications by all three methods confirmed identification of the antagonistic organism as *B. pumilus*. This organism was initially isolated from the chalky soil of this research project supervisor Professor Sally Cutler. *Bacillus pumilus* was also found on two other samples collected from Dagenham area of London with same characteristics.

Chapter 5: Fermentation of antagonistic microbes for their metabolites

5.1 Introduction

Fermentation media optimisation is essential for the growth of microorganism and their primary and secondary metabolites productions. Microorganisms produces various types of secondary metabolites including antibiotics (Malik, 1980). Carbon sources, nitrogen sources, pH, temperatures, incubation periods, agitation, aeration, nutrition are the most essential components in the fermentation media requires optimisation to stimulate the secondary metabolites production for example antibiotics (Cheng et al., 2015; Dubey et al., 2008; Marwick et al., 1999; Singh and Rai, 2012).

5.2 Fermentation methods

Bacillus pumilus was found as antagonistic organism for this study. Conical flask and Duran bottles were used for optimisation of fermentation process in this study to see the differences in container types as this study did not used any fermenters. Two hundred ml of nutrient broth (with 5% glucose) were prepared into 2×conical flasks (500ml), closed with cotton wool and 200 ml of nutrient broth into 2×Duran bottles (500ml) and autoclaved at 121º C for 15 minutes. They were then allowed to cool at room temperature and each flask and bottle were inoculated with 1 ml of 1 McFarland turbid microorganism suspension and incubated up to 7 days. After 7 days incubation fermentation broths were checked for inhibitory metabolite production. Twenty ml of fermented medium were taken into 50 ml universal tube from both bottles and flasks and centrifuged for 15 minutes at 3000 rpm. Supernatant were then tested by the disc diffusion method against *S. aureus* ATCC 25922 and *E. coli* ATCC 25923 and checked for any zone of inhibition.

5.3 Fermentation with and without aeration

As the test organisms was an aerobe, aeration is one of the key factor requires optimisation in fermentation depending on the organism type (Hentges, 1996; Wang and Zhang, 2007). To facilitate the aeration of fermentation media, Duran bottles were loosely capped placing a 0.2 µm pore size filter paper to prevent contamination, whereas Duran bottles and flasks were tightly closed and sealed with parafilm to make stop aeration. Cultures were then incubated for up to 7 days checked for zone of inhibition (chapter-

5.2). Fermentations without aeration were unable to produce any zone of inhibition whereas one with aeration produced zone of inhibition against *S. aureus* ATCC 25922.

5.4 Fermentation with and without agitation

Agitation helps mixing the organisms and temperature throughout the fermentation media (Smith et al., 1990), and has been shown to enhance the fermentation process (Maxon, 1959; Singh et al., 2017; Smith et al., 1990; Wang and Zhang, 2007). To determine if agitation would benefit production, fermentation broths were compared using a shaker incubated at 37º C for 7 days or static aerobic incubator at 37ºC for 7 days. After 7 days incubation, broths were tested for antibiotic production as described in 5.2. Inhibitory zones (9 mm) were seen using *S. aureus* ATCC 25922 in both cases, i.e. with or without agitation.

5.5 Fermentation with different glucose concentrations

Carbon source plays an important role in microbial growth and is an essential part of the growth medium to produce primary and secondary metabolites. Moreover, in many instances carbon sources enhances the biomass formation of primary and secondary metabolites and hence the antibiotic production (Marwick et al., 1999). Not all carbon sources work in the same way and vary in production of secondary metabolites, for example glucose interferes with penicillin production (Sanchez and Demain, 2002), but favours bacilysin production from *Bacillus* species (Ozcengiz et al., 1990), on the other hand, lactose favour penicillin production (Rokem et al., 2007). In this study, 0.5%, 1% and 5% glucose were added to the fermentation nutrient broth and antimicrobial metabolites production were checked as described in section 5.2. No zone of inhibitions was seen in broth without carbon source or in 0.5% glucose, but zones of inhibition were seen in both 1% and 5% glucose contained fermentation broth. Zones of inhibition (9 mm) were only seen using *S. aureus* ATCC 25922.

5.6 Fermentation with addition of nitrogen sources

Like carbon, nitrogen sources thought to influence antibiotic production and different nitrogen sources have been used in fermentation media and vary in their effects (Singh et al., 2017). For example, ammonium triggers antibiotic production by *Streptomyces griseofuocus* whereas suppress the isopenicillin antibiotic production by *Actinomycete*

species (James et al., 1996; Junker et al., 1998). Amino acid also used as nitrogen sources and play important role for certain types of antibiotic production (Marwick et al., 1999). Role ammonium as a nitrogen source was tested as it was readily available in the laboratory, other nitrogen sources were not tested due to lack of budget and study duration. Three different ammonium concentration, 0.5%, 1% and 5% were added to the fermentation media and for this study. After 7 days, metabolites productions were checked as described in section 5.2. Zones of inhibition (9 mm) were seen without addition of ammonium and in 0.5% and 1% of ammonium, but not with 5% ammonium.

5.7 Determination of optimum pH for fermentation medium

Different organisms require different level of pH for their growth and metabolite production. Production of inhibitory zones were tested at pH levels of 3, 5, 7, 9, 11 and evaluated for antibiotic production. pH level was adjusted using HCl and NaOH for acidic and basic pH respectively prior to inoculation and incubation for 7 days, using 200 ml of fermentation media in both conical flasks and Duran bottles. Zones of inhibition were checked as described in section 5.2. No zones of inhibition were seen in pH 3 and 11and 9 mm clear zone were observed for pH 5, 7 and 9.

5.8 Discussion

Bacillus pumilus is an aerobic organism (Logan and Vos, 2015) and found to produce antibiotic only in the aerobic incubation and no antibiotics production in anaerobic condition. *Bacillus pumilus* forms spore in anaerobic condition, hence primary and secondary metabolites are not produced. No effect of agitation seen in this experiment as *B. pumilus* was able to produce antibiotic with or without agitation. *Bacillus pumilus* is highly motile organism (Logan and Vos, 2015) and this could be the reason they did not require any agitation as they were very motile within the fermentation media.

Carbon sources (1%-5%), pH between 5 to 9 and incubation temperature 30º to 40º C in aerobic condition were key in the production of secondary metabolites. Thus, a final fermentation compositions and conditions were determined from this optimisation and used throughout this experimental process for metabolite production.

Chapter 6: Extraction of metabolites, their activities and structure elucidation

6.1 Introduction to the Extraction

Metabolite extractions were done after 7 days incubation of the fermentation broth with 5% glucose at 37ºC under aerobic conditions without agitation. Twenty ml *B. pumilus* fermented broth were collected in a separate 50 ml universal container, spun down at 10000 rpm for 10 minutes and supernatant was collected for disc diffusion test and to compare with organic solvent extracts. Rest of the broth were filtered through a fresh piece of cotton wool as instructed by Dr Rahman, UEL, through personal communication and extracted using organic solvents. Extractions and supernatant were then tested for antimicrobial activities.

6.2 Selection of Solvents

Antibiotics are type of secondary metabolites produced by the microbes. Such secondary metabolites are generally extracted with organic solvents (such as dichloromethane, butanol, methanol) from the fermentation culture filtrates. Selection of solvents are key to isolate target metabolites or compounds as they might have different interactions in polar and nonpolar solvent and can lead to failure of complete or partial isolation. Prior to extraction, solubility of the antibiotic in organic solvent is a very important factor for purification by solvent extraction process. If metabolites are highly soluble in a convenient water immiscible organic solvent, they can be easily extracted from the culture filtrate. To determine the extraction capability of the metabolites, organic solvents such as ethyl acetate, butanol and methanol (from nonpolar to polar) were sequentially used and evaluated using *S. aureus* ATCC 25922 and *E. coli* ATCC 25923.

6.3 Ethyl Acetate Extraction

Filtered broth (200 ml) was taken onto a 500 ml separation funnel followed by addition of 100 ml of ethyl acetate which were then mixed by inverting and shaking several times. It was then placed onto metal stand allowed to separate the solvent mixtures. When a visible separation was seen, it was then collected into a conical flask. Top layer was the ethyl acetate extract whilst the bottom layer was the aqueous fraction from the filtered broth as shown in the Figure 6.1 below. This procedure was repeated 3 times and ethyl acetate were evaporated using a rotary evaporator (Rotavapor®) by BUCHI.

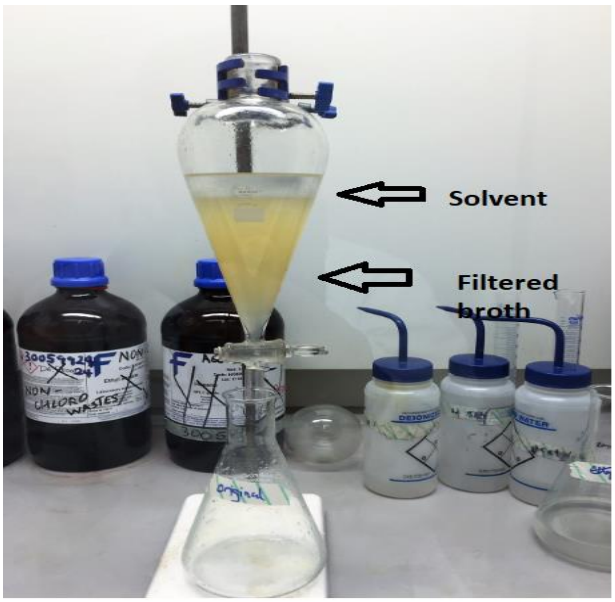

Figure 6.1: Metabolite extraction. Figure shows organic solvent (ethyl acetate) on top layer and aqueous broth at the bottom layer in the separating funnel.

6.4 Butanol Extraction

Followed by ethyl acetate extraction, 200 ml broth was taken into a 500 ml separating funnel and extracted with 100 ml n-butanol following the same method as described in section 6.3. Top layer of the mixture was butanol extract and bottom layer was aqueous fractions from the filtered broth. Similarly, this procedure was repeated 3 times and collected butanol extraction were evaporated using a rotary evaporator (Rotavapor®).

6.5 Methanol Extraction

Like Ethyl acetate and butanol extraction, filtered broth with further extracted with methanol using similar methods described in section 6.3. Similarly, these extractions were repeated thrice followed by evaporation of methanol using the same rotary evaporator. Dried extracts (ethyl acetate, n-butanol and methanol) were used to evaluate the antimicrobial activities following by isolation of compounds.

6.6 Solvents Evaporation

Rotary evaporator (Rotavapor®, R-215 by BUCHI) is consist of vacuum controller (V-850) with a library of 43 specified solvents list, rotation controller, condenser with evaporating flask feed via stopcock, vapour ducting, heating bath (B-491) and distillation chiller.

6.6.1 Evaporation Procedure

Seventy-five ml solvent extracts were taken into a 200 ml round bottom flask and attached to rotavapor ducting for evaporation. Water bath was set to 40º C and increases temperature up to 60º C depending on evaporation rate. Boiling point and required temperature for a solvent system was available in the solvent library of the Rotavapor. Round bottom flask was submerged into water bath for evaporation to take place. Evaporating solvents were selected from the solvent library using vacuum controller (V-850) and rotation of round bottom flask was set to 125 rpm and started evaporation. Most of the solvents were evaporated leaving some liquid in the round bottom flask for collection into a small glass bijou using glass pipette. Collected extractions in glass bijou were left in the laminar airflow for complete dry.

Figure 6.2: Rotary evaporator (Rotavapor®, R-215 by BUCHI) system for solvent evaporation.

6.7 Inhibitory Activities of Extractions

For the isolation and purification of the active metabolites on large scale from the crude solvent extraction of the fermented culture, it was essential to determine the presence of metabolites in the solvent extracts by means of disc diffusion before further purification. In order to find the active metabolites, all the crude extractions were tested by disc diffusion methods against *Staphylococcus aureus* ATCC 25922 and *Escherichia coli* ATCC 25923.

Two×three filter paper discs were prepared with ethyl acetate, butanol and methanol extractions and 2×3 discs with ethyl acetate, butanol and methanol solvent only with equal volume of 25 µl on each disc, then allowed to dry. Two sets of Mueller Hinton agar plates were seeded with *S. aureus* ATCC 25922 or *E. coli* ATCC 25923 to give semiconfluent growth and 3 discs with extractions and 3 discs with solvent only were placed onto the plate and incubated for 24 hours. All tests were duplicated.

After 24 hours incubation plates were checked for any zone of inhibition around the discs. Zone of inhibitions were seen on the plates with *S. aureus* ATCC 25922 fermentation broth supernatant (12 mm) and butanol extracts (10 mm). No zone around ethyl acetate and methanol extracts and no zone on plates seeded with *Escherichia coli* ATCC 25923 were seen. Butanol extract was further assessed with TLC to separate extract constituent compounds.

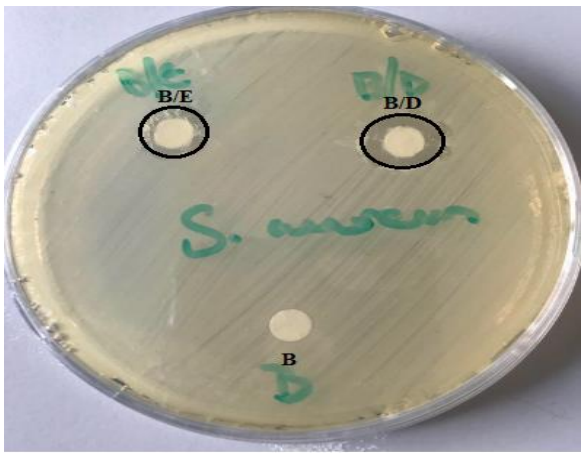

Figure 6.3: Inhibitory effect of metabolite. Figure showing zone of inhibition in *S. aureus* ATCC 25922 (BD=Direct from broth, BE=Butanol extractions and B=Control.

6.8 Chromatographic Analysis of the Crude n-Butanol Extract

Chromatographic analysis was employed to further characterise inhibitory agents present in the crude extract. Various chromatographic systems are available for example, TLC (Thin Layer Chromatography), HPLC (High-Performance Liquid Chromatography), Column Chromatography. For this study, preparative thin layer chromatography (PTLC) technique was adopted using different solvent systems.

6.8.1 Thin Layer Chromatographic (TLC) Analysis of the Extracts

Thin layer chromatography (TLC) is a widely used chromatographic technique which work based on the principle that an analyte move up or across through a solid phase (usually silica gel) with the help of mobile phase (usually, combination of solvents or a single solvent) by the capillary action (Watson, 2012).

6.8.1.1 Analytical TLC

Initially, n-butanol extract was screened using several small cuts of original TLC plates (20 × 20, Merck, Germany) with the following characteristics to determine the number of compounds in the crude extract, solvent systems and R_f values (Aszalos and Frost, 1975).

Plate size: 8 cm × 5 cm

Stationary phase: Thin film of silica gel mesh 60 coated with florescence indicator, F_{254}.

Film thickness: 0.5 mm

Type of development: Ascending and one dimensional.

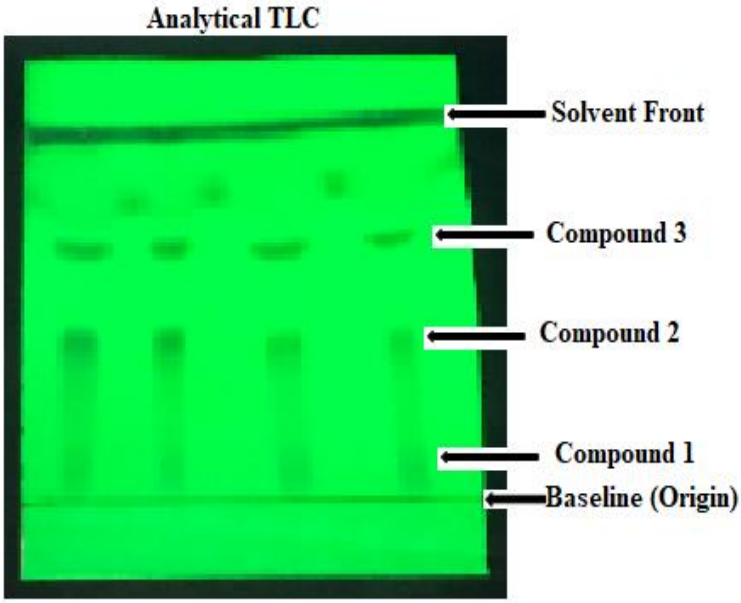

Figure 6.4: Analytical TLC plate. TLC plate showing several compounds under UV lights.

Several solvent system combinations (Table 6.1) with various ratios of solvent mixtures were tested with and without addition of acetic acid. 20 ml of solvent systems were tested for optimisation purposes. A line was drawn above 1 cm from the bottom edge of the plate and n-butanol extract was placed along the line in four different spots. Plate was then placed into TLC tank and removed when solvent reached 1 cm below the top edge of the plate. Plate was then dried and observed under UV light using both shortwave (254nm) and longwave (366nm) and circle the band(s) using a pencil. Plates were then sprayed with 1% vanillin in sulphuric acid to detect non-UV absorbed compound(s). n-Butanol extracts on analytical TLC showed 3 bands for compounds separation and one strong band, referred as compond-3, as shown in Figure 4. Dichloromethane, Methanol and Acetic acid in a ratio of 15 : 5 : 0.02 were found effective mobile phase in the isolation of the compounds.

Table 6.1: Solvent systems used as a mobile phases in TLC for optimisation.

Code	Solvent systems	Ratio
A	Hexane : EtOAc	12 : 8
B	Hexane : EtOAc	16 : 4
C	Hexane : EtOAc	8 : 12
D	Dichloromethane : MeOH	18 : 2
E	Dichloromethane : MeOH	15 : 5
F	Dichloromethane : MeOH : AcOH	15 : 5 : 0.02

6.8.1.2 Preparative Thin Layer Chromatography (PTLC)

A macro scale separation of compounds was done using preparative thin-layer chromatography (PTLC) by applying a thick adsorbent layer of silica gel. PTLC has several advantages such as less run time, small sample volume, easy to use, easy isolation and collection of compounds compared to column chromatography for the isolation of pure compounds (Wing and Bemiller, 1972).

In order to isolate the compounds from the butanol extracts, 20 × 20 cm TLC silica gel 60 F_{254} plates (Merck, UK) were taken and drawn a line 2 cm above the bottom edge of the TLC plate where samples were loaded along with the drawn line using capillary tubes. Plates were then allowed to dry before loading to a TLC glass tank containing mobile phase. Once the tank was covered, the mobile phase moved up the TLC plate by means of capillary action separating target compounds. When mobile phase reached 1 cm below the top edge of the TLC plates, they were then taken out of the tank and allowed to dry. Once TLC plates were dried, they were then observed under the UV light and marked the visible bands using a pencil.

6.8.2 Compound Isolation

Bands of interest marked on the TLC plates under UV light were scrapped off the TLC plates and collected individually in separate beakers. Then approximately 20 ml of mobile

phase (Dichloromethane : methanol = 3:1) was added to the beaker to dissolve the compounds present in the silica gel. Silica gel was stirred using spatula and mixed well with the solvent and left for 5 minutes. A filter paper 125 mm in diameter was used to strain out compound from the silica gel and collected in a round bottom flask followed by evaporation of solvents by using rotary evaporator. Compound was collected in a pre-weighed small bijou, dried out completely in the Laminar flow hood or using nitrogen gas and stored in fridge (4-7° C) for further analysis (antimicrobial assays and NMR analysis).

6.8.3 Determination of R_f (Retardation Factor) Values of the Resolved Fractions

Once bands were marked either using UV light sources or 1% vanillin-sulphuric acid spray, R_f values were calculated using the following formula:

$$R_f \text{ value} = \frac{\text{Distance travelled by the compounds (solute)}}{\text{Distance travelled by the solvent front}}$$

R_f value is the characteristic of a compound in a specific solvent (Watson, 2012).

6.9 Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR is a very powerful tool applied in analytical chemistry to determine molecular structure of an organic compound. NMR works on a principle that when radiation is applied in the radiofrequency region some atoms like protons (^1H) and carbon (^{13}C) spins with the force of magnetic field and aligned either with or against the applied magnetic field. Frequencies ranges used for excitation and the complex splitting patterns are distinctive characters of the chemical structure of the given molecule (Watson, 2012).

6.9.1 NMR Spectral Data Analysis of Compound 3 (RP-3)

Compound 3 was analysed to elucidate its structure using a series of 1D (^1H, ^{13}C, DEPT 135) and 2D (COSY, HSQC and HMBC) NMR spectroscopy at Liverpool John Moore University by Dr Rahman. The ^1H NMR spectrum (CD_3OD, 600 MH_z, Table.7) revealed the presence of a methyl as doublet at δ_H1.38, two olefinic protons at δ_H 5.61 (δ, J = 7.6 H_z), a series of methine and methylene protons resonating between δ_H 2.01 – 4.51 H_z. The ^{13}C NMR (CD_3OD, 125 MH_z) showed the presence of a number of carbons including carbonyls (δ_C 167.5, 169.3, 173.0, 172.1) two olefinic methines (δ_C 101.8 and 143.8 ppm), a methyl (δ_C 15.8 ppm), methylenes (δ_C 23.5, 29.5, 38.4, 46.4, 47.2, 55.3) and methine carbons (δ_C 52.3, 59.0, 60.0 and 69.3 ppm). Based on the interactions/coupling in the

COSY, HSQC and HMBC the compounds confirmed the presence of following structural features

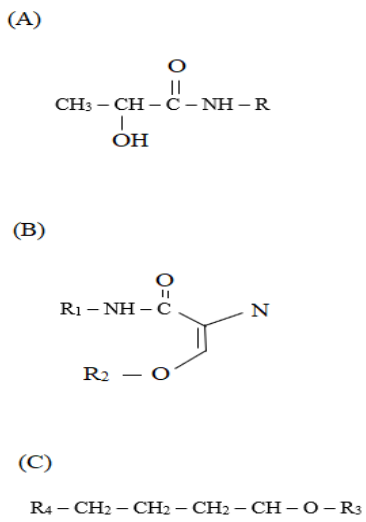

Figure 6.5: Possible structural features of compound 3 represented by NMR.

So, it is concluded that compound 3 (RP-3) is mixture of 3 compounds. Because of time constraints and limitation upon extracted sample, it was not possible to proceed further to scale up the amount and further purification by preparative HPLC and subsequent confirmation of structures by NMR experiments and further mass spectrometric analysis.

Table 6.2: ^{1}H (600 MH_z), ^{13}C (150 MH_z) NMR data of compound 3 and their correlations in the HMBC experiment.

^{1}H	^{13}C (HSQC)	HMBC
7.40, d, j = 7.6 H_z	143.8 (CH)	101.8 (CH), 167.5 (CO), 153.6 (?)
5.61, d, j = 7.6 H_z	101.8 (CH)	143.8, 167.5 (CO)
4.46, t	69.3 (CH)	59.0 (CH), 55.3 (CH_2)
4.22, m	60.0 (CH	169.3 (CO), 15.8 (CH_3) 29.6 (CH_2)
4.51 dd/m	59.0 (CH)	173.0 (CO), 38.4 (CH_2)
3.66 (dd) + 3.43 (d)	55.3 (CH_2)	59.0 (CH), 69.3 (CH), 38.4 (CH_2).
4.22 m	52.3 (CH)	15.3 (CH_3), 29.5 (CH_2).
4.10 d + 3.74 d	47.2 (CH_2)	172.1 (CO), 166.6 (CO)
3.51 – 3.54	46.4 (CH_2)	29.5 (CH_2), 23.5 (CH_2)
2.27 + 2.07	38.4 (CH_3)	173.0 (CO), 69.3 (CH), 55.3 (CH_2), 59.0 (CH)
2.32	29.5 (CH_2)	46.4 (CH_2), 23.5 (CH_2)
2.01	23.5 (CH_2)	
1.38 d	15.8 (CH_3)	52.3 (CH), 169.3 (CO)

6.10 Antibacterial Screening (*in vitro*) of the Compound 3 (RP-3)

The prime objective of performing the antibacterial screening was to determine the susceptibility of the pathogenic microorganisms to the test compound which was isolated using TLC. To determine the antimicrobial activities of the isolated compound 3 (RP-3), both disc diffusion and broth microdilution assay for minimum inhibitory concentration (MIC) were done (Carson and Riley, 1995).

6.11 Microbial Strains Subculture

Microbial test strains evaluated included *S. aureus* ATCC 25922, *E. coli* ATCC 25923, *Klebsiella pneumoniae* UEL strain and ATCC 700603 (Extended spectrum β-lactamase) strain, *Pseudomonas aeruginosa* ATCC 17934, *Candida albicans* ATCC 10231 were subcultured on day before disc diffusion and broth microdilution assays.

6.12 Disc Diffusion Assay

Disc diffusion assays were performed for qualitative or semiqualitative assessment of antimicrobial activity for both the extract and isolated compound. Two MH agar plates for each test organisms were seeded with *S. aureus* ATCC 25922, *E. coli* ATCC 25923 *K. pneumoniae* UEL strain and ATCC 700603 (Extended spectrum β-lactamase) strain, *P. aeruginosa* ATCC 17934, *C. albicans* ATCC 10231. Each MH agar plate included 1 disc with butanol extract, 1 disc with isolated test compound 3 (we call it, RP-3) and 1 disc of butanol on its own as control and incubated for 24 hours (Figure 1). All the discs were prepared using 25 µl Plates were checked after 24 hours incubation and recorded for antimicrobial activity.

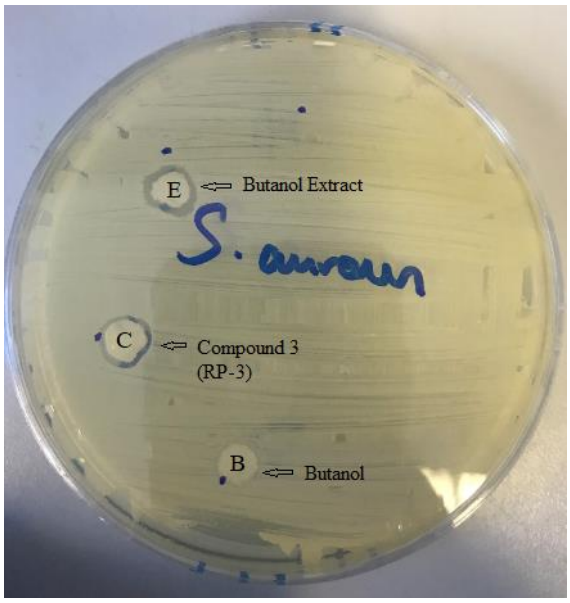

Figure 6.6: Inhibitory effect of extract and compound 3. Disc diffusion showed zone of inhibition in butanol extract (9mm) (E), compound 3 (8mm) (C) and zone to butanol (B).

6.13 Broth Microdilution Assay Procedures

Broth microdilution quantifies the relative potency and the lowest concentration of an antimicrobial agent required to inhibit the growth of the microorganisms *in vitro*.

One hundred µl of MH broth was dispensed to all wells in columns 1 to 11 in 96 well microtiter plate using a multi-channel pipette. A 100 µl of test compound solution was

dispensed to first 2 wells (A and B) of column 1 in 96 well plates and another 100 µl of control antibiotic (Norfloxacin, Sigma-Aldrich, UK) solution was dispensed to next 2 wells (C and D) of column 1 for *S. aureus* ATCC 25922. Similarly, A 100 µl of test compound solution was dispensed to E and F and 100 µl of control antibiotic (Norfloxacin) solution to G and H of column 1 for *E. coli* ATCC 25923.

McFarland turbid (0.5) was added for *S. aureus* ATCC 25922, and saline (0.9% NaCl) solution added to A, B, C and D wells of column 1. Similarly, 0.5 McFarland turbid of *E. coli* ATCC 25923 saline (0.9% NaCl) solution was added to E, F, G and H wells of column 1 in 96 well plate. The contents of column 1 were mixed thoroughly transferred 100 µl to all wells up to column 11 leaving 12th column as sterility control. It was then incubated for 18 hours at 37º C aerobically.

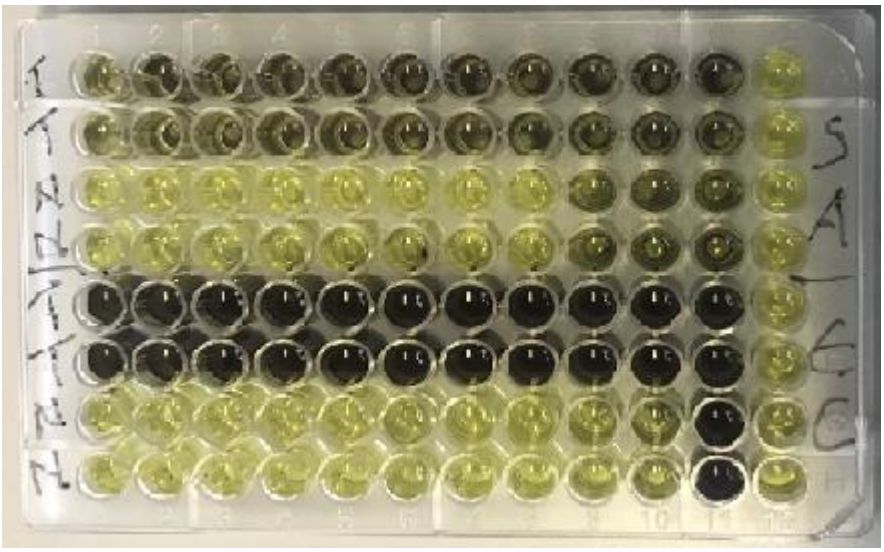

Figure 6.7: MIC. Ninety-six well plate shows column 11 as growth control and column 12 as sterility control. Letter T on the plate denoted for test compound and N for Norfloxacin (known antibiotic) and SA for *S. aureus* and EC for *E. coli*. Light yellow colour indicates no microbial growth and light black for microbial growth.

6.13.1 Incubation and result observation

After 24 hours incubation period, 20 µl of MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) (Sigma-Aldrich, UK) (Rahman et al., 2008a) solution was added to all wells. The plate was then incubated for another 10-20 minutes and observed for the colour changes from light yellow to light black. Light yellow colour indicates no microbial growth and light black for microbial growth.

6.14 Results

In disc diffusion assays, 9 mm zone of inhibition for *S. aureus* ATCC 25922 were seen in butanol extract and 8 mm for compound 3, but no zone for butanol alone. These zone of inhibition measures are mean of three replicates. No zone of inhibition was seen in *E. coli* ATCC 25923, *K. pneumoniae* and ATCC 700603 (Extended spectrum β-lactamase) strain, *P. aeruginosa* ATCC 17934, *C. albicans* ATCC 10231 for compound-3. Having no zone with ethanol and methanol extractions helped to avoid false zone of inhibition during the experimental process.

There were no inhibitory activities seen at 128 µg/ml concentration for both organisms. Subsequently, 512 µg/ml and 1024 µg/ml concentration were tested (data not shown), and no inhibitory activities seen against the test organisms.

Chapter 7: Discussion and conclusion

7.1 Discussion of overall findings of the study

The demand for novel antimicrobials has prompted many to search extreme environments to attempt to fill this need. Given that most microbes surrounding us are non-cultivable, it is possible that there is a vast untapped resource much closer to home. This study used the concept of *in-situ* environmental cultivation using iChip-like devices as well as developed an improved culture medium for the isolation of previously uncultivated microorganisms and screened them for secondary metabolites. Another important aspect was sub-culturing the iChip isolates onto an appropriate medium and "domesticating" them. Here term domesticating means, organisms those were not previous grown in the laboratory culture medium (novel isolate) but grew in iChip or soil supplemented nutrient agar (SSNA) needed to maintain their growth in traditional laboratory medium for further study and their characterisations. This study found that SSNA supported the growth from 90% wells of iChip devices when sub-cultured but only around 50-60% growth was seen on NA and TSA from ichip subcultures. It was believed that soil supplement helped the growth from ichip subcultures as organisms used soil as their main source of nutrition. . Other studies also showed soil extracts enabled to isolate previously uncultivated microorganisms (Hamaki et al., 2005; Nguyen et al., 2018).

SSNA also stopped overgrowth of any microbial species including swarming of *Proteus mirabilis* ATCC 12453 (swarming strain). Impressively, SSNA retained viability of stationary of month-old *S. aureus* ATCC 25922 and *E. coli* ATCC 25923 and *P. mirabilis* ATCC 7002 plates and had reproducibility. These qualities made SSNA the optimal medium for the isolation of soil microorganisms.

This study found an antagonistic microorganism and identified as *Bacillus pumilus*. Cell free supernatant of *B. pumilus* fermented broth showed 12 mm zone of inhibition against *S. aureus* ATCC 25922 and butanol extract and TLC compound-3 showed 9 mm and 8 mm zone of inhibition respectively. Compound-3 had fragments of 3 chemical structures containing peptide bonds and aliphatic chains. Known peptide antibiotic vancomycin also has similar structural feature and active against Gram positive organisms by inhibiting cell wall synthesis of the organisms. Compound found in this study is active against S. aureus which suggest that this might target the cell wall of the organism like vancomycin. Vancomycin is active against MRSA and complete extraction of this study compound

could have similar activities. Further fermentation and extraction optimisations are needed to isolate the compound completely and defined their characteristics and activities. However, novel peptide antibiotic pumilicin 4 by *B. pumilus* WAPB4 strain reported active against MRSA, VRE and other Gram positive bacteria for the first time (Aunpad and Na-Bangchang, 2007).

Bacillus pumilus known to produce various secondary metabolites like antibacterial and antifungal compounds, enzymes, proteases and so on (Dehghanifar et al., 2019). *B. pumilus* are reported as a plant growth promoter (de-Bashan et al., 2010; Gutiérrez-Mañero et al., 2001) and biopesticides (Molina et al., 2010). *B. pumilus* plays an important role in biodegradation of naphthalene (Calvo et al., 2004), carbendazim (Zhang et al., 2009), polyaromatic hydrocarbons (Patowary et al., 2015), chlorpyrifos and its by-products (Anwar et al., 2009) and keratinolytic activity (El-Refai et al., 2005) in the environments. *B. pumilus* can cause cutaneous and subcutaneous infections (Logan and Vos, 2015) and food poisoning (From et al., 2007) in human. Metabolites production can be subjected to several factors or even a single change in fermentation medium can resume secondary metabolites production. Study by (Meyers et al., 1991) showed that when 12 strains *B. pumilus* were cultured in Difco nutrient broth isolates grew poorly and were unable to degrade cyanide. But same strains when cultured in Oxoid nutrient broth under the same conditions, grew well and degraded cyanide rapidly. Beside it, Oxoid nutrient broth made up using deionised water was unable to degrade cyanide whereas same broth made up using tap water was able to degrade cyanide. Further investigation found that tap water contained manganese and addition of 0.01 mg Mn2+ per litre Oxoid nutrient broth with deionised water was able to degrade cyanide. In *B. pumilus* XH195 strain when glucose or sucrose were used as carbon source in the fermentation medium acetoin productions were 63.0g/L or 58.1g/L respectively (Xu et al., 2012). Another study reported that antifungal chitinase from *B. pumilus* was produced only in the presence of chitin in the medium (Ghasemi et al., 2010). Hence, secondary metabolite production might need precursor or triggering compound. So, *B. pumilus* isolated in this study need further experiments to trigger secondary metabolite production.

For this study, extraction solvents ethyl acetate, butanol and methanol were selected based on Dr Rahman's standard protocol and other published studies where these solvents were mostly used (Ciric et al., 2011; Rajan and Kannabiran, 2014; Sapcariu et al., 2014). However, polypeptide compounds produced by *B. pumilus* (NKCM 8905) and *B. pumilus*

*(*AB211228) were isolated by ammonia sulphate precipitation followed by distilled water and hexane extraction (Sawale et al., 2014), Pumilin was isolated by ammonia sulphate precipitation followed by ethanol extraction (Bhate, 1955), antifungal phenazine from *B. pumilus* MTCC7615 was extracted using benzene (Padaria et al., 2016), Iturin from *B. pumilus* HY1 was isolated from the broth supernatant collected in a concentrated HCl and followed by methanol extraction (Cho et al., 2009). Selection of solvents are key to isolate target metabolites or compounds as they might have different interactions in polar and nonpolar solvent and can lead to failure of complete or partial isolation. In this study, compound found were partially identified as a mixture of 3 fragments and this could be due to solvent systems and other parameter within the experiments. Hence solvent selection is very important in secondary metabolites isolation which can be achieved by trial and error experiments and might require excessive time before a perfect solvent is found for any metabolite.

1D, 2D and HMBQ NMR data revealed compound 3 with a mixture of possible 3 compounds or their fragments (Chapter 6; Figure-6.5). Re-isolation of the compound and repeat of NMR was only the approach to answer it but was not possible at this stage of the study due to time constrains. However, presence of numbers of C, H, NH, CO indicate a potential chemical structure or its analogue that could possibly be an antimicrobial compound as it falls in the Lipinski's rule, for example molecular weight, Hydrogen bonding (Benet et al., 2016).

The study highlighted several further directions worthy of exploration, for example, identification of organism was done to a species level, *B. pumilus* by MALDI TOF and not identified to strain level using molecular methods such gene sequencing, DNA hybridization and DNA banding pattern based methods due to lack of fund for this study. Identification of strain was very important as different strains of *B. pumilus* produces different metabolites, antibacterial, antifungal and analgesic. First antibacterial activities of *B. pumilus* was observed by Gilliver in 1949 (Gilliver, 1949) and Pumilin, a non-peptide antibiotic was reported as first antibiotic from this organism (Bhate, 1955). This report was *B. pumilus* species only, not strain involvement in the Pumilin production. Different strains of *B. pumilus* have subsequently been reported with regard to involvement in different antimicrobials and other secondary metabolites (Dehghanifar et al., 2019; Ghasemi et al., 2010; Leifert et al., 1995; Padaria et al., 2016). For example, *B. pumilus* CL27 produces peptide antimicrobial compound and *B. pumilus* CL45 produces

non-peptide antifungal compound and the production of the compound was fermentation composition dependent (Leifert et al., 1995). *B. pumilus* SG2 antifungal chitinase (Ghasemi et al., 2010), *B. pumilus* HY1 antifungal Iturin (Cho et al., 2009), *B. pumilus* E-1-1-1 fungal aflatoxin degrading enzyme, *B. pumilus* (NKCM 8905) and *B. pumilus* (AB211228) both strains reported as polypeptide compounds with antibacterial and antifungal activities (Sawale et al., 2014). *B. pumilus* marine strain reported for its anti-quorum sensing activities (Nithya et al., 2010). However, novel peptide antibiotic pumilicin 4 by *B. pumilus* WAPB4 strain reported active against MRSA, VRE and other Gram positive bacteria for the first time (Aunpad and Na-Bangchang, 2007). Also, *B. pumilus* AQ717 and *B. pumilus* XH195 strain (DSM-16187) were patented for corn rootworm disease and acetoin production, respectively (Sansinenea and Ortiz, 2011; Xu et al., 2012).

Study by Toymentseva et al showed when they analysed two strains of *B. pumilus* 7P and 3-19 using next generation sequencing and search for secondary metabolite clusters within the genome using antiSMASH program, it revealed 11 potential gene clusters for the synthesis of bacilysin, lichenysin, bacteriocin, and other substances (Toymentseva et al., 2019). *Bacillus pumilus* in this study could possibly be completely a new strain with unknown activities yet to discover.

In this study, initial extraction was done by chemical solvent systems and compounds were separated by TLC. Some studies used ammonium sulphate precipitation methods form extractions and then TLC for compound separation (Dehghanifar et al., 2019; Munimbazi and Bullerman, 1998; Sawale et al., 2014). Another extraction process described concentration of cell free supernatant by lyophilization then followed by small volume of methanol extraction (Aunpad and Na-Bangchang, 2007) and this method is not common as it was not reported elsewhere. Hence compound found in this study could not be defined and compare with other findings but open an opportunity for further study on this isolate.

Although, disc diffusion method showed inhibition but MIC even at 1064 µg/ml (highest concentration for MIC determination) failed to show inhibition following purification. As NMR data revealed fraction of compound mixture. Inhibitory activities may be synergic with other compounds present in the crude extract or compound might have lost its activities during solvent separation. Interestingly, previously published work cited did not

show any MIC data for any antibacterial compounds investigated. It is most likely no one found a significant MIC within MIC test parameter (<1064 µg/ml).

iChip devices and SSNA used in this study helped recover previously uncultivated microorganisms. *B. pumilus* isolated in this study well known for secondary metabolites productions by its different strains and identified strain in this study could potentially be one of the novel strains.

7.2 Limitations

Time and funding were the main limitations for this study. Due to lack of time and money many areas could not be explored to answer key finding. This study could investigate further optimise fermentation, compound extractions and purifications techniques. Beside it, the compound isolated from this study was not tested for antifungal activities as UEL microbial stock had no fungal species and could not collaborate with other institute for antifungal studies due to time constrain. Study could also investigate antifungal activities. Spore formations of *B. pumilus* were not checked after fermentation periods as it was too late to repeat all the experiment from fermentation stage. *B. pumilus* can survive in harsh condition due to its spore forming abilities and if fermentation medium does not support their growth, they might form spores. Hence impact on secondary metabolites production

7.3 Conclusion

Development of iChip devices and SSNA medium in this for cultivating previously uncultivated organisms opens further study opportunities for exploration and exploitation of the experimental procedures to find potential new antimicrobial agent. Optimisations in various stages in this study helped gaining new knowledge that could be utilised in further studies and research. Continuing with this study and literature available on *B. pumilus*, further investigation could unlock its potential secondary metabolites.

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Appendices

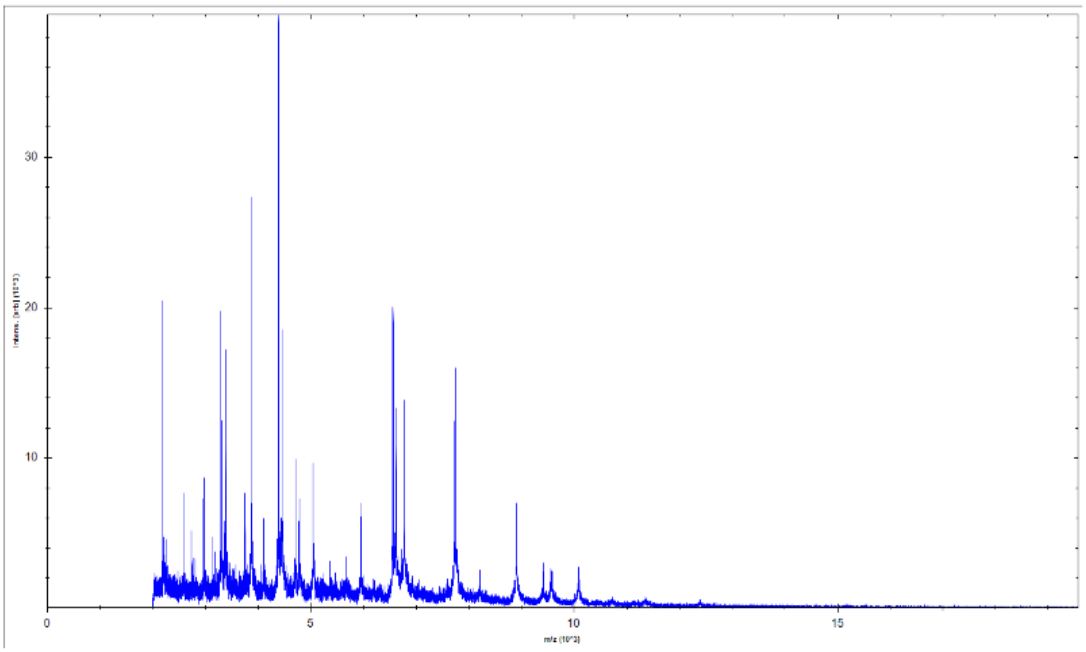

Figure 1: MALDI-TOF spectra of unidentified organism-1. No organism's match found in the extended research database against these spectra.

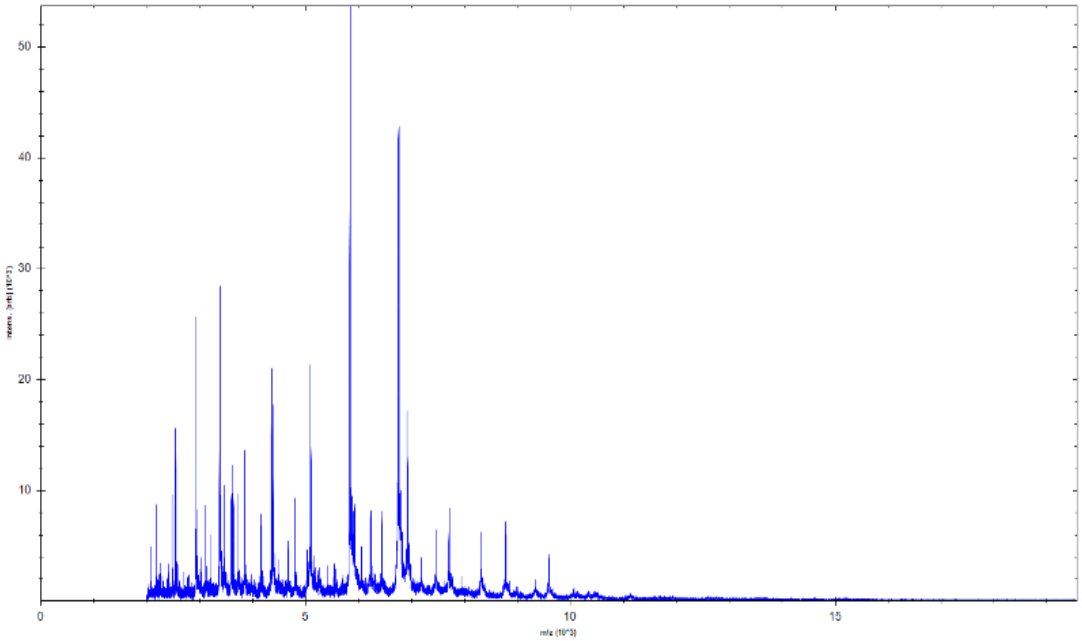

Figure 2: MALDI-TOF spectra of unidentified organism-2. No organism's match found in the extended research database against these spectra.

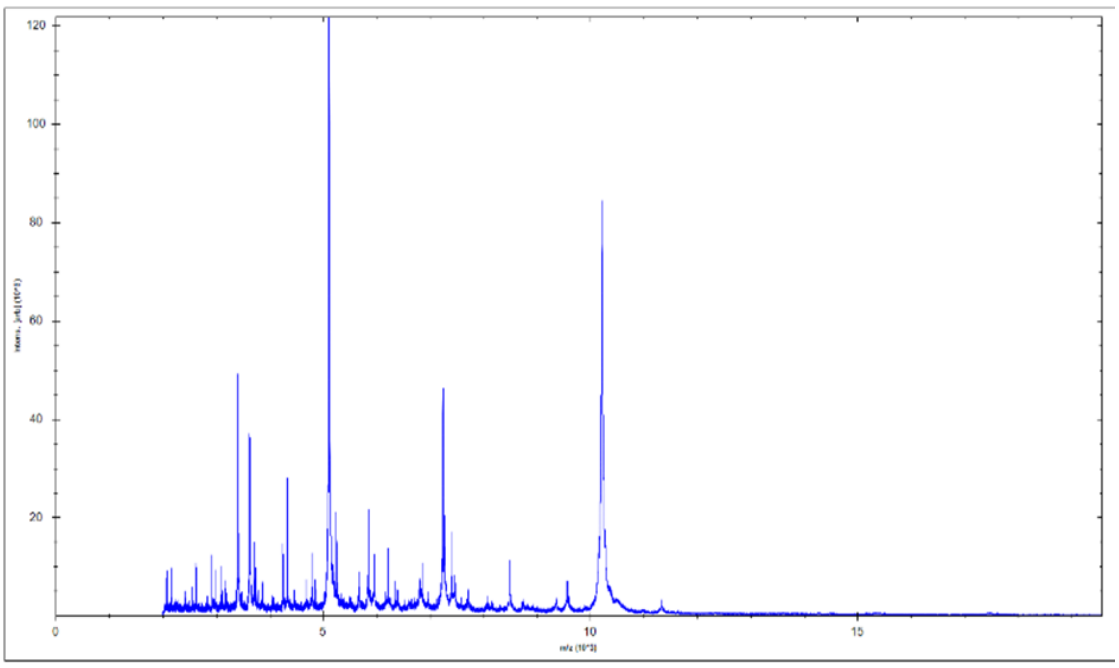

Figure 3: MALDI-TOF spectra of unidentified organism-3. No organism's match found in the extended research database against these spectra.

Above spectra in MALDI-TOF were seen in organisms from iChip subculture which were believed to be novel microorganisms or previously uncultivated microorganisms found in this study. These three spectra are some example, but this study found more organisms those produced spectra but no matches in the database. However, these organisms did not show any inhibitory effect, so they were not followed any further.