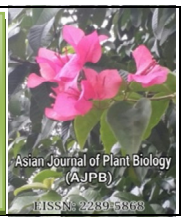
HIBISCUS

ASIAN JOURNAL OF PLANT BIOLOGY

Website: http://journal.hibiscuspublisher.com/index.php/AJPB



Identification of Bacteria from Soils of Cameron Highlands

Parveen Devi Pattiram[1], Baskaran Gunasekaran[2], Norazah Bt Mohammad Nawawi[3*]

[1]Department of Food Technology, Faculty of Food Science and Technology, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

[2]Department of Biochemistry, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia.

[3]Faculty of Sciences and Biotechnology, Universiti Selangor, 45 600 Bestari Jaya, Selangor, Malaysia.

*Corresponding author: n_azahmn@yahoo.com
Norazah Bt Mohammad Nawawi,
Faculty of Sciences and Biotechnology,
Universiti Selangor, 45 600 Bestari Jaya,
Selangor, Malaysia.

Tel: +603 32805123

HISTORY

Received: 11th December 2014
Received In Revised Form: 21st December 2014
Accepted: 25th December 2014
Available Online: 31st December 2014

KEYWORDS

Bacteria
16S rDNA
BLAST
Bacillus pumilus

ABSTRACT

Bacteria are a large group of unicellular microorganisms. Bacteria are ubiquitous in every habitat on Earth for example in soils, radioactive waste, water and as well as in organic matter and the live bodies of plants and animals. They are small cells, found in the environment as either individual cells or aggregated together as clumps. This study was aimed to identify the bacteria from soil sample obtained from Cameron Highlands. Eight samples were inoculated on Nutrient Agar plate. After obtaining pure culture, stock cultures on Nutrient Agar slant and glycerol stock were prepared and the samples were kept in -20˚C. Next the samples were further studied using microscopic observation. From this technique, seven Gram positive bacteria and one Gram negative bacteria were obtained. The Gram positive rod shaped was analyzed further using the molecular technique 16S rDNA using pA and pH primers. Then the sample proceeds to bulk. PCR product of approximately 1kb in size was obtained. The purity value of the DNA sample obtained was 1.16 A260/280. The sample was sent for sequencing to NHK Bioscience and Macrogreen (Korea). Only one sample showed positive for 16S rDNA fragments which were analyzed using Basic Local Alignment Search Tool (BLAST) for bacterial identification. The data analysis of the sequence shows that the bacterium obtained was a *Bacillus pumilus* strain.

INTRODUCTION

Bacteria were first observed by Antonie van Leeuwenhoek in 1676, using a single lens microscope of his own design. The name bacterium was introduced much later, by Christian Gottfried Ehrenberg in 1838. The bacteria are a large group of prokaryotic unicellular microorganisms. Bacteria are ubiquitous in every habitat on Earth for example growing in soil, radioactive waste, water and as well as in organic matter and the live bodies of plants and animals. The size of bacteria cell is small, found in the environment as either individual cells or aggregated together as clumps, and their intracellular structure is far simpler than eukaryotes [1]. Typically a few micrometers in length, bacteria have a wide range of shapes, ranging from spheres, rods and spirals. Bacteria have a single circular DNA chromosome that is found within the cytoplasm of the cell as they do not have a nucleus. Prokaryotic cells contain a plasma membrane, which is usually surrounded by a cell wall and often a capsule. Bacteria are prokaryotes which is single cells that do not contain a nucleus and maintain their genetic material, DNA, in a long circular molecule. Bacteria also contain DNA in small circular molecules termed plasmids. Cells that do contain a nucleus are known as Eukaryotic cells and are advanced cells. The study of bacteria is known as bacteriology, a branch of microbiology [1].

Bacteria can be classified on the basis of cell structure, cellular metabolism or on differences in cell components such as DNA, fatty acids, pigments, antigens and quinones. There are numerous types of bacterial in the world. Before the invention of DNA sequencing technique, bacteria were mainly classified based on their shapes which are sphere shaped bacteria, rod shaped bacteria and spiral bacteria, which is also known as morphology, as well as staining method. Currently, along with the morphology, DNA sequencing is also used in order to classify bacteria [2] Almost 99% bacteria is helpful. Disease is caused by only a few of them. However, there are both helpful and harmful bacteria. Some helpful bacteria present on the skin protect us from the spread of certain fungus. Certain types of helpful bacteria live in the intestines of human beings. Apart from that, the bacteria also enrich the soil by returning these minerals to the soil, rendering it useful once again for plants and animals after breaking down the compounds found in organic waste materials into simpler substances. A number of bacteria cause disease, these are called pathogenic bacteria. Certain harmful bacteria act as pathogens and cause tetanus, typhoid fever, pneumonia, syphilis, cholera, food-borne illness and tuberculosis. Harmful bacteria in food cause botulism, which can cause paralysis or even death if even one millionth of the bacterium is ingested and can create illnesses in humans and can cause food poisoning too [3].

In industrial application, bacteria aid in the production of food products in fermentation processes, such as brewing, baking, cheese and butter manufacturing using bacteria, normally, *Lactobacillus* in combination with yeasts and molds. This have been used for thousands of years in the preparation of fermented foods such as cheese, pickles, soy sauce, sauerkraut, vinegar, wine, and yogurt. Apart from that, in chemical industry, bacteria are most important in the production of enantiomerically pure chemicals such as ethanol, acetone, organic acid, enzymes, perfumes etc.

Besides that, bacteria play an important part in some of the agricultural industries. Specific group of bacteria can degrade metolachlor, propanil and trifluralin which are three commonly used herbicides in the agriculture area. Certain bacteria isolated from soils possess properties that allow them to exert beneficial effects on plants either by enhancing crop nutrition or by reducing damages caused by pathogens or pests. Furthermore, the formation of soil is dependent upon bacteria as well [4]. Common bacteria in the natural environment have been applied in the field of environmental protection such as in the treatment of sewage, wastewater and bioremediation of sediment mud polluted organic matters [5]. Furthermore, in medical applications, photosynthetic bacteria can produce various types of physiological active substance such as vitamin B12, ubiquinone (coenzyme Q10), 5-aminolevulinic acid (ALA), porphyrins and RNA. In particular, photosynthetic bacterial ALA was commercially applied to cancer diagnosis and treatment [6]. Meanwhile, bacteria can also been produced in soil of Cameron Highlands which is located at 5,000 ft (1,500 m) above sea level. It is the highest area on the mainland, with temperatures no higher than 25°C and rarely falls below 12°C. The highlands are rich in fertile soil and the climate is suitable to grow many vegetables such as giant spring cabbages, lettuce, and other temperate and sub temperate vegetables and fruits.

Molecular markers, such as the 16S rDNA gene, have been extensively applied to detect, identify and measure microbial diversity from environmental samples. Ribosomal DNA (rDNA) is a sequence encoding ribosomal RNA. These sequences regulate amplification and transcription initiation and contain transcribed and nontranscribed spacer segments. Perhaps the greatest contribution these studies have made to soil microbiology is sequence based taxonomy. The effects of template concentration on the PCR amplification of 16S rDNA are particularly important in many soil studies where template DNA is often diluted to minimize the effects of humic acid contamination [7]. Therefore, the aim of this study is to identify bacteria from soil of Cameron Highlands using 16S ribosomal DNA (rDNA) via PCR amplification.

MATERIALS AND METHODOLOGY

Pure culture

A total of 8 different types of sample were collected from vegetables soil of Cameron Highlands. The entire samples were recorded in the Table 1. The samples were streaked on nutrient agar (NA) plate from stock culture to obtain a pure colony. Each plate was labeled by appropriate particular and as well as date streaked. the streaked plates are incubated for 24 to 48 hours in temperature of 30°c. The growths are observed.

Table 1: Sample collections

No. Sample	Name of sample	Code
1	Brinjal soil plant (10^{-5})	S11
2	Sweet Potato soil plant (10^{-5})	S12
3	Muscard Leave soil plant (10^{-3})	S13
4	Bharat Tea soil plant (10^{-5})	S14
5	Tomato soil plant (10^{-3})	S15
6	Water Crest soil plant (10^{-3})	S16
7	French Beans soil plant (10^{-5})	P1
8	Cauliflower soil plant (10^{-3})	S18

Preparation of the stock culture

Stock culture is important to preserve the sample for months or even for years. It is also help to prevent the sample from contamination. Usually stock culture were prepared in glycerol stock in bijou bottles and as well as on nutrient agar slants.

Preparation of Nutrient Broth

Each universal bottle is filled with 5 ml of Nutrient broth. 7.5 gram of Nutrient broth were weighed and poured into the Schott bottle. Then, 0.5 liter of distilled water was added in the Schott bottle. The medium is mixed well and the Schott bottle is autoclaved at 121°C for 15 minutes. Once after autoclaved, 5ml of the broth was poured in the universal bottle. This step is done in the biohazard safety cabinet. One single colony is taken using inoculation loop which has been flamed and it is mixed thoroughly in the universal bottle. The culture is shaken thoroughly. This step is done in the present of flame. This step is repeated for the other sample. Finally, the bottles were incubated for overnight at 37°C.

Preparation of Glycerol

2 ml of glycerol is filled in the bijou bottle. Then, autoclaved at 121°C for 15 minutes. After incubation of sample for overnight, pipette 500 µl of the culture from the broth into the bijou bottle containing glycerol. This step is done in the biohazard safety cabinet. The culture is mixed well. The bijou bottle was labeled and stored it in -20°C.

Preparation of Nutrient Agar Slants

Nutrient agar was prepared. The bijou bottles were autoclaved. After that, the bottles were placed on the rack. The molten agar is then poured in the bijou bottles. The bottles are tilted on any solid surface so that the agar in the bottle becomes slant. After the medium become cool and hard as well, the caps were tightened and store it in the chiller or in the room temperature if it is not using. The cultures were streaked on the slant.

Microscopic Technique

The morphology of bacteria is examined using by Gram staining method. Gram staining is a method of differentiating bacterial species into two large groups which are Gram positive and Gram negative. Prepare clean slides and labeled it appropriately. A drop of distilled water is placed on the slides and spread it to get a thin layer. First, a loopful of a pure culture is smeared on a slide and allowed to air dry. Fix the cells to the slide by heat. Do not let the glass become too hot to the touch. Place the slides which need to be stained on the staining tray. Next, crystal violet is then droped on the slides. Leave it for 1 minute. After 1 minute, rinse the slide using tape water. The heat-fixed cells should look purple at this stage. After that, the sample is then treated using iodine. Leave it for 1 minute then rinse using tape water. Later, decolorize the sample by applying 95% of ethanol. Rinse with water immediately to stop decolorization. Finally, rinse the slide with a counterstain (safranin). Rinse with tape water after 45 seconds. Blot gently and allow the slide to dry. Do not smear. Once after the slides dry, it is ready to observe under microscope using immersion oil.

DNA Extraction

Bacterial DNA Extraction Kit

After the growth of the bacteria obtained, to extract DNA, the GF-1 Bacterial DNA Extraction Kit is designed for rapid and efficient purification of DNA from either Gram-negative or Gram-positive bacteria. All steps are to be carried out at room temperature unless stated otherwise. Pellet (1 ml) of bacteria culture was grown overnight and centrifugation at 6,000 rpm for 5 minutes at room temperature. The supernatant was decanted completely. 100ml of Buffer R1 was added to the pellet and the cells were resuspended completely by pipetting up and down.

Next step is, for Gram negative bacteria strains, 10ml lysozyme (50mg/ml) was added into the cell suspension. Meanwhile, for Gram positive bacteria strains, 20ml lysozyme (50mg/ml) added into the cell suspension. Mixed thoroughly and incubated at 37°C for 20 minutes. Pellet digested cells by centrifugation at 10,000 rpm for 3 minutes. The supernatant was decanted completely. Pellets were resuspended in 180ml of Buffer R2 and add 20ml of Proteinase K (20mg/ml).

Thoroughly mixed and incubated at 60°C for 20 minutes in a shaking waterbath or with occasional mixing every 5 minutes. 20ml of RNase (DNase-Free, 20mg/ml) was added to the clear lysate and incubated at 37°C for 5 minutes. 2 volumes (~440ml) of Buffer BG was added and mixed thoroughly by inverting tube several times until a homogeneous solution is obtained. Incubated for a further 10 minutes at 60°C. The mixture cool down for approximately 1 minute. Then, 200ml of absolute ethanol was added. Mixed immediately and thoroughly.

The sample was transfer into a column assembled in a clean 2ml collection tube (provided). Centrifuge at 10,000 rpm for 1 minute. Flow through the decant. The column was washed with 750ml of Wash Buffer and centrifuge at 10,000 rpm for 1 minute. Flow through discarded. Centrifuge the column at 10,000 rpm for 1 minute to remove residual ethanol. The column was placed into a clean 1.5ml microcentrifuge tube. 50 - 100ml of preheated Elution Buffer (10mM Tris-HCl, pH8.5), TE buffer or sterile water was added directly onto column membrane and stand for 2 minutes. Centrifuge at 10,000 rpm for 1 minute to elute DNA. DNA was stored at 4°C or -20°C.

Boiling Method

Nucleic Acid Extraction

Bacteria colony was grown in incubator at temperature 30˚C for overnight to obtain a fresh colony. The sterile 1.5 ml tube was prepared and the sterile distilled water was placed in this tube using pipette. The sterile distilled water was pippeted about 100 µl in each tube. Then the colony of bacteria was taken using a sterile toothpick. The colonies were transferred into the tubes. Then, the tubes were vortexed about 10 seconds to ensure the sample mixed well with the distilled water. All the sample was used the boiling method. The sample was heated in boiling water for 2 minutes and 30 seconds. The sample was cooled under room temperature for 1 minute. After 1 minute, the sample was centrifuge at 13000 rpm for 5 minutes. After that, the tube was kept in ice to proceed with PCR amplification [8].

DNA Quantitation

The DNA concentration was measured by GeneRay UV Spectophotometer at A_{260} absorbance. The calculation formula for DNA concentration is as follows: DNA concentration = A_{260} X 50µg/mL X Dilution factor Dilution factor = DNA sample + Distilled water/ DNA sample. The purity of genomic DNA was measured by absorbance ratio at A_{260} and A_{280}. The calculation formula for DNA purity is: DNA purity= $A_{260/280}$ Where, A= Absorbance

Polymerase Chain Reaction (PCR)

The polymerase chain reaction (PCR) is a technique widely used in molecular biology. It derives its name from one of its key components, a DNA polymerase used to amplify a piece of DNA by enzymatic replication. As PCR progresses, the DNA generated is used as a template for replication. PCR is used to amplify specific regions of a DNA strand. This can be a single gene, a part of a gene, or a non-coding sequence. Most PCR methods typically amplify DNA fragments of up to 10 kilo base pairs (kb), although

some techniques allow for amplification of fragments up to 40 kb in size. The purpose of PCR is to make a huge number of copies of a gene. This is necessary to have enough starting template for sequencing. Table 2 showed the mixture of PCR components. A pair of universal 16S rDNA oligonucleotide primers, which consisted of a forward universal primer, pA (5'- AGA GTT TGA TCC TGG CTC AG -3') and a reverse universal primer, pH (5'- ACG GCT ACC TTG TTA CGA CTT -3') are used. Oligonucleotide primers with specificity for eubacterial 16S rRNA genes, primers pA and pH were used to amplify the 16S rRNA gene fragments with template DNA originating from bacterial isolates and using PCR protocols [9].

PCR Amplification

Table 2: The mixture of PCR component

Concentration	PCR Reagents	Volume (µl)
1X	10X PCR Buffer	1.00
1.5 mM	$MgCl_2$ (50mM)	0.3
0.2 mM	dNTP's (10mM)	0.2
0.25mM	pA (10mM)	0.25
0.25mM	pH (10mM)	0.25
1-5ng	DNA Template	4.00
0.25U	Taq Polymerase	0.25
0.00	Sterile Distilled Water	6.25
Total		10.0

The Polymerase Chain Reaction is an enzymatic way to amplify a DNA template using short oligonucleotide primers and free deoxy nucleotides in replicative cycle of denature, anneal and extend to rapidly achieve more copies of template DNA. A PCR was mix in 0.2 ml of tubes. This mixture was prepared in sterile area to prevent contamination. The PCR component should remain in ice box. The PCR process is done by using Thermal Cycler (Bio Rad, USA). The cycles which are involved are initial denaturation, under temperature 95°C for 30 seconds. The second step is denaturation which is done under temperature of 95°C for 3 to 5 seconds. Next step is annealing which is done at 55-60°C for 1 minute. 1 minute at 72°C for extension and lastly by a last by a final step at 72°C for 7 minutes and finally cooling to 4°C.

Bulk

The positive 16S rDNA has been further amplified in bulk preparation for 100 µl total reaction. The volume has been multiply with 10 µl compare to PCR components. Table 3 showed the mixture of PCR components for bulk.

Table 3: The mixture of PCR component for bulk

Concentration	PCR Reagents	Volume (µl)
1X	10X PCR Buffer	10.0
1.5 mM	MgCl2 (50mM)	3.0
0.2 mM	dNTP's (10mM)	2.0
0.25mM	pA (10mM)	2.5
0.25mM	pH (10mM)	2.5
1-5ng	DNA Template	40.0
0.25U	Taq Polymerase	2.5
0.00	Sterile Distilled Water	62.5
TOTAL		100.0

Agarose Gel Electrophoresis

A 1% gel (1.5 kb) with 0.3 g agarose in 30 ml (1X TBE buffer which is prepared). Then, 30ml of the 1X TBE buffer weighed and poured it into the conical flask containing 0.3g of agarose. The conical flask was placed into microwave, set to high and for 1 minute. Once done, the conical flask carefully took out from microwave and gently shake till it cool. 30mL of TBE Buffer was prepared to run gel. The comb was placed. Once the agarose gel cool down, the gel was poured into the well carefully without forming bubbles. Once the gel become hard, the reuse TBE buffer was poured carefully. 2µl of 6X loading dye buffer and 5µl of sample were mixed well with pippeting. The sample was load from second well onwards. 5µl of VC 1kb was loaded in the first well. Once finish, the electrogel was set, and run in 85 voltages for 45 minutes. After the process finished, the gel is soaked in EtBr for 10 minutes. Then, the gel was soaked in distilled water for 5 minutes.

PCR Product Purification

PCR product purify is done by using GF-1 PCR Clean Up Kit which is manufactured by Vivantis. The volume of sample was determined and adjusted to 100 µl with sterile distilled water. 5 volumes of Buffer PCR were added to the DNA sample and mixed thoroughly by vortexing or inverting tube several times. If the sample turns to orange, pink or red upon solubilization, 5 µl of 3M sodium acetate pH 5.2 was added and mixed thoroughly. The sample should turn yellow. In this step it must make sure that the color of the sample is yellow (pH 7 or below) before proceeding to the next step. The sample transferred was into a column assembled in a clean 2ml collection tube (provided). Centrifuge at 14,000 rpm for 1 minute. The flow through was discarded. The column washed with 750 µl Wash Buffer and centrifuge at 14,000 rpm for 1 minute. The flow through was discarded. Residual ethanol was removed by centrifuge the column at 14,000 rpm for 1 minute. The column was placed into a clean microcentrifuge tube. 30 µl of sterile distilled water was added onto column membrane and stand for 2 minutes. Spin at 14,000 rpm for 1 minute to elute DNA. The DNA stored at 4˚C to 20˚C.

DNA Sequencing

The DNA sequencing was performed by NHK Bioscience and Macrogreen (Korea). The 16s rDNA sequence was compared in the database by using the Basic Local Alignment Search Tool (BLAST) to identify the bacteria.

RESULTS AND DISCUSSIONS

Bacteria Culture

The cultures were streaked on Nutrient Agar (NA) plate through subculture to obtain a pure culture. The streaked plates are incubated for 24 to 48 hours in temperature of 30°C. The growths are observed through the shape of the colony, edge, elevations and the color of the morphology as well. Isolate S11, the color of the culture is yellowish. Meanwhile the shapes of the colony for example whole colony, edge, elevations are circular, lobate, crateriform respectively (Figure 1). Meanwhile, for isolate S12 which is shown in Figure 2, the color is yellowish. The shape of

the colony for whole colony is punctiform, the edge is lobate and the elevations are crateriform. Apart from that, the color for isolate S13 (Figure 3) is pale yellowish. Shape of the whole colony is irregular, edge is entire and elevations are raised. Isolate S14, the color of the culture obtained was dark yellow. Apart from that, the shape of the colony observed was irregular, edge was lobate and raised for elevations (Figure 4). For isolate S15, the color of the culture is pale yellow. Shape of the whole colony is circular, edge is lobate and elevations are crateriform (Figure 5). Isolate S16 (Figure 6), the color of the culture is yellowish. The morphology shape is puntciform, the edge is lobate and the elevations are crateriform. Furthermore, isolate P1, the color of the culture obtained was yellow. Shape of the colony obtained for whole colony, edge and elevations was punctiform, lobate and crateriform respectively (Figure 7). For isolate S18 (Figure 8), yellowish color was obtained. Meanwhile, whole colony shape was irregular, edge shaped entire and raised for elevations.

The term pure culture means that all its constituent cells are descendants of the same individual. A pure culture is therefore genetically pure (although mutations can lead to genetic changes during storage, especially in growing cultures). Streaking is the basic technique to obtain a pure bacterial culture and it is done on Nutrient Agar plate. The culture is then incubated for 24 to 48 hours at 30°C. Normally these pure culture bacteria are free from contamination. However, some culture are easily contaminated by yeast and *Bacillus* or mixed colony of other bacteria also.

Table 4: Eight different samples of bacteria found in soil of Cameron Highlands

No.	Sample	Color	Whole colony	Edge	Elevations
1.	S11	Yellowish	Circular	Lobate	Crateriform
2	S12	Yellowish	Punctiform	Lobate	Crateriform
3.	S13	Pale Yellowish	Irregular	Raised	Entire
4.	S14	Dark Yellow	Irregular	Lobate	Raised
5.	S15	Pale Yellow	Circular	Lobate	Crateriform
6.	S16	Yellowish	Punctiform	Lobate	Crateriform
7.	P1	Yellow	Punctiform	Lobate	Crateriform
8.	S18	Yellowish	Irregular	Entire	Raised

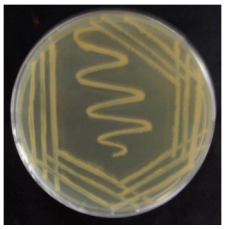


Figure 1: Isolate S11

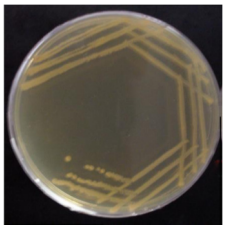


Figure 2: Isolate S12

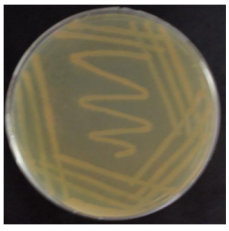


Figure 3: Isolate S13

Figure 4: Isolate S14

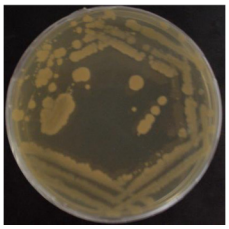


Figure 5: Isolate S15

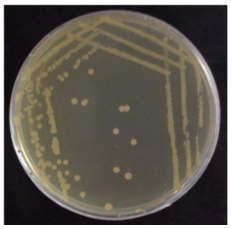


Figure 6: Isolate S16

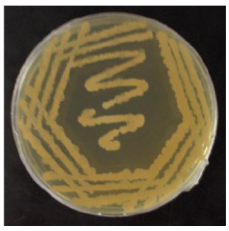


Figure 7: P1

Figure 8: Isolate S18

Microscopic Technique

The Gram Staining technique has divided into many steps. This technique involved four types of reagent that is crystal violet, iodine, 95% of ethanol, as well as safranin. Crystal violet was penetrated through the cell wall and cell membrane of both Gram positive and Gram negative cells. It will give purple color at the beginning. Next the iodine which will interacts with crystal violet and forms large complexes of crystal violet and iodine within the inner and outer layers of the cell. Indirectly it will tight the ion bonding of the crystal violet in the pores. When a decolorizer such as 95% of ethanol is added, it interacts with the lipids of the cell membrane.

A Gram negative cell will lose its outer membrane and the peptidoglycan layer is left exposed. The ionic bonding between the crystal violet and iodine are washed from the Gram negative cell along with the outer membrane. In contrast, a Gram positive cell becomes dehydrated from an ethanol treatment. The large complexes of crystal violet and iodine become trapped within the Gram positive cell due to the multilayered nature of its peptidoglycan.

After decolorization, the Gram positive cell remains purple and the Gram negative cell loses its purple color. Counterstain, safranin which is usually positively charged is applied last to give decolorized Gram negative bacteria a pink or red in color [10]. The microscopic technique is done to identify whether the bacteria are Gram positive or Gram negative. Gram positive bacteria have a relatively thick wall composed of many layers of the polymer peptidoglycan meanwhile Gram negative bacteria have only a thin layer of peptidoglycan, surrounded by a thin outer membrane composed of lipopolysaccharide (LPS). A total of eight isolates were performed microscopic examination and the cells are observed under microscope at 1000X magnification.

Seven isolates are Gram positive bacteria meanwhile another one is Gram negative bacteria. Isolate S11 showed Gram positive rod shape (Figure 9). Isolate S12 showed Gram positive rod shape (Figure 10). Apart from that, for isolate S13 (Figure 11) the results

for Gram stain is Gram negative rod shaped. Meanwhile for isolate S14 is Gram positive rod shaped (Figure 12). Result for Gram stain of isolate S15 obtained was Gram positive rod (Figure 13). For isolate S16 (Figure 14), the result obtained is Gram positive rod and for isolate P1 is Gram positive is rod shaped too (Figure 15). Lastly for isolate S18 (Figure 16) it is Gram positive rod shaped. In overall, all the isolate are in rod shape and this shape is called bacilli. From eight isolate, seven isolates are Gram positive rod and only one isolate is Gram negative rod (Table 5).

Table 5: Microscopic technique for samples

No.	Sample	Gram stain	Shapes
1.	S11	Gram Positive	Rod
2.	S12	Gram Positive	Rod
3.	S13	Gram Negative	Rod
4.	S14	Gram Positive	Rod
5.	S15	Gram Positive	Rod
6.	S16	Gram Positive	Rod
7.	P1	Gram Positive	Rod
8.	S18	Gram Positive	Rod

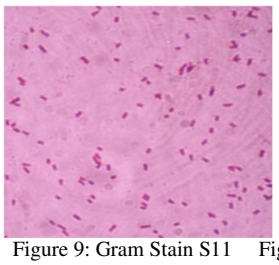


Figure 9: Gram Stain S11

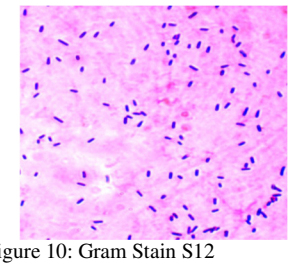


Figure 10: Gram Stain S12

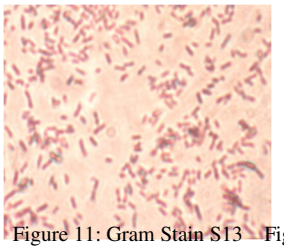


Figure 11: Gram Stain S13

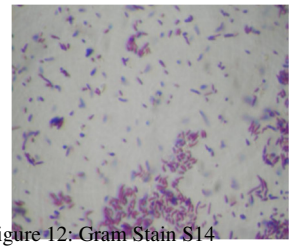


Figure 12: Gram Stain S14

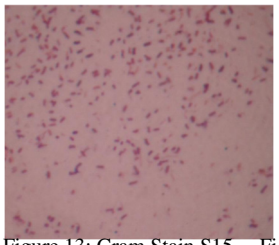


Figure 13: Gram Stain S15

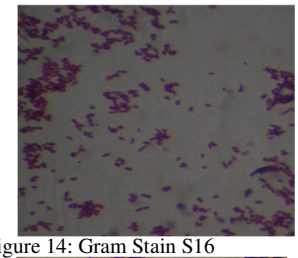


Figure 14: Gram Stain S16

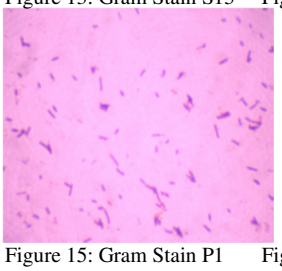


Figure 15: Gram Stain P1

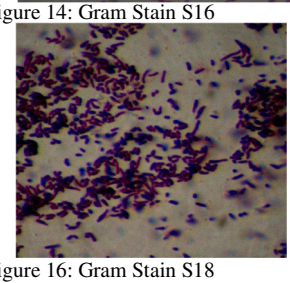


Figure 16: Gram Stain S18

DNA Extraction

Bacterial DNA Extraction Kit

Genomic DNA must be extracted before proceeding to other methods. DNA extraction is the critical point for the molecular technique to obtain DNA that can be applied for further analysis. The selected bacterium was chosen. Bacteria were grown in 5.0 ml of Nutrient broth and kept overnight. The GF-1 Bacterial DNA Extraction Kit is designed for rapid and efficient purification of up to 20 mg of high molecular weight genomic DNA from either Gram negative or Gram positive bacteria. This kit uses a specially treated glass filter membrane fixed into a column to efficiently bind DNA in the presence of high salt. This kit applies the principle of a mini column spin technology and the use of optimized buffers ensure only DNA is isolated while cellular proteins, metabolites, salts and other low molecular weight impurities are subsequently removed during the washing steps.

High purity genomic DNA is eluted in water or low salt buffers and has a $A_{260/280}$ ratio between 1.7 and 1.9 $A_{260/280}$. The purity of the sample obtained was 1.16 $A_{260/280}$ meanwhile the DNA concentration obtained was 948.75 µg/ml. Even though this method is more expensive than any other methodology, it can help in studies using large number of bacteria sample. This bacterial DNA extraction kit is optimized for purification of up to 20 µg of DNA on recommended cell culture volume. Bacteria cultures vary in the number of cells depending on the strain, growth conditions and viability of the cells. Besides that, this method is able to wash particles repeatedly. It is also suitable for qualitative and quantitative analysis. It is simple and does not have precipitation step. Isolate P1 was extracted using GF-1 Bacterial DNA Extraction Kit (Vivantis). The DNA extraction of the bacteria was analyzed with 1% of agarose gel electrophoresis, using Lamda Hind III marker. After the process was finished, the gel was stained using with ethidium bromine for 10 minutes and soak in distilled water for 5 minutes. After staining, the DNA fragment was observed under ultraviolet radiation light using Alpha Innotech Equipment.

In this study, the ratio of the $A_{260/280}$ for total DNA was approximately 1.16. The concentration of total DNA was 948.75 µg/µl according to the absorbance of ultraviolet light at 260nm. A bright band of P1 can be seen clearly [11]. Lambda Hind III marker is suitable for sizing and quantifying linear double stranded DNA molecules of between 125 bp and 23.1 kb. The marker is prepared by completely digesting lambda DNA with Hind III. Therefore, the size of the DNA fragment of sample P1 for DNA extraction is successfully obtained. The results are shown in Figure 17. The concentration of Lambda Hind III are 83ng/ul in 10 mM Tris-HCl (pH=7.5), 1 mM EDTA and with 1 X loading dye solution.

Failure of Boiling Method

Boiling method was done seven times but results cannot be obtained. There are some possible reasons that could explain this. Apparently, boiling method of DNA extraction is less sensitive and less specific as compared to DNA bacterial extraction kit. Normally, boiling method is done because the cells are partially

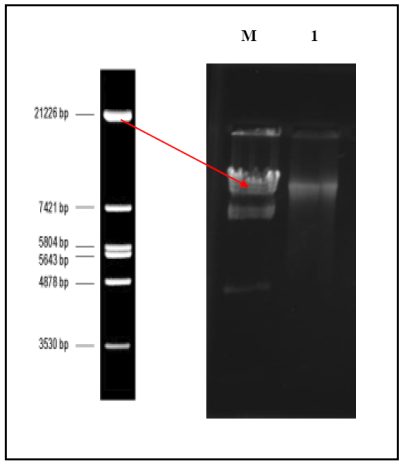


Figure 17: DNA extraction using GF-1 Bacterial DNA Extraction Kit (Vivantis). Run 1% agarose gel electrophoresis at 80V for 45 minutes. Lane M - Lambda Hind III marker; Lane 1- P1.

lysed, allowing plasmid molecules to escape, while most of the genomic DNA is trapped in the cell debris, which is then spined out after boiling for 2 minutes and 30 seconds [12]. This type of bacteria sample is a low temperature bacterium which is found in soil of Cameron Highlands. Due to its characteristic, it cannot be exposed towards higher temperature. When the bacteria were mixed thoroughly, which is heated to 95°C for more than 2 minutes it can attempt to kill the bacteria [8].

Polymerase Chain Reaction Amplification

The polymerase chain reaction (PCR) is a technique widely used in molecular biology. It derives its name from one of its key components, a DNA polymerase used to amplify a piece of DNA. As PCR progresses, the DNA generated is used as a template for replication. PCR is now a common and often indispensable technique used in medical and biological research labs for a variety of applications. These include DNA cloning for sequencing [13].

The basic steps in this PCR research are initialization. This step consists of heating the reaction to a temperature of 94 to 96°C, which is held for 1 to 9 minutes. It is only required for DNA polymerases that require heat activation by hot start PCR. Next is denaturation step. In this step is the first regular cycling event and consists of heating the reaction to 94 to 98°C for 20 to 30 seconds. It causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA. In annealing step, the reaction temperature is lowered to 50 to 65°C for 20 to 40 seconds allowing annealing of the primers to the single stranded DNA template.

Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer template hybrid and begins DNA synthesis [14]. Next is extension step. The temperature at this step depends on the DNA polymerase used, Taq polymerase has its optimum activity temperature 80°C and commonly a temperature of 72°C is used with this enzyme. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction, condensing the 5'-phosphate group of the dNTPs with the 3'-hydroxyl group at the end of the extending DNA strand. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. Next step is final elongation. In this single step is occasionally performed at a temperature of 70 to 74°C for 5 to 15 minutes after the last PCR cycle to ensure that any remaining single stranded DNA is fully extended. Lastly is final hold step. This step at 4 to 15°C for an indefinite time may be employed for short term storage of the reaction [15]. A bacterium sample with positive control, *Brevibacterium casei* and negative control as distilled water was selected for PCR amplification with primers used are pA and pH. The positive control and sample were analyzed with 1% of agarose gel electrophoresis, using VC 1kb DNA ladder. After the process finished, the gel was stained using with ethidium bromine for 10 minutes and soak in distilled water for 5 minutes. After finish staining, the DNA fragment was observed under ultraviolet radiation light using Alpha Innotech Equipment. The results are shown in Figure 18**.**

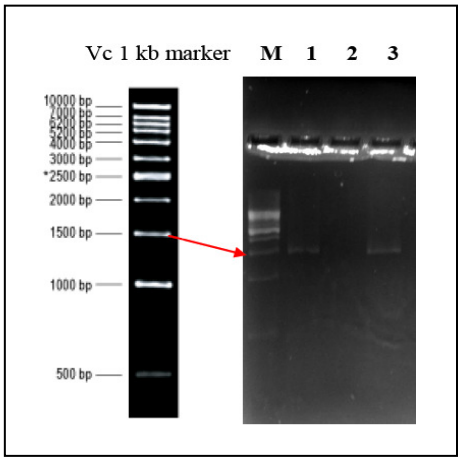


Figure 18: PCR product of bacteria sample using pA and pH primers. Run 1% agarose gel electrophoresis at 80V for 45 minutes. Lane M- Vc 1kb DNA ladder; Lane 1- Positive control, *Brevibacterium casei*; Lane 2- Negative control, distilled water Lane 3- P1.

A bacterium isolate with positive control, *Brevibacterium casei* and negative control as distilled water was selected for PCR amplification with primers used are pA and pH. The average size of the DNA fragment was approximately 1500 bp as showed in Figure 4.18. Vc 1 kb DNA ladder is serves as molecular weight strand for electrophoresis for both agarose and polyacrylamide gel. It is suitable for sizing of PCR product or other double stranded DNA fragment [16].

Bulk

The positive 16S rDNA has been further amplified in bulk preparation for 100 µl total reaction. The volume has been multiply with 10 µl compare to PCR components. Only the sample (P1) was analyzed with 1% of agarose gel electrophoresis, using VC 1kb DNA ladder. After the process finished, the gel was stained using with ethidium bromine for 10 minutes and soak in distilled water for 5 minutes. After finish staining, the DNA fragment was observed under ultraviolet radiation light using

Alpha Innotech Equipment. The size of the DNA fragment for bulk is approximately 1500 bp. The results are shown in Figure 19.

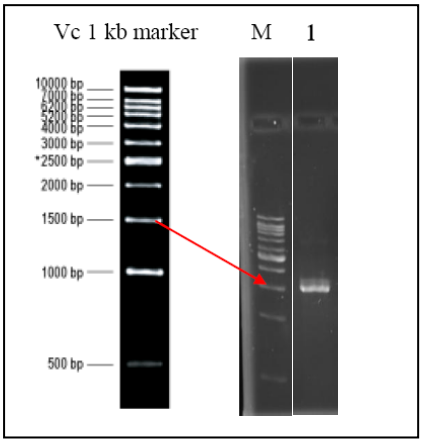


Figure 19: PCR products in bulk preparation (100 µl PCR reactions). Run 1% agarose gel electrophoresis at 80V for 45 minutes. Lane M- Vc 1kb DNA ladder; Lane 1- P1.

Sample Purification

After obtained the bulk result, the sample was purified using GF-1 PCR Clean Up (Vivantis). The sample was prepared to send for sequencing to NHK Bioscience and Macrogreen (Korea). After the sample was purified, the purity of the sample (P1) obtained was 1.55 $A_{260/280}$ meanwhile the DNA concentration obtained was 205.0µg/ml. The purification procedure has been optimized to remove salts, enzymes, nucleotides, mineral oil, agarose, ethidium bromide and other impurities from DNA samples. Specialized binding buffers promoteselective adsorption of DNA fragments and PCR products. The pure DNA is eluted in a small volume of buffer or water, ready to use for any subsequent application.

DNA Sequencing

The term DNA sequencing refers to methods for determining the order of the nucleotide bases, adenine, guanine, cytosine, and thymine, in a molecule of DNA. The first DNA sequences were obtained by academic researchers, using laborious methods based on 2-dimensional chromatography in the early 1970s. Following the development of dye based sequencing methods with automated analysis. DNA sequencing has become easier and orders of magnitude faster. P1 has been sent for sequencing at NHK Bioscience and Macrogreen (Korea). The data analysis sequence for P1 is EU869273 that gave result for *Bacillus pumilus* strain BMI 16S ribosomal DNA gene, partial sequence. The length of the sequence is 1437 bp. Nucleotide sequences of isolate P1 is shown in Table 6.

Table 6: Nucleotide sequences of isolate S17.

Isolates similarity	Accession No.	Most closely related type strain
P1	EU869273	*Bacillus pumilus* strain BMI 16S ribosomal RNA gene, partial sequence

The DNA sequencing was done by Biotechnology Company. The sequences were analyzed using Basic Local Alignment Search Tool (BLAST). *Bacillus pumilus* bacteria are Gram positive, aerobic, spore-forming, rod-shaped prokaryote (Figure 20). They occur in soil, water, air, and decomposing plant tissue. It is also can be found on the developing roots of soybean but it does not harm the plants [17]. Table 7 showed the scientific classification of *Bacillus pumilus. Bacillus pumilus* was found to be the second most dominant species among aerobic spore-forming bacteria. *B. pumilus* isolates showed resistance to H_2O_2 and are thus considered problematic microbes since H_2O_2 is recommended for use in bioreduction components [18]. Appropriate growth media for *Bacillus pumilus* are nutrient agar and nutrient broth. *Bacillus pumilus* can grow at 30ºC. *Bacillus pumilus* is used for alkaline protease production, in environmental decontamination of dioxins, and in the baking industry and it is also used as a pesticide active ingredient. Besides that, *Bacillus pumilus* can cause food poisoning [19].

Bacillus pumilus is commonly isolated from a variety of environmental sources, particularly faeces of animals. *Bacillus pumilus* grows as a smooth colony that becomes yellow with increased incubation. The organism is motile, b-hemolytic on blood agar, catalase and oxidase test are positive, salt tolerant and penicillin susceptible and does not grow under strict anaerobic conditions. *B. pumilus* has toxic properties. Apart from that, it has cytopathic effects in vero cells, β-hemolytic activity, lecithinase production, and proteolytic action on casein. It also has been detected an emetic toxin that can be related to food poisoning incidents [20].

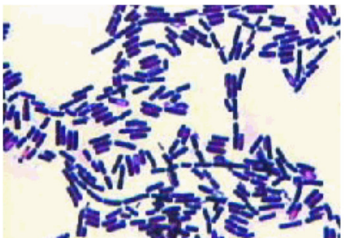


Figure 20: Gram stain of *Bacillus pumilus* [21].

Table 7: Classification hierarchy of *Bacillus pumilus.*

Hierarchy of *Bacillus pumilus*
Kingdom: Bacteria
Phylum: Firmicutes
Class: Bacilli
Order: Bacillales
Family: Bacillaceae
Genus: Bacillus
Species: *Bacillus pumilus*

CONCLUSION

From this study, only the bacteria *Bacillus pumilus* was successfully identified from the soil of Cameron Highlands. The growth of bacteria colonies were observed through its morphology and the color of the culture. The color of the bacteria obtained was yellow. Meanwhile, for the shape of the colony was irregular, entire and raised. Isolate P1 was further analyzed through DNA extraction using GF-1 Bacterial DNA Extraction Kit (Vivantis). The size of the DNA was successfully obtained. The sample was subjected through PCR amplification and the product obtained is approximately 1kb in size and the positive 16S rDNA was further studied to bulk with total volume of 100 µl. The 16S rDNA sequencing was performed to identify the bacteria. Basic Local Alignment Search Tool (BLAST) analysis showed *Bacillus pumilus* is one type of soil bacillus. *Bacillus pumilus* bacteria are Gram positive, aerobic, spore-forming, rod-shaped prokaryote. They occur in soil, water, air, and decomposing plant tissue. They also can be found on the developing roots of soybean but it does not harm the plants. As mentioned earlier, bacteria posses many potential activities such as in degrading soil, producing fertilizers and for making antibiotics. It is suggested that further studies should be done to isolate and identify more bacteria from various sources.

REFERENCES

[1] Porter JR.Tercentenary of his discovery of bacteria. Bacteriol. Rev. 1976; 40:260-9.

[2] Cabeen M, Jacobs WC. Bacterial cell shape. Nat.Rev. Microbiol.2005; 8: 601–10.

[3] Chattopadhyay A, Bhatnagar N, Bhatnagar R. Bacterial insecticidal toxins. Crit. Rev.Microbiol. 2004; 1: 33–54.

[4] Khachatourians G. Agricultural use of antibiotics and the evolution and transfer ofantibiotic-resistant bacteria. J Microbial. 1998; 9: 1129–36.

[5] Sasaki K, Watanabe M, Noparatnaraporn N. Applications of photosynthetic bacterium formedical and environmental fields.Seibutu-Kougaku 2002; 80: 234-236.

[6] Sasaki K, Watanabe M, Suda Y, Ishizuka A, Noparatnaraporn N. Applications of photosynthetic bacteria for medical fields. J Biosci.Bioeng. 2005 ;100: 481-488.

[7] Chandler DP, Li SM, Spadoni CM, Drake GR, Balkwill DL, Fredrickson JK. Use of 16SrRNA and rpoB genes as molecular markers for microbial ecology studies. Appl. Environ.Microbiol. 2003; 73: 278–88.

[8] Adrian HJ. PLC Regulation of PI(3,4,5)P3-mediated Chemotaxis. Mol. Biol.Cell.2000; 18:4772-4779.

[9] Rhodes J, Palmer JL. Complete neuleotde sequence of a 16S rDNA gene from *Escherichia coli*.Proc. Natl. Acad. Sci. USA. 1978; 75: 4801-4805.

[10] Davies JA, Anderson GK, Beveridge TJ, Clark HC. Chemical mechanism of the Gram stain and synthesis of a new electron-opaque marker for electron microscopy which replaces theiodine mordant of the stain. J of Bacteriol. 1983;156: 837–45.

[11] Cullen DW, Hirsch PR. Simple and rapid method for direct extraction of microbial DNA from soil for PCR. Soil Biol. Biochem. 1998; 30: 983-993.

[12] Wilson Q, William H, Joint L, Ian R, Carr G, Nicholas H. Isolation and molecular characterization of five marine cyanophages propagated on synechococcus sp. strain. J of Microb.1992; 8:954-978.

[13] Lee SY, Bollinger J, Bezdicek D, Ogram A. Estimation of the abundance of an unculturedsoil bacterial strain by a competitive quantitative PCR method. Appl. Environ. Microbiol.1996; 62: 3787-3793.

[14] Rychlik W, Spencer WJ, Rhoads RE. Optimization of the annealing temperature for DNA amplification *in vitro*. Nucl. Acids Res. 1990; 18: 145-165.

[15] Zhang S, Goodwin PH. Rapid and sensitive detection of Xanthomonasfragarie by simple alkaline DNA extraction and the Polymerase Chain Reaction. J. Phytopathol. 1997; 145: 267-270.

[16] Cheng S, Fockler C, Barnes WM, Higuchi R. Effective amplification of long targets from cloned inserts and human genomic DNA. Proc. Natl. Acad. Sci. 1994; 91: 5695–5699.

[17] Dickinson DN, La Duc MT, Satomi M, Winefordner JD, Powell DH, Venkateswaran K.MALDI TOFMS compared with other polyphasic taxonomy approaches for the identification and classification of *Bacillus pumilus*spores. J Microbiol.Methods.2004;58:1- 12.

[18] Kempf MJ, Quigley MS, Chen F, Satomi M, Kern R, Vankateswaran K. Isolation and characterization of hydrogen peroxside resistant spores of *Bacillus pumilus*. Spacecraft Assembly Facility. 2005. New York: Protea Press.

[19] Tena D, Martınez-Torres JA, Perez-Pomata MT, Saez-Nieto JA, Rubio V. Cutaneous infection due to *Bacillus pumilus*: Report of 3 Cases. Clin Infectious Diseases2007; 44:40–42.

[20] Benardini JN, Sawyer J, Venkateswaran K, Nicholson WL. Spore UV and acceleration resistance of endolithic *Bacillus pumilus* and *Bacillus subtilis* isolates obtained from Sonoran desert basalt: implications for lithopanspermia. Astrobiology.2003; 3: 709-717.

[21] Xu D, Cote JC. Phylogenetic relationships between *Bacillus* species and related genera inferred from comparison of 3′ end 16S rDNA and 5′ end 16S–23S ITS nucleotidesequences. Int. J Syst.Evol. Microbiol. 2003; 53: 695–704.