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A REVIEW ON MICROBIAL MUTAGENESIS THROUGH GAMMA IRRADIATION FOR AGRICULTURAL APPLICATIONS

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ABSTRACT

Gamma irradiation is widely used in sterilization and mutagenesis, especially for plant breeding and crop protection. Microbial mutagenesis through gamma irradiation is mainly applied in fermentation industry. In agriculture, gamma irradiation is mostly applied in crop improvement. Microbial mutagenesis is mainly applied against fungus and spore-forming bacteria, which are resistant to gamma irradiation. Response of microbes to gamma irradiation varies and depends on various factors. Review of previous works on gamma irradiation for microbial mutagenesis in agriculture may provide some information for the use of this method. The general view on gamma irradiation, its application, and mutagenesis are discussed in this paper. Further investigation on microbial mutagenesis should consider molecular changes, information on which is lacking in previous works. Moreover, studies on microbial mutagenesis are still lacking in Malaysia despite having several gamma irradiation facilities. Therefore, further studies on microbial mutagenesis should be conducted.

Keywords: Agriculture, gamma irradiation, microbial mutagenesis, mutagens, radiation

INTRODUCTION

Beneficial microorganisms are one of the most environmentally friendly alternatives for chemical pesticides and fertilizers to control plant disease and to enhance plant growth. However, the effectiveness of these beneficial microorganisms is affected by the increased resistance of pathogens and by the effects of global warming. Therefore, mutagenesis or genetic modification of these beneficial microorganisms is needed to improve their effectiveness. Gamma irradiation has been used in agriculture to induce mutagenesis in microbes and to ultimately improve their antagonistic effects, as well as to enhance their enzyme production for fermentation purposes. In agriculture, this approach generally focuses on crop protection. Microorganisms can generate new genetic characteristics by two means: mutation and genetic recombination. Genetic recombination improves the efficacy of selected microbes through genetic manipulation of wild-type strains, resulting in genetically modified organisms (GMOs). This approach requires precise knowledge of the mechanisms that drive the microbes, as well as the structure and regulation of relevant genes. Obtaining complete knowledge of these elements remains difficult and is very costly. Furthermore, acceptance of GMOs by the public remains low, and the use of GMOs in some countries are even restricted. In contrast, the use of mutants obtained through mutagenesis is not restricted. Such mutants can be produced by using chemical or physical mutagens. Mutagenesis is also less costly than production of GMOs. Moreover, this approach does not require a vast genetic knowledge to determine the desired features prior to performing the mutagenesis. During mutation, a gene is modified either through spontaneous mutation or through induced mutation (Adrio and Demain, 2006). Chemical mutagenesis primarily produces single-base substitutions and not drastic

mutations, such as large genomic deletions. Physical mutagens include non-ionizing radiation such as ultraviolet (UV) light, as well as ionizing radiation like ion beams and gamma rays (Huma et al., 2012). Ion beams can locally irradiate target organisms. The loss of small energy allows high-resolution control of the penetration depth of relatively low-energy ions, which may induce local structural damage resulting from atomic displacement (Tanaka et al., 2012). UV rays also exert medium effect, inducing pyrimidine dimerization through frame-shift transition from GC to AT base pair. Gamma rays are the most energetic and highly ionizing radiations, causing mutations through single- or double-strand breakage of DNA resulting from deletion or structural change, DNA-protein cross links, oxidation, bases, and basic sites (Huma et al., 2012). Induction of mutagenesis in microorganisms by using ion beam (Chen et al., 2008; Li et al., 2011), gamma radiation (Afsharmanesh et al., 2013), UV (Huma et al., 2012), or chemical mutagens (Li et al., 2014) to produce thermotolerant mutants in the fermentation industry or disease-controlling mutants in the agriculture industry has also been reported. Malaysia possesses gamma irradiation facilities only. In this review, we discuss the type of radiation, principle of gamma irradiation, general application of gamma irradiation and application of gamma irradiation in microbial mutagenesis especially in relation to agriculture. Review of gamma irradiation in microbial mutagenesis focus on effective doses of mutagenesis, effects on various microbes and methods of mutagenesis.

Gamma Irradiation

Three types of radiation are generally released from uranium, namely, α-, β-, and γ-rays. These ionizing radiations were discovered by British physicist Ernest Rutherford in 1903. The entities of α-ray are helium ions (positively charged particles), those of β-ray are electrons (negatively charged particles), and those of γ-ray are photons (ionizing electromagnetic waves). Atoms are electrically neutral because the number of negatively charged electrons they contain is equal to the number of positively charged protons. During ionization, an atom gains or loses electrons and acquires a net electrical charge when energy sources are available. Atom ionization through γ-ray mainly proceeds through Compton effects (Fig. 1), where the γ-ray collides with and transfers some of its energy to a loosely bound electron in an atom. The γ-ray with reduced energy is then scattered in a new direction and ionizes other atoms until it loses sufficient amount of energy that can facilitate ionization reaction. The γ-ray collision with an electron is ejected from its atom and acts as β-ray (negatively charged particle) to create a new ionization that mainly occurred as a result of inelastic collision (Narumi, 2006)

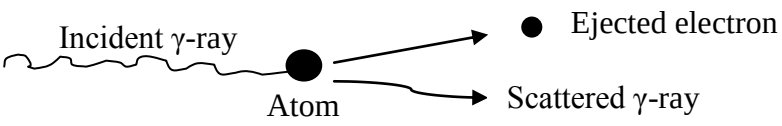

Figure 1: Compton effect

The main effect of exposure to radiation is caused by the alteration of DNA structure, which is a component of chromosome in organism cell. Another indirect effect is formation of free radical where ionising radiation cause excitation, ionisation and breakage of molecules (Fig. 2). This process will lead to biological changes of the cell (Ramli et al., 2002).

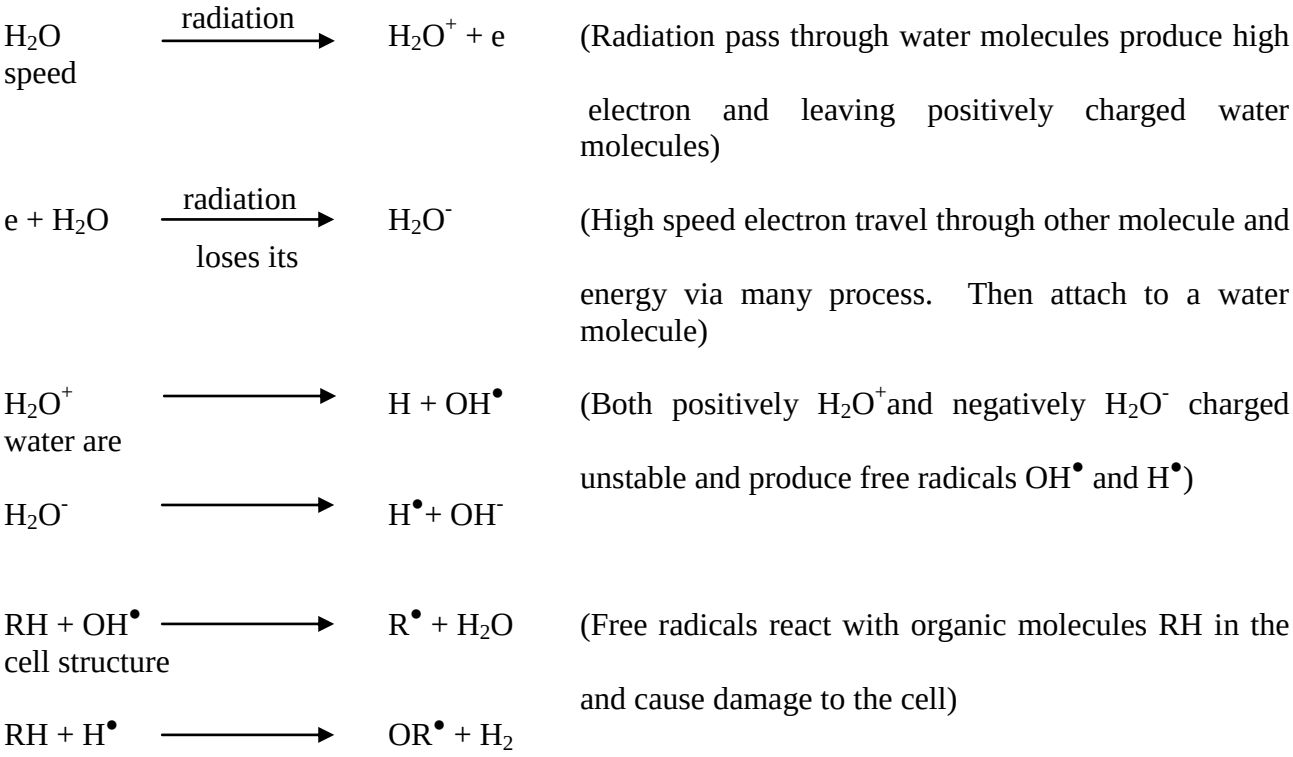

Figure 2: Formation of free radical

Irradiation can lead to changes in physical appearance and metabolism in organisms. Changes that result from irradiation are random events, inheritable, and their stability depends on cell damage after irradiation at molecular levels. Moreover, the degree of radiation resistance and changes in living organisms vary. The type of radiation, duration of radiation, distance of radiation source and others also affect these changes. Two general types of gamma irradiations exist. The first type is chronic irradiation, which is an exposure to ionizing radiation over an extended period (high cumulative dosages with low-dose rate). The second type is acute irradiation, which is an exposure to ionizing radiation for a short period (high cumulative dosages with high-dose rate). In Malaysia, chronic and acute irradiation facilities are both available. At the Malaysian Nuclear Agency, the gamma greenhouse is a chronic irradiation plant (^{137}Cs source), whereas gamma cell (^{137}Cs source) and Sinagama (^{60}Co source) are acute irradiation plants. Synergy Sterilization (M) Sdn. Bhd. is a private gamma irradiation plant. Gamma greenhouse and gamma cells are used for mutagenesis, whereas the other facilities are used for sterilization.

Application of Gamma Irradiation

Gamma irradiation is applied in various ways. Gamma irradiation is commonly used for sterilisation of pharmaceutical and medical products. It is also typically use for decontamination of foodherbs and its product (Aquino, 2012; Narumi, 2006). The most common dose to sterilize medical products is 25 kGy (Aquino, 2012). For example, radiation sterilization at 25 kGy does not cause any undesirable change in the biophysical properties of biological dressing, including human amniotic membranes (Nor Kamalia et al., 2014). Fermentation industries widely use gamma irradiation to produce thermotolerant microbe mutants. *Saccharomyces cerevisiae* mutant that is highly tolerant to ethanol concentrations and temperatures (up to 42°C) was produced through gamma radiation (Mehdikhani et al., 2011). This microorganism is an important microorganism in

the bio-industry, especially in sugar fermentation where high temperature (35 – 45°C) and high ethanol concentration (over 20%) are involved. A thermotolerant *Phialocephale humicola* mutant was also produced through gamma radiation (Huma et al., 2012). This mutant is important in thermostable α-amylase production in starch fermentation.

Actinomycetes, such as *Streptomyces fradiae*, are important in producing tylosin, which is used as veterinary medicine and feed supplement to stimulate the growth of young animals. The combination of gamma irradiation and UV treatment generates a mutant that produces a higher amount of tylosin compared with the wild type (Khaliq et al., 2009).

In cellulase enzyme production, Shahbazi et al. (2014) reported that *Trichoderma reesei* irradiated at 250 Gy of gamma ray produced a maximum amount of cellulose compared with UV-irradiated mutant and wild-type strains. Gamma irradiation at 2 kGy of *Aspergillus niger* also enhances the production of carboxymethyl cellulase and filter paper cellulase (Mostafa, 2014).

Gamma irradiation also plays important role in the biofertilizer industry. Barend and Henri (1981), Shamsuddin (2005) and Phua et al. (2009) reported that gamma irradiation at 50 kGy produces sterile carriers that improve the shelf life of biofertilizers. In plant breeding area, gamma irradiation is widely used in mutagenesis to improve crops and to generate new varieties of them. Improved protein content of *Citrus sinensis* was obtained by irradiating the plantlets at 50 Gy (Anna et al., 2008). Two drought resistant mutants, namely, MR 219-4 and MR 219-9, were produced at the Malaysian Nuclear Agency by using gamma rays at 300 Gy (Ibrahim et al., 2013). The combination of gamma irradiation and ethyl methane sulfonate (EMS) also produced five tomato mutants (Sikder et al., 2013).

In biological control, gamma irradiation was applied to generate pest and disease resistant plants or to improve biological control against plant disease. By using gamma rays and ion beams, the Malaysian Nuclear Agency generated two orchid mutants (*Dendrobium mirbellianum* and *D. jayakarta)* that are resistant to thrips and mites. Banana mutants that show improved qualities (*Fussarium* wilt resistance and high fruit quality) were also obtained though gamma irradiation (Ibrahim et al., 2013). Optimum doses for the application of gamma irradiations are highly dependent on the types of biological materials and the objective of the work. High doses are used for sterilization, medium doses for decontamination whereas low doses are used for mutagenesis.

Application of Microbe Mutagenesis in Agriculture

Microbes are generally more resistant to mutagenesis than animals and plants. This observation can be attributed to the size of microbes, which are small; their nuclei are also considerably small and thus are difficult to target during irradiation. Mutagenesis of microbes is more complicated than that of other organisms. Moreover, microbes demonstrate the capacity for DNA protection and repair. DNA protection includes spore formation (resting stage of cells) and use of radical scavengers, such as catalase, superoxide dismutase, and carotenoids. Some spore-forming bacteria, such as *Bacillus* sp. and *Clostridium* sp., are resistant to irradiation. However, when nutrients are available, the spore germinates to produce a vegetative cell that is much more sensitive to radiation (Narumi, 2006). Prokaryotes (bacteria) and eukaryotes (molds and yeasts) both can repair many DNA breaks. In addition, environmental conditions during irradiation, including temperature, water content, medium, and oxygen, may affect the resistance of microbes to irradiation. Increasing the temperature (above 45°C) also increases the effect of irradiation on vegetative cells. Vegetative microbes are more resistant to radiation at subfreezing temperatures than at ambient temperatures. The diffusion of radicals in their frozen state is also considerably restricted. The presence of oxygen

also increases the lethal effect of irradiation on microbes. In addition, increasing the water content increases the effects of irradiation on microbes; this phenomenon results from the increased formation of free radicals from water molecules caused by radiation. Furthermore, different compositions of medium surrounding the microbes result in varying effects of irradiation (Aquino, 2012). Thus, application of gamma irradiation for microbe mutagenesis in plants is much more complicated than mutagenesis.

The effects of irradiation can be measured via two means, namely, decimal reduction dose (D_{10} value) and 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. D_{10} value varies for different microbes, irradiation conditions, and purposes. In addition, the effects of gamma irradiation on Gram-positive and Gram-negative bacteria differ. The radiation dose required to reduce viable bacteria by 1 $\log_{10}$ (D_{10} value) is 0.31 and 0.35 kGy for *Staphylococcus epidermidis* and *Escherichia coli*, respectively (Trampuz et al., 2006). Another study tested the survival of bacterial isolates under radiation doses of 1 – 10 kGy. *Streptococcus* sp. continued to grow even up to 9 kGy, but all of the isolates died at 10 kGy. Thus, Gram-positive bacteria can tolerate high doses of radiation. In contrast, all Gram-negative isolates, such as *Pseudomonas* sp., died after exposure to 5 kGy (Atique et al., 2013). These studies aimed to test the effect of gamma irradiation that can kill bacterial isolates (for sterilization purposes). Studies on mutagenesis applied the lethal dose (LD_{50}), where 50% of irradiated cells died. Both values can be obtained by plotting the survival curve (Figure 3).

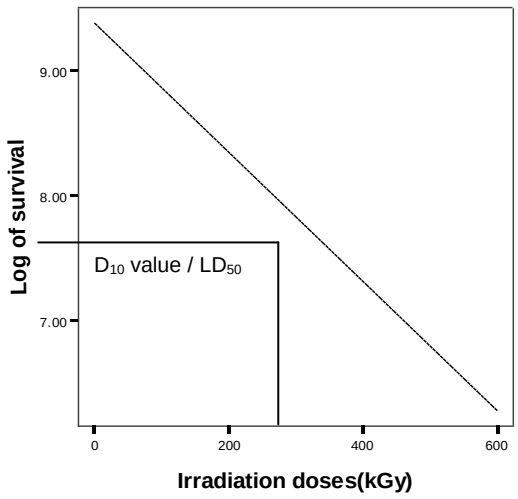

Figure 3: Survival curve for irradiation experiment

In view of the complexity of microbe mutagenesis, gamma irradiation is not widely applied to induce mutagenesis. Microbe mutagenesis is mostly applied in fermentation and cellulose enzyme industries. In agriculture, microbe mutagenesis is mainly applied for biological control. *Trichoderma* sp., *Fusarium* sp., and *Bacillus* sp. are widely used in mutagenesis through gamma irradiation. One possible reason for the wide application of these microbes in mutagenesis study is that they are spore-forming microbes. Another possible reason is that they are well-established microbes, especially as biological control agent in various agricultural studies. With the available background information, mutagenesis in these microbes is easier to study. Since microbe mutagenesis is a complex process and affected by various factors reviewing previous studies is

necessary in order to gain additional information to serve as guidelines, especially on the effective doses, irradiation conditions, and mutagenesis methods.

Haggag (2002) used two biological control agents, namely, *Trichoderma harzianum* and *Trichoderma koningii*, for mutagenesis. Slant cultures (14-day-old) were exposed to 50 krad and 75 krad of gamma irradiation. Conidial suspension was plated on a medium, and selection was performed based on phenotypic variation. The spore-forming microbes were counted by using the diluted plate technique. Subculture was performed seven times to test the stability of the microbes. Inhibition of *Botrytis cinerea* (a pathogen that causes grey mold disease in tomato and cucumber) by mutants and wild-type microbes was performed through in vitro bio-control assay. The mutant and wild-type *B. cinerea* were also tested for chitinase production by using chitinase assay and chemical analysis. Field experiments on tomato and cucumber were also performed. TH12, TH18, TK5, and TK15 (mutants obtained through irradiation at 50 krad) most effectively control grey mold disease in fruits and stems. They also reduce pathogen sporulation and improve yield. Three *Trichoderma* species (T. *harzianum*, *T. viride*, and *T. koningii*) have been used for gamma irradiation mutagenesis to produce a biological control agent for white rot disease caused by *Sclerotium cepivorum* in onion (Haggag and Mohamed, 2002). In this study, sporulation cultures were exposed to gamma irradiation at 20 krad for 75 min or at 50 krad for 180 min. The methods used for mutant selection and antagonistic test were similar to those described by Haggag (2002). Chitinase and other enzymes were detected by using polyacrylamide gel electrophoresis. Moreover, antibiotics and total phenolic compounds were determined by high-performance liquid chromatography. Field experiment on onion plant was also performed. The mutants TK509, TH508, and TV208, which were irradiated at 50 krad for 180 min, produced more isozyme, chitinase, antibiotics, and phenolic compounds compared with the wild type. In addition, no diseases were found in the treatments amended with these mutants. These works revealed that the effective doses to obtain mutants either in sporulation or non-sporulation cultures is 50 krad. Furthermore, *T. harzianum* was irradiated at 0, 50, 150, 200, 250, 300, 350, 400, and 450 Gy. At doses above 450 Gy, spore germination is totally inhibited. Three mutants, namely, T.h M8, T. h M16, and T. h M13, were obtained by irradiating *Trichoderma* at 250 Gy. These mutants inhibited the growth of *Rhizoctonia solani* (soil-borne pathogen) as revealed by dual culture assays. This result suggested that gamma-irradiated mutants exhibit better bio-control than the wild-type microbes (Naseripour et al., 2014). Abbasi et al. (2014) performed an experiment similar to that conducted by Naseripour et al. (2014), and the same results were obtained. The mutants Th1 and Th9 were found to be good bio-control mutants for charcoal rot in melon (*Macrophomina phaseolina*) under laboratory and greenhouse conditions. Baharvand et al. (2014) also reported that *T. viride* irradiated at 250 Gy exhibits improved antagonistic capability against *M. phaseolina.*

An *avrI*-2 mutant of *Fusarium oxysporum* f. sp. was generated by using ^{137}Cs gamma irradiation at 130 Gy. This mutant was expected to demonstrate loss of avirulence; surprisingly, it also displayed reduced pathogenicity toward susceptible tomato plants. Southern analysis on contour-clamped homogeneous electrophoretic field blots demonstrated a translocation of a 3.75-Mb chromosome in this mutant. Random amplified polymorphic DNA and amplified fragment length polymorphism analysis identified at least nine polymorphisms between the wild-type and the mutant isolates. Most of these polymorphisms appeared as extra fragments in the mutant and contained repetitive DNA sequences. These polymorphisms were possibly generated by a mobile element that has been duplicated and inserted in new sites (Mes et al., 1999). Moreover, *Fusarium solani* conidia were irradiated at 0, 60, 90, 120, 150, and 180 Gy. The percentage of spore germination after 18 h, as well as the diameter of generated colonies after 10 days, was scored. Avirulent mutants were selected from greenhouse experiments by using bean seed inoculation method. Bio-control test for

Fusarium root rot were also performed in greenhouse and field experiments. The mutant avr-M23 (irradiated at 130 Gy) is a good bio-control agent against *Fusarium* root rot (Mostafavi et al., 2012).

Mutagenesis of *Isaria fumosorosea* though ionizing radiation by using ion beams or gamma rays or a combination of the two was also reported (Shinohara et al., 2013). Six *I. fumosorosea* mutants demonstrating enhanced resistance against the fungicide benomyl were obtained. These mutants are Ib-34 and Ib-421, which were obtained through ion-beam irradiation and were irradiated at 200 and 300 Gy, respectively; Gr-5 and Gr-22 (both were irradiated at 1000 Gy) obtained through gamma ray irradiation; GrIB-8 and GrIB-9, which were both obtained through dual irradiation at 500 Gy of gamma rays followed irradiation at 200 Gy of ion beams. These mutants also demonstrated enhanced resistance to other fungicides at recommended field application rates. In addition, no differences were observed at the β-tubulin locus (gene involved in benomyl resistance) of the wild-type and mutant isolates, suggesting that the enhancement is not attributable to gene mutations.

Pantoea dispersa was mutated by using UV and gamma irradiation (0.3 – 2.4 kGy), as well as by using chemical mutagens such as ethylmethane sulfonate (EMS), to improve their chitinolytic enzyme production. Gamma mutant no. 8 produces higher chitinolytic enzymes, protease, and β-1,3-glucanase compared with the wild-type. Gamma mutant no. 8 was further mutated through EMS (mutant no. 10). In vitro screening showed that compared with the wild-type strain, mutant no. 10 increases growth inhibition of *Fusarium* sp. and *Macrophomina phaseolina* (Tassi) Goidanich, a root-borne plant pathogen of *Cajanus cajan* (Gohel et al., 2004).

Random mutagenesis in *B. subtilis* UTB1 was conducted by using different doses of gamma irradiation (0.1 – 3 kGy) to improve its antagonistic activity against *Aspergillus flavus* R5. At irradiation doses of 2, 2.5, and 3 kGy showing 3 – 4 log of the microbes were killed. Rep-PCR fingerprinting analysis of bacterial mutants was also performed. In addition, mutant selection was performed based on different polymorphism patterns. Eight mutants were selected from 45 isolates. Six mutants (M419, M425, M455, M464, M562, and M600) demonstrated enhanced production of bio-surfactants on blood agar medium and as revealed by oil spreading technique. They also displayed more robust biofilm compared with the wild-type. These findings suggested that the mutants are promising bio-control candidates against *A. flavus* in pistachio nuts (Afsharmanesh et al., 2013). Further investigation using thin layer chromatograms showed that the production of lipopeptides, including surfaction, fengycin, and iturin families, increased in these six mutants. Furthermore, a considerable inhibition of fungal growth, which is associated with iturin production, was observed using bioautography analysis. *A. flavus* sporulation and aflatoxin content also decreased significantly in pistachio nuts treated with mutant M419 and M 464 compared with those treated with the UTB1 strain (Afsharmanesh et al., 2014). Mutants for *Trichoderma* sp. are 250 Gy, 130 Gy for *Fusarium* sp., and 1000 Gy for *I. fumosorosea*, whereas the optimum doses for *P. dispersa* and *B. subtilis* are 0.3 – 2.4 and 2 – 3 kGy, respectively. Thus, determining the optimum doses for microbial mutagenesis is difficult. In addition, the use of low doses only to produce improved mutants is unnecessary. Survival curve should then be obtained to determine the optimum doses. Moreover, the combination of different types of mutagen also generates good mutants. The studies reviewed in the present paper did not perform significant molecular investigations.

CONCLUSIONS

Various methods as well as optimum doses and conditions are available to generate gamma-irradiated microbial mutants. In Malaysia, studies on gamma irradiation for microbial mutagenesis are still lacking. Malaysia is one of the Asian countries that provide both chronic and acute gamma

irradiation. Thus, further investigation on microbial mutagenesis should be performed. Moreover, molecular information on microbial mutagenesis is insufficient. Thus, further investigation should be performed to determine the molecular changes induced by irradiation.

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