Jurnal Sains Nuklear Malaysia, 2024, 36(No. 2): 23 – 35
ISSN: 2232-0946

DETERMINATION OF MEDIAN LETHAL DOSES (LD_{50}) FOR MUTAGENESIS OF BIOFERTILISER BACTERIA THROUGH GAMMA IRRADIATION

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ABSTRACT

Gamma irradiation for mutation induction in microbes has been utilised to improve antagonistic effects on plant pathogens in agriculture, and to enhance enzyme production during fermentation. Radiation mutagenesis to induce multifunctional activities in biofertiliser microorganisms, is a recent endeavour in Malaysia. Bacteria mutagenesis is complex and is affected by many factors. There is no prior standard or information in this area of research. Therefore, it is crucial to have a standard optimum dose and median lethal dose (LD_{50}) data as a guideline. In this study, eight Gram-negative and four Gram-positive isolates of biofertiliser bacteria, which were isolated from compost, soil, and plants, were subjected to gamma irradiation at Malaysian Nuclear Agency to improve their plant growth-promoting activities, such as N_2 fixation, phosphate, and potassium solubilisation. Gram staining was carried out on the isolates. The isolates were identified as Acinetobacter sp., Klebsiella sp., Enterobacter sp., Pseudomonas sp., Pantoea sp., Bacillus sp. and Priestia sp. by using the 16S rRNA method. These isolates were gamma-irradiated at doses of 50 to 400 Gy. Survival curves revealed that the bacterial levels (log number of cfu/mL) decreased with increasing gamma irradiation doses. The LD_{50} of the Gram-negative isolates ranged from 380 to 500 Gy, while for Gram-positive isolates it ranged from 800 to 1600 Gy. These LD_{50} ranges will be refined further and used for improvement of multifunctional activities of these isolates.

Keywords: Median lethal dose (LD_{50}); gamma irradiation, mutagenesis and biofertiliser

INTRODUCTION

Biofertilisers, also called 'microbial inoculants,' are important for sustainable agriculture and integrated nutrient management. They are applied to seeds, plant surfaces, or soil, and contain living microorganisms that colonise the rhizosphere or the interior parts of plants and promote growth by increasing the supply or availability of primary nutrients to host plants (Vessey, 2003; FNCA, 2018). In the year 2016, the market size of the global biofertiliser market reached USD 1.106 billion, and it is projected to grow at the rate of 14.2% to reach USD 3.124 billion by the end of 2024 (Joshi and Gauraha, 2022). Due to abiotic and biotic effects, an enhancement of the physiology of biofertiliser microbes is much needed. Mutagenesis or genetic recombination of biofertiliser microbes may improve their activities to meet market demands.

Microorganisms can acquire new genetic characteristics through mutation or genetic recombination. In genetic recombination, the efficacy of selected microbes is improved through the genetic manipulation of wild-type strains to produce genetically modified organisms (GMOs). This approach requires a precise knowledge of the mechanisms affecting microbial traits and the structure and regulation of relevant genes (Zeaiter *et al*., 2018). However, a complete understanding of these elements is difficult to obtain and very costly. Furthermore, GMOs have been receiving negative reviews, resulting in low public acceptance; some countries have been restricting the use of GMOs. Conversely, the use of mutants from random mutagenesis is unrestricted. These mutants can be produced by using chemical or physical mutagens. Random mutagenesis is less costly than GMOs. This approach also does not require much genetic knowledge to determine the desired features.

A gene can be modified through spontaneous or induced mutation (Adrio and Demain, 2006; Najafi and Pezeshki, 2013). Chemical mutagen and physical mutagen are two types of mutagens used for mutagenesis (Ram *et al*., 2019). Chemical mutagenesis primarily produces single-base substitutions but not drastic mutations, such as large genomic deletions. Mutagens such as nitrosoguanidine (NTG), 4-nitroquinolone-1-oxide, methylmethane sulphonate, ethylmethane sulphonate (EMS) and hydroxylamine, are the most useful chemical mutagens (Adrio and Demain, 2006; Satoh and Oono, 2019). Physical mutagens, on the other hand, include ionising radiation such as ion beams, ultraviolet (UV) light and gamma radiation (Huma *et al*., 2012).

Ion beams can generate localised irradiation in target organisms. The low energy loss allows a high-resolution control of penetration depth for relatively low-energy ions, which may induce local structural damage caused by atomic displacement (Tanaka *et al.*, 2012; Hase *et al*., 2020). Ultraviolet rays (UV) on the other hand, elicit a moderate effect, which induces pyrimidine dimerisation through frame shift transition from GC to AT base pairs. Among these, gamma rays are the most energetic and highly ionizing form of radiation, which may cause mutations, such as single- or double-strand breakage of DNA through deletion or structural changes, DNA–protein cross-links, oxidised bases, and basic sites (Huma *et al*., 2012;).

Works on microorganisms mutagenesis have been carried out though the use of ion beams (Chen *et al*., 2008; Li *et al.*, 2011), gamma radiation (Afsharmanesh *et al*., 2013; Hing *et al*., 2022), UV light (Huma *et al*., 2012), and chemical mutagens (Adrio and Demain, 2006; Satoh and Oono, 2019) to produce thermo-tolerant mutants in the fermentation industry and disease-control mutants in the agriculture industry.

Among mutagens, ion beam and gamma irradiation are the most used to produce mutants (Hase *et al*., 2020; Ahmad *et al*., 2022; Manikandan *et al*., 2022). In Malaysia, gamma irradiation is utilised since no ion beam irradiation facility is available. Malaysian Nuclear Agency is equipped with acute and chronic irradiation facilities to support this mutagenesis research namely BIOBEAM GM8000 and the Gamma Greenhouse, (Hase *et al*., 2020). These facilities were used for plant mutagenesis on chili, dendrobium orchid, mung bean, groundnut, banana, chrysanthemum, hibiscus and rice (Ibrahim, 2021; Muhammad *et al*., 2021; Ahmad *et al*., 2022; Sherpa *et al*., 2022; Hashim *et al*., 2024). However, radiation mutagenesis on microorganisms are still new in the Malaysia biofertiliser industry (Phua *et al*., 2019b; Hing *et al*., 2022).

Gamma irradiation by gamma cell is a powerful tool for mutagenesis to improve the functionalities of microbes. Microbe mutagenesis is complex and is affected by many factors. Effects of gamma irradiation on Gram-positive bacteria and negative bacteria are different. The effects of irradiation can be measured via two means, namely, decimal reduction dose (D_{10} value) and median lethal dose (LD_{50}). D_{10} value is the radiation dose (kGy) required to reduce the number of microorganisms by 10-fold (one log cycle) or the radiation dose required to kill 90% of the total number of microorganisms (Atique *et al*., 2013; Satoh and Oono, 2019). Studies on mutagenesis applied the LD_{50}, where 50% of irradiated cells died. Both values can be obtained by plotting the survival curve.

The aim of the present study is to establish a standard optimum dose and LD_{50} data as guidelines for gamma irradiation mutagenesis of biofertiliser microorganisms. This data will be useful for future research to improve the multifunctional activities of biofertiliser microorganisms using gamma irradiation.

MATERIALS AND METHODS

Culture collections

Twelve bacterial strains from Malaysian Nuclear Agency (Nuklear Malaysia) and Universiti Putra Malaysia (UPM) culture collections were used in this study. These culture collections were isolated from Natural Farming compost (AP1, AP2 and AP3), Kedah paddy soil (M99 and M100), vanilla plant (V3 and V15), chili plant (C2), oil palm root (UPM10), paddy rice root (UPM06) and Cameron Highlands vegetable farm soil (SB6 and K38).

Screening for multifunctional properties, identification by using 16S rRNA and Gram staining of these isolates have been conducted in earlier studies and the summary is as in Table 1 (Phua *et al*., 2012; Tan *et al*., 2014; Phua *et al*., 2016; Ali-Tan *et al*., 2017; Abdullah *et al*., 2019; Phua *et al*., 2019a; 2019b; Shultana *et al*., 2019; 2020a; 2020b). All culture collections were stored at -20^{o}C.

Table 1. Bacterial designation; organism identification, Gram stain and plant growth promoting activities.

Isolates designation	Organism identification	Gram Stain	Plant growth promoting activities
M100	*Acinetobacter calcoaceticus*	Negative	• N_2 fixation • Phosphate solubilisation • Potassium solubilisation
AP1	*Acinetobacter baumannii*	Negative	• N_2 fixation • Phosphate solubilisation • Potassium solubilisation
AP2	*Klebsiella pneumoniae*	Negative	• N_2 fixation • Phosphate solubilisation • Potassium solubilisation • IAA production (phytohormone)

AP3	*Enterobacteriaceae* bacterium	Negative	• N_2 fixation • Phosphate solubilisation • Potassium solubilisation
C2	*Pseudomonas putida*	Negative	• Phosphate solubilisation • Potassium solubilisation
V3	*Pantoea stewartii*	Negative	• Phosphate solubilisation • Potassium solubilisation
V15	*Pseudomonas putida*	Negative	• Phosphate solubilisation • Potassium solubilisation
M99	*Pseudomonas putida*	Negative	• N_2 fixation • Phosphate solubilisation • Potassium solubilisation
UPM10	*Bacillus sp.*	Positive	• N_2 fixation • Phosphate solubilisation • Potassium solubilisation • IAA production (phytohormone) • Siderophore production • Hydrolyzing enzymes production (cellulase and pectinase)
UPM06	*Bacillus proteolyticus*	Positive	• N_2 fixation • Phosphate solubilisation • Potassium solubilisation • IAA production (phytohormone) • Siderophore production • Hydrolyzing enzymes production (cellulase and pectinase) • Exopolysaccharide (EPS) production • Flocculation yield • Biofilm production • Sodium uptake
SB6	*Priestia megaterium*	Positive	• N_2 fixation • Produce IAA (phytohormone)
K38	*Priestia aryabhattai*	Positive	• Potassium solubilisation • Produce IAA (phytohormone)

Gamma irradiation experiment and determination of LD_{50}

Mutagenesis experiment was done by using gamma cell irradiator, BIOBEAM GM8000 (Gamma Service Medical GmbH, Germany) in Malaysian Nuclear Agency The mutagenesis experiments were carried out in accordance with a modified version of the method used by Rugthaworn *et al.*, (2007).

The isolates were sub-cultured from stock plates. Gram-positive on tryptic soy agar (TSA) and Gram-negative on nutrient agar (NA). The plates are incubated at 28±2 °C for 24 h. All isolates must pass quality control for multifunctional activities. A single colony was picked from the 24 h culture plate and streaked fully on 45 mm diameter plates. Gram-positive bacteria were streaked on TSA plates whilst Gram-negative bacteria were streaked on NA plates. There were four replications for each radiation treatment.

Plates were wrapped with aluminum foil and irradiated at dose 0, 100, 200, 300, 400 and 500 Gy (Fig. 1). Non-irradiated plates were used as control. Dose mapping of gamma cell were done before gamma irradiation was conducted, where Fricke dosimetry was used.

Fig. 1. Culture plates were wrapped with aluminium foil and irradiated in the gamma cell.

After irradiation, plates were incubated 28±2°C for 16 to 18 h. 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 11 times. Plating was started on the 5^{th} dilution until the 11^{th} dilution. 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 h. The first counting was done after 24 h, and second counting was performed after 48 h. 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.

RESULTS AND DISCUSSION

The effects of irradiation can be measured by recording the number of survival samples at different doses after irradiation. Studies on mutagenesis applied the median lethal dose, LD_{50}, where 50% of irradiated cells died. Effects of gamma irradiation on Gram-positive and Gram-negative bacteria were different. A study on the survival of bacterial isolates under radiation doses of 1 to 10 kGy was conducted. *Streptococcus* sp. continued to grow even up to 9 kGy, but all the isolates died at 10 kGy (Atique *et al*., 2013). 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). An investigation on the effect of acute gamma irradiation of Gram-positive bacteria (*Bacillus* sp.) and Gram-negative bacteria (*Escherichia coli*) were conducted (Hing *et al*., 2022). The LD_{50} for *Bacillus megaterium* NMBCC50018, *Bacillus subtilis* NMBCC50025 and *E. coli* were 1.2, 0.2 and 0.03 kGy, respectively. Gram-positive bacteria were more resistant to gamma irradiation in comparison to Gram-negative bacteria (Hing *et al*., 2022).

Results from Fig. 2 and Fig. 3 show the LD_{50} of *Acinetobacter calcoaceticus* (M100) is 445.5 Gy and *Acinetobacter baumannii* (AP1) is 448.5 Gy. The highest LD_{50} of Gram-negative bacteria was of Enterobacteriaceae bacterium (AP3), which was 506.5 Gy (Fig. 5). The second high LD_{50} was of *Klebsiella pneumoniae* (AP2), which was 483.5 Gy (Fig.4). The lowest LD_{50} of Gram-negative bacteria was of *Pseudomonas putida* (V3), which was 381.5 Gy (Fig. 6). Other Gram-negative bacteria LD_{50} was at the average of 400 to 420 Gy (Fig. 6, 8 and 9). They were 417.5, 406.5 and 404.5 Gy for *Pseudomonas putida* (C2, V15 and M99, respectively). These results showed that coccus-shaped Gram-negative bacteria (*Acinetobacter* sp.) was more tolerant to gamma irradiation as compared to long rod-shaped Gram-negative bacteria (*Pseudomonas* sp.). On the other hand, rod shaped Gram-negative bacteria (*Enterobacter* sp. and *Klebsiella* sp.) were the most tolerant to gamma irradiation. Oskouei *et al*. (2022) also reported that *Enterobacter* and *Shigella* were the most resistant bacteria against gamma irradiation which could resist up to 700 Gy. Thus, it was proposed for *Enterobacter* sp., *Klebsiella* sp. and *Acinetobacter* sp. to undergo gamma irradiation mutagenesis to improve their multifunctional properties.

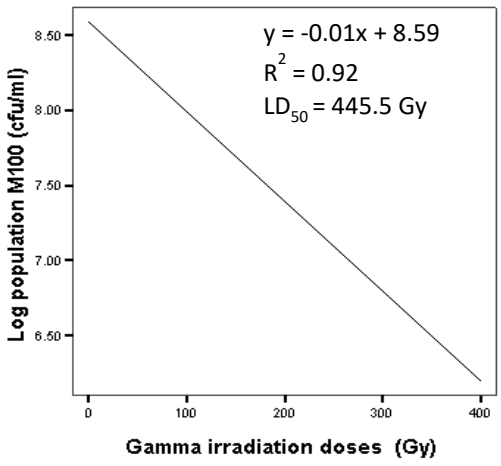

Fig. 2. Log population of *Acinetobacter calcoaceticus* M100 (cfu/mL) for LD_{50}.

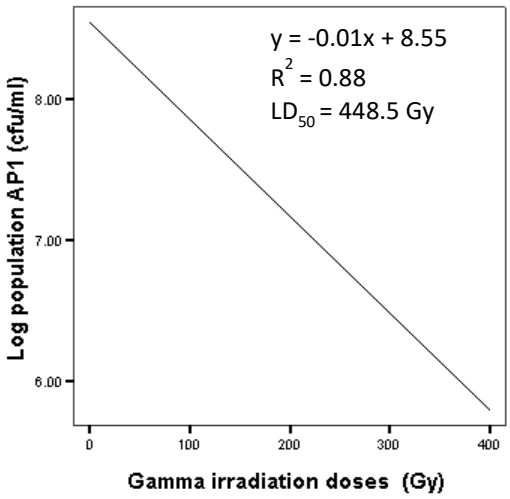

Fig. 3. Log population of *Acinetobacter baumannii* AP1 (cfu/mL) for LD_{50}.

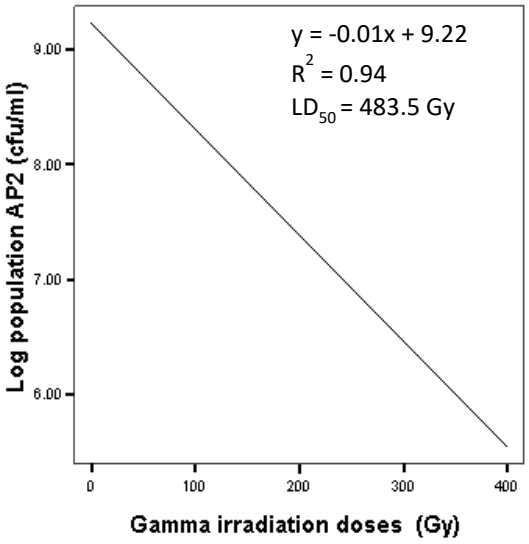

Fig. 4. Log population of *Klebsiella pneumoniae* AP2 (cfu/mL) for LD_{50}.

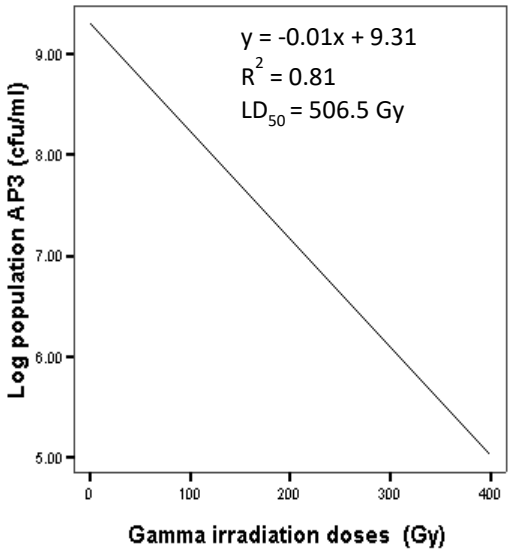

Fig. 5. Log population of *Enterobacteriaceae* bacterium AP3 (cfu/mL) for LD_{50}.

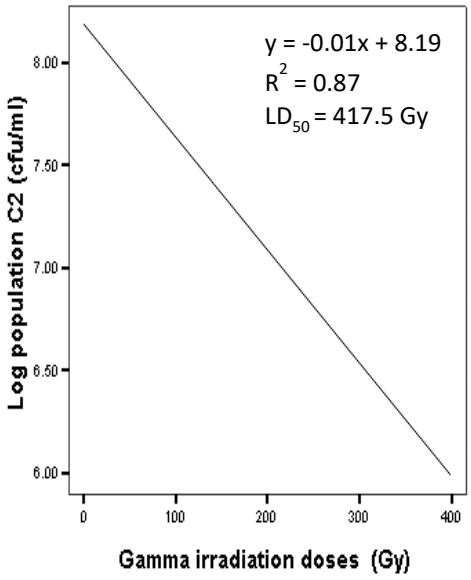

Fig. 6. Log population of *Pseudomonas putida* C2 (cfu/mL) for LD_{50}.

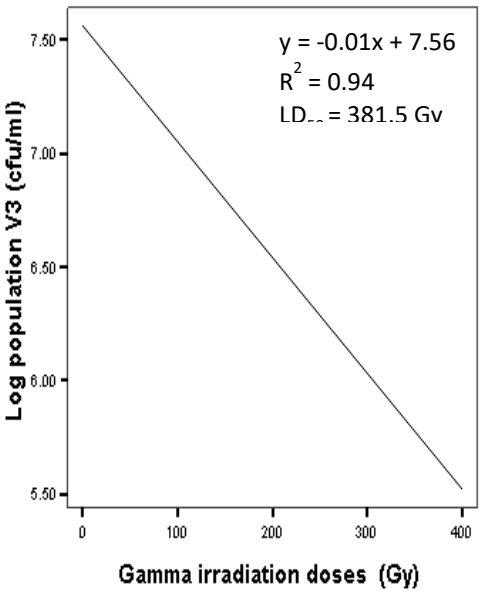

Fig. 7. Log population of *Pantoea stewartii* V3 (cfu/mL) for LD_{50}.

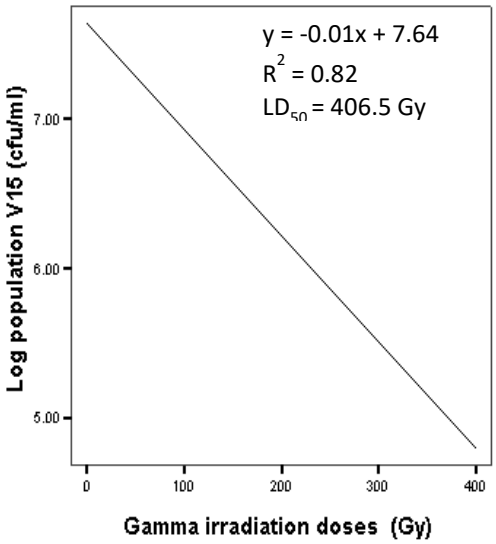

Fig. 8. Log population of *Pseudomonas putida* V15 (cfu/mL) for LD_{50}.

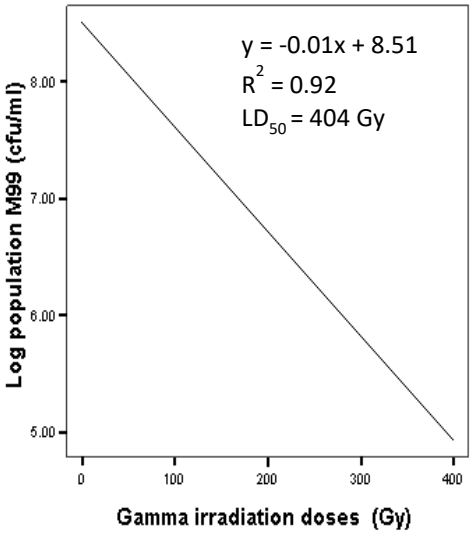

Fig. 9. Log population of *Pseudomonas putida* M99 (cfu/mL) for LD_{50}.

Results from Fig. 10 to Fig. 13 show the LD_{50} of Gram-positive bacteria, namely *Bacillus* sp. (UPM 10); *Bacillus proteolyticus* (UPM 6), *Priestia megaterium* (SB 6) and *Priestia aryabhattai (*K38) as 815; 1154, 1681 and 1061 Gy, respectively. The LD_{50} range of Gram-positive bacteria was slightly broader than those of Gram-negative bacteria. The lowest LD_{50} of Gram-positive bacteria was 815 Gy, whilst the highest was 1681 Gy. This could be due to the type of cell wall they possessed or the endospores they produced. Hence, further investigation on the effects of gamma irradiation on Gram-positive bacteria should be conducted before embarking on improvement or enhancement of their functionalities.

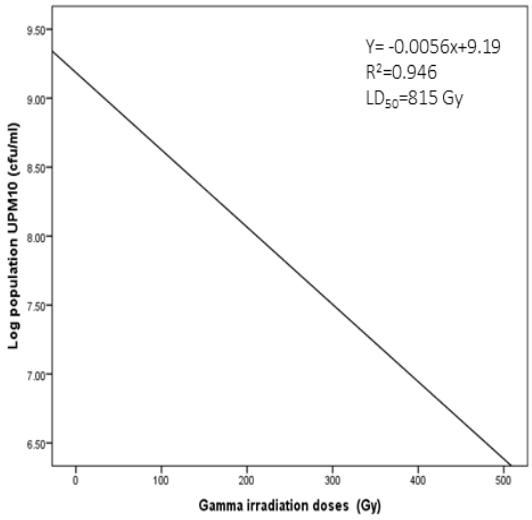

Fig. 10. Log population of *Bacillus* sp. UPM 10 (cfu/mL) for LD_{50}.

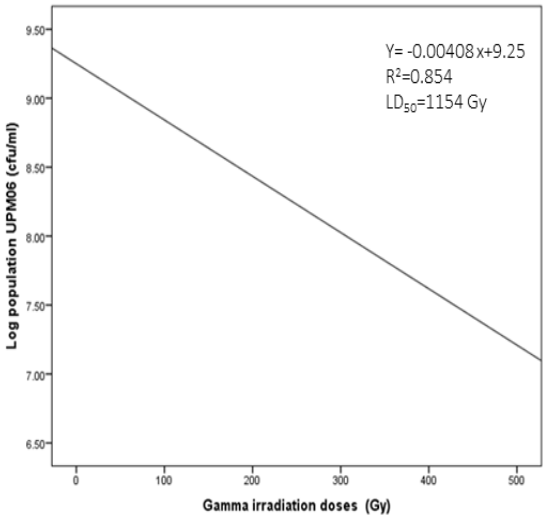

Fig. 11. Log population of *Bacillus proteolyticus* UPM 06 (cfu/mL) for LD_{50}.

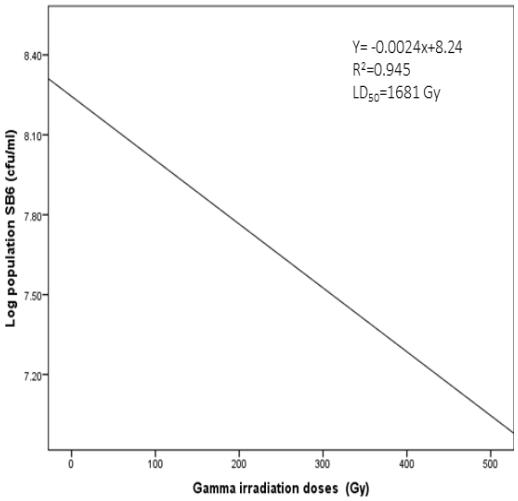

Fig. 12. Log population of *Priestia megaterium* SB6 (cfu/mL) for LD_{50}.

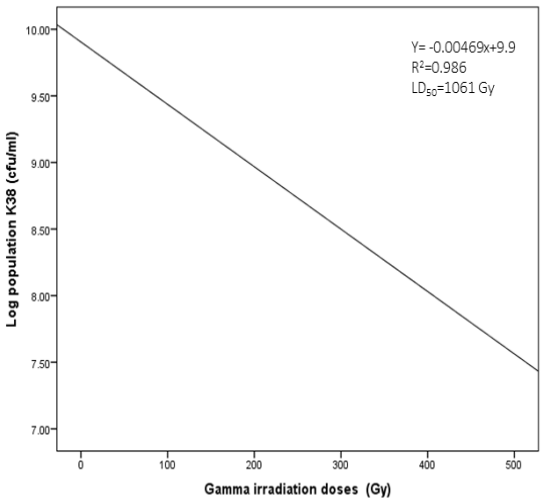

Fig. 13. Log population of *Priestia aryabhattai* K38 (cfu/mL) for LD_{50}.

The LD_{50} for Gram-negative bacteria was in the range of 380 to 500 Gy, while for Gram-positive bacteria it was in the range of 800 to 1600 Gy, thus implying that Gram-positive bacteria were more resistant to gamma irradiation than the Gram-negatives. These results are important as a guide for mutagenesis of biofertiliser bacteria involving gamma irradiation. This study also shows that gamma irradiation at doses of 50–400 Gy did not kill the isolates; mutagenesis could be achieved at these irradiation doses.

The main effect of exposure to radiation is caused by the alteration of DNA structure, which is a component of chromosome in organism cells. Another indirect effect is the formation of free radicals, where ionising radiation cause excitation, ionisation and breakage of molecules. This process will lead to biological changes of the cell (Ramli *et al.*, 2002). Gram-positive bacteria were more resistant to gamma irradiation than the Gram-negatives due to the difference between them in cell wall structure. A Gram-positive bacterium has a membrane that surrounds the cell. The cell wall is primarily made up of peptide glycan layer. This cell wall is also rich in sulfur compounds, which protect the cells from gamma irradiation and free radicals (Abojassim *et al.*, 2016). On the other hand, some of Gram-negative bacteria are also resistant to gamma irradiation, although Gram-negative bacteria do not possess thick cell walls. Peptidoglycan recycling was a metabolic process by which Gram-negative bacteria were able to show resistance (Mayer *et al.*, 2019). Lipopolysaccharide was another important component of Gram-negative bacteria that helps in exhibiting resistance to gamma irradiation. These phosphate groups increase the overall negative charge, which was similar sulfur compounds, help to stabilise the whole structure (Herrera *et al.*, 2010).

Apart from the cell wall factors, DNA repair, genetic factors and environmental factors are possible other reasons that Gram-positive bacteria exhibit resistance to gamma irradiation than Gram-negative bacteria. More studies need to be conducted to gather more information on these correlated factors.

CONCLUSIONS

Twelve biofertiliser bacterial isolates from compost, soil, and plants were irradiated in a gamma cell at doses of 50–400 Gy to determine the median lethal dose, LD_{50}. Eight isolates were Gram-negative, and four isolates were Gram-positive. Isolates were identified as *Acinetobacter* sp., *Klebsiella* sp., *Enterobacter* sp., *Pseudomonas* sp., *Pantoea* sp., *Bacillus* sp., and *Priestia* sp. by using the 16S rRNA method. The LD_{50} of the Gram-negative isolates ranged from 380 to 500 Gy, and for Gram-positive isolates they ranged from 800 to 1600 Gy. Mutagenesis of these isolates for improvement of multifunctional activities is proposed to be conducted within these LD_{50} ranges. Further investigation of the dose responses within Gram-positive and Gram-negative bacteria at molecular levels need to be conducted to gain better information on the effects of gamma irradiation on genes changes.

ACKNOWLEDGEMENT

The authors wish to express their gratitude to Malaysian Nuclear Agency (Nuklear Malaysia) and Ministry of Science, Technology and Innovation, Malaysia (MOSTI) for the financial support, and the co-operation and support from Universiti Putra Malaysia (UPM). The authors thank Dr. Halimi Mohd Saud and Dr. Khairuddin Abdul Rahim for their advice on technical matters.

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