Isolation and characterization of uncultured bacteria from wheat seeds, soil and sponge

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Declaration

I certify that this thesis does not contain material which has been accepted for the award of any degree or diploma; and to the best of my knowledge and belief it does not contain any material previously published or written by another person except where due reference is made in the text of this thesis**.**

SHARANDEEP KAUR

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Thanks All for making this happen!

List of abbreviations

ul, ml	**Microlitre, Millilitre**
uM, mM	Micromolar, Millimolar
%	Percent
bp	Base pair
DNA	Deoxyribonucleic acid
rRNA	Ribosomal ribonucleic acid
min	Minute (s)
^{O}C	Degree Celsius
Approx..	Approximately
OD_{600nm}	Optical Density 600 nanometres
PCR	Polymerase Chain Reaction
temp	Temperature
SARDI	South Australian Research and Development Institute
BLAST	Basic Local Alignment Search Tool
NCBI	National Center for Biotechnology Information

Abstract

Microorganisms are one of the oldest living forms present on the planet and yet microbiologists struggle to culture 90% of genera and study them in *in vitro* conditions. Samples of sponge, wheat seeds and soil for which more than half of their bacterial population cannot be cultured due to various reasons were selected for this study. In this study efforts were made to culture the as yet unculturable bacteria using different techniques. To achieve the growth of the uncultured bacteria in *in vitro* conditions, the dilutions to extinction was done (10^{-12} & 10^{-14}) with the intention of achieving one cell per well and the samples were incubated in the 96-well plates at two different temperature conditions. The samples were subjected to long incubation (10-12 weeks). Other parameters were also tested like liquid v/s agar growth and the ones growing on agar were categorised and selected as per the nature of the growth. The ones which were not growing on agar were hypothesised to be more likely to be novel and therefore selected for sequencing of their 16S rRNA genes to identify the isolates. The bacterial strains growing on agar were categorised and some strains were not showing growth on the solid medium and hence they were suspected to be new bacteria. But after the sequencing of 26 isolates it was observed that the majority of strains belong to already commonly described species.

Chapter 1

Introduction

Introduction

Microorganisms are part of human life, from digestion processes to the production of medicine, humans are surrounded by microorganisms. They are present everywhere but still a major percentage remain uncultured. This research was done with some specific samples to isolate the uncultured novel bacteria from wheat seeds, soils and three different types of sponges collected from Australian waters.

Sponge

Marnie sponges are one of the most ancient animals on the planet having a unique aquiferous system which filters the surrounding sea water, nearly 24 m^{-3} kg^{-1} day^{-1} and they are home for millions of bacteria (Hentschel et al. 2002; Vogel 2008; Zhang et al. 2008). During this filtration process of water, numerous bacteria, microalgae and other organic particles present in the sea get stuck in the sponges and are consumed by phagocytosis and some of them are left undigested and make a symbiosis relationship with sponge. These microbes dwell in the sponges and increase their biodiversity (Zhang et al. 2008). The microorganisms make nearly 35% to 40% of biomass of sponges (Hentschel et al. 2001; Jiang et al. 2007). The range of microorganisms accompanying sponges is extensive as molecular analysis suggests that a discrete sponge host 14 bacterial phyla, two phyla of archaea and numerous types of eukaryotic microbes (Vogel 2008; Yang, Franco & Zhang 2015). The microbes found in the sponge deliver some bacteria specific qualities to sponge like autotrophy, nitrogen fixation and nitrification, apart from this, sponge linked microbes help the sponge in stabilizing the sponge skeleton and also process the metabolic waste compounds (Mehbub et al. 2014).

Microbial ecologists are driven towards sponges because of two reasons (1) to study complex model of symbioses interaction of sponge and associated bacteria, (2) the isolated bacteria from sponges are a good source of bioactive metabolites and they are also known for protecting the sponges from their predators by releasing secondary metabolites which comprises cytotoxic, antibiotic properties (Mehbub et al. 2014; Montalvo et al. 2014; Zhang et al. 2008). There are

various questions about the relation of the sponge and bacteria and to answer those questions both organisms needs to be studied completely. The ecological and genetic diversity of both organisms and the making of natural products in them needs to be explored more. But microbiologists are struggling to explore the sponge and related microorganisms as the range and proportion of uncultured microorganism in sponge is high, making them hard to grow in the laboratory (Achtman & Wagner 2008; Alain & Querellou 2009; Bull et al. 2005; Montalvo et al. 2014).

Marine organisms are known for producing more novel compounds and the trends of isolation of novel compounds are represented in Figure 1.1 during 2001 – 2010 (Figure 1.1 depicts the rate per annum of discovery of new compounds which has increased from 500 products in late 1990s to over 1000 products during 2008 to 2010) and many of those compounds (like anticancer or anti-inflammatory agents) are in clinical and pre-clinical trials (Mehbub et al. 2014; Taylor et al. 2007) and the presence of these bioactive compounds from different sponges led to the conclusion that these compounds are from the microbial origin which raised the hope of cultivating those bacteria *in vitro* and to generate the production of desired metabolites (Piel et al. 2004).

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Figure 1. 1 Novel compounds from marine organisms. The bar graph depicts the total sum of novel compounds isolated from diverse marine organisms since 2001 to 2010 (Mehbub et al. 2014).

The interaction between the marine sponges and bacteria is hard to understand as the symbiosis relation between both the organisms is ancient but with many efforts nearly 28 bacterial phyla have been documented from the sponges using new and old molecular approaches such as 16S rRNA gene, out of 28 phyla 18 are formally described and 10 are candidate phyla which are

recognized only from their 16S rRNA gene sequences (Hentschel et al. 2012). The leading sponge associated bacterial phyla observed so far are Proteobacteria (specifically the classes Alpha-, Gamma- and Deltaproteobacteria), Chloroflexi, Actinobacteria, Acidobacteria, Nitrospirae and the candidate phylum Poribacteria (Figure 1.2 shows the diversity of sponge associated microorganisms) apart from them, Archaea (phylum - Thaumarchaeota), eukaryotic microbes such as fungi, diatoms are also present in abundance in the sponges (Hentschel et al. 2012). The estimated number of existing species are 10^5 to 10^6 but only a small percentage of those have been cultured as only few can be isolated on growth medium in petri dishes. After practising different laboratory methods only a small increase in the number of isolated bacteria was observed (Kaeberlein, Lewis & Epstein 2002). One such example of isolating novel species from sponge (Aplysina aerophoba) is cultivation of uncultured actinobacteria which was also spotted in the original sponge sample (Kaeberlein, Lewis & Epstein 2002).

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Figure 1. 2 The phylogenetic tree of all the known microbial phyla associated with sponge. The figure represents the 16S rRNA gene analysis of sponge microbiota, depicting the representative of the microbial phyla associated with the sponges (Hentschel et al. 2012).

Wheat seeds

Crop seeds are important as they are the source of planting the next generation of the plants and are also the commodity to the agriculture market which emphasises more on the quality of the seeds to make sure their permanency in the food supply and facilitate with good income to the growers. Crop seeds like wheat and maize are populated by microbes and they play an important role in the plant's health, growth and development. The result of these microbes on the seeds yield and soil needs to have comprehensive information of the seed associated microbial community (Links et al. 2014). The bacterial diversity in the wheat seeds is more varied than the surface sterilized sprouts (Huang et al. 2016). Endophytes are found in the

plants interior called endosphere and a mutual collaboration among the plants and microbes takes place without altering the plant's morphology (Podolich et al. 2015). The proportion of bacteria found as endophytes are more limited as compared to the microbes present in the rhizosphere (Podolich et al. 2015). The proportion of uncultured bacteria in wheat seeds is nearly 50% (refer to Figure 1.3) and this amount goes up as they are sown in soil but after 4 weeks the percentage of uncultured microorganisms goes down (Araujo et al. 2019).

Soil

The soil environment is place where different kinds of diversity of physiological and metabolic proficiencies can be witnessed among the soil microbes, also the range of genetic diversity is high among the soil microflora (Sait, Hugenholtz & Janssen 2002). But still a major proportion of the bacterial population is considered uncultured and nonculturable (Alain & Querellou 2009; Araujo et al. 2019; Bull et al. 2005; Janssen et al. 2002; Pham & Kim 2012). Figure 3 depicts the data of uncultured bacteria in soil and wheat seeds(Araujo et al. 2019). Distinct efforts are being made by the microbiologist to solve the mystery and different approaches and techniques have been tried and tested. Molecular biology is one such area to study these microorganisms more closely and with its advent it is surveyed that the diversity of microbes is higher in the natural environment than it was known earlier (Tan, Ward & Goodfellow 2006).

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Figure 1. 3 The uncultured proportion of the microbes in wheat seeds and soil. The pie chart depicts that nearly 50% proportion of the microbes in the seed are still unclassified and this proportion rises to as high 70 % 4 weeks after germination. (Araujo et al. 2019).

There was a time when actinomycetes were not given any importance but presently they are considered of great significance because of the yield of the bioactive compounds (Kumar & Jadeja 2016). *Actinobacteria* are the class of microorganisms which holds the major proportion in the production of bioactive molecules ranging from antibiotics, enzymes,

immunosuppressors to antitumor agents (Qin et al. 2009). They are predominantly found in soil with different population in different habitats. and with the isolation of new actinomycetes, a not only new microorganisms but also new metabolites can be discovered (Kumar & Jadeja 2016; Qin et al. 2009). The amplified interest in finding news metabolites can be observed among the microbiologists and it is evident that the amount of finding novel metabolites from soil streptomyces has nosedived and the proportion of isolation of already isolated metabolites has augmented (Qin et al. 2009). Hugenholtz describes the easily isolated microbes as the "weeds of microbial sphere" and their growth on agar make it quite effortless for microbiologists to study microbes along with working on them, in addition to that, the information it adds to the tank of microbiology knowledge is from the research of microorganisms from the sphere of weed community (Hugenholtz 2002). Comparative phylogenetic analysis of DNA sequences of 16S rRNA genes has depicted that members of four major phylogenetic groups are pervasive in all soil types: the class *Alphaproteobacteria*, and the phyla *Actinobacteria*, *Acidobacteria* and *Verrucomicrobia* (Sait, Hugenholtz & Janssen 2002). In addition to that, the extensively studied and cultivated prokaryotic genera are Proteobacteria, Firmicutes, Actinobacteria and Bacteroides represented in the pie chart (taken from Australian Collection of Microorganisms (https://gold.jgi.doe.gov/distribution) in Figure 1.4, which points out that the bacteria isolated are from these major clusters only and it place another challenge to isolate the bacteria which do not fit these four dominant phyla groups (Hugenholtz 2002).

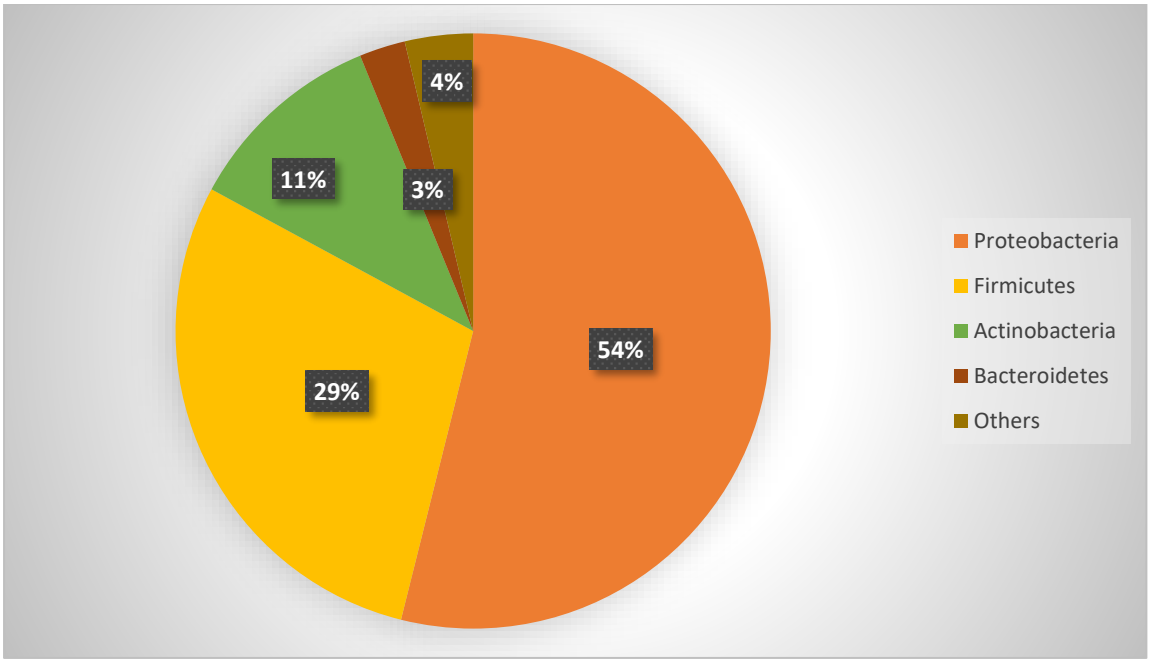

Figure 1. 4 Bacterial phyla. The pie chart represents the major bacterial phyla placed in Australian Collection of Microorganisms as of on November 05, 2019. The other categories include the following phyla Cyanobacteria, Spirochaetes, Tenericutes, Chlamydiae, Fusobacteria and Chloroflexi in very small proportion.

Obstacles in culturing the uncultured

To grow the microorganisms is a challenge when the target is not clear, or the information is very limited about the important parameters required for the growth of these microbes. Simulation of some natural factors are hard to do in the laboratories like, the chemistry of the microbial habitat, effect of the difference in the source temperature and temperature in the labs and many other factors (Alain & Querellou 2009; Pham & Kim 2012; Zengler et al. 2002). Agar based medium can be one of the inhibitory factors that suppress the growth of bacteria (Kamagata & Tamaki 2005). The use of conventional methods to cultivate the uncultured microbes is laborious and the methods are more selective and biased towards the growth of some specific bacteria (Zengler et al. 2002).

Techniques to culture the uncultured

There are different approaches being used by scientists in an attempt to culture the uncultured and some of them are explained further. Specifically, 16S rRNA gene sequencing techniques have unveiled the existence of new bacterial groups that were not detected using the conventional cultivation studies (Joseph et al. 2003). In an attempt to cultivate the uncultured microbes, traditional cultivation methods have been regarded as inadequate and inappropriate, emphasis is on developing and practicing new techniques to isolate the novel microorganism (Joseph et al. 2003; Sait, Hugenholtz & Janssen 2002; Tan, Ward & Goodfellow 2006).

Over the years many methods are being devised and tried like the use of optical tweezers and micromanipulators (Fröhlich & König 2000). ***Manipulators*** are used for isolating the microbial cells and the techniques was proposed nearly 25 years ago. The equipment comes with a pressure device which is mounted onto an inverse phase contrast microscope. In terms of using the machine, the sterile capillary tube which is used for the isolation of the bacterial cells. The sterile capillary tube has a beveled tip placed at 45° angel and the opening of tip is 5-10 μm at anterior end. Posterior end of tube (Bactotip) is sealed with sterile oil. The function of the equipment is shown in Figure 1.5. If the nature of bacteria is to adhere to the glass surface, then the inner side of the tip can be siliconized with dichlorodimethylsilane. There are some benefits of the equipment which attract the microbiologist towards it like the magnification can be adjusted between 400X to 1000X and the size of capillary tip can be adjusted as per the size of the bacterial cell (Fröhlich & König 2000).

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Figure 1. 5 Functioning of Manipulators. Schematic picturization of isolation of single bacterial cell using the micromanipulator. The first diagram shows the bacteria on the cover slip and the bactotip has sterile oil in it. In the second diagram the resuspension of the bacterium is depicted

with the help of sterile oil. The third diagram illustrates the cell suspended in the bactotip (Fröhlich & König 2000).

The optical tweezers are another method which is being used. For this instrument, a neodymium laser is focussed with the help of microscopic objective. A rectangular glass capillary is used as the separation chamber which has fresh medium (90%) in it and the mixed microbial culture (10%). The desired cell is fixed to the laser beam and the separation is done from the mixed culture by moving the microscopic stage (it is controlled by the computer). The capillary is set with a predetermined breaking point and when that point is reached the single cell is isolated to the medium. The culture efficiency was 20 – 100% after the incubation of nearly 5 days. The plus point of this instrument is that the isolation of dead cells can be avoided after applying the fluorescent dye (bis-(1,3-dibutylbarbituric acid) trimethine oxonol) staining viable cells. The method gives promising results in terms of isolation of microbes which can not be in the pure culture with the use of conventional techniques (Fröhlich & König 2000).

Diffusion chamber - One of the big reasons of most bacterial population being uncultivated is because scientists fail to mimic the natural environment of those but with the development of diffusion chambers the natural surroundings can be imitated and isolation of novel bacteria was also accomplished. The microbes were placed in the diffusion chamber after the pre-treatment, dilution and mixing them with the warm agar made with seawater (refer to the Figure 1.6). The first membrane was attached to the chamber base and microbes with agar were poured in and the chamber was sealed using another membrane. The exchange of chemicals between the chamber and environment is possible because of the membranes used but they confine the cells movement. Sealed chambers were placed on the surface of the sediment and kept in a marine aquarium. Large colonies were observed with diverse morphology after the incubation for 1 week. The use of this method leads to the cultivation of uncultured microorganisms and also

the diffusion chambers are referred to be the only way to provide a suitable environment for sustainable growth (Kaeberlein, Lewis & Epstein 2002).

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Figure 1. 6 Diffusion chamber. The set up for cultivation of environment microbes at *in situ* conditions. The figure A represents he formation of chamber using a washer sandwich between two 0.03 μm pore size of polycarbonate membrane. B depicts the incubation of chambers on the surface of the marine sediment (Kaeberlein, Lewis & Epstein 2002).

Cell encapsulation is another interesting and novel way to culture the unculturable. Concentrates cell suspension was diluted for encapsulation. Diluted cells (0.1 ml) were mixed with preheated agarose (0.5 ml). for making the microdroplets the mix was loaded into CellMix emulsion matrix and emulsified at the room temperature using the CellSys 100 micro drop maker. Oil bacterial suspension was cooled with ice resulting in the gel microdroplets (GMD). 10% of GMDs are occupied by single encapsulated cell and it was monitored by the microscopy. GMDs were dispensed into sterile chromatography column containing medium (refer to Figure 1.7). Set of filter membranes were fitted at the inlet and outlet of the column to facilitate to retain GMDs and the washing of free-living cells to avoid the contamination caused by them. Incubation period in the column was nearly 5 weeks and after that the separation was done using the flow cytometry and microscopy (Zengler et al. 2002).

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Figure 1. 7 Encapsulation model. Cells from the environment were encapsulated in the preparation stage. In phase I the GMDs were incubated in the growth column. In phase II the detection of GMDs containing the microcolonies was done using microscopy and flow cytometry was used to separate the cells into 96-well plate having rich organic medium (Zengler et al. 2002).

But these methods are more specialized and will need specific skills to use them (Joseph et al. 2003). The most important technique is the isolation of the singe cells and once this is done techniques required to grow the cells. These techniques could be the result of modifications in the conventional methods like the incubation conditions (temperature, CO_2 concentration and incubation time). The medium used can also affect the growth of microbes. (Janssen et al. 2002; Nunes da Rocha, Van Overbeek & Van Elsas 2009; Pham & Kim 2012; Sait, Hugenholtz & Janssen 2002; Zengler et al. 2002). For example, media with different composition of nutrients, trace elements and pH levels will allow for the optimal bacterial growth with the prolonged incubation periods (Alain & Querellou 2009; Janssen et al. 2002; Nunes da Rocha, Van Overbeek & Van Elsas 2009; Sait, Hugenholtz & Janssen 2002). After the protocols for isolation of uncultured bacteria have been established than the designing of the procedures for isolation and characterization of specific microbes (like actinomycetes) which have biological and commercial value, can be established (Tan, Ward & Goodfellow 2006).

Biotechnology significance

The reason why scientists need to develop the techniques to culture the unculturable is because of the desire to produce the bioactive metabolites from these microbes which can help the mankind.

The production of antibiotics, antitumor agents and other products with therapeutic value can maintain human health therefore: they are of commercial significance as well (Piel 2002; Tan, Ward & Goodfellow 2006). The surge in the drug resistance among many bacterial pathogens and the increase in the fungal infections has also encouraged the scientists to find new compounds (Qin et al. 2009).

Hypothesis & Aim

Hypothesis

During this research two hypotheses were considered.

a. Culturing of the higher dilution of samples to allow isolation of the most abundant microbial genera.

b. Microorganisms that grow only in the liquid media are novel.

Aim

The general aim of the study was to isolate new bacteria from the different samples (soil, wheat seeds and sponge) using the liquid media in the 96-well microtiter flat bottom plates. During the study, the following objectives will also be achieved:

a) To investigate the optimisation of dilution for the samples.

b) To investigate the effect of variable salt concentrations, pH and temperature levels in the media on the growth of bacteria.

c) To determine the characterization of the isolates.

Chapter 2

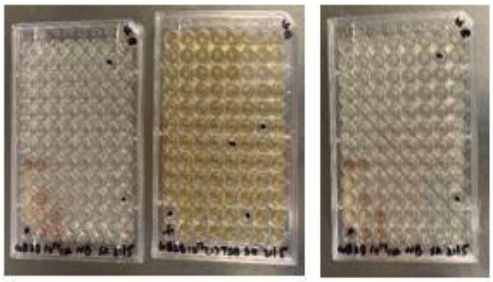
General Materials & Methods

SAMPLE COLLECTION

PRE-TREATMENT & DILUTIONS

SAMPLE PLATING – 96 WELL PLATES

- **MEDIA USED – 4**
- **TEMPERATURE - 27°C & 37°C**
- **REPLICATES – 2 FOR EACH SAMPLE IN EACH MEDIUM**



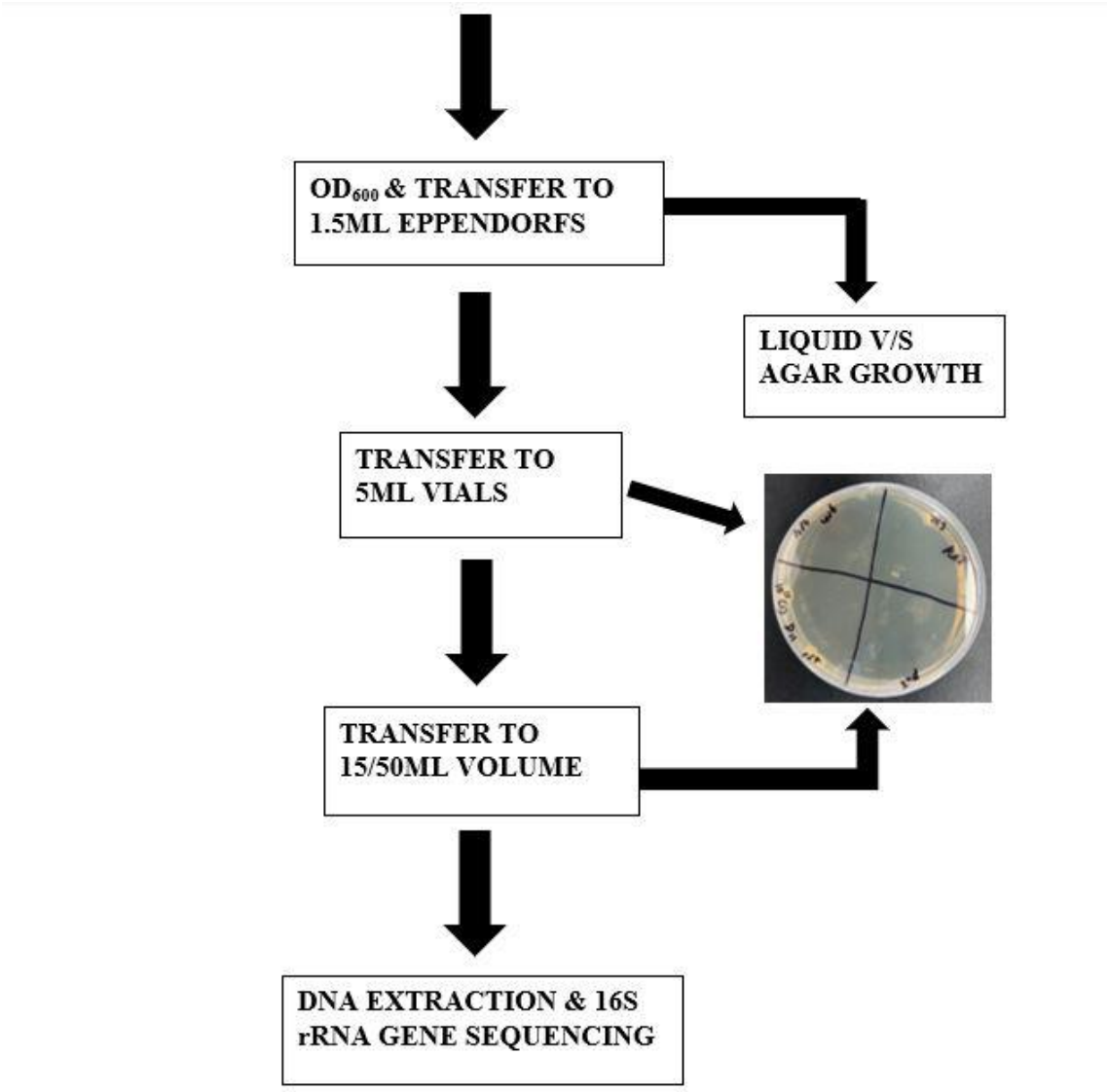


Figure 2. 1 Schematic diagram of research plan. The flow chart describes the steps followed in the study.

2.1 Sample collection & Pre – treatment

There were five different samples used in the study: wheat seeds, soil, three sponge samples as shown in the Table 2.1. The soil and wheat samples were kept at -20°C before and after use. All the sponge samples were kept at -80°C before and after use.

Table 2. 1 Details of samples used in the study.

Sample name	Collection point	Name used in study
Wheat seeds	South Australian Research and Development Institute (SARDI)	LWS
Soil	Turretfield, South Australia, collected in 2016	LCS
Sponge - GB28	Glenelg Beach	LGB
Sponge – RB16	Rapid Bay	LR16
Sponge – RB18	Rapid Bay	LR18

2.2 Pre - treatment of Samples

2.2.1 Pre – treatment of soil

The soil sample was pre-treated using the sonication method with a Sonics Vibra cell sonicator. One gram of soil was weighed and added to a sterile falcon tube containing 9ml of sterile saline. The mixture was vortexed for 2-4 seconds and was sonicated for 15 seconds with a 2 minutes pause – this step was repeated 3 times. The sample was allowed to stand for around 10 minutes after which, the dilutions were carried out.

Another set of soil sample was setup without any pre-treatment, 1 gm of soil was added in the 9 ml of saline solution followed by the gentle mixing after that the sample was allowed to settle for 15-20 minutes, before the dilutions were done.

2.2.2 Pre-treatment of wheat seeds

One gram of wheat seeds was weighed before the surface sterilisation process. The seeds were treated with 70% ethanol for 2 minutes followed by immersion in 4% NaOCl for 4 minutes and washing with sterile RO water twice before adding the 5% $Na_2S_2O_3$ to the seeds for 4 minutes

so that the NaOCl can be neutralized. The final step of sterilization was to wsahthe seeds with sterile water twice.

The seeds were transferred to the sterile mortar and pestle and were crushed until a homogenous paste was made. The wheat seeds paste was added to the 15ml sterile falcon tube with 9ml of sterile RO water.

2.2.3 Pre-treatment of sponge

a) RB16 & RB18

These two samples were obtained from the Medical Biotechnology department which were already diluted to 10^{-1} and the experiments on these two samples was done from these diluted ones. Hence, no pre-treatment was needed for these two samples but the GB28 was pre-treated and the process followed for RB16 & RB18 was similar to that of GB28, which is explained further.

b) GB28

The sponge sample GB28 was stored at -80°C and for pre-treatment process it was kept in sterile natural sea water (as the sea water was autoclaved) at room temperature for an hour. It was done so that the sponge could be cut easily into small pieces. After cutting the sponge (1 cm) with a sterile blade it was weighed (approx. 1gm) and washed 5 times with the sterile sea water.

The sponge sample was transferred to the sterile mortar and pestle and crushed until a smooth paste was obtained and in the process no water was added as the sponge sample will release some water during the process. After that the crushed sponge was transferred into a sterile 15ml falcon tube. The dilution was done using the crude extract of the sponge and rest was stored at -80°C.

2.3 Theoretical underpinnings

The aim of the dilution serial dilution is by 'over diluting' the sample to obtain a single cell in a well. For example, if a soil sample has 10^{11} to 10^{12}cells per g soil then the 10^{12} dilution should give 1 cell per ml. As 200µl of the dilution is added per well, there should be 1 cell per 5 wells in the 96 well plate – or up to 19 wells in which growth should occur. At the 10^{14} dilution there should be 2 well that had a single cell per 96 well plate. The 10^{12} and 10^{14} dilutions were used in case there are more than 10^{12} cells per gram soil.

The rationale for observing growth on agar is that there are some taxa that will not grow on agar. And it is hypothesised that if a culture has not grown on agar or grows poorly it is likely to be new (Albuquerque et al. 2011).

2.3.1 Dilutions

There were several sets of dilutions plated and tested to observe the dilution number at which bacterial growth in a maximum 5 cells per 96 well plate can be observed. The first set of dilution which was plated was 10^{-10} and 10^{-11}. The dilution process was 1 in 9 till 10^{-8}

- For 10^{-10} - 2ml from 10^{-8} was taken and added in 198ml of solution
- For 10^{-11} - 20ml of from 10^{-10} was taken added to 180ml of solution

The another set of dilution which were plated was 10^{-12} and 10^{-14}. To reach those dilution numbers the process was 1 in 9 till 10^{-10}

- For 10^{-12} – 1ml from 10^{-10} + 99ml of solution
- For 10^{-14} – 1ml from 10^{-12} + 99ml of solution

The five samples were pre-treated and diluted, to optimise the dilution number, different sets of dilutions were tested like 10^{-10}, 10^{-11}, 10^{-12}, 10^{-14}.

2.4 Sample plating and Incubation

The dilutions made for all the samples was plated in 4 different media namely **TSB, ISP2, LB, and NB** (recipe in the appendix) made up to 5X strength. **Two temperature** conditions (27°C and 37°C) were used for plating the samples and all the samples after plating were incubated at both the temperature. The **pH** of the media was 7.5 and each sample was plated in the duplicate for each temperature and medium. The diluted samples were added to 96-well plates in each well 200ul of chosen dilution sample was added and 50ul of 5X strength medium. A detailed summary of the plating of the samples in different media and temperature is mentioned in the Table 2.2. Table 2.3 further describes the details of the samples plated during different months which were studied during the research.

The **incubation** period for the sample at the initial stage (in 96 well plates) was 4-5 weeks and OD_{600nm} (optical density) was taken after that using GloMax Explorer (Promega) plate reader and wells were selected randomly with a range of OD_{600nm} (refer to Table 1 in appendix). The selected wells were further transferred to 1.5ml Eppendorf tubes containing a single strength medium and the incubation time varied between 3-4 weeks. The turbid Eppendorf tubes were transferred to the 5ml vials followed by final transfer to 15ml/50ml tubes or flasks and the incubation time for the stages was 2-3 weeks to 1-2 weeks respectively.

Table 2. 2 The tabular chart depicts the detailed summary of all the samples plated under different conditions.

Media	**Temperature**	**pH**	**Samples**	**Dilutions**
TSB (Tryptone Soya Broth)	27°C & 37°C	7.5	Wheat seeds, sponges, soil	10^{-10} & 10^{-11}. 10^{-12} & 10^{-14}
ISP2 (International *Streptomyces* Protocol 2)	27°C & 37°C	7.5	Wheat seeds, sponges, soil	10^{-10} & 10^{-11}. 10^{-12} & 10^{-14}
LB (Luria-Bertani Medium)	27°C & 37°C	7.5	Wheat seeds, sponges, soil	10^{-10} & 10^{-11}. 10^{-12} & 10^{-14}
NB (Nutrient Broth)	27°C & 37°C	7.5	Wheat seeds, sponges, soil	10^{-10} & 10^{-11}. 10^{-12} & 10^{-14}

Table 2. 3 Data of samples plated. The table describes the total number of times samples were plated at which temperature and in which month. This data is for initial plating and it was done in 96 – well plates.

	March/Temp	**April/ Temp**	**May / Temp**	**June/ Temp**
Soil	3 times/ 27°C	3 times/ 27°C + 37°C		1 time/ 27°C + 37°C
Wheat Seeds		3 times/ 27°C + 37°C		
Sponge (GB 28)		1 time/ 27°C + 37°C	1 time/ 27°C + 37°C	
Sponge (RB18)	1 time / 27°C	2 times/ 27°C + 37°C		
Sponge (RB 16)		2 times/ 27°C + 37°C		

2.5 Liquid v/s Agar growth

After at least 5 weeks incubation, the OD_{600nm} was read and the contents of the well which had an O.D. reading of 0.1 and higher in less than 5 wells per 96 well plate were transferred to sterile 1.5 ml Eppendorf tubes. The isolates were tested on agar plates as well to observe the nature of the bacteria. The Eppendorf tubes containing 200µl from the well and 1.3 ml fresh medium were incubated at 27 and 37^0C for 4-5 weeks until turbidity was seen in the tubes. At the same time 20 µl of the content of the 96 plate well was tested for growth on agar by drop plating 2x 10 µl onto the respective agar medium to match the broth medium in which the

growth was observed. Viability on agar was tested at all the stages (96 well plate stage, 1.5 ml Eppendorf tubes, 5ml vials and 15/50ml). To do that each plate was divided into either three or four sections (refer to Figure 2.2).

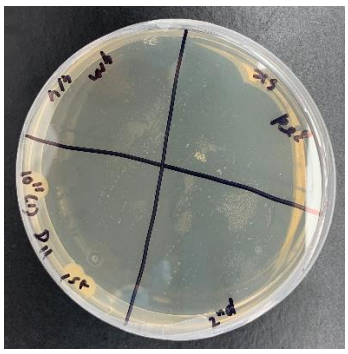


Figure 2. 2 Drop plating. The figure shows the method used for testing the liquid v/s agar growth of the isolates. Two drops of 10µl each was drop plated and incubated to observe for growth on agar.

2.6 Whole cells (Colony) PCR

The DNA of the bacterial cultures which were only growing in liquid media was extracted using the whole cell (colony) PCR technique. For this method the cells were grown in the 15/50ml volume to get a bacterial pellet. 10mM Tris (pH 7.4) was added to the pellet, after mixing the exposure to -80°C and 37°C was done to achieve the freezing and thawing of the samples. It was done twice before setting up the PCR.

2.7 DNA Extraction using beads

Another DNA extraction method was used for some strains. The method is different from the methods used to extract DNA for whole cell(colony) PCR. The method is based on the use of beads and a modified CTAB buffer (Hexadecyltrimethylammonium bromide) (Taylor et al. 2004). Apart from these two methods some other methods (refer to Table 2.4) were also tried to extract the DNA of the strains with more polysaccharide content, but the successful one used Sodium Dodecyl Sulfate (Xiao et al. 2011). This method was used to extract the DNA of **LGB 79** strain from 37°C.

Table 2. 4 The table depicts the data of DNA extraction for the isolates.

	ISOLATES IN LIQUID MEDIA	**ISOLATES IN AGAR MEDIA**
CTAB METHOD (27°C)	21	7
COLONY PCR (27°C)	10	0
CTAB METHOD (37°C)	3	6
COLONY PCR (37°C)	2	0

Polymerase Chain Reaction

Polymerase chain reaction (PCR) was setup for each extracted bacterial DNA (Raeymaekers 2000). The PCR was conducted using a Axygen Maxygene thermocycler and the protocol comprised of an initial denaturation of 94°C for 3 minutes, 40 cycles of 94°C for 1 minute, 56°C for 1 minute, 72°C for 2 minutes and a final extension of 72°C for 10 minutes. The reaction mix in each 200µl PCR tube is described in Table 2.5. The actinobacteria-based primers used for 16S rRNA gene are 27F (5'-AGA GTT TGA TCM TGG CTC AG- 3') & 765R (5'-CTG TTT GCT CCC CAC GCT TTC- 3'). The bacterial based primers used for 16S rRNA gene are 341F (5'-CCT ACG GGI GGC IGC A- 3') & 926R (5'-CCG ICI ATT IIT TTI AGT TT- 3') (Watanabe, Kodama & Harayama 2001) and the PCR protocol for primers with an initial denaturation of 94°C for 3 minutes, 40 cycles of 94°C for 1 minute, 47°C for 1 minute, 72°C for 2 minutes and a final extension at 72°C for 10 minutes. For fungi like strains the 28S rRNA gene was amplified using the primer set LROR (5'- ACC CGC TGA ACT TAA GC-

3') & LR5 (5'- TCC TGA GGG AAA CTT CG- 3'). The PCR protocol for these primers and PCR primer control was similar to the one used with 27F/765R primers.

Table 2. 5 The table describes the reaction mix for each PCR tube. The total volume for the reaction was 50 µl . The reaction mix used is from New England BioLabs.

Contents in PCR tubes	**Quantity (µl)**
Taq 2X Master Mix	24
Injection water	19
$MgCl_2$	1
Primers – forward + reverse (5uM)	2 + 2
DNA	2

Gel electrophoresis

1% agarose gels were used to run the DNA samples. To make 1% gels, scientific agarose gel is mixed TBE solution and heated for 60 seconds in microwave. GelRed (Biotium) is added to visualize the DNA bands before pouring the gel in the tank. Samples (5 µl) were loaded after mixing in the loading dye (1 µl) 6X concentration from the manufacturer New England BioLabs, the marker used was from Thermo Scientific™ GeneRuler 100bp Plus DNA Ladder. The gel was run for 35 minutes at 70V. The DNA in the gel was visualized using a UV transilluminator and the image was photographed.

Sequencing

All the samples were sequenced using sanger sequencing technique (Heather & Chain 2016). The DNA samples were sequenced at Macrogen Inc.in South Korea. After the sequencing

result, the chromatograms were analysed, and the selected base pair sequence of the strain was characterized after their BLAST from NCBI.

Chapter 3

Results

3.1 Dilutions

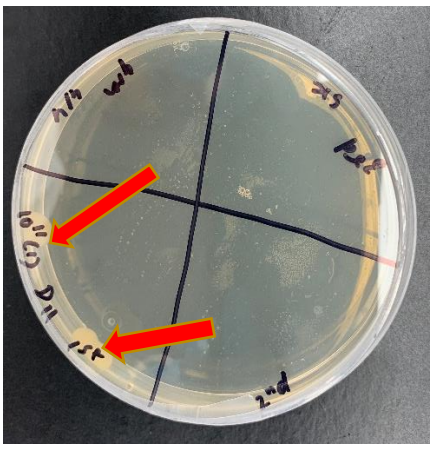
The bacterial growth was observed at the higher dilution number 10^{-12} and 10^{-14}**.** These two dilutions were selected for the further experiments during the research.

3.2 Incubation

It took almost 10 to 12 weeks' time in total from the preparation of the 96 well plates for a culture to reach the stage for DNA extraction. On the basis of growth total of 403 isolates were studied during the project.

3.3 Liquid v/s Agar Growth

The isolates were observed to grow in liquid culture and on agar plates at different stages as illustrated in figure 9. Isolates which were growing on agar are shown in the Figure 3.1. The bacteria which grew only in liquid medium were very limited in number as compared to the isolates growing on agar (Table 3.1) and the incubation time required for their growth was different as per the temperature they were incubated in. The isolates at 37°C took more time to obtain a small pellet as compared to the bacterial isolates kept at 27°C. There were isolates spotted which did not grow on agar until 5ml stage but after testing from the 15ml tubes they were found to grow on the agar plates.

(a) 

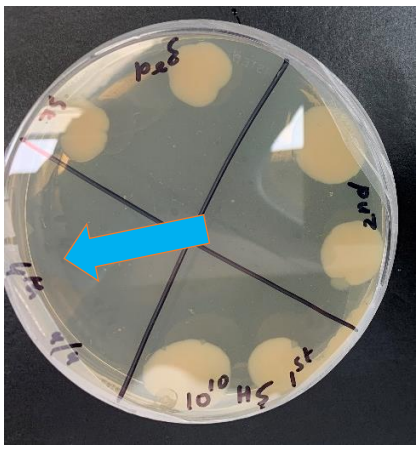
(b) 

Figure 3. 1 Drop plating for liquid v/s Agar. The isolates were plated in divided plate to observe if they grow on agar, it was checked for all 403 isolates. **a** show the growth of bacteria in one

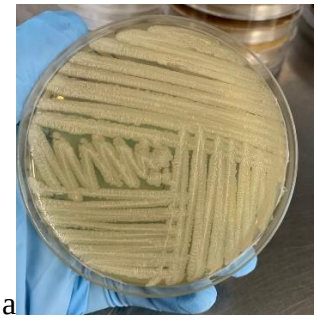
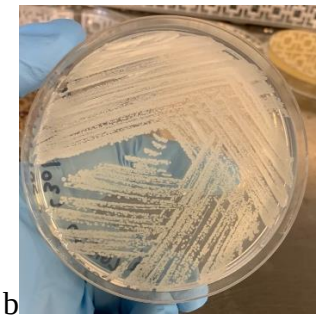
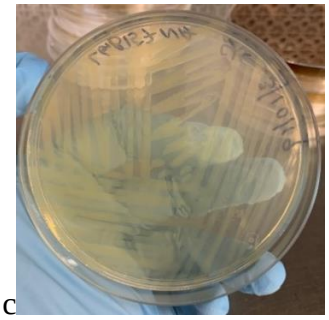
section of divided petri plate (pointed with red arrow); **b** shows the only section without the growth (pointed with blue arrow).

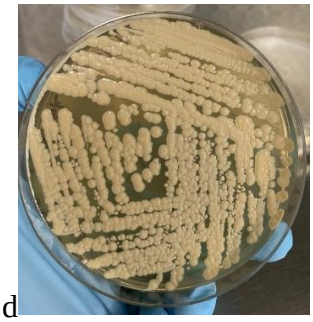
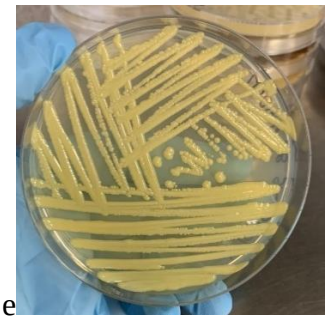
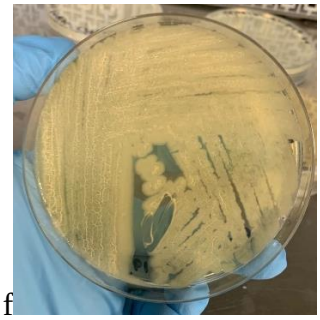
Table 3. 1 The temperature wise summary of isolates. The table depicts the data of all the isolates at different temperatures including the nature of their growth. (* the bacterial isolates were growing after testing from 15ml tubes.)

	27^0C	**37^0C**
Total isolates	287	116
Grows on agar	241	94
Grows in liquid*	33	5
Blanks grown to 5ml stage	2	4
Contaminated	11	13

3.3.1 Isolates growing on agar

The isolates growing on agar were categorized on their colony morphology. The isolates from 27°C and 37°C were categorised into eight and seven different morphotype shown in Figure 3.2 and 3.3 respectively. At 27°C the representatives of each of the eight morpho-types are LCS 172, LGB 170, LGB 157, LGB 146, LGB 164, LCS 71, LGB 313 and LCS 356. The growth types are further detailed in Figure 3.4 and 3.5 which shows the total count of bacterial strains under each category at both the temperatures. At 37°C the 7 morpho-types are LGB79, LR18-218, LGB231, LGB125, LGB232, LGB130 and LGB392.

a  b  c 

d  e  f 

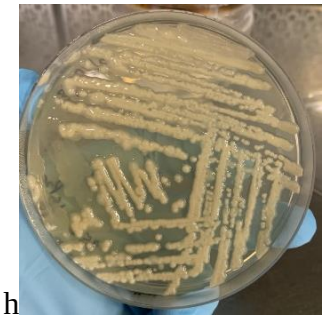
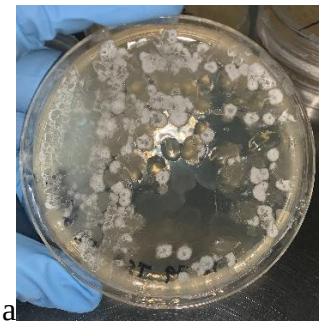
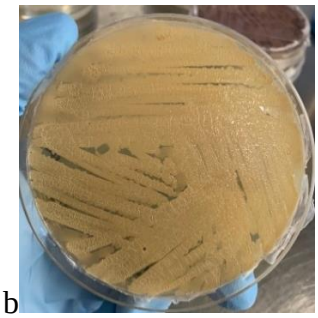
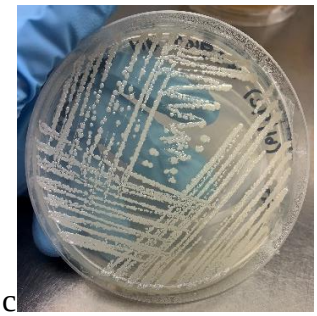
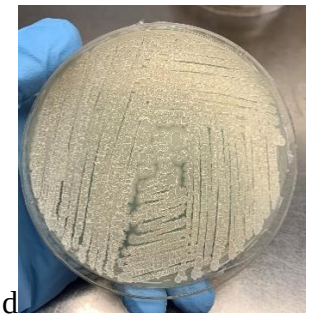
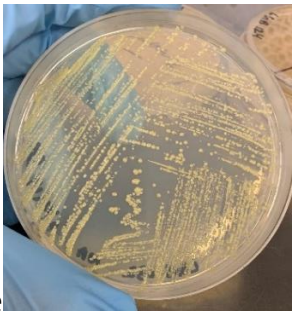
g  h 

Figure 3. 2 Isolates at 27°C. The above figure represents the growth types observed at 27°C. The bacteria growing at 27°C were categorised in the eight types based on the colour and similar morphology growth shown by the strains from all the sample at this temperature. The following pictures shows the representatives from each type. Figure “a” is picture for LCS 172 followed by b - LGB170, c – LGB157, d – LGB146, e – LGB164, f – LCS71, g – LGB313 and h – LCS356.

a  b  c 

d  e  f 

g 

Figure 3. 3 Isolates at 37°C. The above figure represents the growth types observed at 37°C. The growth observed among the strains was grouped into seven different types where the representative was photographed and are shown in the figure. For first type the representative is shown in figure a – LGB79, b – LR18-218, c – LGB231, d – LGB125, e – LGB232, f - LGB130 and g – LGB392.

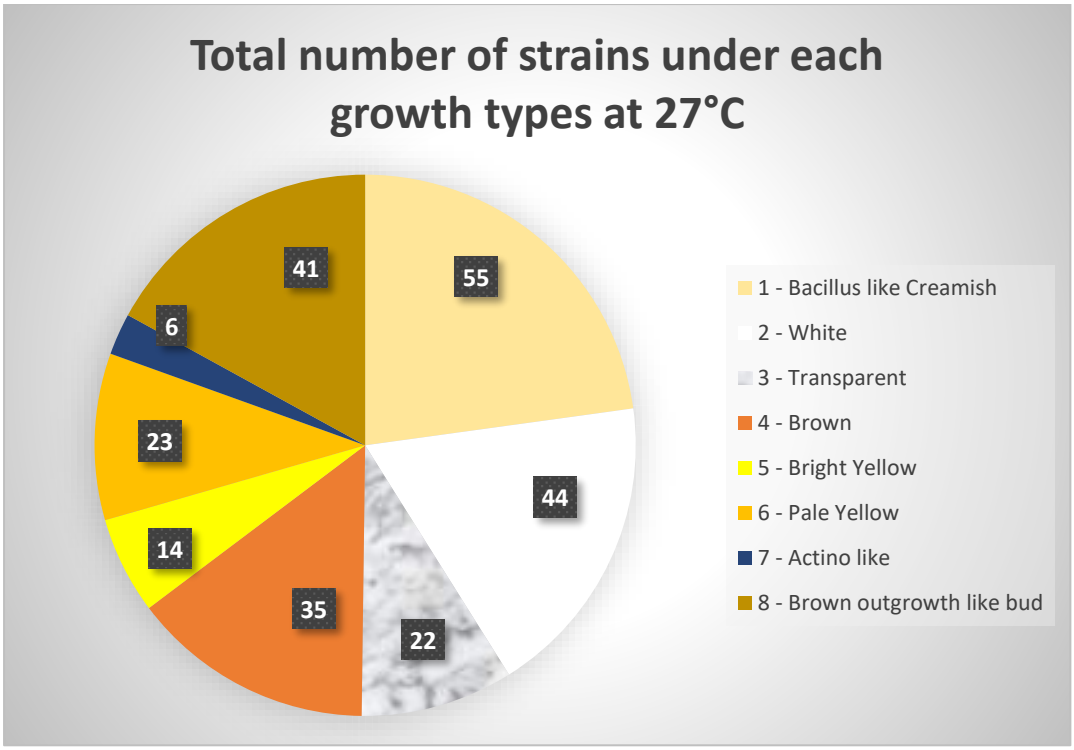


Figure 3. 4 Number of strains at 27°C. The pie chart depicts the total number of strains at 27°C under each growth type. The colours of the pie chart sections are related to the colour of the bacterial growth type and the figure legends are numbered in accordance to the type of growth.

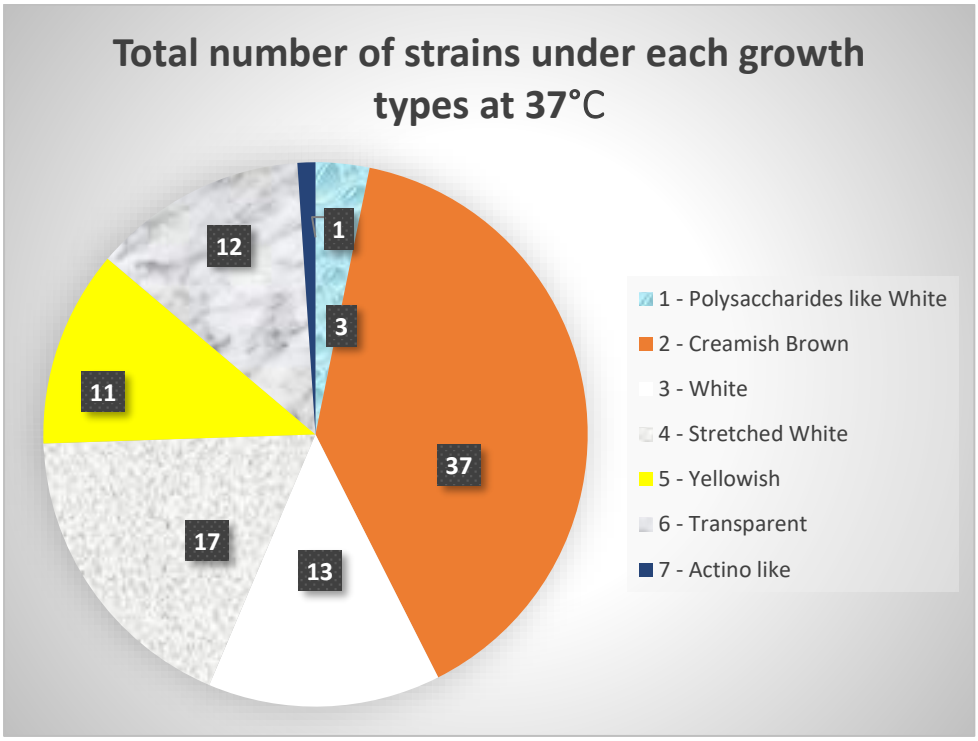
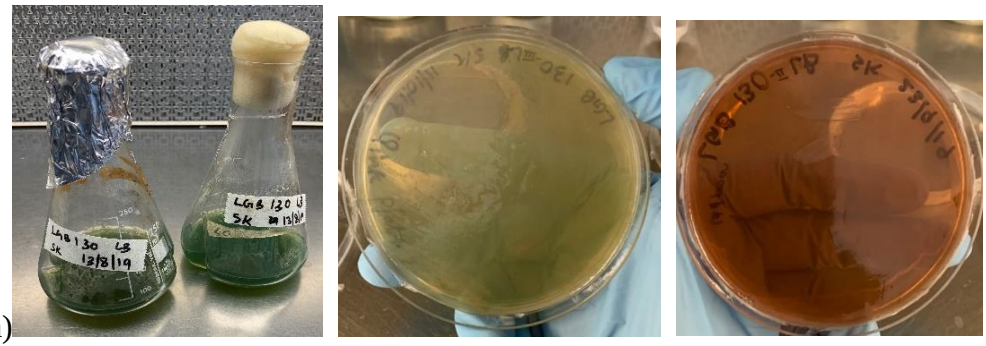
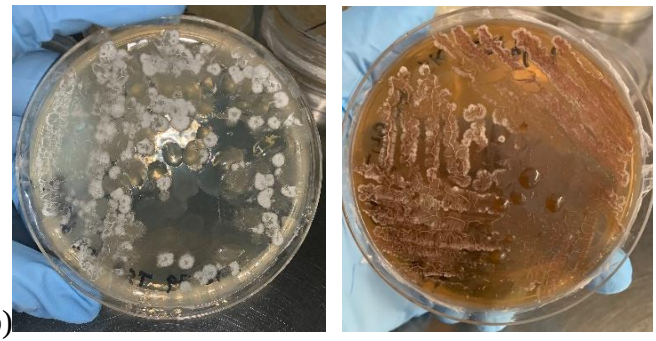


Figure 3. 5 Number of strains at 37°C. The pie chart depicts the total number of strains under each category at 37°C. The bars are colour coded as per the growth colour of the strain. The legend in the right-hand side of the pie chart describes the seven categories at 37°C and the numbers in the pie chart shows the total strains under the respective category.

3.3.2 Isolates showing different growth colours

Some isolates were observed to show different colours in their growth after longer incubation. The other interesting fact noticed here is that the change in colour among the strains was noticed at only 37°C. When the strains were sub-cultured except one all the other isolates were showing the replicative growth. The samples detected with showing varying colours are LGB 79, LGB 231 and LGB 130. The original growth colour of LGB 130 was green in liquid medium but when tested on agar the colour changed from green to red (refer to Figure 3.6 (a)). Another strain with similar trait was LGB 79 (Figure 3.6 (b)) and the colour varied from white to pinkish. LGB 231 had shown not only different colour but also the different growth pattern. During the initial sub-culturing the growth was like normal bacterial strain (refer to Figure 3.6 (c))

a) 

b) 

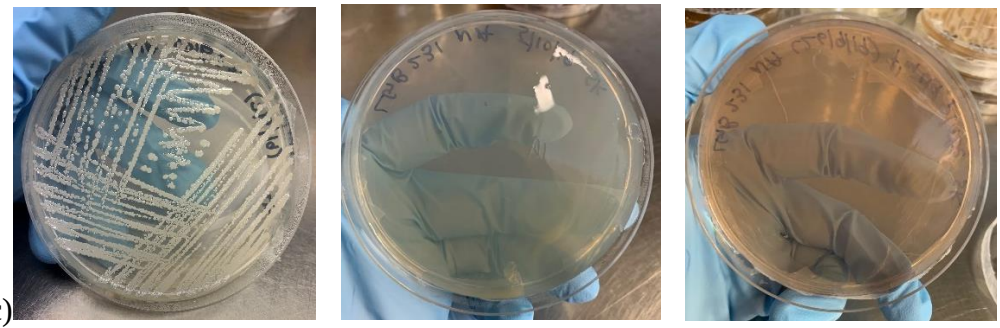
c) 

Figure 3. 6 Isolates showing different growth colours. Different growth colours observed in the isolates, (a) describes the colours noticed in the growth of LGB 130, the colour started varying from green to the red. The isolate was not growing on agar when tested from 5ml but after growth in 50ml the growth on agar can be noticed over the incubation period of 3-4 weeks. (b) LGB 79 was also observed with different growth colours from creamy white to pinkish. The incubation time difference between both the plates was of almost 4-6 weeks as after this time a different colour in the plate was noted but the results of coloured plates was not reproducible with pink replicate after two plates. (c) LGB 231 was showing white coloured growth which changed to light pink at the end and it was noticed after incubation of 6 – 8 weeks.

3.3.3 Isolates growing in liquid

Only a small number of strains were found growing in liquid culture (refer to table 6). The strains were also tested for their growth on agar and till they were plated from 5ml volume there was no growth observed on the plates but when they were plated from 15ml the growth can be noticed from almost all the strains on agar plates. For some isolates the growth was noticed in full plate while some where showing just the traces of growth on agar.

3.4 DNA Extraction & PCR

The DNA extraction was done to amplify the16S rRNA genes in order to characterise the bacteria. The CTAB method was used to extract the DNA for the major proportion of isolates (refer to table 4 in chapter 2). The DNA for isolate LGB 146 from 27°C could not be extracted as all the three methods used in the study were used but no results were obtained. Figure 3.7

shows the bands of extracted genomic DNA using the CTAB method and Figure 3.8 depicts the DNA extraction using colony PCR method. Figure 3.9 shows the bands of the amplified DNA using the 16S rRNA technique with 27F and 765R primers. The gel image of primer as a control is attached in the appendix 2. The results for 28S rRNA gene LROR and LR5 are presented in Figure 3.10, there were no bands observed for all the six strains. But LGB123 and LGB 130 are categorised as *Pseudomonas aeruginosa* after the sequencing while other 4 strains could not be categorised further.

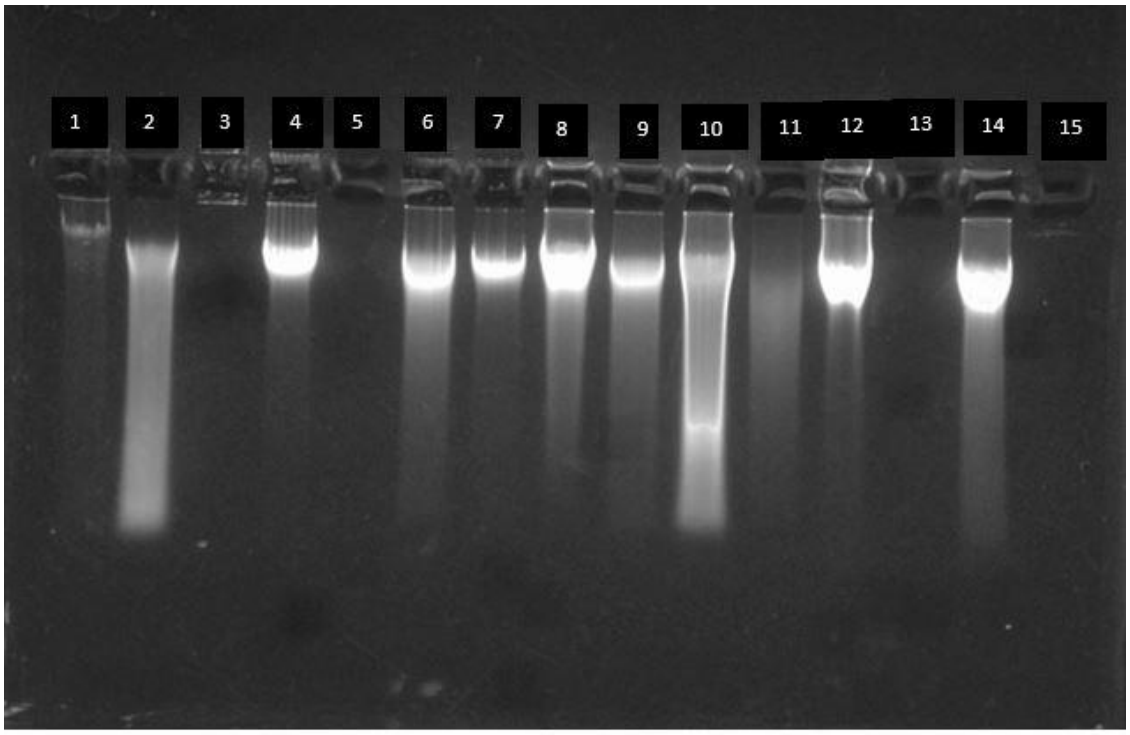


Figure 3. 7 The genomic DNA. The figure depicts the DNA bands after genomic DNA extraction. The lanes are indicated with the arrow. Strains loaded were, lane 1 – LGB79, lane 2 – LGB143, lane 3 – LGB146, lane 4 – LGB152, lane 5 – LGB153, lane 6 – LGB154, lane 7 – LGB155, lane 8 – LWS252, lane 9 – LWS282, lane 10 – LWS290, lane 11 – LWS291, lane 12 – LGB308, lane 13 – LR18-318, lane 14– LCS421, lane 15 – LCS422.

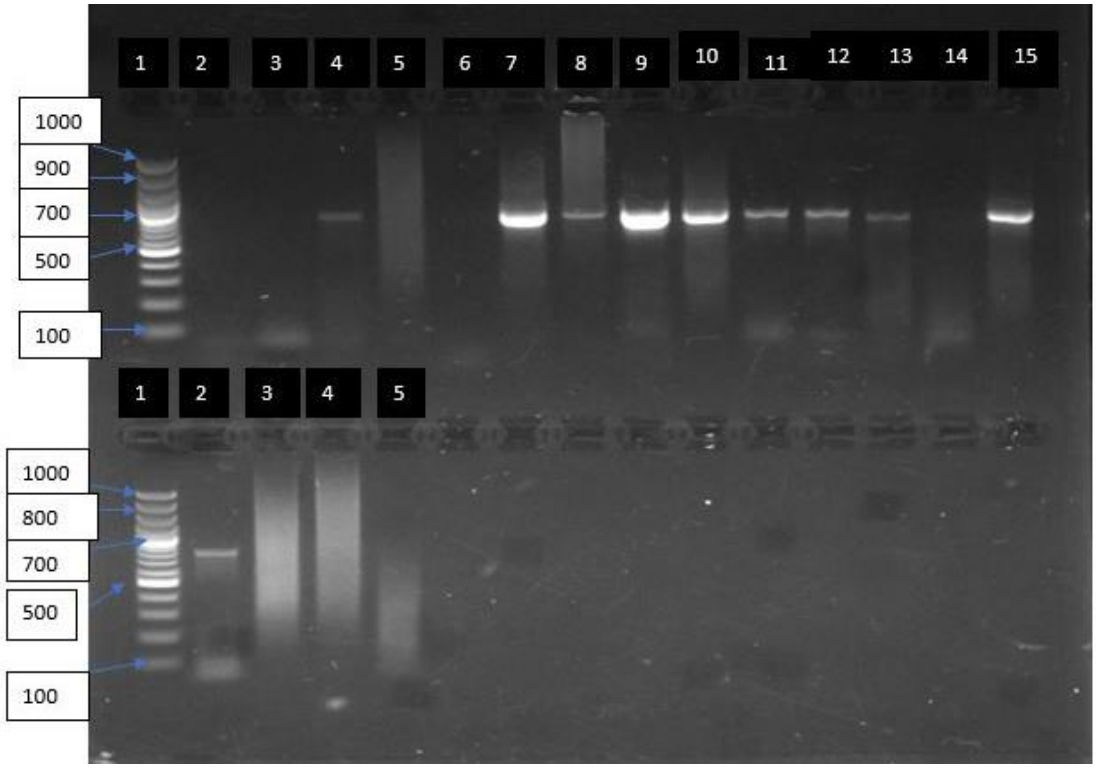


Figure 3. 8 Colony PCR. The DNA for the strains in the gel photo was extracted using colony PCR and 16S rRNA sequencing was done using the primers 27F and 765R. The first row was loaded with following – lane 1 – marker (100bp), lane 2- LGB119, lane 3- LGB121, lane 4- LGB123, lane 5- LR18-217, lane 6- LR18-219, lane 7- LGB246, lane 8- LWS257, lane 9- LCS264, lane 10- LCS271, lane 11- LWS283, lane 12- LWS289, lane 13- LGB304, lane 14- LGB307, lane 15- LR18-315. The second row has marker (100bp) in lane 1, lane 2 - LR18-317, lane 3- LCS402, lane 4- LCS414, lane 5- LGB418.

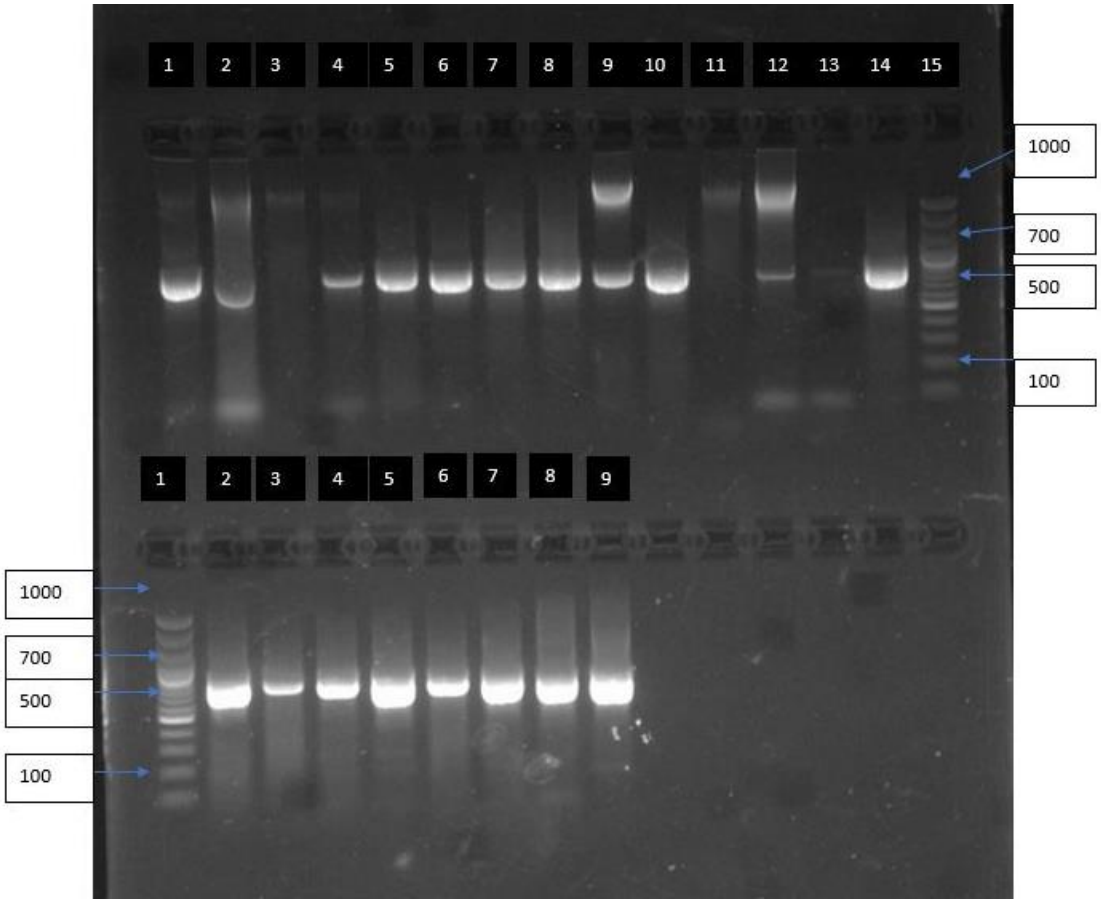


Figure 3. 9 PCR results. PCR result for 16S rRNA sequencing using the primers 27F and 765R. The first row was loaded with following – lane 1 –LGB79, lane 2- LGB130I, lane 3- LGB130II, lane 4- LGB130III, lane 5- LGB143, lane 6- LGB152, lane 7- LGB154, lane 8 – LGB155, lane 9- LGB164, lane 10- LWS290, lane 11- LGB231I, lane 12- LGB231II, lane 13- LGB231III, lane 14- LWS252, lane 15- marker (100bp). The second row has following - lane 1 – marker (100bp), lane 2- LWS282, lane 3- LWS291, lane 4- LGB306, lane 5- LGB308, lane 6- LR18-319, lane 7- LCS421, lane 8 – LCS431, lane 9- LCS432.

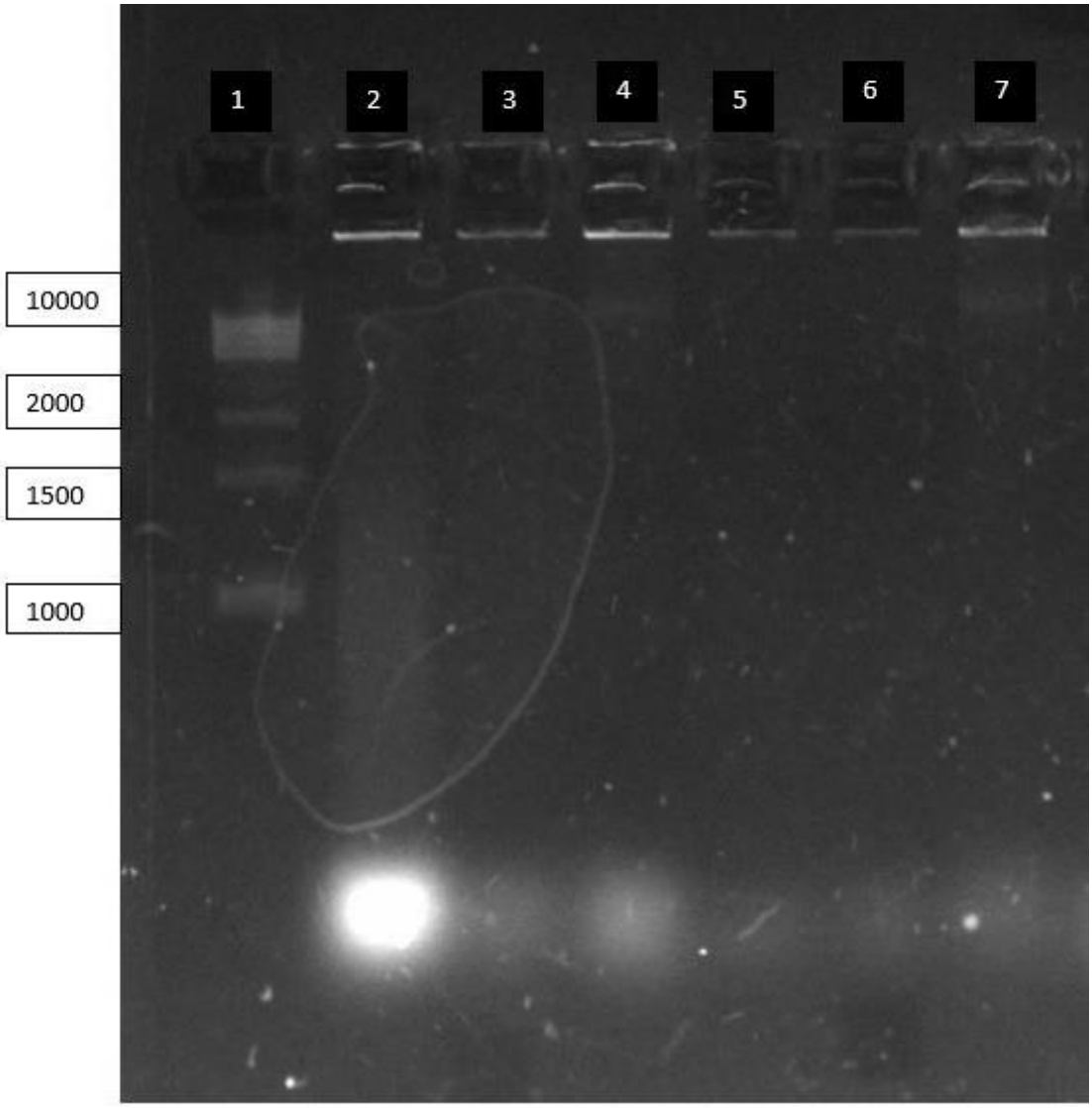


Figure 3. 10 PCR gel image for fungi primers. The gel image shows the result of primers LROR and LR5. There were no bands present which signifies that samples tested do not come from fungi kingdom. Lane 1 had marker (Sigma 1KB), lane2 – LGB117, lane3 – LGB119, lane4 – LGB121, lane5 – LGB122, lane6 – LGB123, lane7 – LGB130.

3.5 Sequencing Results

The purpose of performing 16S rRNA sequencing was to identify the selected isolates to the species level. Total number of strains sent for sequencing were 44 and the results of the chromatograms were checked, the strains showing a clean chromatogram were amplified using the actinobacteria-biased primers. And the chromatograms of 18 strains showed multiple peaks and reliable results cannot be deduced from them. The Table 3.2 describes the results of

sequencing which further characterise the isolates. The sequencing results for LGB 79 and LGB 130 could be obtained, both the strains were of morpho type from 37°C.

Table 3. 2 The sequencing result. The table illustrates the data obtained for the isolates after 16S rRNA sequencing.

Isolate name	**Result**	**% Similarity**
LGB 79	*Pseudomonas aeruginosa*	100%
LGB 123	*Pseudomonas aeruginosa*	100%
LGB 130	*Pseudomonas aeruginosa*	99.69%
LGB 143	*Paenibacillus polymyaxa*	100%
LGB 152	*Bacillus mojavensis*	100%
LGB 154	*Bacillus subtilis*	100%
LGB 155	*Bacillus mojavensis*	99.86%
LGB 246	*Staphyloccocus epidermidis*	100%
LWS 252	*Bacillus subtilis*	100%
LCS 264	*Bacillus subtilis*	100%
LCS 271	*Bacillus subtilis*	100%
LWS 290	*Bacillus subtilis*	100%
LGB 306	*Bacillus subtilis*	100%
LR18 – 315	*Bacillus subtilis*	100%

LCS 100	*Pseudomonas aeruginosa*	100%
LCS 260	*Staphyloccocus epidermidis*	99.82%
LCS 47	*Bacillus subtilis*	99.72%
LGB 53	*Bacillus subtilis*	99.80%
LGB 131	*Pseudomonas aeruginosa*	100%
LR18 – 390	*Pseudomonas aeruginosa*	100%
LWS 288	*Bacillus subtilis*	99.86%
LWS 295	*Bacillus mojavensis*	100%
LWS 275	*Bacillus mojavensis*	100%
LGB 308	*Bacillus mojavensis*	100%
LCS 421	*Bacillus subtilis*	100%

Chapter 4

Discussion

4.1 Discussion

The samples selected for this study were soil, wheat seeds and sponge (three samples of sponges) and the reason of choosing these samples was that they each contained a high proportion (>50%) of uncultivated microbes and hence the chances of isolating one of these strains was higher.

The limiting dilution technique was used in an attempt to culture strains that were hitherto uncultured by allowing a single cell to grow in a nutrient rich medium (Stewart 2012). As the source samples had >50% of uncultured strains the chances of pipetting a new strain was expected to be 1:1. Generally, the dilutions done to grow microbes on the samples range between 10^{-6} to 10^{-9} (Davis, Joseph & Janssen 2005) but in this study the dilution range was 10^{-12} and 10^{-14}, which is quite high, and it indicates the presence of microorganisms at higher dilutions.

The incubation time for the isolates needed was longer than usual as the sample inoculum size of one cell per well had to grow to a level where the O.D. had to increase to a detectable level. The prolonged incubation gave the culture sufficient time for the growth of those microorganisms (Janssen et al. 2002; Sait, Hugenholtz & Janssen 2002).

After the detection of growth at OD_{600nm}, the agar v/s liquid growth was analysed for the selected strains, but contamination was one of the big challenges as the isolates in liquid media were found to be more susceptible to contamination.

The microorganisms growing on the agar at 37°C were showing different colours, this could be due to stress as a result of nutrient depletion or the increase in temperature. Another reason could be the longer incubation time of the plates. As wet paper towels were used to maintain the moisture in the lunch box containing the plates and the inadvertent drying of towel results in less moisture in the growth environment. And less moisture can have also triggered the change in the bacterial growth. The other possibility of observing the change in the colour

could be due to the cross contamination of the sample while plating. The other reason can be that the plates had mixed cultures and the dominant one was able to grow after the longer incubation. The three strains which showed diffusible pigments which were different from the others. The sequenced results for LGB 79 and LGB 130 presented in table 7 showed that they belong to the *Pseudomonas* species which are already known. While for LGB231 no sequencing results could be achieved as thePCR product was not obtained the isolates growing on agar were categorised as per their colour and morphology. The reason for working on them was to identify whether the strains growing on agar plates were novel and to test the second hypothesis.

As a number of isolates produced polysaccharides it made DNA extraction more challenging. Different approaches were used to extract DNA as per the growth type of the isolates and they were used to get a clear DNA band as smearing of DNA was very common issue (refer to figure 15 in results section). In the CTAB methods some minor changes, like reduction in the bead beating time and addition of RNAse incubation step, were done to attain the desirable results. Whole cells (colony) PCR was more efficient in obtaining the amplified 16S rRNA gene from the liquid cultures. The PCR bands for 6 strains were not observed after the use of different set of 16S rRNA gene primers and hence those strains can be novel.

Of the 44 cultures sequenced, 26 strains showed a clean chromatogram. The results indicate that the strains from different samples belongs to only a limited number of bacterial species that is *Pseudomonas, Paenibacillus and Bacillus*. The result for strains growing on agar plates were obtained for two cultures only (LGB79, and LGB130). The sequencing results were similar to that of the growth patterns observed. These results were not expected as no level of novelty was found. Therefore, the reasons for these results must be analysed. The first and foremost reason could be the abundant presence of these species in the natural environment among the microorganisms as the commonly isolates microbes belongs to the phylum

Actinobacteria, members of phyla Proteobacteria (classes *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*), *Bacteroidetes*, and *Firmicutes* (Davis, Joseph & Janssen 2005) (refer to appendix 4 for the results of commonly isolated microbes). It could happen that if those species are present in the sample in large amounts then they were likely to grow, as with dilutions the possibility of growing the abundant microbes are high (Janssen et al. 1997). The percentage of cultivation of *Firmicutes* from sponge is also represented in figure 2 (Hentschel et al. 2012). However, the other reason of contamination is the storage of samples for longer time. There were more manual steps involved in each experiment and the contamination while plating can also be the other reason for this despite maintaining and working in sterile conditions. the medium should be changed so that they can find nutrient which are found in their (samples) natural environment. The last possibility here could be the use of old sponge sample as the sponge used in the study was not freshly collected. The sponge was collected a year ago and it could lead to the contamination by Bacillus. The other source of contamination could be the primers. However, a primer control with no sample DNA template was also run and there were no bands observed in the gel image (refer to appendix 2) which means the primers used were not contaminated. As the sequenced data belonged to a limited microbial species so the phylogenetic tree could not be constructed for them. The clean chromatograms are considered more important in the study because the main focus of the study was to isolate novel microbes. And the chromatograms with multiple peaks could be due to the presence of the mixed cultures in those strains. It is hard to check the presence of the mixed bacterial strains in a liquid culture

5. Conclusion & future directions

During this study total of 44 strains were sequenced from the three samples of sponge, wheat seeds and soil using the 16S rRNA gene. The results of the characterization of 26 strains (which had clean chromatograms), showed that the isolates belonged to the more common species. These bacteria are known to be the most commonly isolated fast-growing bacteria. The growth of bacteria from the liquid culture showed no novelty as the strains growing in liquid were growing on agar plates after a point and all the isolated were already characterized.

For the future study in the project some modifications can be considered to get positive results. At initial stages the low concentration of medium ingredients can be considered followed by the addition of nutrients in at later stages like a fed-batch culture.

As no primers are universal so, new primers for the unamplified strains can be designed as they could be some novel strains.

6. Appendices

Appendix 1: Recipe of media used during the study.

1. **International *Streptomyces* Project (ISP) 2 media**

Per litre RO water	**Quantity**
Malt extract	**10 g**
Yeast extract	**4 g**
Glucose	**4 g**
Agar	**18 g**

2. **Tryptone Soy Broth/Agar**

Per litre RO water	**Quantity**
Tryptone Soy broth	30 g
Agar	15 g

3. **Luria – Bertani Medium**

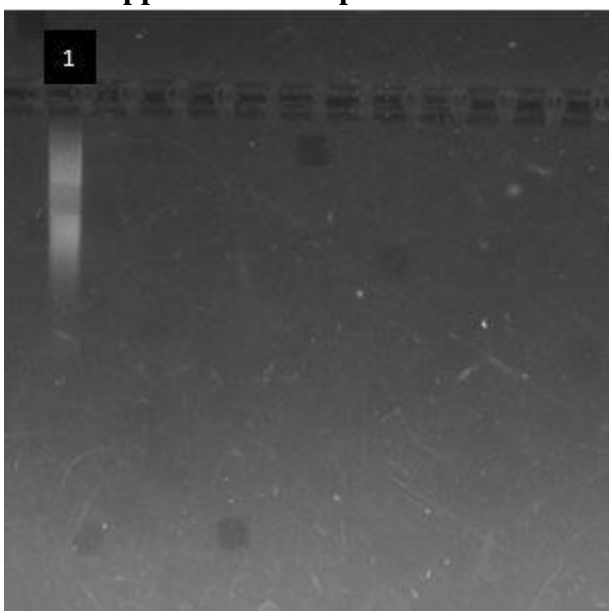
Per litre RO water	**Quantity**
Tryptone	10 g
Yeast extract	5 g
Sodium Chloride	10 g

Agar	20 g

4. **Nutrient Broth/Agar**

Per litre RO water	**Quantity**
Nutrient broth powder	13 g
Agar	15 g

Appendix 2 PCR primer control



Appendix 2: 19 Primer control. The lane 1 in the gel depicts the primer control. It can de illustrated from the gel image that the primer control was negative.

Appendix 3 OD_{600nm} table for selected strains

NAME of the strain	**OD_{600nm}**
LGB143	1.28E-01
LGB152	8.00E-02

LGB153	7.54E-02
LGB154	7.90E-02
LGB155	6.79E-02
LGB246	1.52E-01
LWS252	1.42E-01
LWS257	1.41E-01
LCS260	1.08E-01
LCS264	1.15E-01
LCS271	2.95E-01
LWS275	1.10E-01
LWS277	8.69E-01
LWS283	2.00E-01
LWS287	1.40E-01
LWS288	1.77E-01
LWS289	3.54E-01
LWS290	1.25E-01
LWS291	2.15E-01
LWS295	1.81E-01
LGB306	1.04E+00

LGB307	1.04E+00
LGB308	1.33E-01
LR18-315	1.52E-01
LR18-316	1.78E-01
LR18-317	1.07E-01
LR18-318	1.09E-01
LR18-319	1.83E-01
LGB 418	1.44E-01
LCS421	8.99E-01
LCS422	1.16E-01
LCS423	1.02E-01
LCS 432	1.31E-01
LCS71	1.06E-01
LGB146	1.23E-01
LGB157	1.65E-01
LRB16-164	6.06E-02
LGB170	2.27E-01
LCS172	1.41E-01
LGB313	1.91E-01

LCS356	1.50E-01
LGB118	7.85E-02
LGB120	7.07E-02
LGB124	7.33E-02
RB18-217	1.04E-01
LCS414	1.72E-01
LGB79	1.11E-01
LGB125	6.08E-02
RB18-218	9.19E-02
LGB231	7.16E-02
LGB232	5.96E-02
LGB130	6.33E-02
LGB392	5.33E-02

Appendix 4

Phylogenetic affiliations of 250 isolates cultured from soil for the study (Davis, Joseph & Janssen 2005).

Phylum	**Class, subclass, or subdivision**	**No. of isolates**
Acidobacteriaa	Subdivision 1	9

	Subdivision 2 Subdivision 3 Subdivision 4	1 5 1
Actinobacteria	*Acidimicrobidae*[α] *Actinobacteridae* *Rubrobacteridae*[α]	2 119 7
Bacteroidetes	*Flavobacteria* *Sphingobacteria*	1 5
Chloroflexi[α]	Ellin7237 lineage	1
Firmicutes	"*Bacilli*"	7
Gemmatimonadetes[α]	Subdivision 1	1
Planctomycetes[α]	"*Gemmatae*" "*Isosphaerae*" WPS-1	1 1 2
Proteobacteria	*Alphaproteobacteria* *Betaproteobacteria* *Gammaproteobacteria*	64 19 3
Viridiplantae	*Chlorophyta*	1

α Rarely isolated group.

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