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GAMMA-IRRADIATION RESPONSE OF CASSAVA (VAR. UBI KUNING)

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ABSTRACT

Cassava (Manihot esculenta) is a key staple crop in tropical and subtropical regions, renowned for its ability to thrive in poor soils and withstand drought conditions. In Malaysia, cassava cultivation faces challenges such as low genetic diversity, susceptibility to pests and diseases, and limited yield improvement due to reliance on traditional breeding methods. Despite its economic importance, traditional breeding methods in cassava are challenged by complex polyploid genome and predominantly vegetative propagation. This research aimed to evaluate the acute radiosensitivity of cassava var. Ubi Kuning to gamma irradiation as a preliminary step toward enhancing genetic variability for mutation breeding programs. In this study, evaluation of the acute radiosensitivity of cassava cuttings to gamma irradiation, using doses of 0 (control), 5, 10, 15, 20, 25, 30, 40, 50, and 60Gy was conducted. The cuttings were irradiated with a gamma irradiator (Biobeam GM8000) using Caesium-137 as the source, and subsequently planted in polybags in a glasshouse. The survival data indicated an LD_{50} of approximately 33Gy whilst plant height data showed a GR_{50} of approximately 28Gy. The results show that as the gamma radiation dose increases, both data of the survival and growth of cassava decrease, demonstrating the plant's sensitivity to gamma irradiation. The findings suggest that doses around 28-33 Gy induced significant genetic variability while maintaining sufficient viability for mutation breeding programs. This study emphasizes the importance of optimizing irradiation doses as a foundation for developing improved cassava cultivars through mutation breeding.

Keywords: Acute gamma irradiation; mutation breeding; radiosensitivity test; cassava

ABSTRAK

Ubi kayu (Manihot esculenta) adalah tanaman makanan ruji utama di kawasan tropika dan subtropika, terkenal dengan kemampuannya untuk tumbuh di tanah yang kurang subur dan tahan kepada keadaan kemarau. Di Malaysia, penanaman ubi kayu menghadapi cabaran seperti kepelbagaian genetik yang rendah, kerentanan terhadap perosak dan penyakit, serta peningkatan hasil yang terhad akibat kebergantungan kepada kaedah pembiakbakaan tradisional. Di sebalik kepentingan ekonominya, pembaikbakaan ubi kayu adalah sukar kerana genom poliploid yang kompleks dan hanya dipropagasi secara vegetatif. Penyelidikan ini bertujuan untuk menilai radiosensitiviti akut keratan ubi kayu var. Ubi Kuning terhadap penyinaran gama sebagai langkah awal ke arah meningkatkan kepelbagaian genetik untuk

program pembiakbakaan mutasi. Dalam kajian ini, respon terhadap penyinaran gama menggunakan dos 0 (kawalan), 5, 10, 15, 20, 25, 30, 40, 50, dan 60Gy ke atas keratan ubi kayu dijalankan. Keratan ubi kayu disinarkan dengan sel gama (Biobeam GM8000) yang menggunakan Caesium-137 sebagai punca sinaran, dan seterusnya ditanam dalam polibeg di rumah kaca. Data kelangsungan hidup menunjukkan LD_{50} sekitar 33Gy manakala data ketinggian menunjukkan GR_{50} sekitar 28Gy. Keputusan menunjukkan bahawa apabila dos sinaran gama meningkat, kedua-dua data kelangsungan hidup dan pertumbuhan ubi kayu menurun, yang menunjukkan sensitiviti tanaman ini terhadap sinaran gama. Hasil dari eksperimen ini mencadangkan bahawa dos sekitar 28-33Gy adalah dos yang optima untuk digunakan bagi pembaikbakaan mutasi ubi kayu. Kajian ini menekankan kepentingan mengoptimumkan dos penyinaran sebagai asas untuk membangunkan kultivar ubi kayu yang lebih baik melalui pembiakan mutasi.

Keywords: Penyinaran gama akut, pembaikbakaan mutasi, ujian radiosensitiviti, ubi kayu

INTRODUCTION

Cassava (*Manihot esculenta*) is a vital food crop, providing a primary source of calories for over 800 million people worldwide, particularly in sub-Saharan Africa, Latin America, and Southeast Asia (FAO, 2013). In Malaysia, cassava plays a significant role in both the agricultural and industrial sectors. Traditionally grown as a staple food crop, cassava has increasingly gained importance as a raw material for various industries, including starch production, animal feed, and bioethanol. The crop's ability to thrive in diverse agro-ecological conditions, including marginal soils and drought-prone areas, makes it a valuable resource for Malaysian farmers. Its adaptability to diverse agro-ecological conditions and resilience to abiotic stresses such as drought and poor soils make it a critical crop for food security and economic development in these regions (Nassar & Ortiz, 2010).

Cassava *var.* Ubi Kuning, or yellow cassava, is an economically significant crop in Malaysia, particularly in rural and agricultural communities. Known for its vibrant yellow flesh, Ubi Kuning is a source of income through its diverse applications in food processing and industrial uses. In Malaysia, this variety of cassava is valued for its nutritional richness and versatility, contributing to both food security and economic stability in regions where it is cultivated (Loh *et al.*, 2014). However, cassava production faces numerous challenges, including susceptibility to pests and diseases, and relatively low genetic diversity, which limits its potential for improvement through traditional breeding methods (Ceballos *et al.*, 2010). Recent efforts have focused on improving cassava varieties through breeding programs aimed at enhancing yield, disease resistance, and starch content, thus ensuring the crop's sustainability and economic viability in the face of growing industrial demand and changing climatic conditions (Malik *et al.*, 2020).

Conventional plant breeding, which relies on the natural genetic variation within a species, has been fundamental in improving crop varieties over the past century. However, the inherent genetic limitations and long breeding cycles of conventional methods often hinder the rapid development of new cultivars, especially in crops like cassava with long growth periods and limited genetic variability (Nassar & Ortiz, 2010). Mutation breeding, a technique that involves the induction of genetic mutations to create novel genetic variability, has emerged as a great tool for crop improvement (Ahloowalia *et al.*, 2004). This approach has been used since the early 20th century, with the first classic and successful mutation-induced crop variety being

developed in the 1920s (Foster, 2013). Through the application of physical mutagens such as gamma rays and X-rays, or chemical mutagens like ethyl methanesulfonate (EMS) and sodium azide, plant breeders can induce mutations in seeds or other plant tissues to generate a wide range of genetic diversity (Shu *et al.*, 2012). In cassava, mutation breeding holds significant promise for overcoming its genetic bottlenecks and accelerating the development of superior cultivars (Nkere *et al.*, 2019).

The process typically involves exposing seeds or plant tissues to a mutagen, followed by the growth and screening of mutated populations for desirable traits. This method has led to the development of numerous improved crop varieties with enhanced traits such as disease resistance, improved nutritional content, and increased yield (Oladosu *et al.*, 2016). Notable examples include high-yielding wheat and rice varieties, disease-resistant barley, and improved oil composition in soybean (Ahloowalia *et al.*, 2004).

However, to start a mutation breeding program, radiosensitivity test which is crucial in mutation breeding need to be done as it helps to determine the optimal dose of radiation required for inducing mutations without causing excessive damage to the plant tissues. These tests involve exposing plant materials, such as seeds, cuttings, or tissue cultures, to a series of doses of irradiation and assessing their responses in terms of survival rate, growth inhibition, and frequency of mutations (Roychowdhury & Tah, 2011). The results of radiosensitivity tests guide breeders in selecting the appropriate irradiation dose that maximizes genetic variability while minimizing lethality and detrimental effects. For instance, the lethal dose (LD_{50}), which is the irradiation dose at which 50% of the treated population is expected to die, is a critical parameter often determined during the test (Mustapha *et al.*, 2019). Additionally, sublethal doses that induce a significant mutation rate without severely affecting plant viability are identified to optimize mutation breeding efforts.

This paper aims to study the response of cassava *var*. Ubi Kuning against acute gamma irradiation to determine its optimal dose for subsequent mutation induction work in the future.

PROCEDURE

Plant material

Cassava *var*. Ubi Kuning cuttings were sourced from Taman Kekal Pengeluaran Makanan (TKPM), Ulu Chuchoh, Sepang Selangor. For consistency and reliability in the study, only cuttings with a diameter of 5cm and a length of 15cm were selected. These cuttings were used to ensure uniformity in the experiment and to facilitate accurate comparisons across different irradiation treatments.

Experimental design and irradiation of cuttings

The experiment was conducted using a Completely Randomized Design (CRD) to evaluate the effects of gamma irradiation on cassava cuttings. The cuttings were subjected to gamma irradiation using a gamma irradiator (BioBeam GM 8000, Germany) at Nuklear Malaysia, with Caesium-137 as the radiation source. A range of gamma irradiation doses was applied to the cuttings, specifically 5, 10, 15, 20, 25, 30, 40, 50, and 60 Gy. A control group was included, which consisted of cuttings that were not exposed to gamma irradiation. Each dose and the

control have three (3) biological replicates and ten (10) technical replicates to ensure the reliability and reproducibility of the results.

Soil preparation and cuttings planting

Mix organic soil containing topsoils, coconut and rice husk, compost and microbial fertilizer was filled in 12x15 inches polybags and prepared with the same total number of irradiated and control cuttings. Each irradiated and control cuttings was planted in the polybags at the glasshouse in slanted mode and watered every two days.

Data collection and analysis

Data collection were carried out at eight weeks after planting. Key parameters measured included survival rate, plant height, number of leaves, and number of buds. Additionally, leaf morphology was documented to assess the effects of gamma irradiation on leaf structure. Statistical analysis was performed using Analysis of Variance (ANOVA) with Statistical Analysis System (SAS) version 9.2 to determine significant differences ($p \leq 0.001$) among the various treatment groups. The Least Significant Difference (LSD) test was employed to assess mean differences between irradiation doses. For radiosensitivity analysis, the best-fit model from Curve Expert 1.3 was used to estimate the LD_{50} (lethal dose for 50% of the population) and GR_{50} (growth reduction dose for 50% of the population).

RESULTS AND DISCUSSION

Lethal Dose 50

The percentage of survived plants (survival rate) of cassava under different gamma irradiation treatments was investigated. The number of survived plants was scored after 8 weeks of planting in order to determine the Lethal Dose 50 (LD_{50}). The treatments ranged from 0Gy to 60Gy, with significant differences observed among the groups as illustrated in Table 1. Specifically, treatments sharing the same letter (e.g., a) in the t Grouping are not significantly different from each other, while treatments with different letters represent significantly different survival rates. From the results obtained, treatments with 10Gy and 20Gy showed similar survival rates with control which is 0.9 or 90% and were not significantly different from each other. The highest survival rate was observed in the 15Gy treatment, which had a mean survival rate of 1.0 which is 100% of survival. This suggests that these doses are within a tolerable range for cassava, allowing high survival rates. Similar findings have been reported in previous studies where moderate doses of gamma irradiation have been shown to not causing significant harm to the population (Mba *et al.*, 2010).

Table 1. Cassava *var*. Ubi Kuning survival rate at 8th week after planting

Dose (Gy)	Survival rate (%) (Means)	t Grouping		
0 (Control)	90	b	a	
5	80	b	a	c
10	90	b	a	
15	100		a	
20	90	b	a	
25	70	b		c
30	60			c
40	10		d	
50	0		d	
60	0		d	

However, a notable drop in survival rate was observed in the 5Gy treatment group with 80% of survival rate. Although this dose is lower than the aforementioned groups, it resulted in a statistically significant decrease in survival rate, indicating a possible threshold for beneficial effects of low-dose gamma irradiation. Lower survival rates at this dose may be due to the activation of stress responses in the plants. The survival rates continued to decrease with increasing doses of irradiation, with the 25Gy and 30Gy treatments having mean survival rates of 0.7 or 70% and 0.6 or 60%, respectively. These doses appear to be approaching the upper limit of cassava's tolerance to gamma irradiation. This trend aligns with other studies where higher doses of gamma irradiation have been shown to cause detrimental effects on plant health, such as reduced growth and increased mortality (Kodym & Afza, 2003).

The most severe reductions in survival rates were observed at 40Gy with only 10% of survival rate whilst 50Gy and 60Gy showed no survival. These results demonstrate that high doses of gamma irradiation are lethal to cassava, causing significant damage that the plants cannot recover from. This finding is consistent with established study on the effects of high-dose radiation on plants, which can lead to cellular damage and death (Kim *et al.*, 2019).

The fitted curve extracted from Curve Expert 1.3 for survival rate against dose as shown in Fig. 1, with a high correlation coefficient (r = 0.977), indicates a strong fit to the observed data, suggesting the model accurately represents the relationship between gamma irradiation doses and cassava survival rate. The LD_{50} which is the dose at which 50% of the cassava plants survive in comparison with control population, was estimated to be around 32Gy. This value indicates the dose at which half of the cassava plants do not survive, providing a benchmark for safe and effective doses in mutation breeding programs for cassava *var*. Ubi Kuning. The information obtained from this result can guide the application of gamma irradiation in cassava breeding, ensuring the selection of doses that maximize mutation induction while minimizing plant mortality.

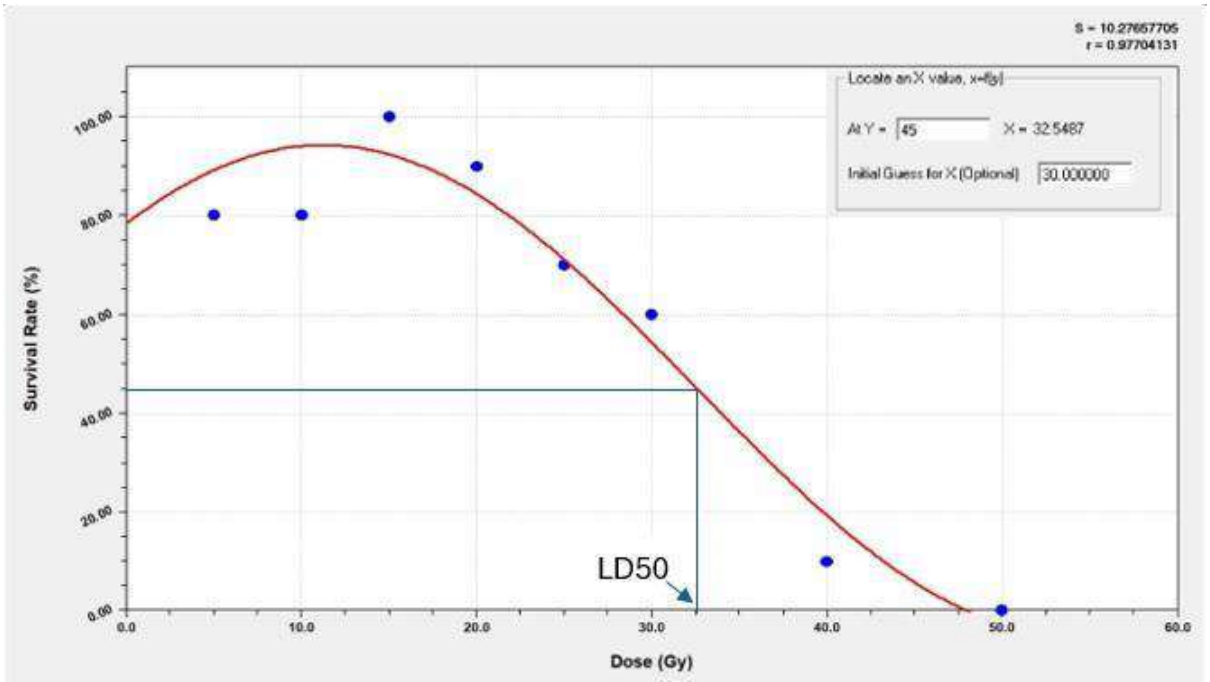

Figure 1. Survival rate (%) of irradiated cassava and control against dose (Gy) at 8th week after planting

Growth Reduction Dose 50

Growth reduction dose 50 (GR_{50}) can be calculated from the plant height data. The statistical analysis revealed significant differences in plant height among the various treatment groups as shown in Table 2. Treatments assigned the same letter (e.g., a) in t Grouping indicate no significant difference in plant height, whereas treatments assigned different letters represent statistically significant differences in plant height. The highest mean plant height was observed in the 15Gy treatment group which is 86.1cm, followed closely by the 10Gy treatment group (82.4cm), with both groups showing no significant difference from each other but has significant different in comparison with control. Even though treatment at 5Gy and 20Gy has no significant different compared to control, but the means of plant height treated with both doses is a bit high in comparison with control. This is consistent with findings in other mutation breeding studies where low to moderate doses of gamma radiation have been reported to enhance growth traits in various crops such as reported by Jaipo *et al.* (2019). A classic study by Pitirmovae (1979) mentioned that stimulation of cell division can affect the synthesis of nucleic acid at low dose of gamma irradiation that might rouse the growth of plant.

Table 2: Cassava *var.* Ubi Kuning plant height (cm) at 8th week after planting

Dose (Gy)	Plant height (cm) (Means)	t Grouping	
0 (Control)	54.7	c	
5	57.6	c	
10	82.4	a	b
15	86.1	a	
20	68.4	c	b
25	22.6	d	
30	18.9	d	
40	1.0		e
50	0		e
60	0		e

Furthermore, as irradiation dose increased from 25Gy to 60Gy, plant height drastically decreased, with the 40Gy resulting in mean plant heights of 1.0cm only whilst both 50Gy and 60Gy treatments killed the population. These high-dose groups demonstrated low plant height, aligning with previous research that highlights the detrimental effects of high gamma radiation doses on plant development as mentioned by Omar *et al* (2008) where DNA damage and inability to repair them is the reason of the severe growth inhibition or complete lethality.

Similarly with LD_{50}, growth reduction dose (GR_{50}) refers to the growth reduction by 50%, which is a measure used to determine the dose of a mutagen that reduces the growth of a plant growth e.g. plant height, number of leaves, number of buds etc by 50%. As illustrated in Fig. 2, the GR_{50} for cassava based on plant height data was determined to be approximately 28Gy, providing a critical reference point for optimizing gamma irradiation doses in cassava *var.* Ubi Kuning. As mentioned above in the LD_{50} discussion, this value underscores the plant's relative tolerance to radiation and highlights the importance of carefully selecting irradiation doses to balance mutagenic effectiveness with plant viability.

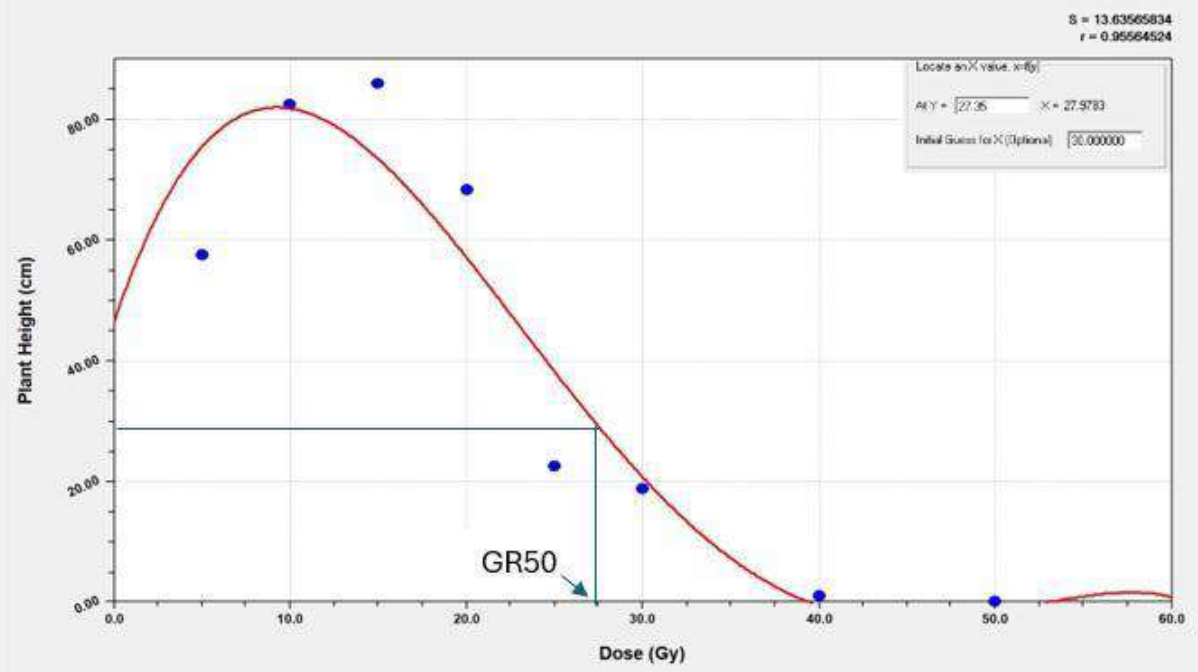

Figure 2. Plant height (cm) of irradiated cassava and control against dose (Gy) at 8th week after planting

Effect of acute gamma irradiation on number of shoots and number of buds of cassava *var.* Ubi Kuning

The impact of gamma radiation on cassava plants demonstrates significant effects on both the number of leaves and number of buds. Treatments labeled with the same letter (e.g., a) in t Grouping denote no statistically significant differences in the number of leaves, while those labeled with different letters indicate significant variations in leaf count. For number of leaves as shown in Table 3, the control group (0Gy) and lower dose groups i.e. 5Gy, 10Gy, and 15Gy displayed comparable leaf numbers, with the population irradiated with 15Gy exhibiting the highest mean which is 7.8. This suggests that low doses of gamma radiation might not adversely affect, and could even slightly enhance leaf development. However, as the radiation dose increased beyond 20Gy, there was a notable decline in leaf numbers. At 40Gy the mean leaf numbers dropped drastically to 0.4, whilst doses above 40Gy showed no development of leaves, indicating severe damage to the plant's ability to produce leaves due to high levels of irradiation (Kovalchuk *et al.*, 2003; Kim *et al.*, 2010).

Table 3. Number of leaves of cassava *var.* Ubi Kuning at 8th week after planting

Dose (Gy)	Number of leaves (Means)	t Grouping		
0 (Control)	7.3	b	a	
5	7.2	b	a	
10	7.1	b	a	
15	7.8		a	
20	6.4	b	a	c
25	4.6	b		c
30	3.7			c
40	0.4		d	
50	0		d	
60	0		d	

Similarly, the data on the number of buds in cassava *var.* Ubi Kuning also reveals a pronounced effect of acute gamma irradiation as illustrated in Table 4. Treatments sharing the same letter (e.g., a) in t Grouping indicate no statistically significant differences in buds count, whereas treatments assigned different letters represent significant differences in the number of buds. The control group (0Gy) had a mean bud number of 1.9. Low irradiation doses up to 20Gy did not significantly reduce the bud number, with the 15Gy treatment showing the highest mean of bud number which is 3.0. This indicates a potential stimulatory effect of low-dose radiation on bud development (Wang *et al.*, 2007). However, higher doses of irradiation which were 25Gy and above, led to a significant reduction in bud numbers. At 40Gy, the mean of number of buds decreased drastically to 0.1 and similarly with number of leaves, no formation of buds was observed at 50Gy and 60Gy due to the higher irradiation dose that can affect the cell growth.

Table 4. Number of buds of cassava *var.* Ubi Kuning at 8th week after planting

Dose (Gy)	Number of buds (Means)	t Grouping		
0 (Control)	1.9	b	d	c
5	1.9	b	d	c
10	2.6	b	a	
15	3.0		a	
20	2.2	b	a	c
25	1.5		d	c
30	1.3		d	
40	0.1		e	
50	0		e	
60	0		e	

These observations highlight the dual effects of gamma radiation on cassava agronomic characteristics. Low doses may promote certain growth parameters, such as leaf and bud development, while higher doses generally inhibit growth due to cellular damage. This information is crucial for optimizing gamma irradiation doses in mutation breeding programs aimed at enhancing cassava traits.

Leaves morphology

The impact of gamma irradiation on leaf morphology varies across different plant species and doses applied. Leaves play a crucial role in the life of a plant, serving as the primary sites for photosynthesis, the process by which plants convert light energy into chemical energy stored in glucose. This function is essential for the growth and energy needs of the plant. Leaves are also vital for gas exchange, containing stomata that open and close to regulate the intake of carbon dioxide and the release of oxygen and water vapor. Changes to leaf morphology can have significant implications for a plant's overall health and functionality. Generally, gamma irradiation can cause a range of morphological changes in leaves, including alterations in leaf size, shape, colour, and structure. In this study, various changes on the leaf's morphology of cassava *var*. Ubi Kuning were observed.

Control leaves 5Gy – Extra lobes of leaves 5Gy – Leaves abnormality

Figure 3. Effect of gamma irradiation on leaf morphology

As illustrated in Fig. 3, cassava plants subjected to a 5Gy gamma irradiation treatment exhibited a notable increase in the number of leaves compared to the control group, which had only five lobes. The irradiated plants not only had a higher leaf count but also demonstrated robust and healthy leaf development. This observation suggests a stimulatory effect of low-dose gamma irradiation on leaf production. The increased leaf number can be attributed to the activation of growth-promoting genes and hormonal changes induced by the irradiation. At lower doses, gamma irradiation has been known to enhance leaf area and improve photosynthetic activity, leading to overall better plant vigor and productivity (Kovács & Keresztes, 2002).

Despite these positive effects, it is important to note that some of the irradiated leaves exhibited irregular and distorted shapes as shown in Fig. 3 as well. This irregularity indicates that while low-dose gamma irradiation can enhance leaf production, it may also induce unintended morphological abnormalities. These abnormalities may arise due to the inherent randomness of mutation and the plant's repair mechanisms. The variability in response to irradiation underscores the complexity of mutation breeding, where beneficial effects are sometimes accompanied by adverse changes in plant morphology.

On the other hand, higher doses of gamma irradiation presented more pronounced detrimental effects on leaf morphology. As shown in Fig. 4, plants exposed to higher irradiation doses exhibited reduced leaf size, chlorophyll degradation, and necrosis (death of leaf tissue). These changes are indication of severe damage to cellular structures, including chloroplasts, which are essential for photosynthesis. Higher doses of gamma irradiation can inflict significant damage to DNA, proteins, and lipids, leading to cell death and tissue necrosis. The impairment

of chloroplast function further contributes to decreased chlorophyll content, which ultimately hinders the plant's ability to produce energy and grow effectively (Wi *et al.*, 2007).

The observed effects underscore the dose-dependent nature of gamma irradiation. While low doses can positively influence plant growth and morphology by promoting leaf production, higher doses can have adverse impacts by disrupting critical physiological processes. The extent of these changes is influenced by the species' sensitivity to radiation and the specific dose administered. Understanding this dose-response relationship is crucial for optimizing gamma irradiation protocols in plant mutation breeding to balance beneficial outcomes with the potential risks of morphological and physiological damage.

Control leaves

10 Gy – Chlorophyll degradation

15 Gy – Crinckle leaves

20 Gy – Chlorophyll degradation

25 Gy – Leaf abnormality

Figure 4. Leaf mutation observed in irradiated cassava *var*. Ubi Kuning at different doses compare to the Control

CONCLUSION

In conclusion, this study demonstrates the acute radiosensitivity of cassava *var*. Ubi Kuning to gamma irradiation, with significant implications for mutation breeding program. The determined LD50 of approximately 33Gy and GR50 of approximately 28Gy provide critical benchmarks for the optimal irradiation doses that induce genetic variability while maintaining sufficient plant viability. Thus, doses between 28-33Gy can be used for mutation induction. The results indicate a clear dose-dependent response, where survival and growth rates decrease with increasing gamma radiation doses, highlighting the importance of carefully selecting appropriate doses to balance mutagenic effectiveness with plant health. These findings contribute to the broader understanding of radiation-induced mutagenesis in cassava, offering valuable insights for developing improved cultivars through mutation breeding.

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LEVEL OF TOTAL ARSENIC AND MERCURY IN SELECTED BIVALVE SPECIES FROM MELAKA AND PAHANG STATES OF MALAYSIA

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ABSTRACT

Arsenic and mercury levels in marine organisms are critical environmental and public health concerns. These toxic elements can accumulate in bivalves and pose significant consumer risks. Monitoring of these elements is essential to ensure the safety of seafood consumption. In this study cockles, clams and mussels were collected from Melaka and Pahang states for the determination of total arsenic and total mercury levels. The bivalve tissues were digested using microwave digester and analyzed using inductively coupled plasma-mass spectrometer (ICP-MS). The total arsenic in the bivalve samples ranged between 0.85 ± 0.04 mg kg^{-1} and 1.60 ± 0.27 mg kg^{-1}. Meanwhile, the total mercury in the bivalve samples was from 0.004 ± 0.002 to 0.007 ± 0.002 mg kg^{-1}. The result of the elements was compared with the local and global guidelines. This research provides the latest information for food safety and risk assessment studies in the regional and national levels.

Keywords: bivalvia, arsenic, mercury, inductively coupled plasma-mass spectrometer.

ABSTRAK

Paras arsenik dan merkuri dalam kerang-kerangan adalah antara perkara kritikal yang dibimbangkan pada alam sekitar dan kesihatan awaml. Unsur-unsur toksik ini boleh berkumpul dalam organisma marin dan boleh memberi risiko yang ketara pada pengguna. Pemantauan unsur-unsur ini adalah penting untuk memastikan keselamatan pengambilan makanan laut. Di dalam kajian ini kerang, lala dan kupang telah diambil dari negeri Melaka dan Pahang untuk menentukan jumlah arsenik dan jumlah merkuri. Tisu kerang-kerangan ini telah dicerna menggunakan pencerna gelombang mikro dan dianalisis menggunakan spektrometer jisim plasma (ICP-MS) yang digabungkan secara induktif. Jumlah arsenik dalam sampel kerang berjulat antara 0.85 ± 0.04 mg kg-1 dan 1.60 ± 0.27 mg kg-1. Manakala, jumlah merkuri dalam sampel kerang daripada tidak dapat dikesan kepada 0.01 ± 0.002 mg kg-1. Hasil daripada elemen tersebut dibandingkan dengan garis panduan tempatan dan global. Penyelidikan ini menyediakan maklumat terkini untuk kajian keselamatan makanan dan penilaian risiko di peringkat serantau dan nasional.

Kata kunci: *kerang-kerangan, arsenik, merkuri, spektrometer jisim plasma yang digabungkan secara induktif*

INTRODUCTION

Bivalve, as filter feeders, play a significant role in the marine ecosystem by filtering water and accumulating various substances, including contaminants. Among these contaminants, arsenic and mercury are of particular concern due to their toxicity and potential health impacts on both marine life and humans. Arsenic (As) is a naturally occurring element found in the Earth's crust, and its presence in marine environments can result from natural processes such as volcanic activity and the weathering of rocks, as well as from anthropogenic sources like industrial discharges and agricultural runoff (Luvonga et al., 2020). Similarly, mercury (Hg), primarily released into the environment through industrial activities such as coal combustion and mining, poses severe ecological and health risks due to its ability to bioaccumulate in marine organisms and biomagnified through the food web (Fui et al., 2022; Haris et al., 2020).

The study of As and Hg in bivalve samples is crucial for understanding the extent of marine pollution and its implications for food safety. Bivalve are considered good bioindicators for monitoring the presence of heavy metals in marine environments due to their sedentary nature and ability to accumulate contaminants in their tissues over time (Alina et al., 2012; Lucero Rincón et al., 2023). This makes them an excellent model for assessing the levels of these toxic elements and the potential risks they pose to human health when contaminated mussels are consumed. Monitoring these levels helps in the implementation of regulations and policies aimed at reducing pollution and protecting marine and human health.

Recent studies have shown varying levels of As and Hg in bivalvia samples, reflecting the diversity of contamination sources and environmental conditions (Al-Sulaiti et al., 2022; Luvonga et al., 2020). For instance, recent research in Klang River, Malaysia revealed significant accumulation of Hg in gastropod samples, highlighting the impact of local industrial activities and urbanization towards marine environment (Haris et al., 2020). Similarly, investigations in other coastal regions have demonstrated that bivalvia populations in proximity to urbanized and industrialized areas tend to exhibit higher concentrations of these metals compared to those from less impacted areas (Alina et al., 2012).

The quantification of the total As and Hg in biological samples such as bivalve often employs the use of microwave digestion followed by Inductively Coupled Plasma Mass Spectrometry (ICP-MS) for precise quantification. The microwave digestion technique is utilized to efficiently break down the organic matrix of the samples, ensuring complete extraction of the metals. ICP-MS is then used for its high sensitivity and ability to detect trace levels of As and Hg. To ensure method accuracy and reliability, analysis of Standard Reference Materials (SRMs) and/or certified reference material (CRM) is essential to validate the performance of the digestion and quantification procedures. This practice ensures that the results obtained are accurate and precise.

In Malaysia, the guidelines of As and Hg levels in bivalves are established based on organic As and methyl-Hg. According to the Malaysian Food Regulation 1985, the Malaysian Food Regulations 1985, the maximum allowable limit for organic As is 1.0 mg kg^{-1}, meanwhile for methyl-Hg is set at 0.5 mg kg^{-1}. Due to the high cost and tedious analytical methods for speciation analysis, the routine bivalve samples analysis for organic As and methyl-Hg were screened for total As and Hg levels prior to speciation analysis in the majority of laboratories. Therefore, the precise and accurate determination of total As and Hg was vital to identify the level of these metals and understand their distribution and behavior in the environment. This

study aims to determine the level of total As and Hg in selected bivalve samples from Melaka and Pahang states and compare with the local and global guidelines.

METHODOLOGY

Sample collection and preparation

A total of 20 to 40 samples of three different bivalvia species (cockle, clam and mussel) were purchased from the local fisherman at Kuala Sungai Baru, Melaka and Kuantan, Pahang (Figure 1). The locations were selected based on the abundance of bivalvia from the two states. The three bivalvia samples were cockle, clam and mussel (Figure 2). The lengths of the bivalvia samples were 2-3 cm, 3-4 cm and 6-7 cm respectively. The samples were purchased from local fishermen between July and December 2022. The bivalvia samples were stored in the plastic zip lock bag and kept chilled at 4°C during transportation. In the laboratory the samples were stored frozen below 0°C prior to extraction and analysis.

Figure 1. Sampling locations

Figure 2. The bivalvia samples in this study. Cockle (i), clam (ii), and mussel (iii)

Digestion of samples using microwave digester

Frozen bivalvia samples were thawed at room temperature and dissected using plastic forceps. The soft edible tissues were pooled in between 10 and 20 samples for each bivalvia species. The pool bivalvia samples were ground using stainless steel grinder and stored in propylene containers. The bivalvia tissue samples were digested according to the Association of Analytical Collaboration (AOAC) Official Method 2015.01 Heavy Metals in Food. In brief, 1.0 g of wet-weight soft bivalvia tissues (n=3) were weighted using analytical balance and transferred to microwave digester vessels. The tissues were added with 10ml concentrated nitric acid and allowed to stand for 10 minutes.

The tissue samples were digested using microwave digester (MARS 6, USA). The digestion program was set at 200°C for 15 minutes ramping followed by 15 minutes holding times. The pressure was set at 800 psi and the power was between 900-1050 W during the entire digestion. The digested tissue samples were allowed to cool and mark up to 25 ml using deionized water. The samples were stored in polypropylene tube. In every batch of digestion procedure, two method blanks and standard reference materials (SRMs) were digested for method performance analysis. The SRM used in this study are trace elements and methylmercury in mussel tissue (National Institute of Standard and Technology (NIST) SRM 2976), Dogfish liver certified reference material (CRM) for trace metals and other constituents (National Research Council Canada (NRCC) DOLT-5) and oyster tissue (NIST SRM 1566b). The limit of detection (LOD) was determined through the analysis of 23 method blanks and calculated as 3 times the standard deviation (SD) of the method blanks results.

Quantification of arsenic and mercury in the bivalvia samples

The determination of As and Hg in the bivalvia tissue samples was performed using ICP-MS. As was quantified using kinetic energy discrimination (KED) mode using helium gas with 3.5 ml min^{-1} flow and Hg was determined using standard mode. To compensate for the percentage of acidity in samples and the carbon buffer content in the samples, the internal and calibration standards were prepared in 20% acid and 15% methanol. The calibration standards were prepared using As and Hg single-element standards (CPA Chem, Bulgaria). The internal standard rhodium (Rh) and Iridium (Ir) mixture was prepared at 0.020 mg L^{-1} and 0.040 mg L^{-1} respectively for instrument internal standardization. Three calibration points consisted of 0.010 mg L^{-1}, 0.025 mg L^{-1}, and 0.050 mg L^{-1} were established with correlation coefficient (R^2) of more than 0.995. Calibration verification (quality control standards) was analyzed for every ten samples and the values ranged between 90 and 110%. The concentration of samples was reported in mg kg^{-1} (wet weight) units by following formula:

$$mg\ kg^{-1} = mg\ L^{-1}\ x\ fv\ (L)\ x\ \frac{1}{ws\ (kg)}$$

Where *fv* is the final volume mark up after digestion and *ws* is the weight of sample. The LOD of As and Hg for this method was 0.004 and 0.002 mg kg^{-1} for As and Hg respectively. The spatial difference of As and Hg in both locations were plot using ArcGIS software and statistical analysis (t-test and *F*-test) was performed using Excel software.

RESULTS AND DISCUSSION

Method performance using certified reference material (CRM)

Table 1 shows the concentration obtained from this research analytical procedures, the certified values of the SRM/CRM and the percentage recovery. The percentage recovery (%) for As was between 85.17 ± 5.35 and 113.83 ± 3.24%. The percentage recovery (%) for Hg in SRM/CRM was between 76.5 ± 9.17 and 91.56 ± 1.59. Based on the percentage of recovery results, the microwave digestion and the quantification procedures were in good performance, produce satisfactory precision and reliable results.

Table 1. SRM and CRM recovery results

SRM/CRM	Arsenic (As)			Mercury (Hg)		
	Obtain result (mg kg^{-1})	Certified Value (mg kg^{-1})	Recovery (%)	Obtain result (mg kg^{-1})	Certified Value (mg kg^{-1})	Recovery (%)
Mussel Tissue NIST SRM 2976 (n=2)	15.14 ± 0.43	13.3 ± 1.8	113.83 ± 3.24	0.056 ± 0.001	0.061 ± 0.04	91.56 ± 1.59
Dogfish Liver NRCC CRM DOLT-5 (n=2)	29.32 ± 0.99	34.6 ± 2.4	87.4 ± 2.86	0.36 ± 0.02	0.44 ± 0.18	80.64 ± 5.2
Oyster Tissue NIST SRM 1566b (n=2)	6.52 ± 0.40	7.65 ± 0.65	85.17 ± 5.35	0.028 ± 0.003	0.037 ± 0.0013	76.56 ± 9.17

Level of arsenic (As) and mercury (Hg) in the bivalvia samples

Figure 3 displays the level of As in the bivalvia samples from Kuala Sungai Baru (i) and Kuantan (ii). In Kuala Sungai Baru, As concentrations in cockle, clam and mussel samples were 1.48 ± 0.10, 1.23 ± 0.09 and 0.94 ± 0.02 mg kg^{-1} respectively. Meanwhile, in Kuantan, the level of As in cockle, clam and mussel samples were 1.04 ± 0.10, 1.60 ± 0.09, and 0.85 ± 0.02 mg kg^{-1} respectively. The As in the bivalvia samples may derived from natural input and also anthropogenic activities such as mining, industrial discharge and agricultural input runoff

(Luvonga et al., 2020). Based on the study by Alina et al. (2012), the As level in their cockle samples (0.88 ± 0.02 mg kg $^{-1}$) from the Strait of Malacca was lower than the As level in the cockle samples from Kuala Sg Baru Melaka. The increase of As in bivalvia samples from the same region of sampling locations and after more than ten years of study may indicate the increase of this element may derive from the urbanization and industrialization nearby the sampling locations (Salam et al., 2021)

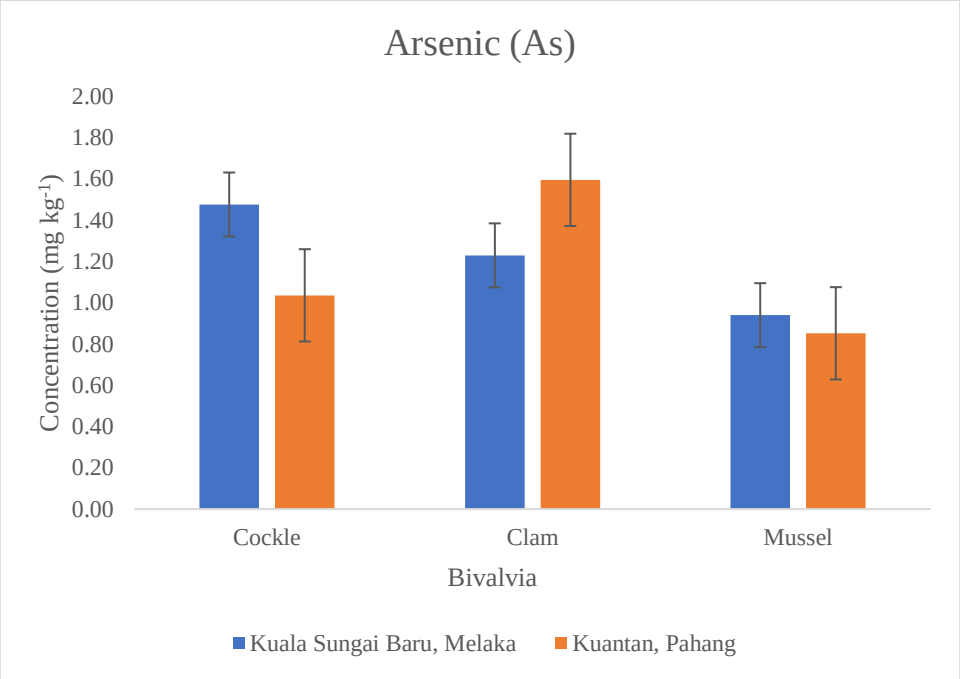

Figure 3. As level in the bivalvia from Kuala Sungai Baru, Melaka and Kuantan, Pahang

Figure 4 shows the level of Hg in bivalvia samples from Kuala Sungai Baru (i) and Kuantan (ii). In Kuala Sungai Baru, the concentration of Hg in cockle, clam and mussel samples were 0.006 ± 0.06, 0.007 ± 0.07, and 0.007 ± 0.003 mg kg^{-1} respectively. Meanwhile in Kuantan, the level of Hg in cockle, clam and mussel samples were 0.006 ± 0.001, 0.005 ± 0.001, and 0.004 ± 0.0003 mg kg^{-1} respectively. The Hg concentration in bivalvia samples in both locations was similar with the exception to the mussel sample in Kuantan region. Similar to As, the bioavailability of Hg in bivalvia samples may derived from natural and anthropogenic sources (Al-Sulaiti et al., 2022). The Hg natural sources originated from earthquake activities, volcanic eruptions, and geothermal springs meanwhile mining, smelting, coal burning, and oil refinery were due to Hg anthropogenic activities. Based on the previous study by Fui et al. (2022), the Hg in their bivalve samples (0.23 mg kg-^{1}) at Setiu, Terengganu was higher than the Hg in the bivalve of Kuantan. The difference of Hg concentration in the bivalve samples may due to the Hg availability in the water ecosystem, the hydrodynamic of the environment and also detoxifying capabilities of the bivalves (Fui et al., 2022; Haris et al., 2020).

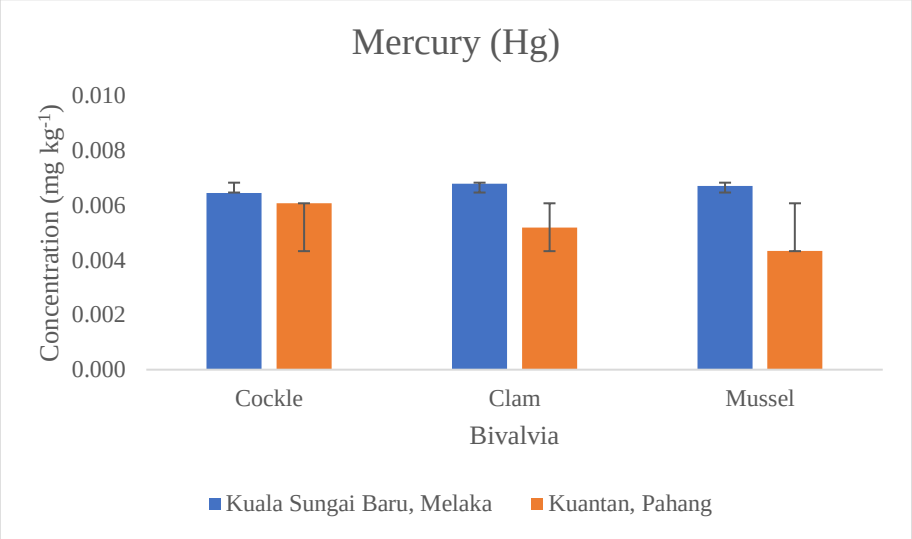

Figure 4. Hg level in the bivalvia from Kuala Sungai Baru, Melaka and Kuantan, Pahang

Spatial difference of arsenic (As) and mercury (Hg) between Melaka and Pahang states

Based on the *t*-test (Table 2), the level of As and Hg in all bivalvia samples showed insignificant differences between the two sampling locations ($p>0.05$). The similar distribution of As and Hg in all bivalve samples from both sampling stations may due to the similar sampling location (muddy coastal ecosystem) with moderate urbanization areas. Kuala Sungai Baru, Melaka and Kuantan, Pahang areas were occupied by medium industrialization with shipping port, petroleum and tourism industries.

Table 2. Spatial difference of As and Hg in all three bivalvia species based on *t*-test

Bivalvia	As		Hg	
	Melaka	Pahang	Melaka	Pahang
Cockle	$p > 0.05$		$p > 0.05$	
Clam	$p > 0.05$		$p > 0.05$	
Mussel	$p > 0.05$		$p > 0.05$	

Based on *F*- test, the level of As in both locations was significantly difference between bivalvia samples ($p<0.05$) (Table 3). The difference in As accumulation between bivalvia samples was due to the biological parameters such as body sizes, growth, reproductive status and genotype (Harsono et al., 2017). In contrast, based on the *F*-test, the concentration of Hg in both locations was similar between bivalvia samples ($p>0.05$) (Table 3).

Table 3. F-test result for As and Hg difference in all three bivalvia species in Melaka and Pahang

Location	As			Hg		
	Cockle	Clam	Mussel	Cockle	Clam	Mussel
Melaka	$p<0.05$			$p>0.05$		
Pahang	$p<0.05$			$p>0.05$		

Comparison of arsenic (As) and mercury (Hg) levels with guideline

The Malaysian Food Act 1985 guidelines specify the maximum permissible level of organic As at 1.0 mg kg^{-1}. Based on the analysis of total As in bivalve samples from both locations, the clam and cockle samples exceeded the total of 1.0 mg kg^{-1} level. Therefore, it is compulsory and recommended for further As speciation analysis for clam and cockle samples from both locations to investigate the species of As in the samples as the amount of As detected may derived from the total As. However, according to the Food and Agricultural Organization (FAO) of the United Nations, guidelines for total As in bivalvia samples have not been established. Meanwhile, for Hg, all bivalve samples in both locations were below the methyl-Hg guideline from the Malaysian Food Act 1985 and lower than the international guideline for total Hg in bivalvia sample based on FAO (0.5 mg kg^{-1}).

CONCLUSIONS

As for conclusion, the distribution of total As and Hg levels of cockle were examined for the clam, cockle and mussel samples from Kuala Sungai Baru, Melaka and Kuantan, Pahang. The As and Hg distribution patterns for all three bivalve samples were similar due to the similar geological and urbanization regions. However, the different levels of these elements between bivalve samples may due to biological factors. It is recommended to perform further As speciation analysis for clam and cockle samples to understand the safety risk of bivalve consumption in the local areas. The further analysis of Hg in the food chain also was important to investigate the potential of Hg biomagnification across the food chain. This study newest data on As and Hg food safety and risk assessment in the bivalve samples from local areas of Melaka and Pahang states. Furthermore, it is recommended to conduct the food contaminants monitoring program more frequently in the study area to ensure food safety compliance and address human risk potential.

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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.

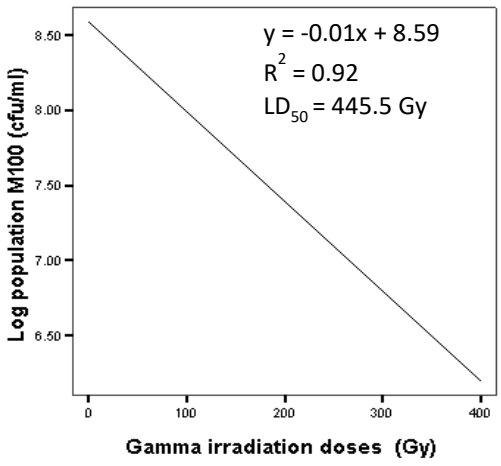

Fig. 2. Log population of *Acinetobacter calcoaceticus* M100 (cfu/mL) for LD_{50}.

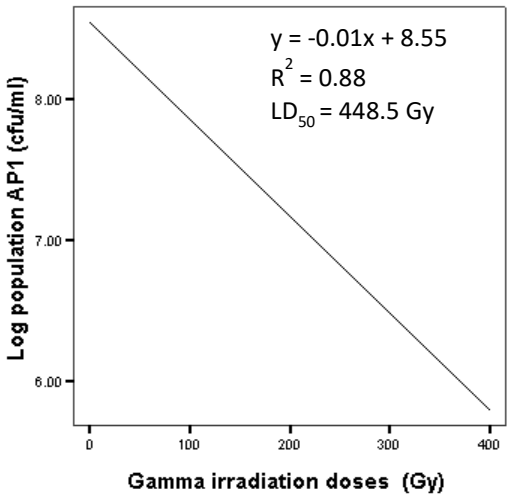

Fig. 3. Log population of *Acinetobacter baumannii* AP1 (cfu/mL) for LD_{50}.

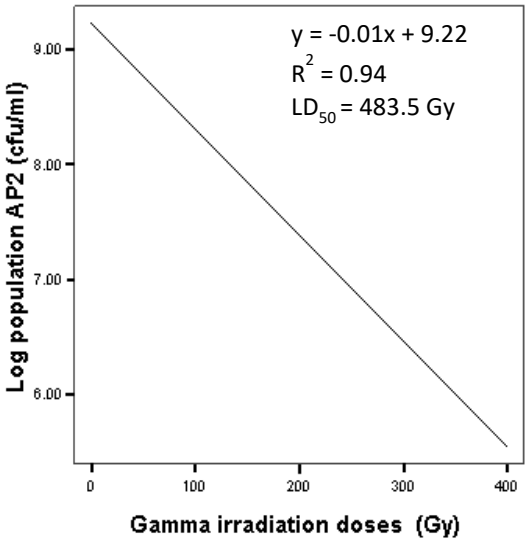

Fig. 4. Log population of *Klebsiella pneumoniae* AP2 (cfu/mL) for LD_{50}.

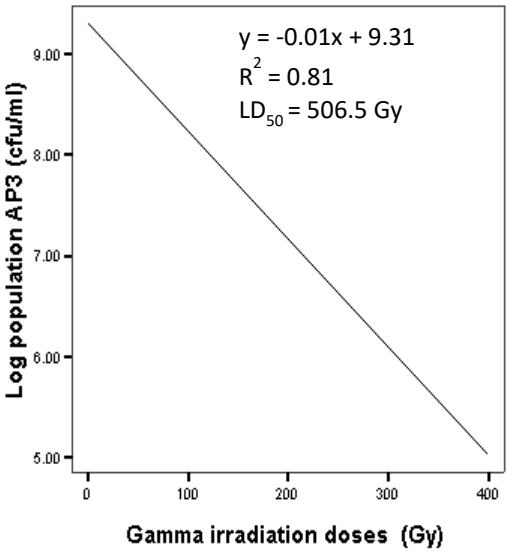

Fig. 5. Log population of *Enterobacteriaceae* bacterium AP3 (cfu/mL) for LD_{50}.

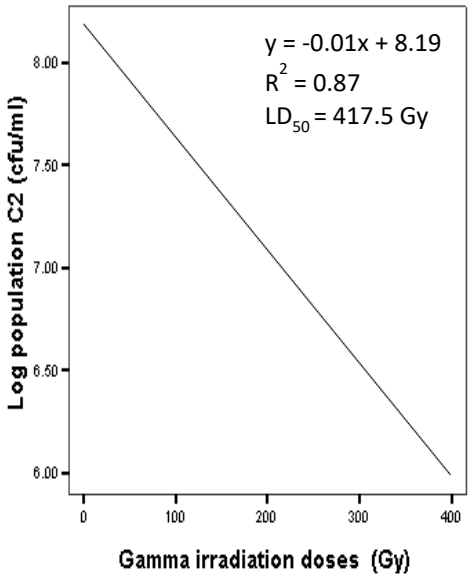

Fig. 6. Log population of *Pseudomonas putida* C2 (cfu/mL) for LD_{50}.

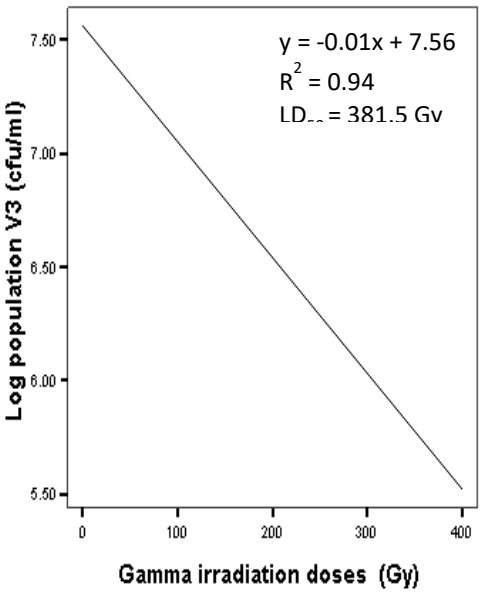

Fig. 7. Log population of *Pantoea stewartii* V3 (cfu/mL) for LD_{50}.

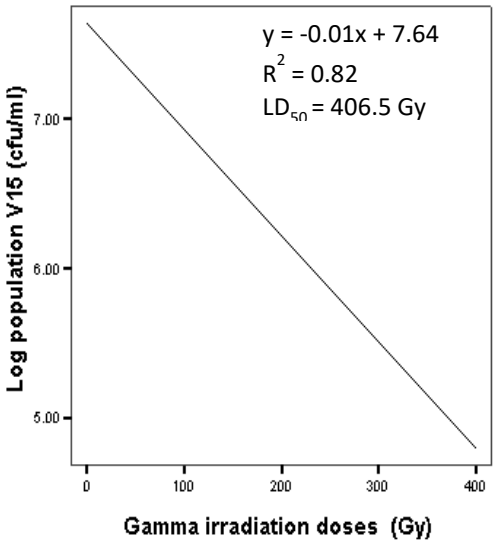

Fig. 8. Log population of *Pseudomonas putida* V15 (cfu/mL) for LD_{50}.

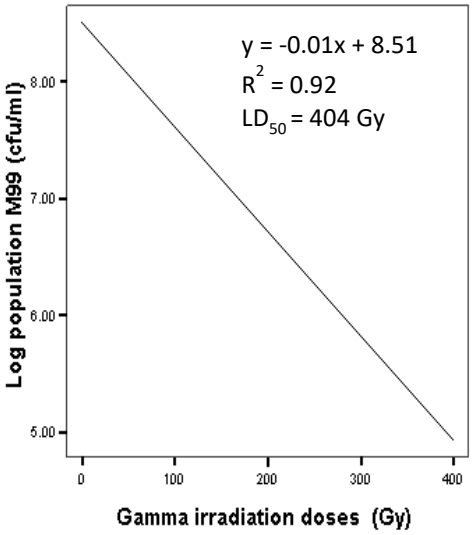

Fig. 9. Log population of *Pseudomonas putida* M99 (cfu/mL) for LD_{50}.

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.

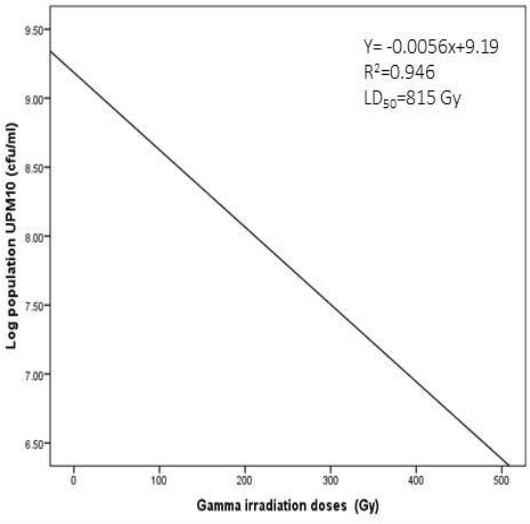

Fig. 10. Log population of *Bacillus* sp. UPM 10 (cfu/mL) for LD_{50}.

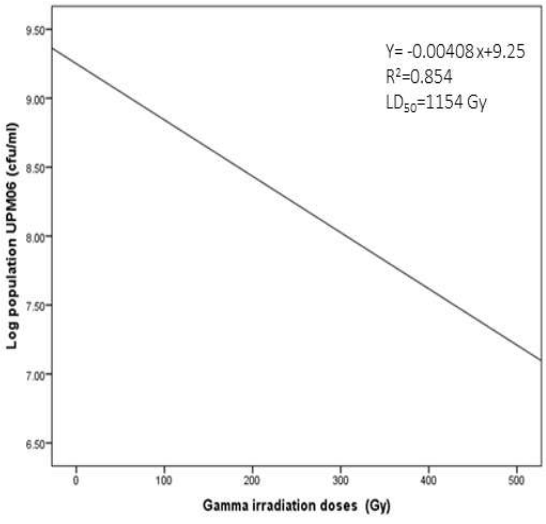

Fig. 11. Log population of *Bacillus proteolyticus* UPM 06 (cfu/mL) for LD_{50}.

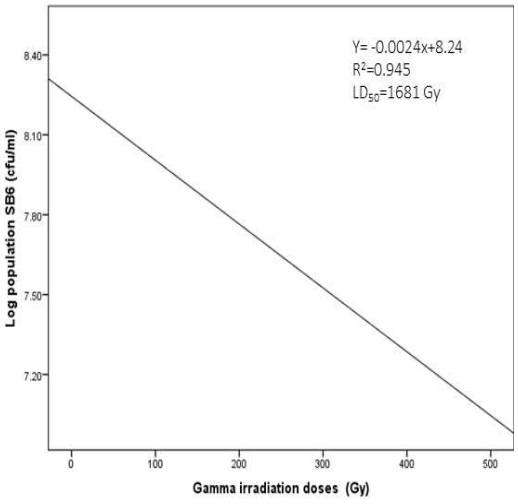

Fig. 12. Log population of *Priestia megaterium* SB6 (cfu/mL) for LD_{50}.

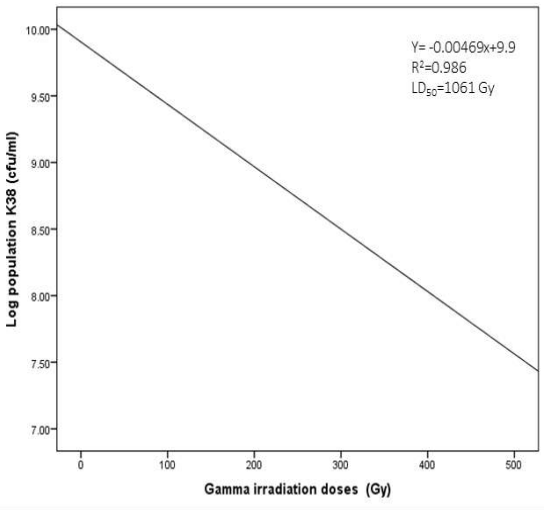

Fig. 13. Log population of *Priestia aryabhattai* K38 (cfu/mL) for LD_{50}.

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.

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PROFICIENCY TEST RESULTS FOR STRONTIUM-90 ANALYSIS IN ENVIRONMENTAL MATRIX AT RADIOCHEMISTRY AND ENVIRONMENT LABORATORY, MALAYSIAN NUCLEAR AGENCY

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ABSTRACT

Strontium-90 (Sr-90) is a radioactive isotope with significant health risks due to its long half-life and bone-seeking properties. Accurate analysis of Sr-90 is essential for environmental monitoring and safety assessments. This study presents the proficiency test (PT) results conducted at the Radiochemistry and Environment Laboratory, Malaysia Nuclear Agency, in 2021 until 2023. A total of nine samples, including spiked water, simulated contaminated surfaces, and seawater, were analyzed by using a gas proportional counter for beta counting. The acceptability of results was rated based on the trueness and precision determined by the relative bias and P values. Four results fell within the acceptable range, one was in the warning range, and four were deemed unacceptable. The main causes of the unacceptable results were due to the lack of Y-90 correction, loss of Sr-90 during sample preparation, and possible incomplete separation of Sr-90. These findings emphasize the need for method refinement, enhanced quality control, and continuous laboratory training to improve the accuracy and reliability of Sr-90 analysis.

Keywords: strontium-90, proficiency test, radiochemistry

INTRODUCTION

Strontium-90 (Sr-90) is a radioactive isotope that is produced as a by-product of nuclear fission in reactors and nuclear weapon detonations. Due to its similarity to calcium, Sr-90 can replace calcium in bones, leading to serious health issues such as bone cancer and leukemia. Its long half-life of approximately 28.8 years means Sr-90 remains a persistent environmental contaminant, posing long-term risks to human health and the environment. The accurate detection and quantification of Sr-90 in various matrices is crucial for environmental monitoring, public health protection, and regulatory compliance.

The analysis of Sr-90 involves complex radiochemical procedures due to its low-energy beta emissions and the presence of other radionuclides in environmental samples. It can be measured using various techniques, i.e. beta counting, liquid scintillation spectrometry, and mass spectrometry (Vajda & Kim, 2010). Laboratories must employ precise and accurate methodologies to ensure reliable results. Proficiency testing (PT) is an essential quality assurance tool that evaluates the performance of laboratories in conducting specific analyses, providing an objective

measure of their analytical capabilities. Participation in PT schemes enables laboratories to benchmark their performance against known values and other laboratories, identifying strengths and areas for improvement. The findings from these tests provide valuable insights into the analytical processes at the participating laboratory and highlight the importance of continuous quality control and method validation.

This study presents the results of PT conducted at Radiochemistry and Environment Laboratory (RAS) from the year 2021 to 2023 through two PT programmes, Analytical Laboratories for the Measurement of Environmental Radioactivity (ALMERA) and World-Wide. ALMERA PT program was provided by the IAEA Terrestrial Environmental Radiochemistry Laboratory (TERC) on determination of anthropogenic and natural radionuclides in water, soil and simulated contaminated surface samples. On the other hand, the World-Wide PT program was organized by the Radiometrics Laboratory (RML) of the IAEA Environment Laboratories to test the performance of participating laboratories in an analysis of radionuclides in a seawater sample.

The PT results highlight the critical role of accurate Sr-90 analysis in environmental monitoring and public health protection, as well as its significance at the national level for regulatory compliance, radiation safety, and policymaking. National agencies such as the Ministry of Health (MOH), the Department of Environment (DOE), and the Atomic Energy Department (JTA) rely on precise Sr-90 data for assessing contamination risks, ensuring food and water safety, and guiding radiation protection measures. This study serves as a benchmark for RAS's performance and guides future improvements.

MATERIALS AND METHODS

A total of nine samples were received from the International Atomic Energy Agency (IAEA) through two PT programmes, ALMERA and World-Wide. The details of the samples received are shown in Table 1. The spiked water was prepared using drinking water sourced from Seibersdorf, Austria and gravimetrically spiked with known amounts of a prepared standard solution, containing a mixture of certified radionuclides and acidified to < pH 2 (0.05M HNO_3) for stability.

The simulated contaminated surface with Sr-90 spiked magenta ink printed on the surface was prepared by the IAEA using in-house printing technique (International Atom Energy Agency, 2023). For seawater, 5 L of filtered Mediterranean Sea water was spiked by the IAEA with radionuclides H-3, Sr-90, Ba-133, Cs-134, Cs-137 and two undisclosed γ-ray emitters (International Atomic Energy Agency, 2024). All samples were sent to the laboratory via courier delivery.

Table 1. Details of the samples received

Year	PT Code	Sample No.	Sample Type
2021	IAEA-TEL-2021-04 Part II	2	Spiked water
2021	IAEA–RML–2021–01	S21N020	Seawater
2022	IAEA-TERC-2022-02 Part II	1, 2	Spiked water
2022	IAEA–RML–2022–01	S22N20	Seawater
2023	IAEA-TERC-2023-02	2	Spiked water
2023	IAEA-TERC-2023-02	5,7	Simulated contaminated surface
2023	IAEA–RML–2023–01	S23N020	Seawater

Sample Pre-treatment and Preparation

A volume of 10 mL of liquid PT sample (spiked water and seawater) was diluted with distilled water to 100 mL. For spiked water, 1.0 g of Sr^{2+} carrier (5 mg/g) was added to the 100 mL solution. For seawater, 0.175 mL of concentrated HNO_3 was added before the addition of Sr^{2+} carrier. The preparation of seawater was continued by adding 2 M NaOH until the pH of the solution increased to between pH 8 and 9. Next, about 1.0 g of Na_2CO_3 was added and the solution was left overnight to allow precipitation. The collected precipitate was dissolved in concentrated HNO_3 and then evaporated until dryness. After that, 100 mL distilled water was added into the solution to dissolve the residue and the sample was further acidified until pH 2 by using 3.0 M HNO_3. Simulated contaminated surface paper was cut and stuck to a 2.5-inch diameter planchet and was ready for counting.

Radiochemical Separation and Purification

Radiochemical separation and purification processes were carried out on the prepared spiked water and seawater samples. Radiochemical separation was done by using 2 mL of cation exchange resin, Dowex 50W-X8 (Na^+ form), 100-200 mesh. The resin was pre-conditioned by using 0.1 M HNO_3 before the sample was loaded into the resin. The beaker containing the sample was then rinsed with 5 mL of 0.1 M HNO_3 for three times followed by elution of Sr-90 using 8 M HNO_3. The solution collected was then evaporated to dryness and the residue was re-dissolved with 10 mL of 8 M HNO_3 and proceeded to the purification process.

Purification was done by using 0.7 g of Eichrom Sr resin (100 – 150 µm) that was pre-conditioned by using 5 mL of 8 M HNO_3. After the sample was loaded into the column, 5 mL of 3 M HNO_3– 0.05 M oxalic acid solution was added. Then, the column was rinsed with 5 mL of 8 M HNO_3. Sr-90 was then eluted by using 10 mL of 0.05 M HNO_3. The Sr purified solution was transferred to the counting planchet and dried.

Sample Counting

The sample was counted for 100 minutes, in 3 cycles by using a gas proportional counter of Automatic Low Background Alpha/Beta Counting System (S5XLBPF, Canberra Inc., USA) as shown in Figure 1. The alpha and beta plateau of the system were set using Am-241 and Sr-90 plated disc standard sources, respectively, to establish the measurement conditions. The counting efficiency of Sr-90 was determined by using a series of Sr-90 standards prepared at different activities (0.5 Bq – 4 Bq).

Figure 1. Low background gross alpha beta counting system

Data Analysis

The data were analysed based on the report by Wan Mahmood & Abdullah (2020). The chemical yield for Sr based on residual weight resulting from the addition of a stable Sr carrier, Sr^{2+} ($Sr(NO_3)_2$) was calculated using equation below:

$$y_{Sr}(\%) = \frac{[(Rd)\,(x/z)]\,(1000)\,(100)}{(Cc)(wc)} \qquad (1)$$

where,

Y_{Sr}:	chemical yield for strontium (%)
Rd:	dried weight residue in the planchet (g)
x:	molecule weight of strontium (87.62 g/mol)
z:	molecule weight of $Sr(NO_3)_2$ (211.63 g/mol)
Cc:	concentration of strontium carrier, Sr^{2+} (mg/g)
wc:	weight of added strontium carrier, Sr^{2+} (g)

The Sr-90 activity concentration calculated in the liquid sample is expressed in Bq/L. All nuclear data used in the calculation of Sr-90 activity concentration are taken from the data suggested in the Chart of the Nuclides (Magill et al, 2006). Sr-90 activity concentration is calculated using the count per minute (CPM) values for the sample and the background obtained from the beta counting data generated. The Sr-90 activity concentration reported in the results is a specific activity concentration during the reference date and it is calculated based on the equation below:

$$A = \frac{(CPMgs - CPMbg)(e^{\lambda t})(1000)}{(60)(Esr)(Ysr)(Wdw)} \quad (2)$$

where,

A:	Measured activity concentration of Sr-90 at sampling date (Bq/kg dry wt. or Bq/L)
CPMgs:	CPM value for Sr-90in the sample (CPM)
CPMbg:	CPM value for background (CPM)
λsr:	decay coefficient = ln 2/t1/2 (28.5 years)
t:	elapsed time from the reference date and time to the counting date and time (year)
Esr:	Sr-90 counting efficiency
Ysr:	chemical yield of strontium
Wdw:	sample dry weight

The calculation of the combined uncertainty value for the Sr-90 activity concentration measurement in the sample on the reference date is based on equation (2) as discussed above. Thus,

$$\frac{U\,(A) =}{(A)\,[\sqrt{[(U(n^{s}_{90Sr})/n^{s}_{90Sr})^2 + (U(t)/t)^2 + (U(\lambda_{Sr})/\lambda_{Sr})^2 + (U(E_{Sr})/E_{Sr})^2 + (U(Y_{Sr})/Y_{Sr})^2 + (U(W_{dw})/W_{dw})^2]} \quad (3)$$

Where,

U(A):	uncertainty value for Sr-90 activity concentration measured at reference date (Bq/L)
A:	Sr-90 activity concentration measured at reference date (Bq/L)
$U(n^{s}_{90Sr})$:	uncertainty value of net CPM of Sr-90 (CPM)
n^{s}_{90Sr}:	net CPM of Sr-90 (CPM)
U(t):	uncertainty value for elapse time from the date and time of sampling to date and time of counting (year)
t:	elapse time from the reference date and time to date and time of counting (year)
$U(\lambda_{Sr})$:	uncertainty value for decay coefficient of Sr-90 (year-1)
λ_{Sr}:	decay coefficient of Sr-90 (year-1)
$U(E_{Sr})$:	uncertainty value for Sr-90 counting efficiency
E_{Sr}:	Sr-90 counting efficiency
$U(Y_{Sr})$:	uncertainty value for a chemical yield of Sr-90
Y_{Sr}:	chemical yield of Sr-90
$U(W_{dw})$:	uncertainty value of sample dry weight
W_{dw}:	sample dry weight

Result Evaluation

The evaluation follows the methodology applied for the annual IAEA proficiency testing schemes in the areas of radionuclide measurements and trace element analysis. The trueness of the result was based on the relative bias calculated from equation:

$$Bias_{relative} = \frac{Value_{reported} - Value_{target}}{Value_{target}} \; x \; 100\% \quad (4)$$

The relative bias is the relative difference between the reported and the target value and is compared to the Maximum Acceptable Relative Bias (MARB) which has been determined for each property value, considering the analytical methods, the analyte level in the sample and the complexity of the analysis. The result will be rated "Accepted (A)" for trueness when the value of relative bias is lesser than MARB.
The precision was evaluated based on the P value that corresponds to the relative combined uncertainty of the relative bias, calculated in Equation (4).

$$P = \sqrt{\left(\frac{U_{target}}{A_{target}}\right)^2 + \left(\frac{U_{reported}}{A_{reported}}\right)^2} \times 100\% \quad (5)$$

Where,
U_{target}: uncertainty of target value
A_{target}: activity of target value
$U_{reported}$: uncertainty of reported value
$A_{reported}$: activity of reported value

The relative bias is then compared to the expanded uncertainty of the relative bias:

$$|Bias_{relative}| \leq k \times P \quad (6)$$

Where,
k: the coverage factor, 2.58 for a level of confidence of 99%
P: P value from Equation (5)

When the above criterion is fulfilled, the reported result is not significantly different from the target values considering the uncertainties associated with both values. The reported uncertainty of measurement is large enough to cover the bias of the method. Aside from that, the P value is also compared to the MARB and must be less than the MARB. When both criteria related to the measurement uncertainty are fulfilled, the reported result for precision is rated "accepted (A)". The result is rated "Not Accepted (N)" for precision if either of the two conditions are not fulfilled.
The final score is assigned according to the detailed evaluation described above. The possible scores are "Accepted (A)" when both, trueness and precision were rated "Accepted", "Not Accepted (N)" when the trueness rating is "Not Accepted" and "Warning (W)" when the trueness rating is "Accepted" but the precision rating is "Not Accepted".

RESULTS AND DISCUSSION

The PT results for Sr-90 analysis in seawater samples from 2021 to 2023 are summarized in Table 1. The table includes the assigned values, reported values, relative bias, P values, and final scores for three seawater samples received in 2021 (S21N020), 2022 (S22N020), and 2023 (S23N020).

Table 1. Sr-90 analysis results for seawater samples

Sample No.	Assigned Value (Bq/L)	Reported Value (Bq/L)	Relative Bias (%)	P value (%)	Final Score
S21N020	0.618 ± 0.005	0.744 ± 0.045	20.4	6.0	Warning
S22N020	0.816 ± 0.007	0.770 ± 0.050	-5.6	6.5	Accepted
S23N020	0.708 ± 0.006	0.446 ± 0.027	-36.9	6.1	Not Accepted

The PT results for the seawater samples show varying levels of performance in Sr-90 analysis. The seawater samples were prepared using the precipitation method, which may have influenced the recovery of stable Sr carrier and contributed to the observed biases in reported activities. The reported value for sample S21N020 of 0.744 Bq/kg was 20.4% higher than the assigned value of 0.618 Bq/kg, resulting in a final score of "Warning." The P value of 6.0% indicates a marginally acceptable performance, suggesting that the laboratory's methodology may need adjustments to improve accuracy. The significant positive bias suggests potential issues in sample preparation or measurement processes, and the precipitation method used for sample preparation might have contributed to reduced recovery, leading to an overestimation of activity.

In contrast, the reported value of 0.770 Bq/kg in sample S22N020 was within 5.6% of the assigned value of 0.816 Bq/kg, and this result was accepted with a P value of 6.5%. This sample demonstrated the laboratory's ability to produce accurate and reliable results for Sr-90 in seawater. The minor negative bias indicates that while the results are close to the assigned value, there may still be slight underestimation that needs to be addressed.

The reported value of 0.446 Bq/kg in sample S23N020 showed a significant negative bias of -36.9% compared to the assigned value of 0.708 Bq/kg, resulting in a final score of "Not Accepted." The P value of 6.1% indicates an unsatisfactory performance, highlighting substantial inaccuracies in the laboratory's analysis for this sample. This significant underestimation suggests issues such as incomplete radiochemical separation or overestimation of the analysis efficiency. Additionally, the precipitation method used in sample preparation may have led to reduced recovery, further contributing to the underestimation of activity.

The PT results for Sr-90 analysis in spiked water samples from 2021 to 2023 at RAS are summarized in Table 2.

Table 2. Sr-90 analysis results for spiked water samples

Sample No.	Assigned Value (Bq/L)	Reported Value (Bq/L)	Relative Bias (%)	P value (%)	Final Score
2/2021	146.8 ± 8.4	248.5 ± 14.9	69.28	8.29	Not Accepted
1/2022	26.4 ± 0.007	23.66 ± 2.91	-10.38	13.71	Accepted
2/2022	7.42 ± 0.45	6.64 ± 0.82	-10.51	13.76	Accepted
2/2023	14.2 ± 0.7	13.92 ± 0.88	-2.0	8.02	Accepted

The reported value of 248.5 Bq/kg in Sample 2/2021was 69.28% higher than the assigned value of 146.8 Bq/kg, resulting in a final score of "Not Accepted" with a P value of 8.29%. This significant positive bias indicates substantial overestimation, suggesting possible issues in sample preparation, contamination, or measurement errors. This result highlights a critical need for method refinement and strict quality control to prevent such discrepancies in future analyses.

For the PT result in 2022, the reported value of 23.66 Bq/kg in sample 1/2022 showed a relative bias of -10.38% compared to the assigned value of 26.4 Bq/kg, with a final score of "Accepted" and a P value of 13.71%. Although the result is within acceptable limits, the negative bias indicates a slight underestimation, pointing to potential areas for improvement in the laboratory's analytical processes to enhance accuracy further. Similar to Sample 1/2022, the reported value of 6.64 Bq/kg for Sample 2/2022 showed a negative bias of -10.51% relative to the assigned value of 7.42 Bq/kg. With a P value of 13.76% and an "Accepted" score, this result indicates acceptable performance but suggests the need for minor adjustments to reduce bias and improve the accuracy of low-level Sr-90 measurements.

In 2023, the result for spiked water showed a significant improvement. The reported value of 13.92 Bq/kg closely matched the assigned value of 14.2 Bq/kg, with a relative bias of -2.0% and a P value of 8.02%. This result was "Accepted," demonstrating the laboratory's improved accuracy and precision in Sr-90 analysis. The minor negative bias suggests a successful enhancement of analytical methods and quality control procedures over the testing period.

The proficiency test results for Sr-90 analysis in simulated contaminated surface samples from 2023 are summarized in Table 3.

Table 3. Sr-90 analysis results for simulated contaminated surface samples

Sample No.	Assigned Value (Bq/sample)	Reported Value (Bq/sample)	Relative Bias (%)	P value (%)	Final Score
5/2023	2.25 ± 0.10	8.37 ± 0.11	272.0	4.63	Not Accepted
7/2023	4.52 ± 0.22	10.19 ± 0.12	125.4	5.01	Not Accepted

The proficiency test results for the simulated contaminated surface samples indicate significant discrepancies in the reported Sr-90 values compared to the assigned values, resulting in "Not Accepted" scores for both samples. The reported value of 8.37 Bq/sample in Sample 5/2023 was 272.0% higher than the assigned value of 2.25 Bq/sample, resulting in a P value of 4.63% and a final score of "Not Accepted." For Sample 7/2023, the reported value of 10.19 Bq/sample was 125.4% higher than the assigned value of 4.52 Bq/sample, with a P value of 5.01% and a final score of "Not Accepted". This substantial positive bias indicates a severe overestimation in the Sr-90 analysis.

A key factor contributing to this overestimation is the presence of Yttrium-90 (Y-90), the decay product of Sr-90. Y-90 has a half-life of approximately 64 hours and reaches secular equilibrium with Sr-90 in about three weeks. At equilibrium, the total beta activity measured will be nearly double the activity of Sr-90 alone because both Sr-90 and Y-90 contribute equally to the beta emissions. If Y-90 is not properly accounted for, the reported Sr-90 activity can be significantly overestimated.

Unlike liquid samples, which require radiochemical separation to isolate Sr-90, surface samples are analyzed by direct counting using a gas proportional counter. Since no chemical separation is performed, the analysis entirely depends on the instrument's efficiency and the ability to consider all factors affecting the determination of Sr-90 radioactivity from the beta counts. Proper consideration of Y-90 equilibrium with Sr-90 is crucial to prevent overestimation. Since Y-90 contributes to beta activity, its presence must be accounted for when interpreting results. Other important factors that strongly affect the determination of Sr-90 in surface samples is the difference in calibration source and sample geometry. Calibration was carried out by using distributed plated surface while surface samples is printed paper which have a different surface composition, thickness, and uniformity. Differences in material properties can affect beta energy attenuation and scattering causing over- or underestimation of beta counts. Aside from that, ensuring accurate background subtraction is essential, as fluctuations in background radiation can influence the measured beta activity.

The mixed results from the sample analyses indicate the need for continuous improvement in the laboratory's procedures. The accepted result demonstrates that the laboratory at RAS has the potential to perform accurate Sr-90 analyses. However, the warning and not accepted scores highlight inconsistencies that must be addressed.

CONCLUSION

The proficiency test results for Sr-90 analysis at RAS revealed that only 4 out of 9 samples met the acceptance criteria. While this demonstrates the laboratory's capability to achieve accurate Sr-90 measurements under certain conditions, the results also highlight critical areas that require improvement. The instances of warnings and failures indicate inconsistencies in analytical procedures, potential issues in radiochemical separation, and limitations in measurement processes that must be addressed to enhance overall reliability and accuracy. To improve performance, RAS should refine its analytical techniques to minimize errors, particularly in radiochemical separation methods for liquid samples and correction for Y-90 interference in direct counting of surface samples. Additionally, enhancing calibration procedures to accurately reflect real sample conditions will help reduce systematic biases. Continuous participation in PT schemes and adherence to international standards will be crucial in maintaining and improving the laboratory's analytical performance.

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OPTIMIZATION OF FLY ASH-BASED GEOPOLYMER FOR IMMOBILIZATION OF SPENT RESIN FROM LOW LEVEL EFFLUENT TREATMENT PLANT (LLETP), MALAYSIAN NUCLEAR AGENCY

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ABSTRACT

Radioactive waste management is an important element in ensuring the peaceful use of nuclear and its related technology. In Malaysia, Malaysian Nuclear Agency is solely responsible for the management of radioactive waste in the country and its responsibilities include collection, transportation, treatment, storage and disposal of the radioactive waste. Treatment process is crucial in immobilizing the radionuclides from leaching out from the waste form. This project aims at assessing the effectiveness of fly ash-based geopolymer in the immobilization of radioactive waste in particular spent resin. In this project, spent resin from the Low-Level Effluent Treatment Plant (LLETP) will be treated with geopolymer blended from fly ash obtained as a by-product from a coal power plant in Malaysia. The spent resin from the LLETP becomes unusable after several cycles of usage and thus needs to be replaced. The spent resin is considered to be problematic and, in many cases, requires special approaches and precautions during its treatment to meet the waste acceptance criteria for disposal. This investigation serves to support the pursuit for effective and viable option to immobilize spent ion exchange resin at WasTeC in ensuring long-term safety.

Keywords: geopolymer, spent resin, immobilization

INTRODUCTION

Radioactive waste is produced in various fields such as agriculture, medicine, industry, and others. Nuclear Malaysia's Waste Technology Development Center (WasTeC), is the only agency responsible for handling the management of radioactive waste generated from the use of nuclear technology in Malaysia. Radioactive waste management includes collection, treatment and conditioning, storage and disposal. The facilities and equipment at WasTeC were mostly developed since 1982 and became fully operational since 1984. One of the facilities available is the Low-Level Effluent Treatment Plant (LLETP) for treatment of low-level liquid waste and storage of organic liquid waste. The plant has 4 collection tanks with a total capacity of 80 m^3 for storage of radioactive liquid waste obtained from laboratories in Malaysian Nuclear Agency.

In 2019, Tank 3A has been added to the LLETP and this collection tank is designed to collect the effluent from the newly installed mineral processing plant under Thorium Project that is yet to start operation. Tank 3A is specialized for liquid waste containing alpha emitters anticipated to be produced from the mineral processing activity. The Thorium Project aims to recover thorium, uranium, and rare earth elements in the monazite and xenotime which are by-products of tin mining processing. Extraction processes include acid leaching and selective precipitation that resulted in thorium, uranium and rare earth recovery. In this process, secondary effluent will be produced. The effluent contains physical, chemical or biological pollutants that often exceed the limit that has been set by the authorities. Figure 1 shows the flowchart for treatment of aqueous waste containing alpha emitters at the LLETP.

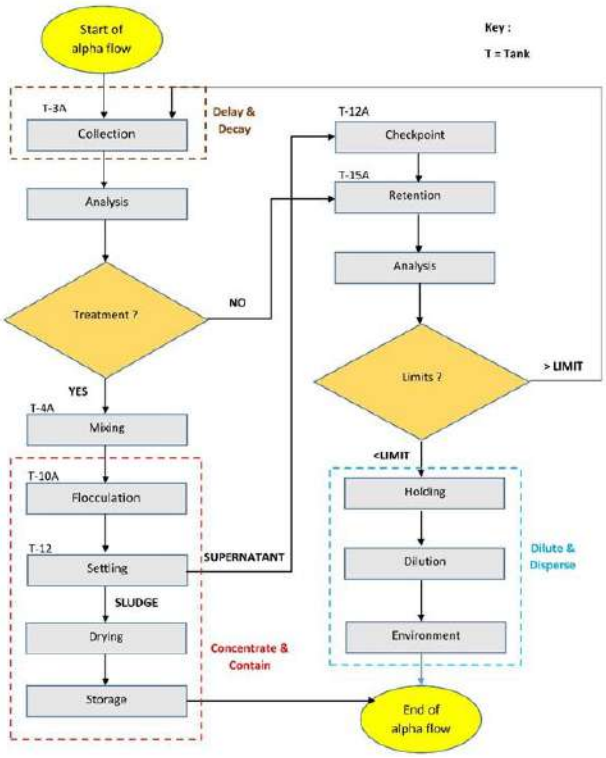

Fig. 1. Flowchart for Treatment of Aqueous Liquid Waste containing Alpha Emitters at Low Level Effluent Treatment Plant (LLETP), Nuclear Malaysia.

Treatment of effluent containing radioactive material is conducted using chemical flocculation-coagulation process. This process is commonly used in water and wastewater treatment in which compounds such as iron chloride and/or polymers are added to the wastewater to stabilize the colloidal material and cause small particles to accumulate into larger floats (Amuda et al., 2007; Tatsi et al., 2003; Abel- Shafy et al. 1991).

The effluent also contains physical, chemical or biological pollutants that often exceed the limit that has been set by the authorities. There are four parameters that usually exceed the limit: Chemical Oxygen Demand (COD), conductivity, total solid and the radioactivity for alpha emitters. The effluent discharge limit set by the regulator was successfully compiled by introducing an ion exchanger in the effluent treatment. Table 1 shows the analysis result for treatment of aqueous liquid waste containing alpha emitter.

Spent ion exchanger or spent resin requires special handling and treatment method due to its high radioactivity. This spent resin becomes no longer useful after several cycles of usage and needs to be replaced. Currently, approximately 640 kg spent resin from Nuclear Malaysia's research reactor were stored at WasTeC storage facility. Prolonged waste storage may pose risk to the human health and environmental due to potential leakage, which may contaminate the storage area. Currently, WasTeC is investigating treatment and disposal options for spent resin.

Table 1 Analysis Result for Treatment of Aqueous Liquid Waste containing Alpha Emitters.

Parameter	**Discharge Limit (DOE & SHE-MS)**	**Coagulation and flocculation treatment**	**After Treatment (ion exchanger)**
pH	6-9	<	<
Total Suspended Solid	50 mg/l	<	<
Temperature	40 ^{0}C	<	<
B.O.D	50mg/l	<	<
C.O.D	25 mg/l	> 25 mg/l (36 mg/l)	< 25 mg/l (6 mg/l)
Conductivity	1000 ⌈S/cm	>1000 ⌈S/cm (4392 ⌈S/cm)	< 1000 ⌈S/cm (103.7 ⌈S/cm)
Total Solid	1000mg/l	> 1000 mg/l (2843 mg/l)	< 1000mg/l
Radioactivity for alpha emitters	1.5 Bq/l	>1.5 Bq/l (27.7 Bq/l)	< 1.5 Bq/l (0.4 Bq/l)

*< = below discharges limit
*> = above discharges limit

Geopolymers are made by adding aluminosilicates to concentrated alkali solutions for dissolution and subsequent polymerization to form a solid (Perera et al., 2004). The advantage of this technology is that it can be performed at room temperature and does not require complex technology such as vitrification. The term 'geopolymer' was first introduced by Davidovits in 1978 to describe a family of mineral binders with a chemical composition similar to zeolites but with an amorphous microstructure (Rajamane et al., 2011).

Unlike ordinary Portland/pozzolanic cement, geopolymers do not form a calcium silicate-hydrates (CSHs) for matrix formation and strength but utilize the polycondensation of silica and alumina precursors to attain structural strength (Olawale et al., 2013). Two main constituents of geopolymers are source materials and alkaline liquids. Among hydraulic binders common in our modern world, Portland cement remains the most used. However, the production of Portland cement is a resource-exhausting, energy intensive process that releases large amounts of greenhouse gas CO_2 into the atmosphere (Guo et al., 2010).

The Geopolymer Institute in France recommended that rock based geopolymer cement as ideal for environmental applications, such as the permanent encapsulation of radioactive and other hazardous wastes, toxic metals, as well as sealants, capping, barriers, and other structures necessary for remedying toxic waste containment sites (Geopolymer Alliance, 2009). Geopolymer binder behaves similarly to Portland cement. It can set and harden in a room temperature and can gain reasonable strength in a short period. Some proportions of geopolymer binders have been tested and proved to be successful in the field of construction, transportation, and infrastructure applications. Any current building component such as bricks, ceramic tiles, and cement could be replaced by geopolymer.

Advantages of polymerization technology include (Jihui et al., 2021):

1. Fast hardening rate and high strength.
2. Good thermal stability and high temperature resistance
3. Strong material interface bonding ability.
4. Good corrosion resistance and durability

METHOD

Materials

Spent resin samples generated from the water treatment process of LLETP were prepared. The spent resins were scooped and filled in clean 3L sealed plastic containers for characterization. Direct immobilization and encapsulation of spent resin was used in this study. The fly ash used in this study was obtained from Sultan Salahuddin Abdul Aziz Power Plant, Kapar Selangor. To activate silica and alumina elements contains in fly ash, sodium hydroxide and sodium silicate were added. Super plasticizer was also added on the geopolymer mixture to obtain a good workability.

Particle Distribution, Morphological Imaging and Phase Identification.

Particle size analysis was performed using a PCScope PCS 81x digital microscope and Microtrac X-100 particle size analyzer. Phase identification was done by X-Ray Diffraction analysis using PANalytical X'Pert PRO MPD.

Analysis of Radionuclide Content

The fly ash samples were packed into 350 ml cylindrical plastic containers and measured using Ortec hyper-pure germanium (HpGe) gamma spectrometer system with 30% relative efficiency and a resolution of 1.74 keV at 1.33 meV of ^{60}Co. The detector efficiency calibration was performed using a multinuclides standard source in 350 ml plastic container (containing certified concentrations of ^{241}Am, ^{109}Cd, ^{57}Co, ^{123m}Te, ^{51}Cr, ^{113}Sn, ^{85}Sr, ^{137}Cs, ^{88}Y, and ^{60}Co), purchased from Isotopes Product Laboratories (IPL, USA). Gamma Vision analysis software was used to analyze the samples. Gamma-ray energies of 1460Kev (K-40) for 40K, 1764.5 Kev (Bi214) and 351.9 Kev (Pb-214) were used to determine the concentration of ^{226}Ra. The activity of ^{226}Ra was assumed to be in equilibrium as its parent ^{238}U. Gamma-ray energies of 911.2, 964.6, and 969.0 KeV (Ac-228), were used to determine the concentration of ^{232}Th. While for spent resin, the samples were also packed into 320 ml cylindrical plastic container and were sent to Radiochemistry and Environmental Technology Group (RAS) for analysis of radionuclide content.

Mixture Proportions

A number of experiments were designed to test the influence of the variables on the workability and the strength of monolith geopolymer. The variables include workability, method of concentration and ratio of NaOH and Na_2SiO_3 and the effect of water and superplasticizer on strength. Based on this, the optimum geopolymer mixture was determined by casting pervious geopolymer monolith cube in order to obtain optimum ratios of fly ash to alkaline activator, sodium hydroxide (NaOH) to sodium silicate to solution (Na_2SiO_3), concentration of NaOH in molar, volume of superplasticizer, water and spent resin. The materials were prepared according to the given ratio and mixed in the Hobart mixer for 2 minutes. The slurry was poured into a 50 mm x 50 mm x 50 mm stainless steel cube mould. The samples were then vibrated to release any residual air bubbles and compact the sample. During the hardening of the geopolymer cement paste, the samples were covered with a thin film of polyethylene to avoid water evaporation and then kept for 24h in ambient condition of the laboratory before demoulding. Each sample were prepared in duplicates. The compressive strength determination of the sample was based on BS 1881-116:1993 standard. A total of 9 cube samples (50 mm x 50 mm x 50 mm) were prepared and tested at 3 different curing times (7, 14 and 28 days).

RESULTS AND DISCUSSION

Physical and Chemical Properties of Fly Ash.

The particle distribution of the samples was determined with a laser diffraction analyzer (Microtrac-X100). Table 2.0 shows that Kapar fly ash has a particle size range between 1.06-209.3 μm and the median particle size of 20.64 μm (Nurul Wahida et al.2021). Particle size of fly ash needs to be 80-90% lower than 45 μm to obtain the optimum binding effect (Fernandez-Jimenez et al., 2003).

Table 2. Granulometric Data for Fly Ashes

Samples	Percentages % of particles with diameter			Mean Diameter (μm)
	d_{10}	d_{50}	d_{90}	
Kapar fly ash	3.75	20.64	73.29	20.64

Mineralogy composition

There are two classes of fly ash which is defined by the ATM C618 which is Class F fly ash and Class C fly ash. The main difference between the classes is based on the amount of calcium, silica, alumina, and iron content in the ash. Based on the XRF result (Table 3.0), showed that the Kapar fly ash had a class C fly ash due to the total percentage of SiO_2, Al_2O_3 and Fe_2O_3 is less than 70% and the SO_3 content is less than 5% (Nurul Wahida et al., 2021). To obtain the optimum properties for binding, fly ash as source material should have a low calcium content, and material that does not burn less than 5%. In addition, Fe_2O_3 content should not be more than 10% and silica content should be between 40-50% (Suhana et al., 2015).

Table 3. Chemical Composition of Fly Ash

Oxide	Kapar fly ash	ASTM C618 Class C	ASTM C618 Class F
SiO_2	44.39	-	-
Al_2O_3	19.90	-	-
Fe_2O_3	3.75	-	-
Total SiO_2, Al_2O_3 & Fe_2O_3	68.04	Min. 50%	Min. 70%
CaO	5.44	-	-
SO_3	0.49	Max. 5%	Max. 5%

Radionuclides Content

The average radionuclide activity concentrations for fly ash are reported in Table 4.0 (Nurul Wahida et al., 2021). Activity concentration ranges from 96.5 to 338.5 Bq/kg, 139 to 422.7 Bq/kg, and 251.8 to 422 Bq/kg for ^{226}Ra, ^{232}Th, and ^{40}K, respectively. Coal that contains natural uranium and thorium is classified as Naturally Occurring Radioactivity Material (NORM) (Suhana et al., 2015). In the Second Schedule of Atomic Energy Licensing (Radioactive Waste Management) Regulations 2011 (P.U(A)274), the clearance limit set for activity concentration of radionuclides of natural origin for ^{226}Ra, ^{232}Th and ^{40}K shall not exceed 1000 Bq/kg, 1000 Bq/kg and 10,000 Bq/kg, respectively. The activity concentrations for sample fly ash from Kapar are below than the regulatory limit set by legislation.

Table 4. The Activity Concentration of ^{226}Ra, ^{232}Th, ^{40}K in Bq/kg for Kapar Fly Ash

Sample	**Activity Concentration (Bq/kg) (Nurul Wahida et al., 2021)**		
	^{226}Ra	**^{232}Th**	**^{40}K**
Kapar fly ash	338.5±9	422.7±24	251.8±9
P.U(A) 274	1000	1000	10000

The results of the gamma spectrometric analysis of spent ion exchange resins are shown in Table 5.0. The result shows the highest activity concentration value for spent resins was found for ^{228}Th (6.24±0.45 Bq/kg) and the lowest was for ^{234}U (2.49±0.09 Bq/kg) respectively. The activity concentrations of this spent resin are below than the clearance level set in the Second Schedule Radioactive Waste Clearance Level.

Table 5. The Activity Concentration for Spent Resin

Element	**Activity Concentration (Bq/kg)**
^{228}Th	6.24±0.45
^{230}Th	4.89±0.36
^{232}Th	5.42±0.39
^{234}U	2.49±0.09
^{235}U	< 0.2
^{238}U	< 0.2

*< = minimum detection activity

Optimum Mix Design for Immobilization of Spent Resin using Geopolymer Based Fly Ash

Table 6.0 shows the optimum value obtained for mix proportions based on the work that has been carried out in which the parameters such as the ratio of fly ash to alkaline solution, the ratio of Na_2SiO_3 to NaOH, NaOH molarity, volume of superplasticizer, water and waste loading has been considered for each of these parameters.

The highest compressive strength on day 7 was 15.4 MPa compared to the previous study 6.1 MPa (Nurul Wahida et al.2015). According to International Atomic Energy Agency (IAEA), (1983) the minimum standard for compressive strength for monolithic radioactive waste immobilization after reaching the age of 28 days was 3.2-70 MPa and while Comissão Nacional De Energia Nuclear (CERN-NN), (2002) the compressive strength at age of 28 days must be greater than or equal to 10 MPa. Based on the result, the values obtained are within the recommended value by the IAEA and CERN-NN. Higher molar concentration of NaOH promotes the dissolution of aluminosilicate in early age, which lead to the increase of strength in early stage (Wei et al., 2019). The composition of the spent resin made up of the monolithic geopolymer was 14 % (wt). According to Natsuda et al., (1992) and Brookhaven National Laboratory, (1981) the solidification of spent resin into Ordinary Portland Cement (OPC) should be restricted to less than 20% to prevent the formation of cracks at higher loadings, which will result in an unstable waste product that tends to deteriorate in water when the waste loading is higher.

Table 6. The Optimum Mix Design for Immobilization of Spent Resin using Geopolymer Based Fly Ash

Details of Mix Proportion		
Parameter	**(Nurul Wahida et al., 2015)**	**Current study**
Ration fly ash to alkaline activator	2.0	2.5
Ratio Na_2SiO_3 to NaOH	2.5	3.0
NaOH (M)	12	14
Distilled water (%)	-	8
Superplasticizer (%)	6.0	2.0
Spent resin (%)	10	14
Curing Temperature	Room temperature	Room temperature
Curing time (hrs)	24	24
Compressive strength (MPa) on 7^{th} day	6.1	15.4

A new parameter, water was added compared to the previous study. Based on table 6.0, shows an increase in compressive strength compared to the study conducted by Nurul Wahida et al., 2015. The workability of the geopolymer concrete mixture can be enhanced and improved by adding more water (Subhash et al., 2013). However, an excessive amount of water will result in reduction of compressive strength. Sagoe-Crentsil et al., (2013) revealed that water takes part in the dissolution, hydrolysis and polycondensation reactions during geopolymer synthesis. It offers a medium for the dissolution of aluminosilicates and the transfer of various ions, hydrolysis of $Al^{3+}$and Si^{4+} compounds and polycondensation of different aluminate- and silicate-hydroxyl species. As a result, water has great effects on the geopolymer formation, structure of the geopolymer gels and properties of the products. According to Chindaprasirt et al., (2007) using extra water to improve workability of fly ash geopolymer had higher compressive strength than adding superplasticizer. In this study, the optimum water percentage obtained was 8%. Superplasticizer was added to improved and enhanced workability properties, strength and

durability of the monolith geopolymer (Potluri et al., 2024). The inclusion of superplasticizer more than 2% resulted in bleeding as well as segregation of fresh geopolymer mixture. It also reduced the compressive strength of the monolith geopolymer

Fig. 2. Samples of Monolith Geopolymer

CONCLUSION

Results from the study shows that conditioning of the spent resin together with fly ash at room temperature can produce geopolymer with reasonable strength properties. The composition of the spent resin that make up the monolith geopolymer was 14 % (wt.), with the monolith geopolymer compressive strength of 15.4 MPa is well within the internationally acceptable value. Adding water in the geopolymer mixture can increase the compressive strength of the monolith geopolymer. This research should be put in a continuous effort in other method of testing such as leaching and durability test. In this way, more reliable data could be produced to support the use of geopolymer technology as an option for immobilization of radioactive waste. Furthermore, immobilization could enhance the handling and transportation of radioactive waste. Solidified waste is less prone to leakage, spillage, or accidental release during handling and transport operations, could reduce the risks to the workers and environment

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