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GUIDELINE

on Mutagenesis of Biofertiliser Bacteria Using Gamma Irradiation

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GUIDELINE ON MUTAGENESIS OF BIOFERTILISER BACTERIA USING GAMMA IRRADIATION/ PHUA CHOO KWAI HOE, ALI TAN KEE ZUAN, CHONG SAW PENG, AHMAD ZAINURI MOHD DZOMIR, NUR HUMAIRA' LAU ABDULLAH

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Declaration
Similar to studies on mutagenesis of plants, there is no "ethics issues" in mutagenesis of microorganisms. This contrasts with studies involving manipulation, including mutation induction of animal cells, where "ANIMAL ETHICS" is strictly adhered to.

FOREWORD

It is with great honour that I present this foreword for the publication titled “Guideline on Mutagenesis of Biofertiliser Bacteria Using Gamma Irradiation.” As the Director General of the Malaysian Nuclear Agency, I am proud to support this significant contribution to the field of agricultural science and biotechnology, for the benefit of agroindustry, especially in the region.

The journey of the FNCA Biofertiliser Project has been transformative since its inception in 2002, evolving under the guidance of highly dedicated scientists and researchers. This project exemplifies our commitment to harnessing nuclear technology for the benefit of agriculture, emphasizing the role of ionising irradiation in enhancing the quality and effectiveness of biofertiliser products.

Biofertilisers, derived from pure cultures of indigenous beneficial microorganisms, play a crucial role in sustainable agriculture by enhancing soil health and nutrient availability. The meticulous work documented in this publication highlights innovative approaches to mutagenesis, enabling us to develop microbial strains with superior capabilities. By advancing our understanding of these processes, we can significantly improve crop yields and promote environmental sustainability.

I commend the collective efforts of all contributors to this publication, whose dedication has culminated in this valuable resource. While it serves as an important foundation, it is also a call to action for continued research and collaboration within the FNCA network and beyond. Together, we can drive further innovations that will enrich the agro-industrial landscape and contribute to food security in our region.

I extend my sincere congratulations to all involved in this endeavour and wish everyone success in future undertakings.
Thank you.

Thank you.

ROSLI BIN DARMAWAN, Ph.D.
Director General
Malaysian Nuclear Agency

PREFACE

The e-book “Guideline on mutagenesis of biofertiliser bacteria using gamma irradiation” is an effort by Malaysian scientists involved in biofertiliser projects to share a treatise of steps and procedures taken for traits improvement from existing strains of biofertiliser microorganisms, bacteria in particular. Like radiation mutagenesis of plants for desired characteristics, there is no ethics issues for bacteria and other microorganisms.

Gamma irradiation of bacteria to improve their functionalities involves a series of steps. A prerequisite to achieving this is the need for a good starting bacterial strain. The dose response of Gram-positives and Gram-negatives differs due to their structure. The introduction dwells on the description of biofertilisers, the principles of radiation mutagenesis using gamma irradiation, and followed by description of the gamma irradiation equipment and the necessity of dose mapping for mutagenesis. The next section outlines the standard operating procedures (SOP) on staining of bacterial cells, followed by screening procedures for the main functions of biofertiliser bacteria. The last section of the e-book is on laboratory safety, especially when handling microorganisms isolated from the natural environment. Multifunctional biofertiliser bacteria were isolated from soils, composts, and plants by Dr. Phua Choo Kwai Hoe and Dr. Ali Tan Kee Zuan. Preparation of bacteria for mutagenesis involved Gram staining and identification using 16S rRNA, conducted by Chong Saw Peng, Dr. Ali Tan Kee Zuan, and Dr. Phua Choo Kwai Hoe. Dose mapping and gamma irradiation of biofertiliser bacteria were performed by Dr. Ahmad Zainuri Mohd Dzomir. Screening tests for nitrogen (N_2) fixation, phosphate and potassium solubilisation of irradiated biofertiliser bacteria were conducted by Dr. Phua Choo Kwai Hoe. Laboratory safety for mutagenesis bacteria is crucial for the entire process, and the laboratory safety guide was prepared by Nur Humaira’ Lau Abdullah.

This publication provides the general issues to be covered when conducting irradiation mutagenesis of bacteria for the improvement or enhancement of their functionalities. Other microorganisms may have specific requirements. This publication provides room for improvement, especially from the biofertiliser research communities, locally and from abroad, especially from members of the FNCA Biofertiliser Project.

Thank you.

Khairuddin Abdul Rahim, Ph.D.

Chapter : 1

Introduction

1.1 Biofertiliser

Ali Tan Kee Zuan
Universiti Putra Malaysia

The world's population is facing the dangers of starvation and malnourishment due to issues pertaining to food security (Gliessman, 2014). Malaysia has a population of 33.57 million, with a 15.4% increment from 2010 to 2021 (World Bank, 2022). To fulfil the growing demand the United Nations Food and Agriculture Organization (FAO) has established a target of doubling the global food output by 2050 (FAO, 2018).

Use of chemical fertilisers is a quick and convenient means to improve crop yield, and conventional farmers are inclined to overuse. Nevertheless, this approach leads to endanger the environment, in addition to fertiliser loss through leaching, volatilisation, and surface runoff. These inorganic fertilisers will be deposited into the river due to leaching and runoff, significantly contributing to eutrophication. Nitrous oxide (N_2O), a potent greenhouse gas (GHG) that destroys the ozone layer, is produced when denitrification occurs during volatilisation (Gruber and Galloway, 2008). Chemical fertiliser use has been linked to several issues, including threats to human health from occupational and non-occupational exposures, farm animal death, and environmental changes (Mazumder et al., 2021). The efficacy of most chemical inorganic fertilisers, including urea as the primary source of nitrogen, is low — generally, around 30% to 40%; this loss has led to an increase in the input cost (Choudhury and Khanif, 2001; Choudhury and Kennedy, 2005).

An environmentally friendly strategy is needed to increase crop output while minimizing chemical fertiliser usage. Biofertilisers consisting of beneficial bacterial inoculants have been reported to improve the development and productivity of several crops and are getting avid attention of researchers. Numerous beneficial microbes, including bacteria such as *Azospirillum, Acetobacter, Bacillus*, and *Herbaspirillum,* have been utilized as biofertilisers (Boddey et al., 1995; Mia et al., 2010). Many previous studies have shown that applying biofertiliser increases the crop's growth and output, especially when a minimum rate of inorganic fertiliser is used.

Plant growth-promoting bacteria (PGPB) is a type of biofertiliser comprising free-living beneficial soil microorganisms that can colonize plant roots and stimulate plant growth (Vessey, 2003; Remans et al., 2008). There is evidence that PGPB adds N to the soils through symbiotic and asymbiotic N_2 fixation, with approximately 175 million tons of N transferred annually (Orhan et al., 2006). Naher et al. (2009) found that bacterial strains inoculation in rice possessed more nitrogen (4.47%), with the biomass rising by 36%. In addition, Mia et al. (2012) found that bacterial application leads to better seedling germination, emergence, vigour, and root growth, which in turn benefits crop growth and yield. Thus, applying PGPB with optimum chemical fertilisation rates could meet the N demand for vegetables, leading to improved growth and crop yield. PGPB has been known to promote nodulation and N_2 fixation in leguminous plants (Figueiredo et al., 2008), but it has received little attention in non-legumes. Injudicious use of agrochemicals poses a severe problem to the crop cultivation system (Villarreal-Romero et al., 2010). The use of PGPB could reduce the demand of plants for chemical fertilisers because the bacteria can enhance the size and volume of the plant roots, which in turn, makes the plants capable of obtaining more water and nutrients, and

extending their life span (Pešaković et al., 2013). They can also fix atmospheric nitrogen (N_2) and can be used to reduce N fertiliser application in agriculture (Masood et al., 2020). For instance, Woodard & Bly (2000), Ratti et al. (2001), Sundara et al. (2002), and Zahir et al. (2012) have reported that plant growth improved with PGPB inoculation in combination with a minimal amount of chemical fertilisers in maize, ginger grass, and sugarcane, respectively. It can be concluded that PGPB can be used as a biofertiliser as an eco-friendly approach to promoting a sustainable crop cultivation system.

1.2 Mutagenesis of Biofertiliser Bacteria Using Gamma Irradiation

Phua Choo Kwai Hoe
Malaysian Nuclear Agency

In recent years, an increasing number of growers use biofertiliser as one of their agriculture inputs. Users often expect improvement in the functionalities of the biofertiliser products. Mutagenesis of microorganisms is one of the means to improve microbial activities to meet market needs (Satoh and Oono, 2019; Hing et al., 2022; Manikandan et al., 2022). The advantage of mutagenesis is it does not require vast knowledge in genetics to determine the desired features.

Mutagenesis is a process of mutation induced by a mutagenic agent. The two types of mutagens used for microorganism mutagenesis are chemical mutagen and physical mutagen (Ram et al., 2019). Gamma ray or radiation is a powerful physical mutagen with a short wavelength and high penetration power resulting from the nuclear disintegration of certain radioactive substances such as cobalt-60 (Co-60 or ^{60}Co) and caesium-137 (Cs-137 or ^{137}Cs).

At the Malaysian Nuclear Agency, the Biobeam gamma cell (^{137}Cs as gamma source) is an acute gamma irradiation facility, whereby the ionizing radiation, gamma, was exposed to the target for a short period (high cumulative dosages with high-dose rate). Thus, gamma irradiation from a gamma cell is a powerful tool for mutagenesis to improve the functionalities of microbes. In agriculture, microorganism mutagenesis is mainly applied for biological control. *Trichoderma* sp., *Fusarium* sp., and *Bacillus* sp. are widely used in mutagenesis through gamma irradiation (Haggag, 2002; Haggag and Mohamed, 2002; Baharvand et al., 2014; Naseripour et al., 2014; Manikandan et al., 2022).

Microorganisms are generally more resistant to mutagenesis than animals and plants. This observation can be attributed to the size of microorganisms, which are small; their nuclei are also considerably small and thus are difficult to target during irradiation. Mutagenesis of microorganism is more complicated than that of other organisms (Satoh and Oono, 2019). Moreover, microorganisms demonstrate the capacity for DNA protection and repair. DNA protection includes spore formation (resting stage of cells) and use of radical scavengers, such as catalase, superoxide dismutase, and carotenoids. Some spore-forming bacteria, such as *Bacillus* spp. and *Clostridium* spp., are resistant to irradiation (FNCA, 2006).

The effects of irradiation can be measured via lethal dose (LD_{50}). Studies on mutagenesis applied the lethal dose (LD_{50}), where 50% of irradiated cells died. Effects of gamma irradiation on Gram-positive and Gram-negative bacteria are different. A study on the survival of bacterial isolates under radiation doses of 1 kGy to 10 kGy was conducted. *Streptococcus* sp. continued to grow even up to 9 kGy, but all the isolates died at 10 kGy. Thus, Gram-positive bacteria can tolerate high doses of radiation. By contrast, all Gram-negative isolates, such as *Pseudomonas* sp., died after exposure to 5 kGy (Atique et al., 2013). Random mutagenesis in *B. subtilis* UTB1 was conducted by using different doses of gamma irradiation (0.1–3 kGy) to improve its antagonistic activity against *Aspergillus flavus* R5. At irradiation doses of 2, 2.5, and 3 kGy 3–4 log

of the microorganisms was killed (Afsharmanesh et al., 2013). An investigation on the effect of acute gamma irradiation of Gram-positive bacteria (*Bacillus* sp.) and Gram-negative bacteria (*Escherichia coli*) were conducted. The LD_{50} for *Bacillus megaterium* NMBCC50018, *Bacillus subtilis* NMBCC50025 and *E. coli* were 1.2 kGy, 0.2 kGy and 0.03 kGy, respectively. Gram-positive bacteria were more resistant to gamma irradiation in comparison to Gram-negative bacteria (Hing et al., 2022). In view of these results, there has been an absence of a standard protocol in mutagenesis study on microorganisms. It is important to have a standard optimum dose and LD_{50} data as guidelines for mutagenesis of microorganisms involving gamma irradiation.

1.3 Dose Mapping of Gamma Cell

Ahmad Zainuri Mohd Dzomir
Malaysian Nuclear Agency

The type of radiation used for mutagenesis in bacteria may consist typically of gamma, X-ray, and ultra-violet (Nagata et al., 2003; Dong et al., 2011; Manikandan et al., 2022). As for the current procedure, mutagenesis of biofertiliser bacteria is accomplished using gamma rays of caesium-137 (Cs-137 or ^{137}Cs) from self-contained gamma cell irradiator, Biobeam GM 8000 (Gamma Service Medical GmbH, Germany). It has a mechanism to rotate the beaker from the loading (shielded) position to irradiation position. The beaker is made of stainless steel to hold the sample during irradiation. When a sample is ready to be irradiated, it is placed in suitable containers and arranged accordingly to certain load configuration inside a 7.5 litre stainless steel beaker (BB75-4). The beaker is then placed in the irradiation chamber while it is in the loading position. After the program and timer are set to deliver the pre-selected dose, the chamber is automatically rotated to the irradiation position by pushing a start button on the touchscreen panel.

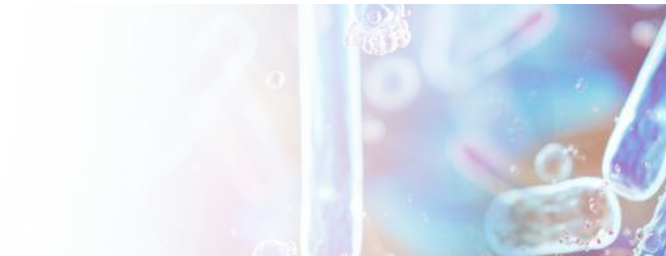
In many circumstances, the dose rate within the irradiation chamber will vary depending on the positioning of the isotopic ^{137}Cs pencil source. Because of this, there exist a systematic pattern of dose variation within the beaker; thus, resulting in samples receiving variable doses throughout their geometry. In radiation studies the primary parameter is dose. To investigate dose distribution, dosimeters are placed at several pre-determined locations across the beaker, which this exercise term it “dose mapping.” Dose mapping is achieved through an established dosimetry system to measure absorbed dose accurately, and to provide an estimation of the maximum and minimum radiation absorbed doses given during the irradiation process.

A dosimetry system includes dosimeters, measuring equipment along with their associated reference standards and procedures on how to use them. Dosimetry is performed using Fricke dosimeters and analysed by UV spectrophotometer. Dosimeters are devices that when irradiated exhibit a quantifiable change in a particular property that can be related to the absorbed dose. In this case when a Fricke dosimeter is irradiated it cause change in colour that can be detected by UV spectrophotometer at 500 nm, thus providing information on the dose within the beaker, including areas of maximum and minimum doses, the dose uniformity ratios, location where the dose rate is relatively uniform and determine the dose rate at a reference position within the radiation field (IAEA, 2013).

Ideally, for research purpose, it is desirable to obtain doses as close as possible to target doses with the smallest possible dose variation within the sample being irradiated. Therefore, the dose is measured throughout the sample being irradiated for certain and specific load configuration, including load size, shape, and its position within the radiation field to determine the point of highest and lowest doses. Once this known, further exposures can be monitored by measuring the dose only at reference position, provided the sample size and positions remain the same. When characterising dose-response following routine irradiation, sample placement in similar container shape and material is always consistent in the position for which the dose distribution has been measured.

Chapter : 2

Standard Operating Procedure on Mutagenesis of Biofertiliser Bacteria Using Gamma Irradiation



2.1 PREPARATION OF BIOFERTILISER BACTERIA

2.1.1 Gram Staining

Ali Tan Kee Zuan
Universiti Putra Malaysia

I. Principle

Gram staining is a microscopic technique used to differentiate bacteria, based on their structure, into Gram-positives and Gram-negatives. A Danish scientist and physician Hans Christian Joachim Gram, discovered it in 1884. The procedure is based on the ability of microorganisms to retain the colour of the stains used during the Gram stain reaction. Gram-negative bacteria are decolourized by alcohol, losing the colour of the primary stain, purple. Gram-positive bacteria are not decolourized by alcohol and will remain purple. After the decolourization step, a counterstain is used to impart a pink colour to the decolourized Gram-negative organisms.

The Gram stain is an essential preliminary step in the initial characterization and classification of bacteria. It is also necessary to identify bacteria based on staining characteristics, enabling the bacteria to be examined using a light microscope. When viewed using a light microscope, the bacteria in an unstained smear are invisible. Once stained, the bacteria's morphology and arrangement may also be observed. Furthermore, it is also an essential step in screening infectious agents in clinical specimens such as direct smears from a patient.

The Gram stain procedure enables bacteria to retain the colour of the stains based on the differences in the chemical and physical properties of the cell wall.

II. Equipment and materials

- A fully-grown (24 hours) bacterial culture
- Bunsen burner
- Clean glass slides
- Distilled water
- Immersion oil
- Inoculating loop
- Lens paper and lens cleaner
- Light microscope
- Tissue paper

III. Chemicals and reagents

- Crystal violet (primary stain)
- Decolourizer (acetone or 95 % ethyl alcohol)
- Iodine solution/Gram's Iodine (mordant that fixes crystal violet to the cell wall)
- Safranin (secondary stain)
- Water

IV. Procedures

Prepare a glass slide by wiping it off with alcohol. After cleaning, dry the slides and place them on laboratory towels until ready for use. Drawing a circle on the underside of the slide using a glassware-marking pen may be helpful to clearly designate the area in which you will prepare the smear. You may also label the slide with the initials of the name of the organism on the edge of the slide. Care should be taken that the label should not be in contact with the staining reagents.

With a sterile cooled loop, place a loopful of the broth culture on the slide. Spread by means of the circular motion of the inoculating loop to about 1 cm in diameter. Excessive spreading may result in disruption of cellular arrangement. A satisfactory smear will allow examination of the typical cellular arrangement and isolated cells. With a sterile cooled loop, place a drop of sterile water or saline solution on the slide. Sterilize and cool the loop again and pick up a small sample of a bacterial colony and gently stir into the drop of water/saline on the slide to create an emulsion. Roll the swab over the cleaned surface of a glass slide. Heat fixes the sample to the slide by carefully passing the slide with a drop or small piece of sample on it through a Bunsen burner three times.

Add the primary stain (crystal violet) to the sample/slide and incubate for 1 minute. Rinse slide with a gentle stream of water for 5 seconds to remove unbound crystal violet. Add Gram's iodine for 1 minute this is a mordant or an agent that fixes the crystal violet to the bacterial cell wall. Rinse the sample/slide with acetone or alcohol for ~3 seconds and rinse with a gentle stream of water. The alcohol will decolourize the sample if it is Gram-negative, removing the crystal violet. Add the secondary stain, safranin, to the slide and incubate for 1 minute. Wash with a gentle stream of water for a maximum of 5 seconds.

If the bacteria are Gram-positive, they will retain the primary stain (crystal violet) and not take the secondary stain (safranin), causing it to look violet/purple under a microscope. If the bacteria are Gram-negative, they will lose the primary stain and take the secondary stain, causing it to appear red when viewed under a microscope. Blot dry the slide with tissue paper and view the smear using a light microscope under oil immersion.

V. Precautions:

- It is crucial to prevent preparing thick, dense smears which contain an excess of the bacterial sample. A very thick smear diminishes the amount of light that can pass through, thus making it difficult to visualize the morphology of single cells. Smears typically require only a small amount of bacterial culture. An effective smear appears as a thin whitish layer or film after heat-fixing.
- Do not let the decolourizing agent (alcohol) remain on the sample for too long, as it may decolorize Gram-positive cells.
- Always wear gloves and a laboratory coat.
- Tie your hair properly to prevent contamination from your working culture.
- After entering the lab, make sure that the microscope is working correctly. Oculars and objective lenses should be cleaned with lens paper before and after each use.

- Adjust the illumination before using the microscope.
- Prepare your workspace (Laminar Air Flow Cabinet) or laboratory bench by disinfecting the area.
- Properly adjust the flame of the Bunsen burner. The proper flame is a small blue cone; it is not a large plume or orange.
- Wipe the glass slide with ethanol and wave the slide over the Bunsen burner to remove any unwanted microorganisms in the slide.
- Label one side of the glass slide with a. Initials, and b. Date
- While flaming the inoculation loop, be sure that each metal segment glows orange/red-hot before you move the next segment into the flame.
- Once you have flamed your loop, do not lay it down, blow on it, touch it with your fingers, or touch it to any surface other than your inoculums. If you touch the tip to another surface or blow on it, you will have to re-flame the loop before proceeding to your experiment.
- Allow your loop to cool before you try to pick up your organism. If you pick up an organism with a hot inoculation loop, your cells will be killed, affecting your results.
- Always keep the caps in your hand when removing the caps from tubes. Never set them on the table, as they could pick up contaminants.
- Always handle open tubes at an angle near the burner's flame; never let them point directly up since airborne or other environmental organisms could fall into the tube and cause contamination.
- Flame your loop as soon as you transfer the organism into the slide. Never place a contaminated tool on your workbench.
- Try to prepare a single cell layer of the organism (a thin smear). Otherwise, all cells will appear as gram-positive in a wide area.
- Do not over warm the cells. Dry the slide thoroughly before heat fixing.
- Use young, vigorous cultures rather than older cultures for your experiment.
- The decolourization step should not exceed the time limit.
- While washing the slide after staining, do not let the water stream fall directly on the smear. This may disrupt the smear. Let the stream of water flow slowly along the surface so only the stain is flooded, and the smear is intact.
- Always observe under 10X first. This will give you an idea of the location of a good area for observation. After this, you may prefer to switch over to 40X.
- Do not observe a specimen at 100X without immersion oil.
- While focusing the microscope, glass slides should be handled carefully to avoid chipping or breaking.
- After the observation, wipe the microscopic lens with absorbent paper and cover the microscope properly.
- Discard all contaminated materials, return your supplies to the proper storage locations, and clean up your working area.

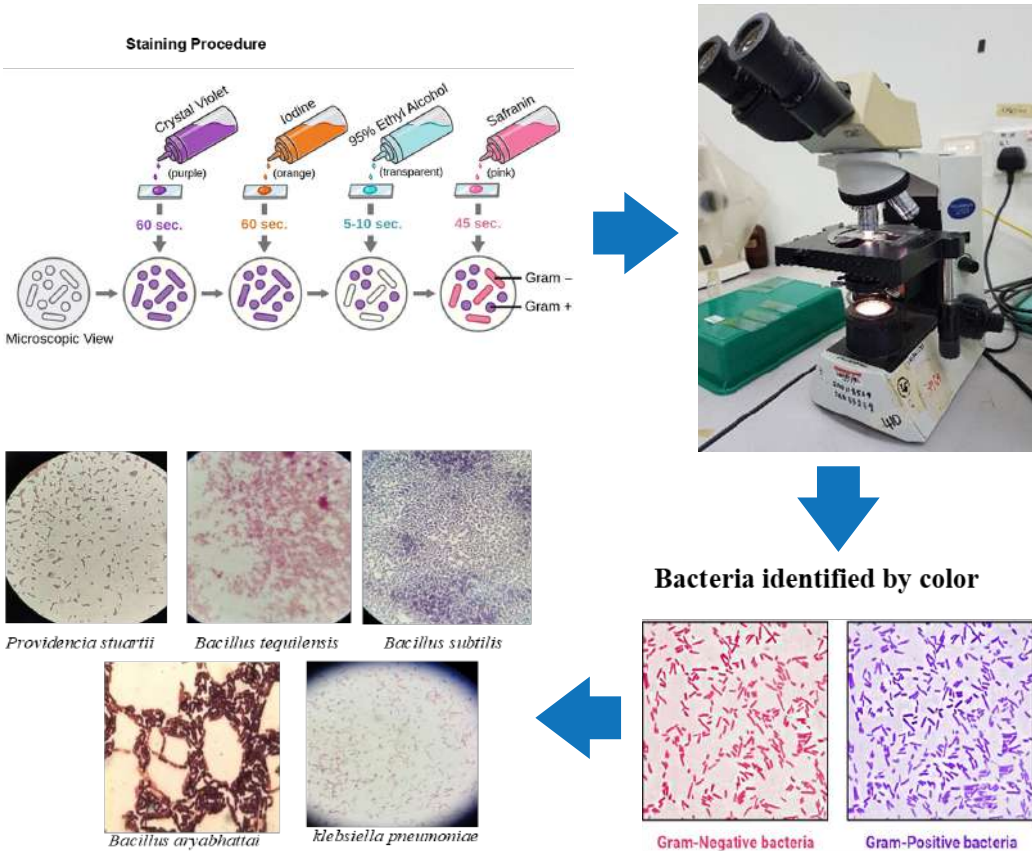


Figure 1. Gram staining procedure

2.1.2 Bacteria Identification by Using 16S rRNA

Chong Saw Peng
Malaysian Nuclear Agency

I. Principle

Before 16S ribosomal RNA (rRNA) sequencing method was introduced in bacterial identification, bacteria were identified using conventional microbiology methods, such as growth on specific media, microscopy, biochemical and serological tests, and antibiotic sensitivity assays. Molecular microbiology evolved rapidly in recent decades, and 16S rRNA sequencing becomes a popular and reliable method in bacterial identification. The 16S rRNA sequencing method is not only more rapid and precise than conventional methods, but it also allows the identification of bacterial strains at the molecular level, enabling discrimination between phenotypically identical bacteria (Johnson et al., 2019).

16S rRNA is an RNA component, together with a complex of 19 proteins to form a 30S subunit of a prokaryotic ribosome. The genes coding for it are the 16S rRNA gene, which is present and highly conserved in all bacteria due to its essential function in ribosome assembly. The 16S rRNA gene is about 1500 bp long, with nine variable regions interspersed between conserved regions. Variable regions of the 16S rRNA gene are frequently used for the phylogenetic classification of genera or species in diverse microbial populations (Clarridge et al., 2004).

II. Equipment and materials

- Autoclave
- Balance
- Biosafety cabinet
- Blue light transilluminator
- Fluorometer
- Gel electrophoresis machine
- Heating block
- Incubator
- Incubator shaker
- Magnetic stirrer
- Micropipette (0.5, 2, 10, 100, 200, and 1000 µL)
- PCR Thermal cycler
- Refrigerated centrifuge
- Refrigerator or chiller
- Vortex mixer
- Water bath

III. Chemicals and reagents

- 100 bp ladder
- 10X PCR buffer
- 16S rRNA primers
- 6X gel loading dye
- Agarose gel
- Deoxynucleotide triphosphate (dNTP)
- DNA extraction kit

- DNA stain (Fluorosafe DNA stain)
- Ethanol (70%)
- Ethanol (absolute)
- Ethylenediaminetetraacetic acid (EDTA)
- Florosafe DNA stain
- Glacial acetic acid
- Lysis buffer
- Magnesium chloride ($MgCl_2$)
- Nuclease-free water
- Nutrient agar (NA)
- Nutrient broth (NB)
- PCR purification kit
- Proteinase K
- Sterile water
- TAE buffer
- Taq DNA polymerase
- Tris

IV. Procedures

a) Bacterial culture

Bacteria from either glycerol stock kept at -20°C freezer or any bacterial culture plate were streaked onto a new NA plate to isolate a single colony of bacteria. The plate was incubated at 28°C in an incubator for 16 to 24 hours. A single colony was picked from the streak plate using the bacterial inoculation loop and inoculated into 100 mL of NB media in a 250 mL conical flask. The bacteria inoculum was incubated at 28°C in an incubator shaker for 16 hours before being harvested for genomic DNA extraction (Lagier et al., 2015).

b) Genomic DNA extraction

The bacteria's genomic DNA was extracted using a DNA extraction kit (Haendiges et al., 2020). The fresh bacteria cells were harvested from the 16 hours of bacteria inoculum (maximum 2 x 10^9 cells) by pipetting 1000 µL of inoculum into a 1.5 mL microcentrifuge tube using the micropipette. The microcentrifuge tube was centrifuged for 10 minutes at 5000 x g (7500 rpm) at room temperature using a refrigerated centrifuge. The supernatant after centrifuging was discarded. The bacteria pellet was resuspended in 180 µL of tissue lysis buffer (30-50% guanidine hydrochloride and 0.1-1% maleic acid) by pipetting up and down using the micropipette to disburse cells into the buffer.

Proteinase K was added to the cell suspension and mixed thoroughly by vortexing using a vortex mixer. The cell suspension was incubated at 56°C in a water bath or a heating block for 1 to 3 hours until the tissue was completely lysed. The sample was vortexed occasionally during the incubation to disperse it. After the incubation, the sample was vortexed for another 15 seconds. An additional 200 µL lysis buffer was added to the sample and mixed thoroughly by vortexing, followed by 200 µL ethanol (96 – 100%) and mixed thoroughly by vortexing.

The mixture above was pipetted into a mini spin column placed in a 2 mL collection tube and centrifuged at 6000 x g (8000 rpm) for 1 minute at room temperature. The flow-through together with the collection tube was discarded. The spin column was

placed in a new 2 mL collection tube. Approximately 500 µL wash buffer I (70% ethanol and 30% lysis buffer) was added to the spin column and centrifuged at 6000 x g (8000 rpm) for 1 minute at room temperature. The flow-through together with the collection tube was discarded. The spin column was placed in a new 2 mL collection tube again. Approximately 500 µL wash buffer II (70% ethanol and 30% RNase-free water) was added to the spin column and centrifuged at 20,000 x g (14,000 rpm) for 3 minutes at room temperature to dry the column membrane. The flow-through together with the collection tube was discarded.

For the DNA elution, the spin column was placed in a clean 1.5 mL microcentrifuge tube and 100 µL elution buffer (10 mM Tris-Cl and 0.5 mM EDTA; pH 9.0) was added to the column membrane. The tube was incubated at room temperature for 1 minute, then centrifuged at 6000 x g (8000 rpm) for 1 minute to elute the DNA. The extracted DNA has then measured the concentration and purity using a microvolume spectrophotometer or fluorometer and visualized using gel electrophoresis (Figure 2). The extracted genomic DNA was stored at 4°C for further use.

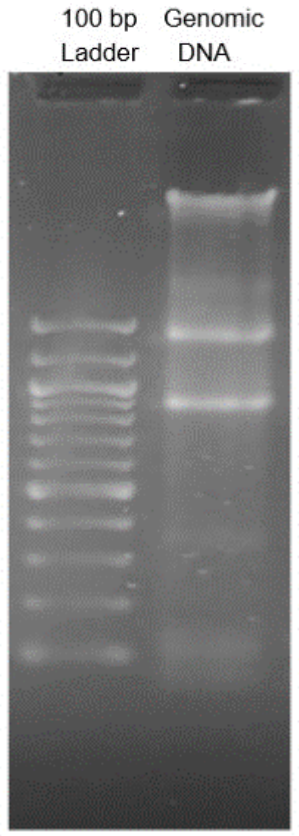


Figure 2. The genomic DNA extracted from bacteria.

c) 16S rRNA gene PCR amplification

The 16S rRNA gene amplification was conducted using the polymerase chain reaction (PCR). PCR is a powerful technique used in molecular biology to amplify specific DNA sequences. This technique was first developed in 1983 by Kary Mullis, who was awarded the Nobel Prize in Chemistry in 1993 for his contribution to the development of PCR. PCR is used to amplify specific fragments of DNA through a series of cycles that include several steps like denaturation, annealing, and extension.

In the first step, denaturation, the DNA double helix is heated to a high temperature (usually around 95°C) to separate the two strands of the DNA molecule. This creates single-stranded DNA templates for the next step. In the second step, annealing, the temperature is lowered to allow short, synthetic DNA primers to bind to complementary regions of the single-stranded DNA template. These primers are designed to flank the region of interest that is to be amplified. In the final step, extension, the temperature is raised to around 72°C, which is the optimal temperature for the DNA polymerase enzyme to extend the primers and synthesize new DNA strands. The DNA polymerase enzyme adds nucleotides to the 3' end of the primers, resulting in the amplification of the targeted DNA sequence. These three steps are repeated in a cyclic manner, with each cycle doubling the amount of DNA present. After 30 to 40 cycles, millions of copies of the target DNA sequence are produced, allowing for its detection and analysis (Rosselli et al., 2016).

One of the most common applications of PCR is in bacterial identification. PCR can detect the DNA of bacteria using the 16S rRNA universal primers. The universal primer pairs used to amplify the 16S rRNA gene in bacterial species were designed based on the conserved regions of the 16S rRNA gene. Some of the primer pairs can amplify most of the 16S rRNA gene (~1500 bp). Examples of commonly used 16S rRNA universal primers for bacteria are shown in Table 1.

Table 1. 16S rRNA universal primers for bacteria.

Primer name	Sequence (5'–3')	Reference
27F	AGAGTTTGATCMTGGCTCAG	Suzuki and Giovannoni (1996)
518R	GTATTACCGCGGCT CTGG	Muyzer et al. (1993)
27F	AGAGTTTGATCMTGGCTCAG	Suzuki and Giovannoni (1996)
1492R	CGGTTACCTTGTTACGACTT	Lane et al. (1991)
8F	AGAGTTTGATCCTGGCTCAG	Juretschko et al. (1998)
1492R	CGGTTACCTTGTTACGACTT	Lane et al. (1991)
785F	GGATTAGATACCCTGGTA	Andersson et al. (2008)
907R	CCGTCAATTCCTTTRAGTTT	Muyzer et al. (1993)
27F	AGAGTTTGATCMTGGCTCAG	Suzuki and Giovannoni (1996)
336R	ACTGCTGCSYCCCGTAGGAGTCT	Weidner et al. (1996)
515F	GTGCCAGCMGCCGCGGTAA	Caporaso et al. (2011)
806R	GGACTACVSGGGTATCTAAT	Caporaso et al. (2011)

To start a PCR amplification, a PCR mixture needs to be prepared. A total volume of 25 µL reaction was needed per sample, which included a DNA template, Taq DNA polymerase, dNTPs, 10X PCR buffer, magnesium chloride, 16S rRNA forward and reverse primers and sterile water (JoVE Science Education Database, 2023). The PCR mixture ingredients was shown in Table 2.

Table 2. The PCR mixture ingredients.

Ingredients	Volume (µL)
Taq DNA polymerase (5U/µL)	0.25
2.5 mM dNTP	0.5
10X PCR Buffer	2.5
25 mM $MgCl_2$	0.75
Forward primer (100 pmol/µL)	0.5
Reverse primer (100 pmol/µL)	0.5
DNA (100 ng/µL)	2
Sterile water	18
Total volume	25

Throughout the preparation time, the PCR mixture was placed on ice to keep it in a cold condition before putting it inside the thermal cycler. The PCR program used for 16S rRNA gene amplification was shown in Figure 3.

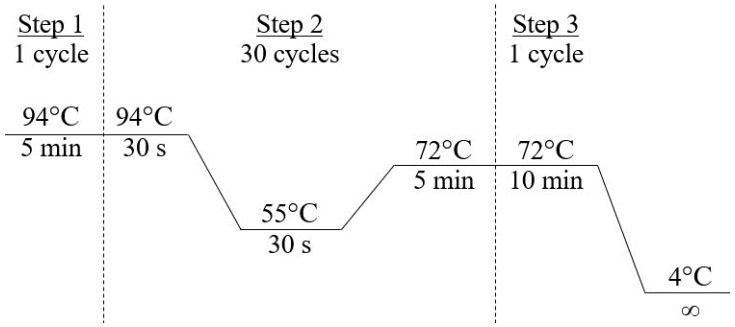


Figure 3. The PCR cycle program for 16S rRNA gene amplification.

The amplified PCR product was evaluated using the agarose gel electrophoresis. Electrophoresis is a basic and essential technique used in the laboratory for separating DNA fragments based on their size. DNA is negatively charged, and it can be moved through an agarose matrix by means of electric current. DNA fragments are loaded onto the gel, and they migrate through the gel based on their size and charge. The smaller the fragments, the faster they move through the gel. This results in the separation of DNA fragments based on their size, which can be visualized by staining the gel with a dye, such as ethidium bromide or other safe DNA stains (Armstrong et al., 2015).

To prepare a 1% agarose gel for electrophoresis, first, a 10X Tris-acetate-EDTA (TAE) buffer was prepared by adding 48.5 g tris, 11.4 mL glacial acetic acid, 20 mL 0.5M EDTA, and topping up to 1 litre with distilled water and adjusted the pH to 8.0 then autoclaved it at 121°C for 15 minutes. Approximately 50 mL 1X TAE buffer was diluted from the 10X TAE buffer and 0.5 g agarose was added to it. The agarose/ buffer mixture was heated up in a microwave until all the agarose was completely dissolved. Due to ethidium bromide being a carcinogen, we used other DNA stains such as the Florosafe DNA stain to stain the gel. Approximately 1-2 µL DNA stain was added to the agarose solution at a temperature of 50-65°C. The gel tray was placed into the casting apparatus to form a mould and an appropriate comb was placed into the gel mould to create the wells. The molten agarose was poured into the gel mould and allowed to be set at room temperature. The comb was removed once the gel was set and placed the gel in the gel box and immersed with 1X TAE buffer.

A 6X gel loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol) was added to the PCR product. Loading dye helps to track the DNA sample that has travelled and allows the sample to sink into the gel. Cover the surface of the gel with enough 1X TAE buffer. The PCR product was loaded slowly and carefully into the well. An appropriate DNA size marker (100 bp ladder) was loaded along with the PCR product. Once the lid was placed on the gel box and the electrodes were plugged into the power supply, turned on the power and run the gel until the dye has migrated to an appropriate distance.

When electrophoresis had completed, turn off the power supply and removed the lid of the gel box. The gel was removed from the gel box and the gel tray was then placed onto the ultraviolet (UV) or blue light transilluminator box to expose the gel to UV or blue light. DNA bands should show up as orange fluorescent bands. A picture of the gel was taken. For the 16S rRNA gene amplified using 16S rRNA primer pair F27 and R1492, a PCR product of about 1.5 kb had been amplified (Figure 4).

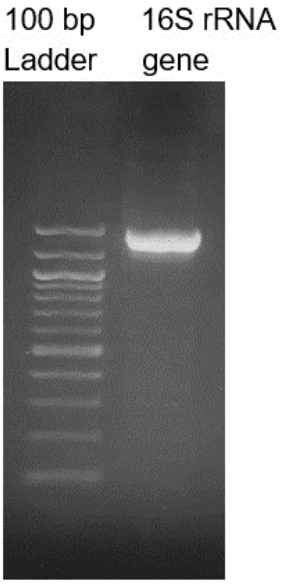


Figure 4. A 1.5 kilo base PCR product of the 16S rRNA gene.

d) **PCR product purification**

The PCR product needs to be purified before sequencing. A commercial PCR purification kit (Wizard® PCR Clean-Up System, Promega) was used to clean up the PCR product (Tamaki et al., 2011). An equal volume of membrane binding solution was added to the PCR reaction. One spin column was placed in a collection tube for each PCR reaction. The prepared PCR product was transferred to the spin column and incubated for 1 minute at room temperature. After that, the spin column set was centrifuged at 16,000 x g (14,000 rpm) for 1 minute. The spin column was removed and the liquid in the collection tube was discarded. The spin column was returned to the collection tube to proceed with the washing step. Approximately 700 µL of membrane wash solution was added to the spin column and centrifuged for 1 minute at 16,000 x g (14,000 rpm). The collection tube was then emptied, and the spin column was placed back in the collection tube. The washing step was repeated with another 500 µL of membrane wash solution added and centrifuged for 5 minutes at 16,000 x g (14,000 rpm).

The spin column set was removed from the microcentrifuge. The collection tube was emptied and recentrifuged the spin column set for 1 minute with the microcentrifuge lid open to allow evaporation of any residual ethanol. The spin column was transferred to a clean 1.5 mL microcentrifuge tube. Approximately 50 µL of nuclease-free water was added directly to the centre of the column without touching the membrane with the pipette tip. The spin column was then incubated at room temperature for 1 minute and followed by centrifuging for 1 minute at 16,000 x g (14,000 rpm). Finally, the spin column was discarded and the eluted PCR product in the microcentrifuge tube was stored at 4°C or -20°C for further use.

e) 16S rRNA sequencing

16S rRNA sequencing is a molecular technique used to identify and classify bacterial species. The 16S rRNA gene is made up of several regions that are highly conserved across all prokaryotes, as well as hypervariable regions that are unique to specific groups of bacteria. By sequencing these hypervariable regions, it is possible to identify and classify bacterial species based on their genetic signatures (Johnson et al., 2019).

The 16S rRNA sequencing can be performed using a variety of techniques, such as Sanger sequencing or next-generation sequencing (Slatko et al., 2018). The samples were outsourced to a service-provided sequencing Laboratory to sequence the DNA. The resulting sequence data is then analyzed using bioinformatics tools to identify the bacterial species present in the sample.

f) Sequence editing and blasting

The bioinformatics analysis involved here was sequence editing and blasting. Sequence editing involves the process of checking a DNA sequence chromatogram by adding, deleting, or substituting nucleotides. This is often done using DNA sequence editing tools that allow for visualizing and manipulating the sequence (Dear et al., 1991).

Blasting, on the other hand, refers to a bioinformatics technique for comparing a sequence of interest to a database of known sequences to identify homologous sequences. The technique is named after the Basic Local Alignment Search Tool

(BLAST), which is a popular program for performing these comparisons. Blasting allows researchers to identify related sequences and infer functional and evolutionary relationships (Lobo, 2008). Examples of the sequence editing and blasting were shown below in Figures 5, 6, and 7.

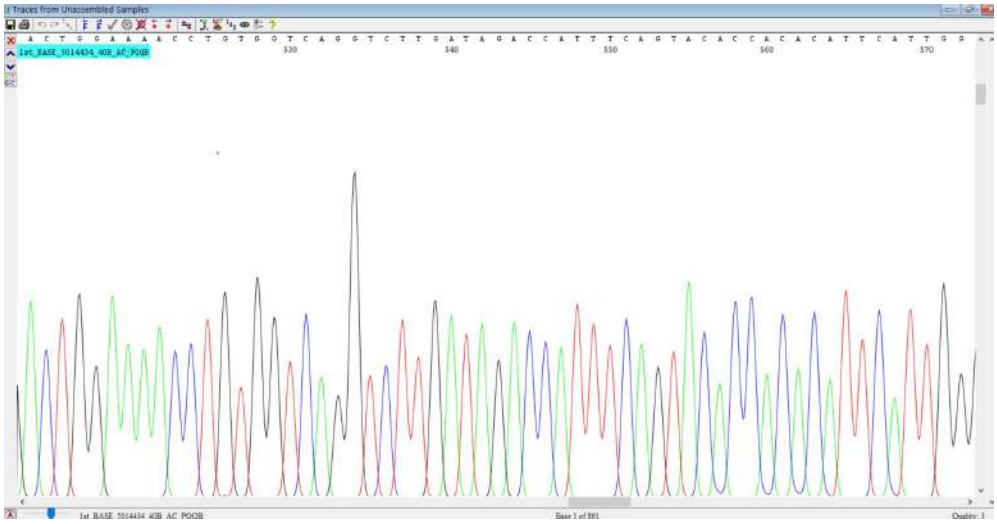


Figure 5. The DNA sequence chromatogram was visualized using the sequence editing tool (Codon Code Aligner).

>Sample.seq

CCGTTGATCCAAAGTGGTAAGCGCCCTCCCGAAGGTTAAGCTACCTACTTCTTTTGCAACCCACT
CCCATGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGTAGCATTCTGATCTA
CGATTACTAGCGATTCCGACTTCATGGAGTCGAGTTGCAGACTCCAATCCGGACTACGACGCAC
TTTATGAGGTCCGCTTGCTCTCGCGAGGTCGCTTCTCTTTGTATGCGCCATTGTAGCACGTGTGT
AGCCCTACTCGTAAGGGCCATGATGACTTGACGTCATCCCCACCTTCCTCCAGTTTATCACTGGC
AGTCTCCTTTGAGTTCCCGGCCGAACCGCTGGCAACAAAGGATAAGGGTTGCGCTCGTTGCGG
GACTTAACCCAACATTTCACAACACGAGCTGACGACAGCCATGCAGCACCTGTCTCAGAGTTCC
CGAAGGCACCAATCCATCTCTGGAAAGTTCTCTGGATGTCAAGAGTAGGTAAGGTTCTTCGCGTT
GCATCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCATTTGAGTTTTAAC
CTTGCGGCCGTACTCCCCAGGCGGTCGACTTAACGCGTTAGCTCCGGAAGCCACGCCTCAAGG
GCACAACCTCCAAGTCGACATCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTTGCTCC
CCACGCTTTCGCACCTGAGCGTCAGTCTTTGTCCAGGGGGCCGCCTTCGCCACCGGTATTCCTC
CAGATCTCTACGCATTTCACCGCTACACCTGGAATTCTACCCCCCTCTACAAGACTCTAGCCTGC
CAGTTTCGAATGCAGTTCCCAGGTTGAGCCCGGGGATTTCACATCCGACTTGACAGACCGCCTG
CGTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGTATTACCGCGGCTGCTGG
CACGGAGTTAGCCGGTGCTTCTTCTGCGGGTAACGTCAATTGCTGAGGTTATTAACCTCAACACC
TTCCTCCCCGCTGAAAGTACTTTACAACCCGAAGGCCTTCTTCATACACGCGGCATGGCTGCATC
AGGCTTGCGCCCATTGTGCAATATTCCCCACTGCTGCCTCCCGTAGGAATCTGGACCGGGTCTC
AGTTCCAGGGTGGCTGGGCATCCTCTCAGACCAGCTAGGGATCTTCGCCTAAGGGGAACCGTT

Figure 6. The edited sequence was presented in FASTA format.

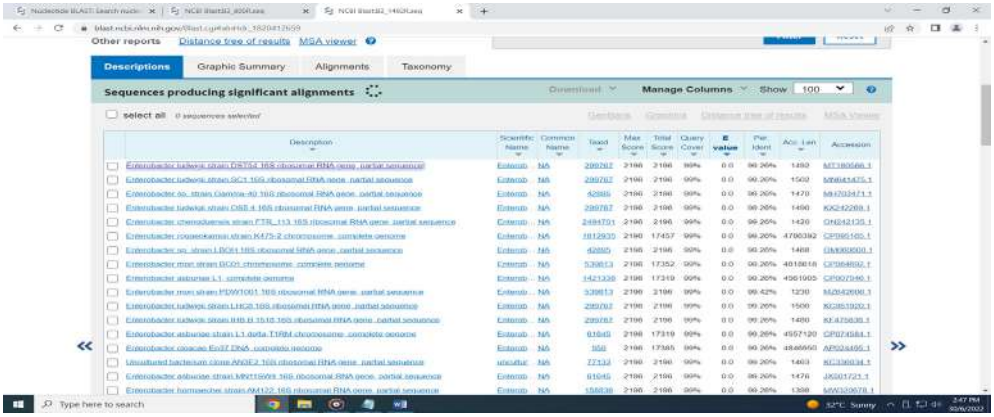


Figure 7. The sequence blast search result using Basic Local Alignment Search Tool (BLAST).

2.2 GAMMA IRRADIATION OF BIOFERTILISER BACTERIA

2.2.1 Determination of LD_{50}

Phua Choo Kwai Hoe and Ahmad Zainuri Mohd Dzomir
Malaysian Nuclear Agency

I. Principle

Gamma radiation is the electromagnetic radiation of short wavelengths emitted by radioactive isotopes when an unstable nucleus breaks up and decays to reach a stable form. Gamma irradiation is widely used for sterilization purpose. Gamma irradiation can also be administered to induce mutagenesis and improve the characteristics of organisms for certain purposes. The effects of irradiation can be measured via lethal dose (LD_{50}), where 50% of irradiated cells died. The values can be obtained by plotting the survival curve (Figure 8). The LD_{50} for different microorganisms, irradiation conditions, and purposes varies. In addition, the effects of gamma irradiation on Gram-positive and Gram-negative bacteria differ. Gram-positive bacteria can tolerate high doses of radiation. For example, *Streptococcus* sp. continued to grow even up to 9 kGy. By contrast, Gram-negative isolates, such as *Pseudomonas* sp., died after exposure to 5 kGy (Atique et al., 2013). Therefore, determination LD_{50} was important to set the range of irradiation doses.

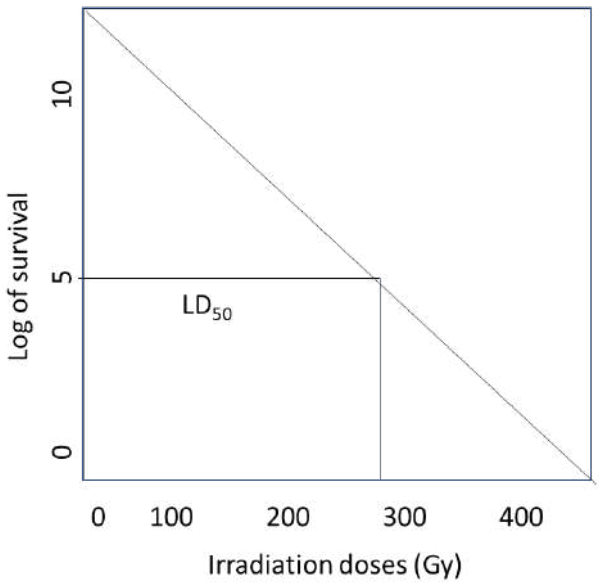


Figure 8: Typical survival curve of gamma irradiated microorganism for determination LD_{50}

II. Equipment and materials

- 15 mL bottle
- Analytical balance
- Autoclave
- Autoclave tape
- Beaker
- Biosafety cabinet
- Conical flask
- Sterile distilled water
- Incubator
- Measurement cylinder
- Petri dish (diameter 45 mm)
- Pipette (100 and 1000 µL)
- Pipette tips (100 and 1000 µL)
- Spatula
- Weighing boat

III. Chemicals

- 20 g nutrient agar (Merck, USA)
- 40 g tryptic soy agar (Oxoid, UK)
- Distilled water

IV. Procedures

a) Media preparation

Nutrient agar (NA) or tryptic soy agar (TSA) powder was weighed using a spatula, a weighing boat and an analytical balance and dissolved with 1 L distilled water. The mixture was poured into 1 L medium bottle and autoclaved at 121 °C for 15–20 minutes. The medium was mixed well by slight shaking and poured into a Petri dish (diameter 45 mm and 90 mm) inside the biosafety cabinet. Thickness of all media plates must same, which was approximately 5 mm (Figure 9).

Figure 9. Thickness of media plates, which was approximately 5 mm.

b) Determination of LD_{50}

Day 1: The isolates were sub-cultured from stock plates (Gram-positive on TSA and Gram-negative on NA) and incubated at 28±2 °C for 24 hours. All isolates must pass quality control for multifunctional activities.

Day 2: A single colony was picked from the 24 hours culture plate (NA or TSA) and streaked full on the 45 mm diameter plates. Gram-positive bacteria were streaked on TSA plates while Gram-negative bacteria on NA plates. There were four replications for each radiation treatments. The plates were incubated at 28±2 °C for 16 to 18 hours.

Day 3: Plates were wrapped with aluminium foil and irradiated at dose 0, 100, 200, 300, 400 and 500 Gy by using self-contained gamma cell irradiator, Biobeam GM 8000 (Gamma Service Medical GmbH, Germany) in Malaysian Nuclear Agency (Figure 10). Non-irradiated plates were used as control. After irradiation, plates were incubated 28±2 °C for 16 to 18 hours.

Figure 10. Self-contained gamma cell irradiator, Biobeam GM 8000 (Gamma Service Medical GmbH, Germany)

Day 4 - 6: The cultures were suspended in sterile distilled water and adjusted to the same concentration at OD_{620nm} = 0.2 (Gram-negative bacteria) and OD_{620nm} = 1.5 (Gram-positive bacteria) with a spectrophotometer (Shimadzu UV mini-120, Japan), which is approximately 10^8 cfu/mL. Population was determined by 10-fold serial dilution via plate counting method. The culture was serially diluted 12 times. Plating was started on the 5^{th} dilution until the 12^{th} dilution (Figure 11 and 12). The plating was conducted via the spread plating technique wherein 100 µL of the suspension was spread on the surface of the agar plate by using a sterile L-shaped rod and incubated at 28 °C for 24–48 hours. First counting was done after 24 hours, and second counting was performed after 48 hours. Three replications of dilution and plating were performed. The LD_{50} was determined by plotting survival graph (Linear regression) by using Statistical Package for the Social Sciences (SPSS) software version 22.

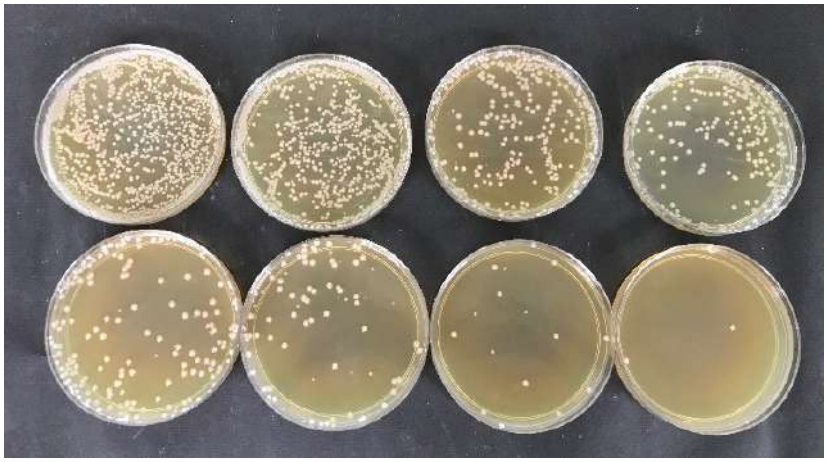


Figure 11. Culture plating

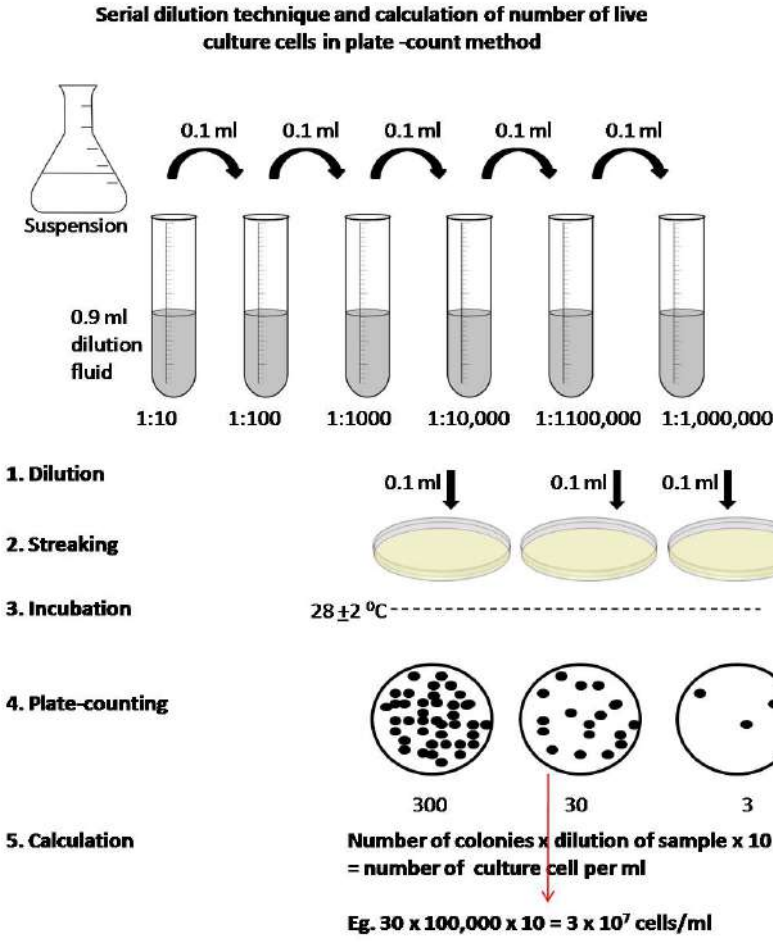


Figure 12. Serial dilution of culture (Ho, 2000)

2.3 SCREENING OF IRRADIATED BIOFERTILISER BACTERIA

2.3.1 Nitrogen (N_2) fixation

Phua Choo Kwai Hoe
Malaysian Nuclear Agency

I. Principle

The nitrogen (N_2) fixing activity of bacteria culture is screened by culturing isolates on yeast extract mannitol agar (YMA) containing 25 µg/mL bromothymol blue (BTB) (Swelim et al., 2010; Phua et al., 2019). Positive nitrogen fixation produces a blue zone on YMA agar plates.

II. Equipment

- Analytical balance
- Autoclave
- Autoclave tape
- Beaker
- Biosafety cabinet
- Sterile distilled water
- Incubator
- Measurement cylinder
- Media bottle
- Petri dish
- Pipette (100 and 1000 µL)
- Pipette tips (100 and 1000 µL)
- Spatula
- Weighing boat

III. Chemicals

- 10 g of mannitol
- 0.2 g of magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$)
- 0.1 g of sodium chloride (NaCl)
- 0.5 g of dipotassium phosphate (K_2HPO_4)
- 0.2 g of calcium chloride monohydrate ($CaCl_2 \cdot H_2O$)
- 0.01 g of iron (III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$)
- 1 g of yeast extract
- 20 g of agar powder
- 25 µg/mL of bromothymol blue (BTB)
- 1 L of distilled water

IV. Procedures

All chemicals were weighed using a spatula, weighing boat and an analytical balance. Except for BTB, the chemicals were mixed and dissolved with 1 L distilled water. The mixture was poured into a 1 L medium bottle and autoclaved at 121 °C for 15–20 minutes. BTB was added to cool the media and poured into a Petri dish inside the biosafety cabinet. Thickness of all media plates must same, which was approximately 5 mm.

After 24 hours incubation after irradiation, the irradiated and non-irradiated cultures were suspended in 9 mL sterile distilled water and adjusted to the same concentration at OD_{620nm} = 0.2 (Gram-negative bacteria) and 1.5 (Gram-positive bacteria) with a spectrophotometer (Shimadzu UV mini-1240, Japan), which is approximately 10^8 cfu/mL. The tested culture (20 µL) was spotted on the screening medium agar plates (Figure 13). Six spots per plates (0, 100, 200, 300, 400 and 500 Gy). Each culture had four replications. The plates were incubated at 28±2 °C for 24 hours.

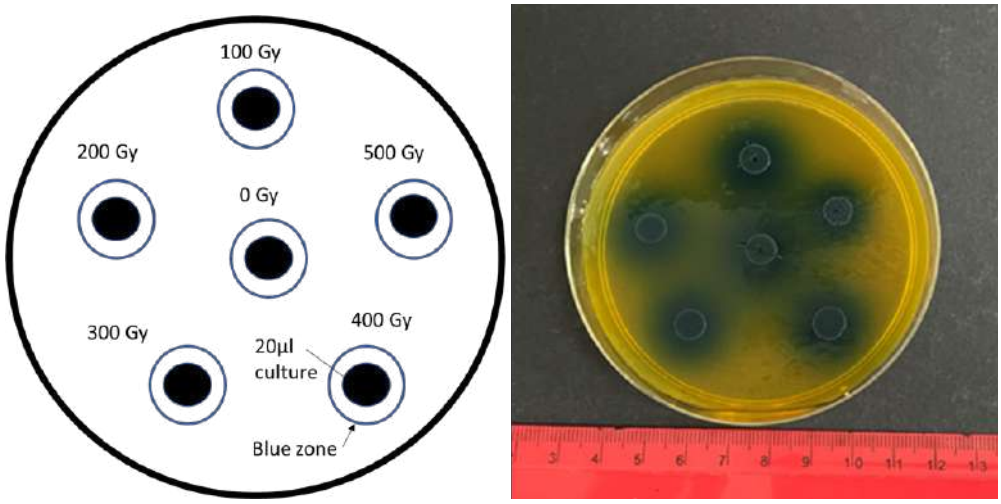


Figure 13. Quick screening for N_2 fixation activity

Positive cultures that showed blue zones indicated atmospheric nitrogen (N_2) fixation activity. The colonies that produced larger blue zones than the non-irradiated colonies were selected and kept as stock. The N_2 fixation activity of irradiated and non-irradiated colonies were determined using N_2 fixation screening media. Bacterial culture suspensions (approximately 10^8 cfu/mL) as method mention above. Briefly, 20 µL of each suspension was spotted at the centre of the screening media agar plate (Figure 14). Each culture had four replications. The plates were incubated at 28±2 °C for 24 hours. The diameter of the clear zone and the growth of the bacterial cultures were measured before and after 2 days of incubations. Data were analysed by ANOVA with the means separated by Duncan's Multiple Range test ($P \leq 0.05$) by using SPSS software. Same steps were repeated for five generation. After five generations screening, the stable culture was considered as potential mutant. Keep it as stock culture.

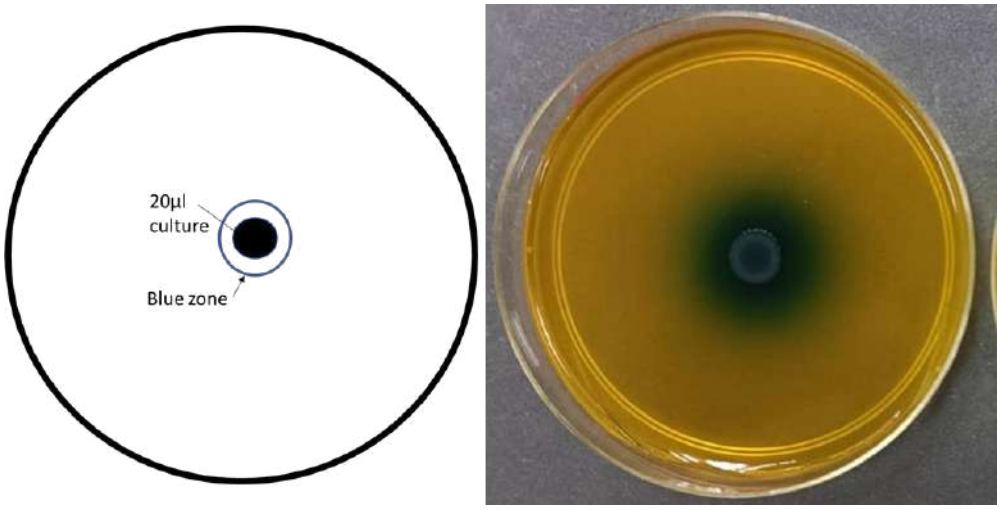


Figure 14. N_2 fixation screening test

2.3.2 Phosphate Solubilisation

Phua Choo Kwai Hoe
Malaysian Nuclear Agency

I. Principle

Phosphate solubilisation activities are screened on phosphate agar plates (PDYA) (Freitas et al., 1997; Phua et al., 2019). The appearance of clear zones around the cultures after 24 hours of incubation at 28±2 °C indicates positive phosphate solubilisation.

II. Equipment

- Aluminium foil
- Analytical balance
- Autoclave
- Autoclave tape
- Beaker
- Biosafety cabinet
- Conical flask
- Sterile distilled water
- Incubator
- Measurement cylinder
- Medium bottle
- Petri dish
- Pipette (100 and 1000 µL)
- Pipette tips (100 and 1000 µL)
- Spatula
- Weighing boat

III. Chemicals

- 39 g of potato dextrose agar (Merck, USA)
- 1 g of yeast extract
- 850 mL of distilled water
- 50 mL of dipotassium phosphate (K_2HPO_4) (10% w/v)
- 100 mL of calcium chloride ($CaCl_2$) (10% w/v)

IV. Procedures

All chemicals were weighed using a spatula, a weighing boat, and an analytical balance. Except for K_2HPO_4 and $CaCl_2$, the chemicals were mixed and dissolved with 850 mL distilled water. The mixture was poured into a 1 L medium bottle and autoclaved at 121 °C for 15–20 minutes. Calcium phosphate mixture was obtained by separately autoclaving 50 mL of K_2HPO_4 (10% w/v) and 100 mL of $CaCl_2$ (10% w/v). The calcium phosphate mixture was added to potato dextrose yeast agar before the resulting liquid medium was poured onto agar plates. The media were mixed well by slight shaking and then poured into a Petri dish inside the biosafety cabinet. Thickness of all media plates must same, which was approximately 5 mm.

Irradiated and non-irradiated cultures were incubated 24 hours at 28±2 °C. The irradiated and non-irradiated cultures were suspended in 9 mL sterile distilled water and adjusted to the same concentration approximately 10^8 cfu/mL at OD_{620nm} = 0.2 (Gram-negative bacteria) and 1.5 (Gram-positive bacteria) with a spectrophotometer (Shimadzu UV mini-1240, Japan). The 20 µL cultures were spotted on the screening medium agar plates (Figure 15). Six spots per plates (0, 100, 200, 300, 400 and 500 Gy). Each culture had four replications. The plates were incubated at 28±2 °C for 24 hours.

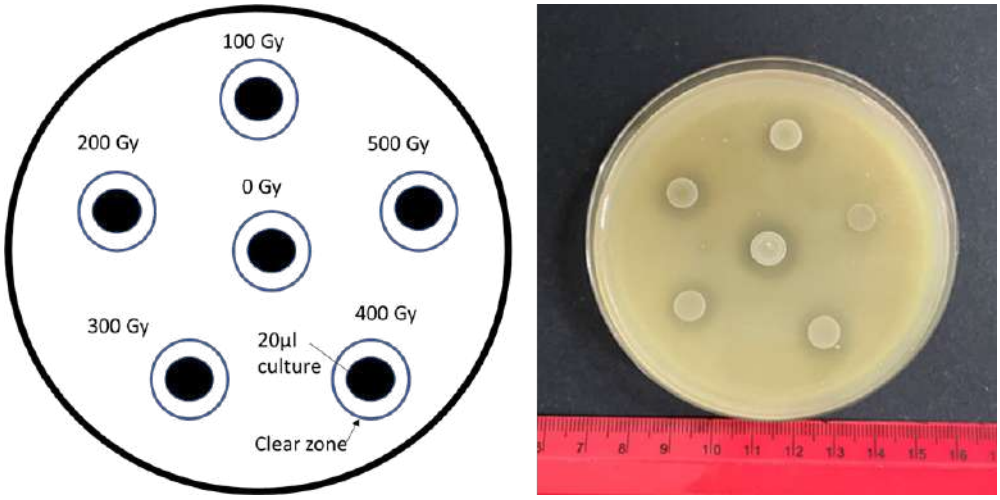


Figure 15. Quick screening for phosphate solubilisation activity

Positive cultures of phosphate solubilisation showed clear zones. The cultures that produced larger clear zones than the non-irradiated cultures were selected and kept as stock. Further confirmation of the phosphate solubilisation activities of irradiated and non-irradiated cultures were determined by using phosphate solubilisation screening media. Approximately 10^8 cfu/mL of culture suspensions were prepared by using spectrophotometer as method mention above. Each culture suspension (20 µL) was spotted at the centre of the screening media agar plate (Figure 16). Each culture had five replications. The plates were incubated at 28±2 °C for 24 hours. The diameter of the clear zone and the growth of the bacterial cultures were measured before and after 2 and 7 days of incubations. Data were analysed by ANOVA with the means separated by Duncan's Multiple Range test ($P \leq 0.05$) by using SPSS software. Same steps were repeated for five generation. After five generations, the screened stable culture was considered as potential mutant. Keep it as stock culture.

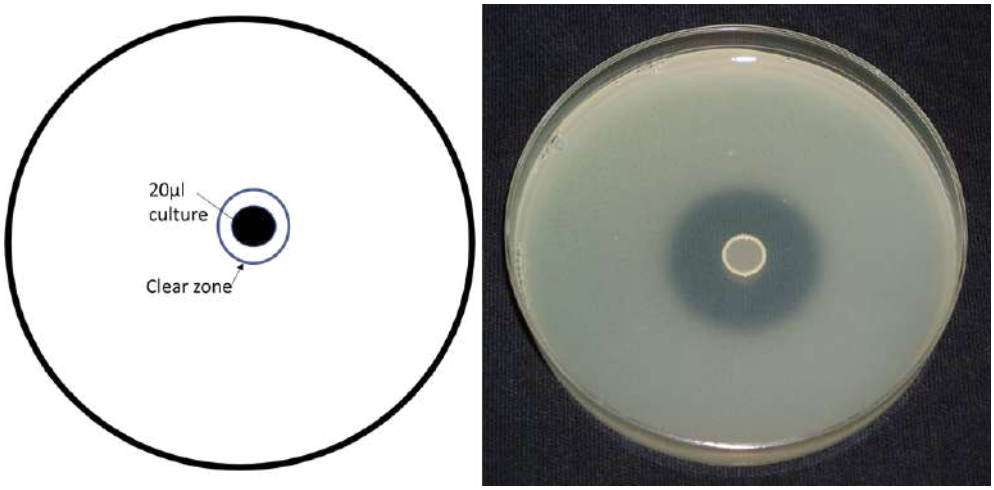


Figure 16. Phosphate solubilisation screening

2.3.3 Potassium Solubilisation

Phua Choo Kwai Hoe
Malaysian Nuclear Agency

I. Principle

Potassium solubilisation activities are screened on Aleksandrov's medium agar plates (MA) (Hu et al., 2006; Phua et al., 2019). The appearance of clear zones around the cultures after 24–48 hours of incubation at 28±2 °C indicates positive potassium solubilisation.

II. Equipment

- Analytical balance
- Autoclave
- Autoclave tape
- Beaker
- Biosafety cabinet
- Sterile distilled water
- Medium bottle
- Measurement cylinder
- Petri dish
- Pipette (100 and 1000 µL)
- Pipette tips (100 and 1000 µL)
- Spatula
- Weighing boat

III. Chemicals

- 5 g of glucose
- 0.005 g of magnesium sulphate heptahydrate ($MgSO_4 \cdot 7H_2O$)
- 0.1 g of iron (III) chloride ($FeCl_3$)
- 2 g of calcium carbonate ($CaCO_3$)
- 2 g of muriate of potash (MOP)
- 2 g of calcium phosphate ($Ca_3(PO_4)_2$)
- 29 g of agar powder
- 1 L of distilled water

IV. Procedures

All chemicals were weighed using a spatula, a weighing boat, and an analytical balance. The chemicals were mixed and dissolved with 1 L distilled water. The mixture was poured into a 1 L medium bottle and autoclaved at 121 °C for 15–20 minutes. The medium was mixed well by slight shaking and poured into a Petri dish inside the Biosafety cabinet. Thickness of all media plates must same, which was approximately 5 mm.

Irradiated and non-irradiated cultures were incubated 24 hours at 28±2 °C. The irradiated and non-irradiated cultures were suspended in 9 mL sterile distilled water and adjusted to the same concentration approximately 10^8 cfu/mL at OD_{620nm} = 0.2 (Gram-negative bacteria) and 1.5 (Gram-positive bacteria) with a spectrophotometer (Shimadzu UV mini-1240, Japan). The 20 µL cultures were spotted on the screening medium agar plates (Figure 17). Six spots per plates (0, 100, 200, 300, 400 and 500 Gy). Each culture had four replications. The plates were incubated at 28±2 °C for 24 hours.

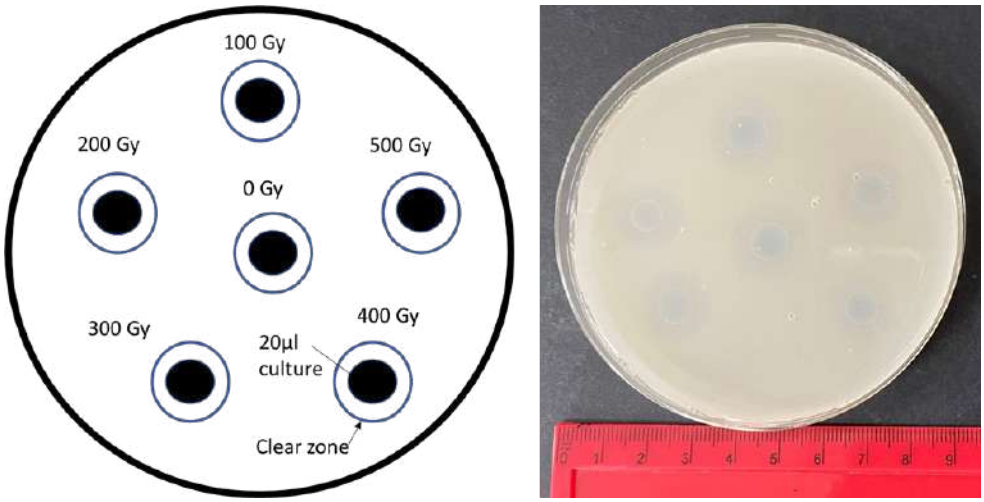


Figure 17. Quick screening for potassium solubilisation activity

Positive cultures of potassium solubilisation showed clear zones. The cultures that produced larger clear zones than the non-irradiated cultures were selected and keep stock. Further confirmation of the potassium solubilisation activities of irradiated and non-irradiated cultures were determined by using Aleksandrov's medium agar plates. Approximately 10^8 cfu/mL of culture suspensions were prepared by using spectrophotometer as method mentioned above. Each culture suspension (20 µL) was spotted at the centre of the screening media agar plate (Figure 18). Each culture had five replications. The plates were incubated at 28±2 °C for 24 hours. The diameter of the clear zone and the growth of the bacterial cultures were measured before and after 2 and 7 days of incubations. Data were analysed by ANOVA with the means separated by Duncan's Multiple Range test ($P \leq 0.05$) by using SPSS software. Same steps were repeated for five generation. After five generations, the screened stable culture considered as potential mutant. Keep it as stock culture.

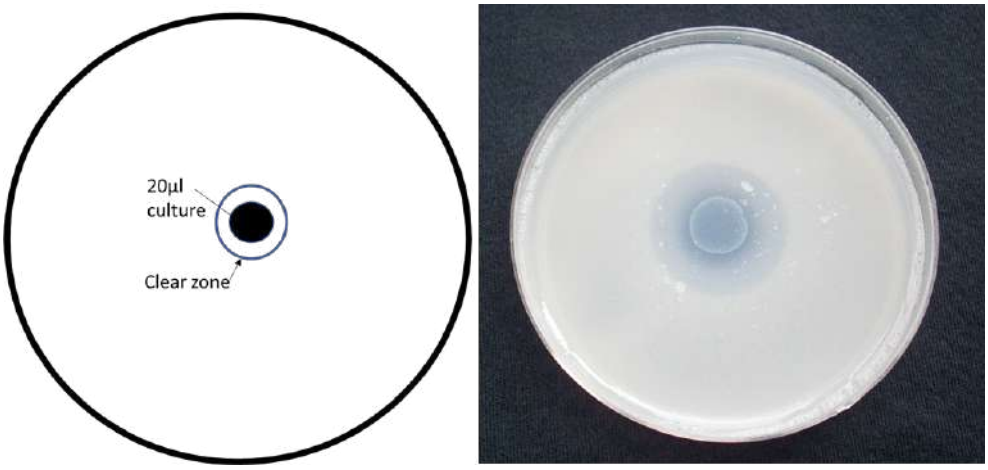


Figure 18. Potassium solubilisation screening

Chapter : 3

Safety

3.1 Laboratory Safety for Mutagenesis of Bacteria

Nur Humaira' Lau Abdullah
Malaysian Nuclear Agency

Laboratory biosafety activities are fundamental to protect the laboratory workforce and the wider community against unintentional exposures or releases of pathogenic biological agents. These activities are implemented through the development of safety culture which is needed to ensure a safe workplace where adequate measures are applied to minimize the likelihood and severity of any potential exposure to biological agents.

I. General Procedures

- Employees or external parties must ensure that all work which involves the handling of materials is done in a controlled condition and followed this procedure.
- The handling of all biohazard materials must with care to prevent spills and contamination. All work must be done in the Biohazard Cabinet (BHC) to prevent cross-contamination and avoid the spread of microorganisms to other places. Infection and contamination by microorganisms into the body can occur through breathing, food, and skin.
- Always use disposable or plastic utensils. If reusable materials ae used, make sure they are washed using 70% ethanol or liquid disinfectant.
- Keep a complete inventory of the use of biohazardous materials and notify the Health Physics Group (KFK) of any biohazard infection/material contamination on materials used, including storage location.

II. Biological Material

- All laboratories dealing with biological materials must be clearly marked/labelled with the BIOHAZARD label at the front door.

III. Safety of Employees

- Employees or external parties must wear personal protective equipment (PPE) depending to the risk group of microorganisms faced. This includes the use of disposable items such as face masks, disposable needles, rubber gloves and others to protect direct contact with items suspected to contain biohazard agents.
- Practice personal hygiene. Wash hands immediately with soap and disinfectant after working in the laboratory, after removing gloves or protective clothing and when contaminated with biohazard materials.
- It is forbidden to use cosmetics, eat, drink or store food in the laboratory.
- Do not perform mouth pipetting.
- Remove contaminated clothing immediately after being contaminated with biohazard materials.
- If injured, treat the injury immediately and notify the Work Supervisor immediately.

IV. Safety of Workplace Environment

- Wash and clean table surfaces, biohazard cabinets and workplace before, after using and immediately in case of biohazard material spillage by using 70% ethanol or disinfectant liquid.

- Waste material should be disinfected by autoclaving or incineration before disposal.
- Limit the entry of not related person to the laboratory where biohazard materials are used of stored.
- The laboratory must be ensured to be safe before allowing visitors to visit or there should be enough protection to visitors if laboratory biohazard potential exists.
- Dispose all contaminated disposables in autoclave bags. All culture media and microorganisms should be autoclaved before disposing as regular rubbish.

V. Emergency Involving Biohazard Materials

- Notify all employees and external parties who are in the laboratory about any spillage.
- Prohibit employees from entering the spill area.
- Remove all contaminated clothing and put into decontamination bag.
- If skin is contaminated, wash with soap and water immediately.
- In case of exposure, seek treatment immediately.
- Wear clean gloves and face shield.
- Place an absorbent pad over the spill area.
- Spray the pad with antifouling.
- Contact the Area Supervisor and KFK by phone and report the incident.

VI. Sterilization and Decontamination of Biohazard Materials

- Laboratories involved in the use of biohazard materials must decontaminate all cultures, stocks and materials used in trial/studies before disposal.
    - Biohazard decontamination technique that can be used for waste materials and reusable materials is autoclaving at 121°C, 15 psi for 15 minutes.
    - Wash reusable materials with detergent and wrapped in heat resistant plastic. Autoclave this package before reuse.
    - Waste that has been decontaminated can be disposed of as normal waste.

VII. Disposal Method

- Biological waste material such as media that has been used, need to be autoclaved before disposed as normal solid waste (Agensi Nuklear Malaysia, 2019a).
- Non-toxic waste materials such as plastics and others can be done disposed as normal solid waste (Agensi Nuklear Malaysia, 2019a) or (Agensi Nuklear Malaysia, 2019b) after autoclaving to ensure safety and prevent environmental pollution.

VIII. Labelling and Container Requirements

- Reusable containers
    - Contaminated waste collection and transport containers must be resistant to leaks, breaks and tears in the situation of normal handling and use. It needs to be decontaminated before reuse.
- Labelling Requirements
    - Biological hazardous waste accumulated in containers and bags must be clearly labelled with symbols and/or marked with the words 'Biohazard Waste.'

- The Biohazard label must be displayed above the door of any laboratory that uses it Biohazard materials or high-level research. Preferably attach the Biohazard warning label to refrigerators, cold containers, and others to warn about the hazard.

Chapter : 4

Facilities and Procedures of Gamma Irradiation Mutagenesis

4.1 Facilities and Procedures for Gamma Irradiation Mutagenesis

Phua Choo Kwai Hoe
Malaysian Nuclear Agency

Mutagenesis of biofertiliser bacteria is carried out at two facilities, which are microbiology laboratory and gamma irradiation facility. Mutagenesis of biofertiliser bacteria requires at least Class II microbiology laboratory. Basic equipment such as biosafety cabinet Class II; autoclave; incubator; microscope; analytical balance; blue light transilluminator; fluorometer; gel electrophoresis machine; heating block; magnetic stirrer; PCR thermal cycler; refrigerated centrifuge; refrigerator; water bath; vortex mixer; incubator shaker; spectrophotometer and pH meter must be in place at the microbiology laboratory. Gamma irradiation facility should be operated by radiation workers led by a radiation protection officer or radiation protection supervisors. Radiation survey meter and personal dosimeters must be provided to radiation workers in this facility for safety and personal dose monitoring.

Procedures for gamma irradiation mutagenesis of biofertiliser bacteria start from isolation and identification of bacteria. This can be obtained from an earlier work or it can start from the beginning. Before starting, media will be prepared by measuring of media materials and chemicals, followed by sterilisation using the autoclave, pouring into petri dishes in a biosafety cabinet. Working stock of biofertiliser bacteria is cultured on media and incubated in an incubator.

Preparation of biofertiliser bacteria involves Gram staining and bacteria identification by using 16S rRNA methods. Gamma irradiation of biofertiliser bacteria for determination LD_{50} include culture preparation, dose mapping and irradiation of biofertiliser bacteria. Screening of irradiated biofertiliser bacteria for atmospheric nitrogen fixation, phosphate and potassium solubilisation start from media preparation, follow by culture preparation and test on selective media. Potential mutants will be screened for five generations and kept as stock for molecular determination to identify gene changes. The following figures or photographs are instrumental in the process of mutagenesis of biofertiliser bacteria by using gamma irradiation.

Microbiology laboratory

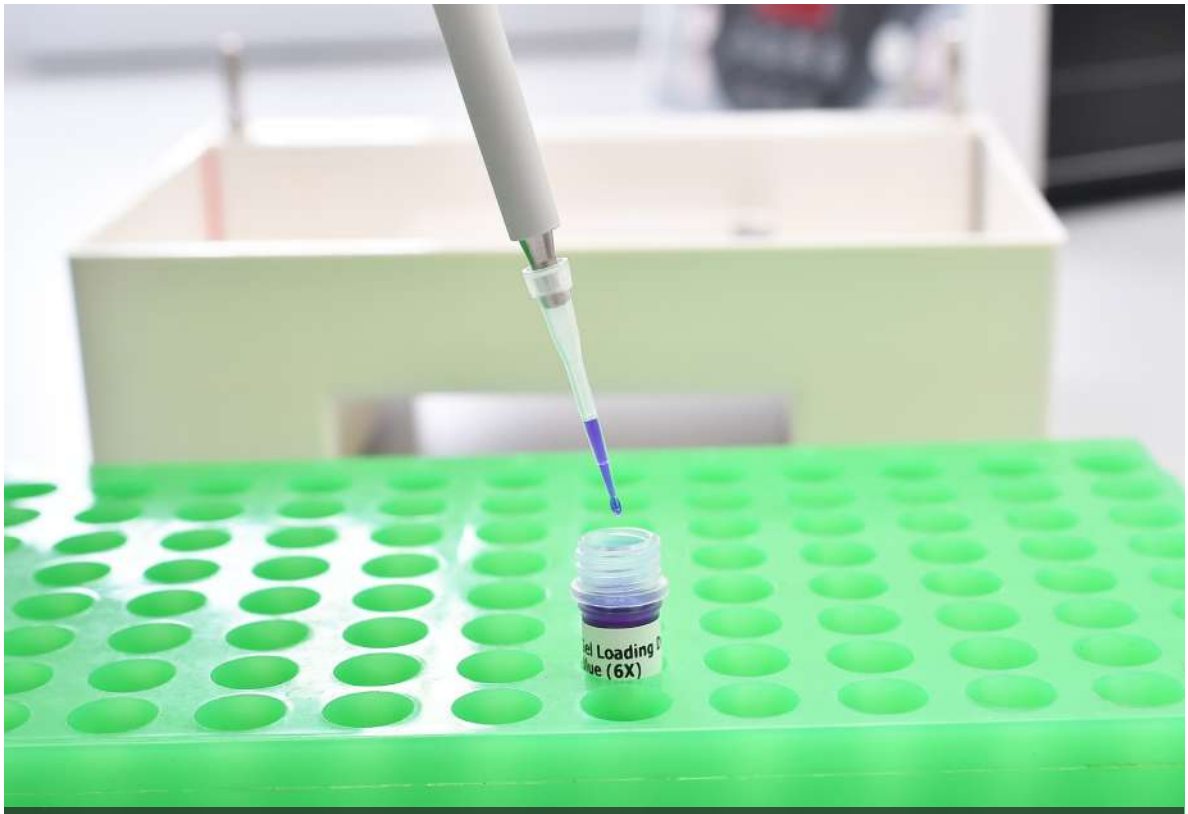
Autoclave

Media preparation

Culture incubation

Microscopy for Gram staining

Gel preparation for electrophoresis



Dye loading for electrophoresis

Thermal cycle set up

The 16S rRNA gene amplification

Self-contained gamma cell irradiator, Biobeam GM 8000 (Gamma Service Medical GmbH, Germany)

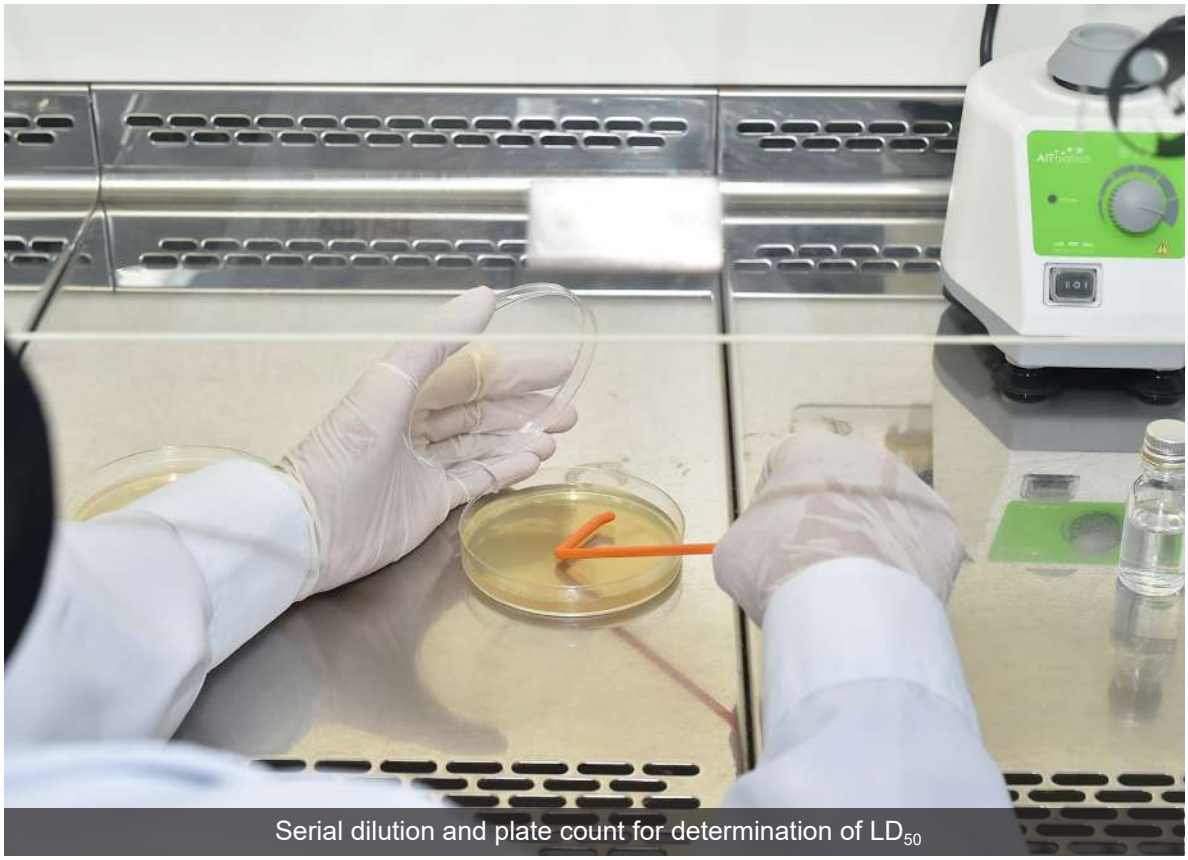
Fricke dosimetry

Dose mapping

Culture plates in container ready for irradiation

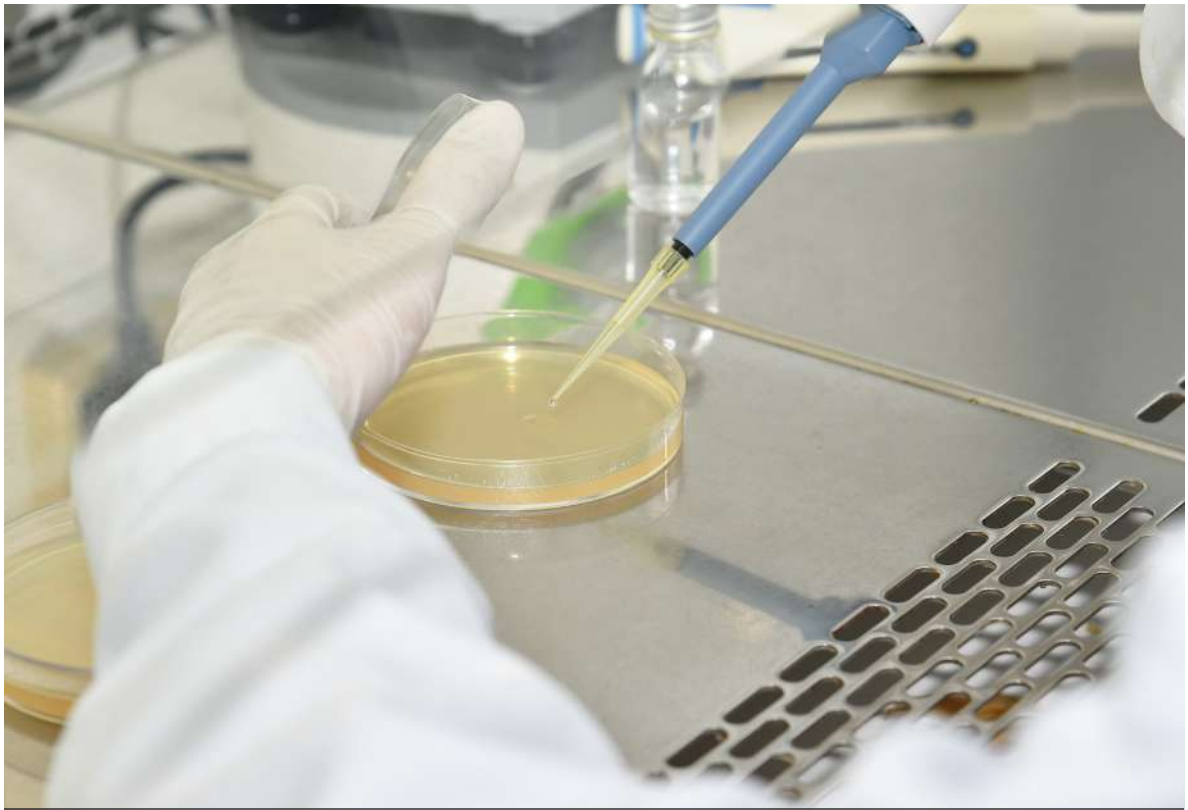
Gamma irradiation on bacteria cultures

Determination of optical density using a spectrophotometer



Serial dilution and plate count for determination of LD_{50}

Preparation of gamma irradiated suspension



Screening of irradiated bacteria on selective media

Appendix

Example of log population (cfu/mL) for LD_{50} for Gram-negative bacteria

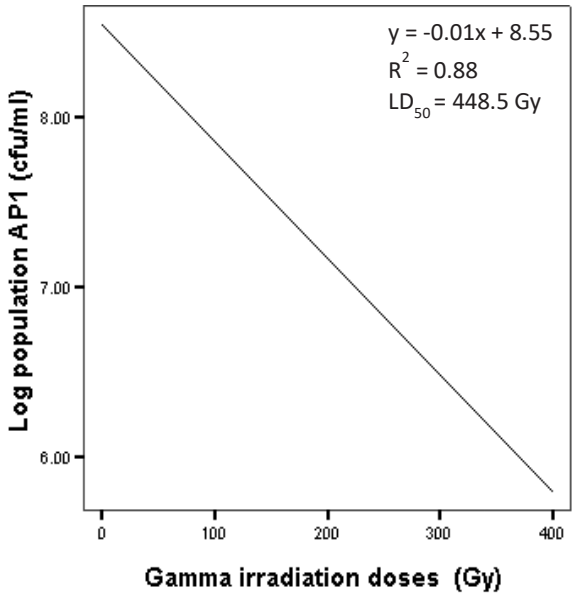


Figure A1. Log population Acinetobacter *baumannii* AP1 (cfu/mL) for LD_{50}

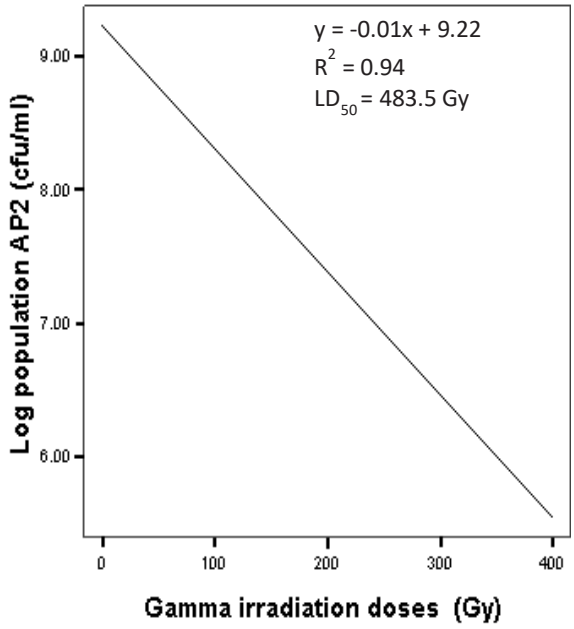


Figure A2. Log population *Klebsiella pneumoniae* AP2 (cfu/mL) for LD_{50}

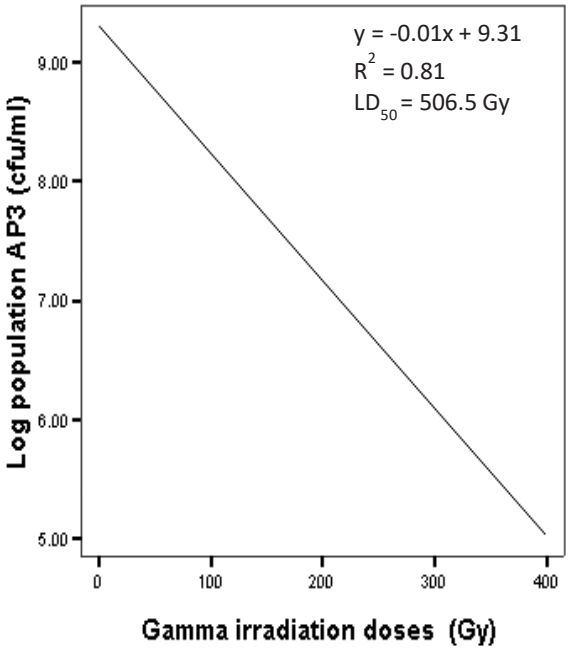


Figure A3. Log population *Enterobacteriaceae bacterium* AP3 (cfu/mL) for LD_{50}

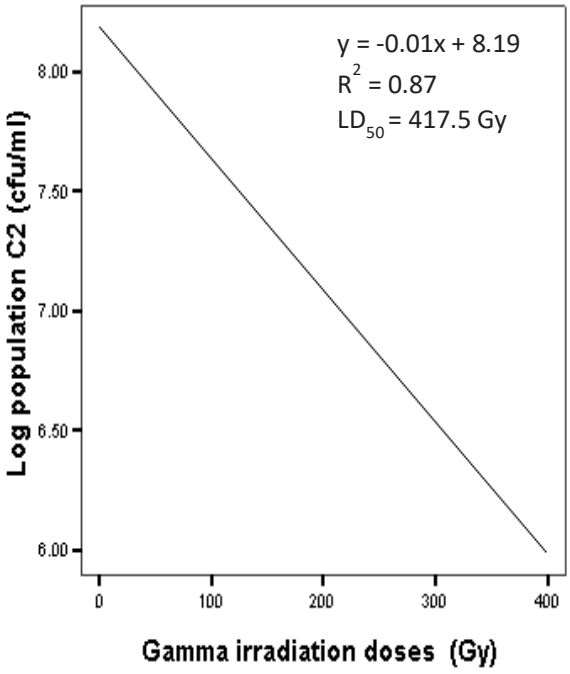


Figure A4. Log population *Pseudomonas putida* C2 (cfu/mL) for LD_{50}

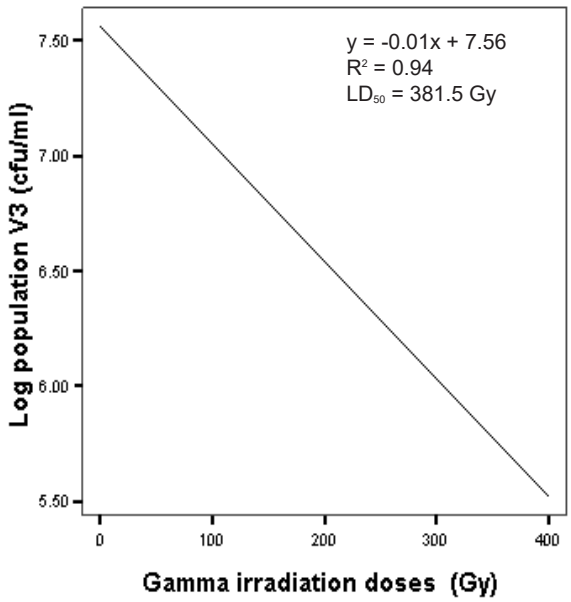


Figure A5. Log population *Pantoea stewartii* V3 (cfu/mL) for LD_{50}

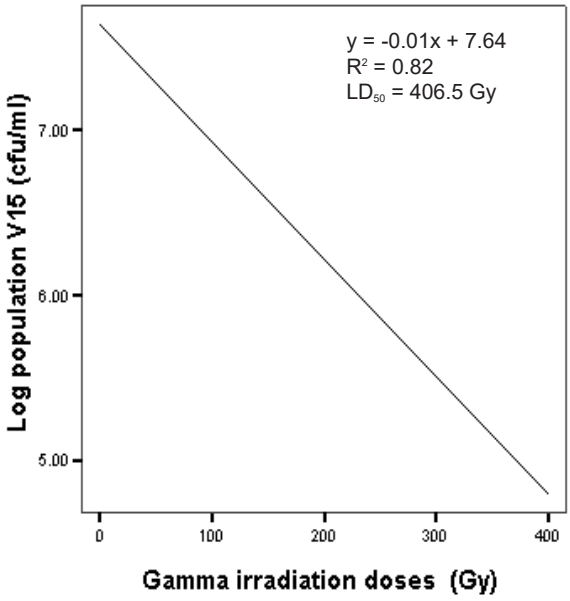


Figure A6. Log population *Pseudomonas putida* V15 (cfu/mL) for LD_{50}

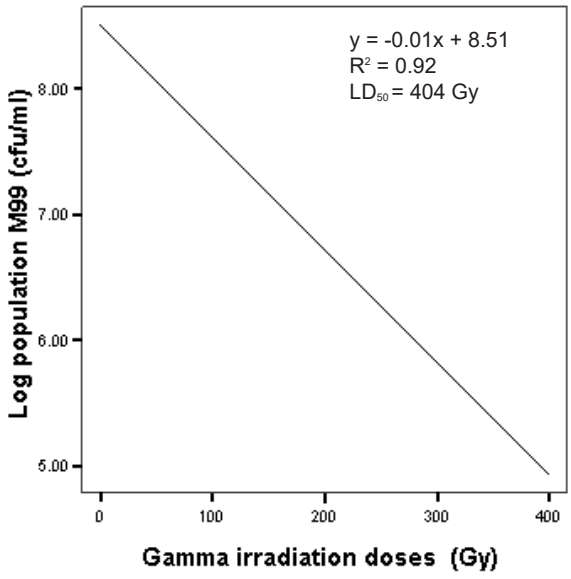


Figure A7. Log population *Pseudomonas putida* M99 (cfu/mL) for LD_{50}

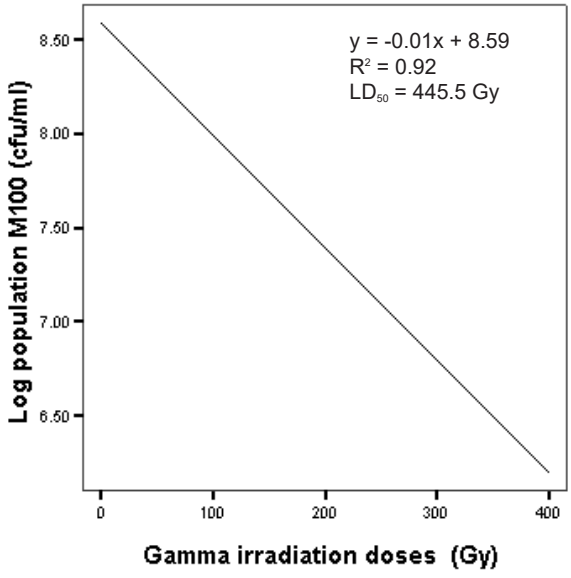


Figure A8. Log population *Acinetobacter calcoaceticus* M100 (cfu/mL) for LD_{50}

Example of log population (cfu/mL) for LD_{50} for Gram-positive bacteria

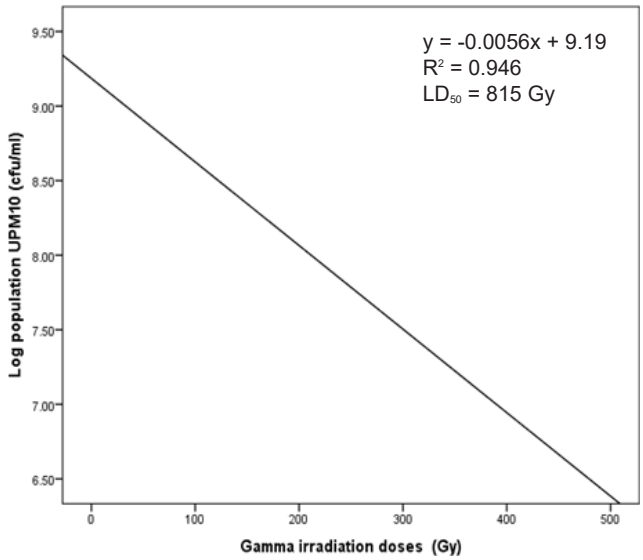


Figure A9. Log population *Bacillus sp.* UPM 10 (cfu/mL) for LD_{50}

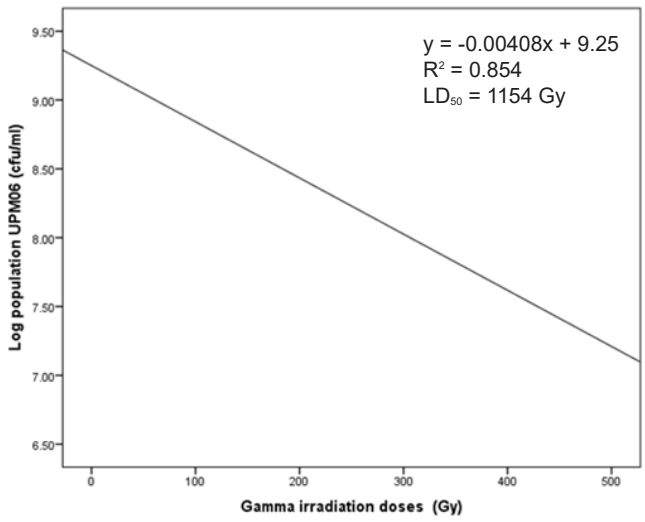


Figure A10. Log population *Bacillus proteolyticus* UPM 06 (cfu/mL) for LD_{50}

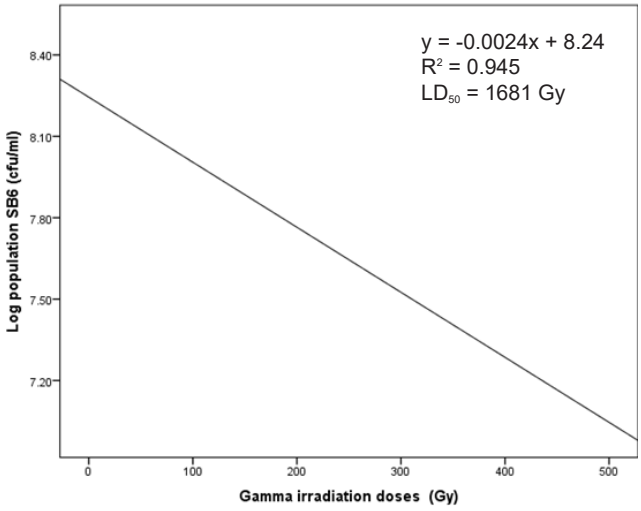


Figure A11. Log population *Priestia megaterium* SB6 (cfu/mL) for LD_{50}

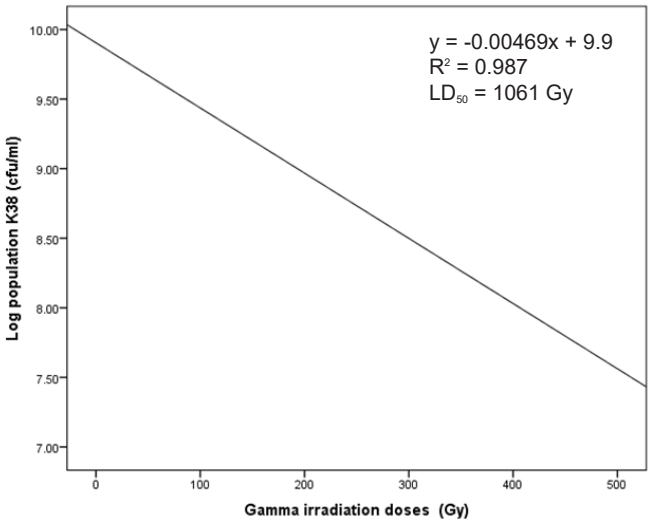


Figure A12. Log population *Priestia aryabhattai* K38 (cfu/mL) for LD_{50}

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