Radiation-induced mutation of Pleurotus sajor-caju for the improvement of mycelial growth, productivity of fruiting bodies and generation of bioactive compounds with radioprotective properties

A Dissertation Submitted to the University of Fukui for the Degree of Doctoral of Engineering

Radiation-induced mutation of *Pleurotus sajor-caju* for the improvement of mycelial growth, productivity of fruiting bodies and generation of bioactive compounds with radioprotective properties

菌糸の成長、子実体の生産性、および放射線防護特性を持つ生理活性物質の生成を改善するための放射線照射によるハイイロヒラタケの突然変異誘発

2025 March

Rosnani binti Abdul Rashid

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Chapter 1: General Introduction

1.1 Introductory remarks

The International Atomic Energy Agency (IAEA) actively promotes the use of radiation and radioisotopes in research and their applications in industry, agriculture and medicine.[1)] Knowledge on radiation effects on plants, microbes and living organisms a prerequisite in radiation application in these socio-economic sectors. To date a considerable number of studies or works on the radiation application in these field have been reported.[2)] In agriculture, application of radiation for mutation induction of crops quite common amongst breeders and researchers. Mutation induction by radiation is important for improving useful traits and selected characteristic of plants as well as mushrooms due to market niche and problem faced by the industries. The aim of this study to investigate and clarify the effects of the gamma radiation prior to mutagenesis of *Pleurotus sajor-caju* with the improvement characteristics such as better size, high productivity and bioactive compounds. The improvement characteristics and findings from this study have a great potential and application to support and expanding the agriculture and medicine nuclear field.

The combination of irradiation and fungal biodegradation was used for bioconversion of natural polymer into useful products has been reported. Mushrooms were chosen as the biodegradation agents due to their edible feature as the product output of the process were for animal feed and human consumption.[3)] The interest in mushrooms mutation induction arises early in the year 2000. Following this interest, mushroom mutation induction project was initiated, primarily to find way to overcome challenges faced by the local mushroom industry in Malaysia, which include slow growth, low productivity, and high percentage of contamination.[4)] In the present study, the aim are to obtain new varieties of *P. sajor-caju* mushroom with better morphology, high productivity and high bioactive compounds content through radiation mutation induction. These bioactive compounds will be studied on their potential as protective agents. *P. sajor-caju* mushroom was chosen due its high demand and being the most commercially cultivated in Malaysia.[5)] Extracts from fruiting bodies and mycelia of *Pleurotus* spp. have been reported to have antioxidant, antitumour and immunomodulating effects.[6)]

In this study, the effects of gamma radiation of *P. sajor-caju* on mycelial growth and productivity was conducted at Malaysian Nuclear Agency. Irradiation of *P. sajor-caju* mycelia was carried out at the Biobeam GM 800 radiation facility using caesium-137 as the gamma source at LD50 dose of 2.2 kGy, with dose rate of 0.227 Gy s-1. The LD_{50} of *P. sajor-caju* mushroom

irradiated with gamma rays was determined to be at 2.2 kGy has been reported.[7] The LD_{50} value is one of the parameters to predict radiosensitivity level and determine the optimum effect of mutation in plants.[8, 9, 10] It is particularly important to determine the effects of LD_{50} prior to inducing mutation.[11] Irradiated mycelia were screened and selected based on mycelia growth performance. The surviving irradiated mycelia with the fastest growth characteristic were selected and used for preparation of liquid seeds for mushroom cultivation. From the cultivation, results of productivity and morphology characteristics of fruiting bodies such shape of cap, diameter of cap and length of stipe were obtained. Results showed that growth of gamma irradiated mycelia were slower than that of non-irradiated mycelia. However, the productivity from irradiated mycelia was higher than the non-irradiated, as shown by the high number and size of the fruiting bodies. Studies on increasing of mushroom productivity by radiation on mycelia and basidiospores for mushroom have been reported.[12, 13, 14, 15]

Many mushrooms have high antioxidant contents, which enable them to scavenge free radicals and prevent cell damage.[16] Numerous studies have been conducted to investigate bioactive compounds in mushrooms, including studies on the effects of bioactive compounds from mushroom extracts on cancer patients. For example, the dietary intake of mushrooms was shown to minimize undesirable side effects after chemotherapy and radiation therapy.[17] Radioprotective agents have been reported to minimize the effects of radiation such as hypotension, vomiting, nausea, sneezing, and hot flashes. [18] The discovery and development of antioxidant radioprotectors with less toxicity is also essential. For example, the radioprotective effect of the *Cordyceps militaris* mushroom has been reported.[19]

Phenolics and flavonoids are bioactive compounds, known as antioxidants, that have gained interest among researchers due to their benefits for human health. The total phenolic content (TPC) of the extracts was determined by Folin–Ciocalteu assay and the total flavonoid content (TFC) was determined by aluminum chloride assay. The antioxidant activities were determined by performing 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP). The results suggested that the possibility of gamma radiation increased the bioactive contents of *P. sajor-caju* as well as their antioxidant activities. The radioprotective effect of an extract of *Pleurotus ostreatus* mushroom on mice exposed to gamma radiation has been reported. [20] In this present study, the bioactive compounds from *P. sajor-caju* were studied for their potential as radioprotectant on yeast cells. The aim of this study was to investigate and gain better

understanding on the protective effect of bioactive compounds extracted from *P. sajor-caju* against the oxidative stress induced by gamma radiation on yeast cells. Yeast was selected to be used in this study due to it being a model organism in radiation biology studies, especially on responses to ionizing radiation. It has a short life cycle and has the same characteristic as a human cell. [21)]

Ionizing radiation, for example gamma radiation can trigger the formation of free radicals, which induced biological damage even at an extremely low dose. [22)] This ionization process will lead to production of short-lived free radicals, which will interact further with other biological molecules in the cell including DNA. [23)] Therefore, to protect the cells from damage and to support cell functions, antioxidants are needed to scavenge these free radicals. The understanding of biological effects on yeast cells by ionizing radiation and protection effects of bioactive from mushroom will help for development of radioprotection agent from natural sources especially for medicinal or clinical use.

1.2 Objectives

The objectives of this present studies are:

1. To evaluate the effects of LD_{50} gamma radiation on mycelium growth, morphologies and productivity fruiting bodies of *P. sajor-caju*
2. To evaluate the effects of LD_{50} gamma radiation on bioactive compounds of *P. sajor-caju*
3. To investigate the protective effects of bioactive compounds from irradiated *P. sajor-caju* on yeast cells

1.3 Outline of thesis

The present thesis consists of seven chapters including this as an introductory remark and other chapters dealing of radiation application in mushroom research studies. especially the effects on morphology characteristics and bioactive compounds and its application as radiaoprotective agents on living organisms. The living organism are used in this study are yeast cells wild type S288c.

In **Chapter 1**, a background of radiation application in mushroom study and reasons why the authors were interested to study the LD_{50} gamma radiation effects on mycelium growth,

productivity and fruiting bodies, bioactive compounds and its potential as radioprotectant agent on yeast cell, represented in this thesis are described.

Chapter 2, provides a background of the mushrooms industry and radiation application in studies of mushroom such as for sterilization mushroom substrate for cultivation, sterilization fruiting bodies after postharvest and mutation induction.

Chapter 3, gives a review on studies or works on radiation protection against with addition of radioprotective agent was discussed and summarized. This chapter also includes introduction of radiobiology study, effects of radiation on biological samples, types of biological samples, free radical, radiation protection, natural radioprotector and natural sources for natural radioprotector.

In **Chapter 4**, the effects of LD_{50} gamma irradiation on mycelium growth, morphology and productivity fruiting bodies are described. The study was conducted at the Biobeam GM 800 radiation facility, Malaysian Nuclear Agency using caesium-137 as the gamma source at LD50 dose of 2.2 kGy, with dose rate of 0.227 Gy s-1. Non-irradiated mycelia were used as control. The irradiated mycelia of *P. sajor-caju* were measured after radiation for 12 days with 3 days interval. The fastest growing and healthy mycelia were selected for the cultivation in baglogs of saw dust substrate. At this stage, the mycelial growth also was measured once a week until the mycelia fully covered the substrate. The baglogs substrate with fully covered by mycelia were opened for fruiting. Data for morphologies and productivity were recorded. The results on mycelial growth, morphology and productivity was obtained and discussed.

In **Chapter 5**, the effects of LD_{50} gamma irradiation on bioactive compounds as well as total phenolics content (TPC), total flavonoid content (TFC) and antioxidant activities from *P. sajor-caju* are investigated and discussed. The bioactive compounds of *P. sajor-caju* was extracted by water, ethanol and azeotropic (mixture of water and ethanol) solvents. The yield of crude extract from irradiated *P. sajor-caju* mushroom is higher compared to non-irradiated *P. sajor-caju* for all types of extraction solvent. The azeotropic (mixture of water and ethanol) solvent gave the highest yield of extract. The TPC was determined by Folin-Ciocalteu assay and TFC by aluminium chloride assay. The antioxidant activities of *P. sajor-caju* was determined by 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) and Ferric Reducing Antioxidant Power (FRAP) assay. The effects of LD_{50} on bioactive characteristics of *P. sajor-caju* mushroom were discussed based on TPC, TFC and antioxidant activities obtained.

Chapter 6, discusses the protective effect of bioactive compounds extracted from *P. sajor-caju* mushroom against the oxidative stress induced by gamma radiation on yeast cells are described. This study was conducted to investigate the potential of bioactive compounds from *P. sajor-caju* as radioprotectant on irradiated yeast cells. The yeast cell was exposed to gamma radiation at doses up to 150 Gy. The effects were discussed based on percentage of survival and mutant frequency obtained.

In **Chapter 7**, the conclusion from the present study is summarized.

1.4 References

1) S. Ravindra. Applications of radioisotopes in agriculture. *Times of agriculture*. 16: 88-90 (2021)
2) Y. Amano. Radiation technology for a more prosperous and sustainable future. *IEAE Bulletin*: Radiation Technology the industrial revolution behind the scenes. Vienna, Austria (2017)
3) T. Kume, S. Matsuhashi, H. Ito, S. Hashimoto, I. Ishigaki, A. Mat Rasol, J. Muhamad Lebai, O. Zainon, A. Foziah, W. H. Wan Badrin, and M. Hassan Hamdani. Study on upgrading of oil palm wastes to animal feeds by radiation and fermentation processing. *JAERI-Research*. 98-013 (1998)
4) A. Mat Rasol, A. R. Rosnani, M. Azhar, M. Hassan Hamdani and M. Mohd Meswan, Mushroom liquid seed. *MOSTI Year Commercialization Melaka* (2014)
5) A. R. Rosnani, M. Mohd Meswan, S. Shuhaimi, A. N. Mohd Hafiz and H.M.H. Hamdani Hassan Mutaat. Biological effects of LD_{50} gamma radiation on fruiting bodies of *Pleurotus* sp. mushroom. *Konvensyen Teknikal Nuklear Malaysia* (2021)
6) C. O. Bamigboye, I. O. Omomowo, M. B. Alao, J. A. Elegbede and E. A. Adebayo. Free radical scavenging ability, mechanisms of action and health implications of oyster mushrooms (*Pleurotus* species). *Journal of Microbiology, Biotechnology and Food Sciences.* 10 (4): 636-647 (2021)
7) A. R. Rosnani, D. Fauzi, A. Mat Rasol, M. Hassan Hamdani, S. Sahidan, M. Azhar, M. Mohd Meswan and K. Abdul Rahim. Evaluation of mycelia growth, morphology and yield for low dose gamma irradiated grey oyster mushroom *Pleurotus sajor-caju. International Journal of Innovation and Scientific Research*. 24 (2): 332-336 (2016)

8) S. S. Harding and O. Mohamad. Radiosensitivity test on two varieties of Terengganu and Arab used in mutation breeding of roselle (*Hibiscus sabdariffa* L.). *African Journal Plant Science*. 3 (8): 181-183 (2013)

9) L. Horn and H. Shimelis. Radio-sensitivity of selected cowpea (*Vigna unguiculata*) genotypes to varying gamma irradiation doses. *Scientific Research and Essays*. 8(40): 1991-1997 (2013)

10) A. Kumar, S. Paul and G. Thakur. Determination of lethal dose (LD50) and effects of gamma rays and ethyl methane sulphonate (EMS) induced mutagenesis in linseed (Linum usitatissimum L.). *International Journal of Current Microbiology and Applied Sciences*. 9 (10): 2601-2608 (2020).

11) I. R. Dewi, Herman, and F. Isro. Lethal dose 50 (LD50) of mungbean (Vigna radiata l. wilczek) cultivar kampar. *SABRAO Journal of Breeding and Genetics*. 47 (4): 510-516 (2015)

12) I. Djajanegara and A. Harsoyo. Mutation study on white oyster mushroom (*Pleurotus floridae*) using gamma (^{60}CO) irradiation. *Journal of Chemical and Natural Resources Engineering*. 4 (1): 12-21 (2000)

13) P. H. F. Beejan and R. Nowbuth, Use of irradiation for the induction of mutations in oyster mushrooms for improvement of strains. In. Induced Plant Mutations in the Genomics Era. Q. Y. Shu (ed.). *Food and Agriculture Organization of the United Nations, Rome*: 289-292 (2009)

14) E. A. Adebayo, J. K. Oloke, A.K. Bordoloi, M. Barooah and T. C. Bora. Improvement of *Pleurotus pulmonarius* Lau 09 through mutation for yield performance. *Research Journal of Mutagenesis*. 4 (1): 1-13 (2014)

15) R. Sharma and B. M. Sharma. Strain improvement in *Pleurotus ostreatus* using UV light and ethyl methyl sulfonate as mutagens. *African Journal of Microbiology Research*. 8 (5): 432-436 (2014)

16) C. Sánchez. Modern aspects of mushroom culture technology. *Applied Microbiology Biotechnology*. 64 (6): 756–762 (2004)

17) M. Jayachandran, J. Xiao and B. Xu. A Critical Review on Health Promoting Benefits of Edible Mushrooms through Gut Microbiota. *International Journal of Molecular Sciences.* 18 (1934): 1-12 (2017)

18) A. Shirazi, E. Mihandoost, S.R. Mahdavi and M. Mohseni. Radio-protective role of antioxidant agents. *Oncology Reviews*. 6 (e16): 130-134 (2012)

19) M.H. Jeong, Y.S. Park, D.H. Leong, C.G. Lee, C. G., J. S. Kim, S. J. Oh, S. K. Jeong, K. Yang and W.S. Jo. In vitro evaluation of Cordyceps militaris as a potential radioprotective agent. *International Journal of Molecular Medicine.* 34: 1349-1357 (2014)

20) G. Llaurado´, H. J. Morrisa, V. Tamayo, Y. Lebeque, Y. Beltra´n, J. Marcos, S. Moukhae, E.E. Creppy and R.C. Bermu´dez. Haematopoiesis radioprotection in Balb/c mice by an aqueous mycelium extract from the Basidiomycete *Pleurotus ostreatus* mushroom. *Natural Product Research*: 1-5 (2014)

21) G. Cao, M. Zhang, J. Mioa, W. Li, J. Wang, D. Lu and J. Xia. Effects of X-ray and carbon ion beam irradiation on membrane permeability and integrity in *Saccharomyces cerevisiae* cells. *Journal of Radiation Research.* 56: 294–304 (2015)

22) K. N. Prasad. Rationale for using multiple antioxidants in protecting humans against low doses of ionizing radiation. *The British Journal and Radiology*. 78: 485-492 (2005)

23) C. Borek. Antioxidants and radiation therapy. *American Society for Nutritional Sciences. Journal Nutritional.* 13: 3207S-3209S (2004)

Chapter 2: Radiation Application in Studies of Mushrooms

Chapter 2 gives a review and provides a background on radiation, radiation application in studies of mushroom, such as sterilization of mushroom substrate for cultivation, postharvest sterilization of fruiting bodies, and mutation induction. This chapter will also review details on findings as reported by other researchers, including on the effects or radiation and the resulting improvement of mushroom characteristics s achieved through irradiation.

Abstract

Radiation is widely used in our life due to its benefits and advantages. The background of radiation, radiation application including in the mushroom industry, will be described and discussed. This chapter provides an introduction of radiation application in studies of mushroom such as for sterilization of mushroom substrate, sterilization for postharvest treatment of mushroom fruiting bodies and mutation induction. In this review, the status of mutation induction studies and research on mushroom for the development of varieties with desired traits such as high yield, high temperature tolerance, low-sporing, shelf-life prolongation and high bioactive compounds are discussed. The radiation application on mutation induction for morphology and chemical characteristics will be elaborated in detail and applied in subsequent chapters and this present study.

2.1 Introduction

The demand for mushrooms is rapidly rising due to their healthy, nutritious, and medicinal values. The global mushroom market size was valued at USD 50.3 billion in 2021 and is expected to expand at a compound annual growth rate (CAGR) of 9.7% from 2022 to 2030.[1)] In Asia, mushrooms were first cultivated in the 12th century. The first mushroom species cultivated was *Agaricus bisporus*, followed by shiitake mushroom.

China is the largest producer of cultivated and edible mushrooms in the world followed by the United States of America, the Netherlands, Poland, Spain, and France. Japan was the world major producer of *Lentinus edodes* (shiitake in Japanese or xianggu in Chinese) until the mid-1980s.[2)] Initially, mushroom was grown on hardwood oak logs. Later, *Pleurotus* spp. were cultivated on straws, shiitakes on logs and enoki in bottle cultures.

In Malaysia, mushrooms have been cultivated since the early 1970s and it is still considered a small and growing industry. Seventeen types of cultivated mushrooms have been recorded. However, of these, only eight species are preferred, where the most popular is *Pleurotus* spp. due to their high demand for consumption, and due to favourable temperature and humidity for growth of these mushrooms.[3)] *Pleurotus sajor-caju* was selected to be studied in the present work due it being the most cultivated and has high market demand in Malaysia.

Since early 80's, radiation has Since then, research and development activities in radiation technologies have been explored and applied in many industries, including in agriculture and the food sectors. Radiation technology is considered as a clean or green technology due to less waste generation and is capable to operate in bulk capacity in a short time. Agroindustry is one of the industries where radiation technology is used, for example in soil and water management, pest control, food safety and mutation induction.

Mutation induction is also known as mutagenesis, which can be extended to mutation breeding. It is an alternative and a complementary technique in breeding programmes for the introduction of genetic changes and the establishment of new genetic resources.[4)] Mutation induction has been investigated and utilized for a long time in many countries. [5)] Radiation mutation breeding has been used for around 100 years and has successfully for improved crops by increasing genetic variations.[6)]

Mutation induction technology began in the early 1930s, mainly using X-ray as an agent of mutation. Since 1950s, this technology has been widely used specifically in crops with low genetic variability, and those that are not amenable to improvement through conventional breeding methods. [4)] The number of physical and chemical mutagens are numerous and rising. Examples of physical mutagens include gamma rays, beta particles, neutrons, electron beams and ion beams, while examples of chemical mutagens include sodium azide, ethylmethanesulfonate and colchicines.[7, 8, 9)]

In Japan, mutation induction programmes accelerated since the construction of the gamma field at the Institute of Radiation Breeding (IRB) in 1960.[10)] Meanwhile, Malaysia has been involved in plant breeding and improvement through mutagenesis programmes since 1980s, many of which were led by Malaysian Nuclear Agency (Nuklear Malaysia), which was formerly known as Malaysian Institute for Nuclear Technology Research, or MINT.[4)]

Through mutation breeding, many plant varieties have been generated with improved traits such as high yield and productivity, superior quality are the principal goals for the improvement of crops, including mushrooms.[11] According to International Atomic Energy Agency (IAEA) Mutant Varieties Database (http://www-mvd.iaea.org) published on 22 March 2022, 3,402 mutant varieties have been registered and more than 1,000 new varieties have been used, promoted, or commercialized worldwide. [6] Of these, four (4) mutant varieties of *Pleurotus* spp. mushrooms have been produced by Mauritius, and the improved characters include high yield, fruit size and growth habits.

Mushrooms have been used as food source as well as for medicinal purposes due to the nutritional and benefits to health. Mushrooms are also known as fungi, of which mostly belong to the Class of Basidiomycetes and to a lesser extent the Class of Ascomycetes. China is the main producer of cultivated and edible mushroom in the world, with 40% of total world mushroom are exported from China.[2, 12] In Malaysia, the mushroom industry is still small and is expanding, out of domestic demand.[13] Therefore, developing new varieties with high yield and novel characteristics is critical for the enhancement of the local mushroom industry.

In this chapter, the radiation application especially in studies of mushroom for sterilization of mushroom substrate, sterilization for postharvest treatment of mushroom fruiting bodies and mutation induction are deliberated. The status of mutation induction studies and research on mushroom for the development of varieties with desired traits such as high yield, high temperature tolerance, low-sporing, shelf-life prolongation, and high bioactive compounds from findings and reports by researchers worldwide will be reviewed and summarized.

2.2 Radiation

Radiation is the emission or transmission of energy through space or through medium. Radiation emits or transmits the energy, and radiation can be divided into the forms of waves and particles. The examples of radiation in the forms of waves and particles are shown in Table 2.1.

Table 2.1 Types of radiation in the forms of waves and particles

Wave	Particle
Electromagnetic radiation • Radio wave • Microwave • Infrared • Visible light • Ultraviolet • X-rays • Gamma rays (γ rays)	Particle radiation • Alpha radiation (α) • Beta radiation (β) • Neutron radiation (n)
Acoustic radiation • Sound wave • Ultrasound	

Based on quantum mechanics theory, electromagnetic radiation has both types of radiation, waves, and particles characteristic at the same time, so it may also be viewed as a particle radiation especially in the form of photon. Depending on the energy, radiation can be divided into non-ionizing radiation and ionizing radiation. Ionization is the process when the atom or a molecule breakdown or separated. For the separation process, one part acquires negative charge, and the other part acquires positive charge by gaining or losing electrons to form ions.

When the energy of incoming radiation is not sufficient to knock an electron out from the orbit, this is considered as a non-ionizing radiation. Thus, the absorption of energy from non-ionizing radiation could only make an electron move to higher orbit. This is known as excitation. If the radiation carries enough energy to knock an electron out form the atom or molecules, or to break chemical bonds, thus will ionize atoms or molecules. This is called ionizing radiation. The energy spectrum of non-ionizing and ionizing radiation are shown in Figure 2.1.

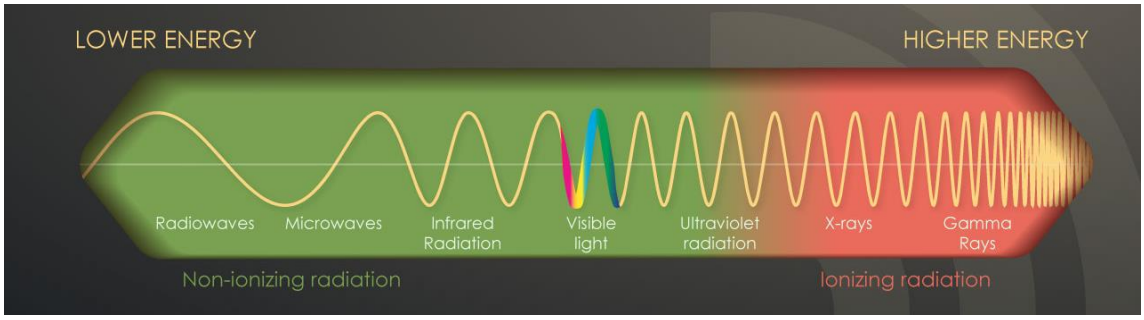

Figure 2.1 The energy spectrum of non-ionizing and ionizing radiation

Radiation has been used in many industries and studies for several purposes based on their beneficial application. Radiation application related to human activities include nuclear medicine and nuclear industry. However, this chapter focuses on ionizing radiation application in studies or research on mushroom.

2.3 Mushroom cultivation in Malaysia and Japan

Mushrooms have been cultivated in Malaysia since early 1970s, of which seventeen types predominated. However, presently only eight (8) mushroom species are commercially cultivated on a large scale, where the majority is *Pleurotus* spp. due to their demands from consumers, and the conditions for growth, especially temperature and humidity are favourable. Species of popular oyster mushrooms commercially grown include *Pleurotus sajor-caju, Pleurotus florida, Pleurotus cystidiosus* and *Pleurotus ostreatus*.[14)] The topmost cultivated and popular mushrooms in the world are the button (*Agaricus bisporus*) and shiitake (*Lentinus edodes*) mushrooms.[15, 16)]

The domestic demand for mushrooms is on an upward trend, from consumer awareness on their health benefits and factored by extensive promotion by public agencies and non-governmental organizations (NGOs) over their merits. However, the domestic production is unable to meet the huge demand. Thus, Malaysia has been importing more than 5 tons of fresh and dried mushrooms yearly since 2009.[17)]

Several species of edible mushrooms are cultivated artificially in Japan. Many of them are wood-rotting fungi and grow on dead trunks of wide leaf trees in the forest. Shiitake is the most cultivated mushroom due to the suitable climate and is mostly grown on natural log of shii tree (*Castanopis cuspidate*).[18)] In addition to shiitake, *Flammulina velutipes* (Enokitake), *Pholiota glutinosa* (Nameko), *P. ostreatus* (Hiratake), *Glifola frondosa* (Maitake), *A. bisporus* and

Lyophyllum ulmarium (Shirotamogitake) are also cultivated commercially, using sawdust cultures in bottle or bags in temperature and humidity regulated rooms.[19)]

2.4 Application of irradiation in mushroom study or research

Radiation technology is versatile tools that have a vital role to support of sustainable development.[20)] To date the radiation technology is gaining acceptance by many industries. This technology has proven to enhance the industrial efficiency, productivity and improve product quality, competitiveness, environmentally friendly.[21)] Radiation technology can be applied in several industrial sectors such as medical, food, manufacturing, and agriculture.

In agriculture especially in the mushroom industry, radiation also can be applied for several of purposes, such as for sterilization of mushroom substrate for cultivation and postharvest and mutation induction. The details for each purpose will be described as below.

2.4.1 Radiation sterilization of mushroom substrate

Mushrooms need a substrate as a medium for cultivation. A mushroom substrate is normally from agroindustry waste contains cellulose, hemi-cellulose, and lignin.[18)] These wastes are produced in enormous quantities causing disposal problems and consequently environmental pollution and health risks.[22)] Therefore, utilization of these wastes in mushroom cultivation can help reduce these problems. Lignocellulose agro-wastes such as rice straw, rice husk, wheat straw, cotton straw, tea leaves, banana leaves, coffee pulp, sawdust, barley straw, corncob, coconut coir, cassava peels, wood chips and oil palm empty fruit bunch can be used as a substrate for mushroom cultivation.[23, 24, 25)]

The mushroom cultivation substrate must be completely sterilized before inoculating with mushroom seeds or spawn. The sterilization can be done by heat, which is using autoclave or steam and by irradiation.[26)] Mushroom substrate requires to be fully sterilized, which means treating the substrates to eliminate pathogenic and competitive microorganisms to enhance the mushroom mycelial growth.

In the 1990s, the production of autoclaves for the sterilization in bulk sizes began in China, and subsequently in Korea. Trials on sterilizing substrates to produce wood mushrooms ensued. However, sterilization by autoclave often takes a long time, and has limitation for bulk production. Hence, radiation sterilization of substrates for mushroom cultivation was introduced for bulk

production. Gamma irradiation for sterilization of lignocellulosic materials, as substrates for mushroom cultivation has been since reported. Radiation sterilization at doses of up to 15 kGy was found to be efficient for composted sawdust, and dose of 25 kGy was suitable for oil palm empty fruit bunch in bags have been reported.[23, 26)]

2.4.2 Radiation sterilization for postharvest treatment of mushroom fruiting bodies

Fresh mushrooms can only be stored for a few days, and thereafter ensued loss of freshness and quality. Browning, cap-opening, weight-loss, and microbial spoilage are the most common postharvest changes in mushrooms, often resulting in low market price.[27)] Therefore, postharvest management of mushroom is a key factor that must be overseen to guarantee the quality before introducing them into the market. The postharvest technology is to guarantee a longer storage period and to upkeep a high quality of mushrooms fruiting bodies.

Biologically, mushrooms even after harvesting continue to grow, respire, mature, and undergo senescence process. Mushrooms have a short shelf-life after harvest. Therefore, understanding the proper postharvest handling is especially important to maintain the quality and extending the shelf life of mushrooms. Postharvest handling also affects the colour, taste, and biochemical compounds of mushrooms, and may extended their shelf life.[27)] According to Rai and Arumuganathan,[28)] two most common postharvest practices of mushrooms are proper packaging and storage to maintain the freshness. In mushroom industry, sun drying is the oldest and simplest methods to dry fruiting bodies, and this practice was used a long time ago. In general, the moisture content of mushrooms ranges from 85 to 95% of their fresh weight.[27)] Therefore, complete drying of mushroom fruiting bodies will take a long time. As technology becomes more advanced, new food preservation technologies have been developed, such as cooling and refrigeration, vacuum cooling, ice bank cooling and irradiation, to improve the shelf life and consumption of mushrooms.

Radiation processing technology has been developed through worldwide R&D efforts of more than four decades. Irradiation processing of foods may be considered one of the techniques in food industries for preservation, with exposure of food to ionizing radiations such as gamma and electron beam. This technology is a safe and cost-effective way to enhance shelf life while also ensuring hygienic and sensory quality.[29)] The softening and browning process associated with the ripening of certain fruits and vegetables such as in mushrooms can be delayed by utilizing irradiation.[27)] Moreover, postharvest treatments such as refrigeration at 4°C can be combined with

low dose gamma irradiation to increase shelf life of fresh mushrooms with minimum quality losses.[30)]

According to Kashif and Kwon,[27)] in addition to enhancing the shelf-life, irradiation improves the hygienic and sensory quality of mushroom. Radiation dose of 0.5 kGy can improve the sensory quality of fresh mushrooms and provide an increase in the shelf life of two (2) days at ambient temperature. However, the dose of approximately 1 kGy was found most effective to inhibit stem growth and cap opening, leading to increased shelf-life.[31)] Irradiation at 2 kGy of *Agaricus bisporus* fruiting bodies extended shelf life for 3-4 days when stored at 15°C.[27)]

2.4.3 Radiation mutation induction of mushroom

Mutation has long been exploited in crop breeding programmes, including for mushrooms to improve quality and productivity. Various methods of inducing mutation may be used, including chemical, biological, and physical agents such as ultraviolet (UV) and gamma irradiations.[32)] Radiation mutation induction has been used for around 100 years and has reported successful improved crops either for morphology or chemical characteristic.[6)] The process of radiation mutation induction involve the interactions between radiation and biological target structure, which will cause direct and indirect effects (Figure 2.2). Direct effect of radiation happens when ionizing radiation energy directly interact with biological target structure to cause ionization and thus initiating the chain of events that could leads to biological damage. For example, in DNA, if the incoming radiation energy is sufficient to remove an electron from the DNA molecule, bond will be broken, and this can break one or both of DNA strand.

The absorption of energy depends on the abundance of material in the path of the radiation. In the living organisms water is the most predominant molecule; about 80% of the mass of a cell is water. Therefore, indirect effects of ionizing radiation happen when incoming ionizing radiation energy interact with water within the cell first before damaging the cell. Water hydrolysis or decomposition of water is a result from interaction of ionizing radiation with the water. This interaction could produce many free radicals, which eventually damage critical biological processes within the cell, through breaking of chemical bonds and producing chemical changes that lead to biological damages. However, amount of direct and indirect effects of radiation depends on the Linear Energy Transfer (LET) of the incoming ionizing radiation. LET depends on the type of radiation and the energy of radiation.

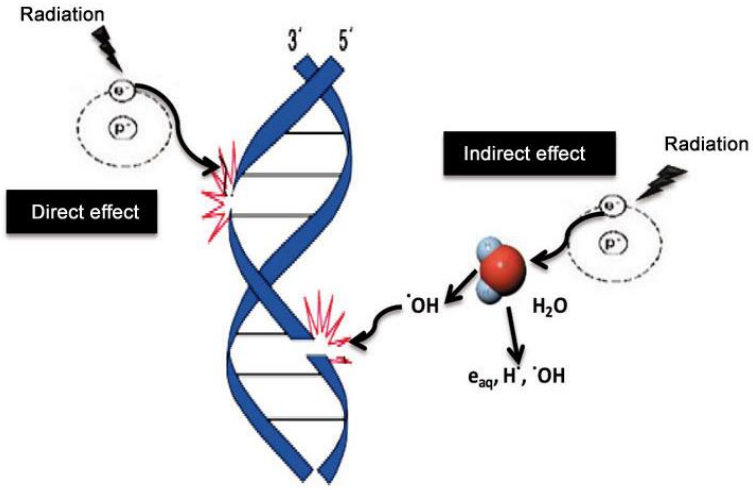

Figure 2.2 Illustration of direct and indirect effects of radiation

Mutation induction is applied to mushrooms to obtain better quality and productivity such as high yield or biological efficiency, elevated temperature tolerance, low-sporing, and shelf-life prolongation.[33)] Gamma rays have been proven to have positive and negative effects on mushroom for increasing genetic variations. The positive effect of radiation resulted in improvement of desired traits of the new varieties being developed.[12, 34)] The studies of strain improvement by radiation either by gamma or UV-rays on several mushroom species such as *Pleurotus* sp., *Agaricus* sp., *Volvariella volvacea* and *Auricularia auricula-judae* has been reported.[35, 36, 37, 38, 39, 40, 41, 42, 43)]

2.4.3.1 Effect on mycelia growth

Mycelium has a porous structure composed of tubular filaments called hypha. The components of hypha wall are including chitin, beta-glucans, and proteins (Figure 2.3). Typically, hyphae have diameters on the order of 1 μm to 30 μm, depending on the species and growth environment, and lengths ranging from a few microns to several meters. The growth rate of a mycelium depends on the formation of clamps (Figure 2.4), which enables the exchange of genes and extension to more areas as compatible mated mycelia and resulting in faster growth mycelium.[44, 45)] Clamp connections are the combination of two hyphal cells that have high affinity (positive polarity) towards each other.[42)]

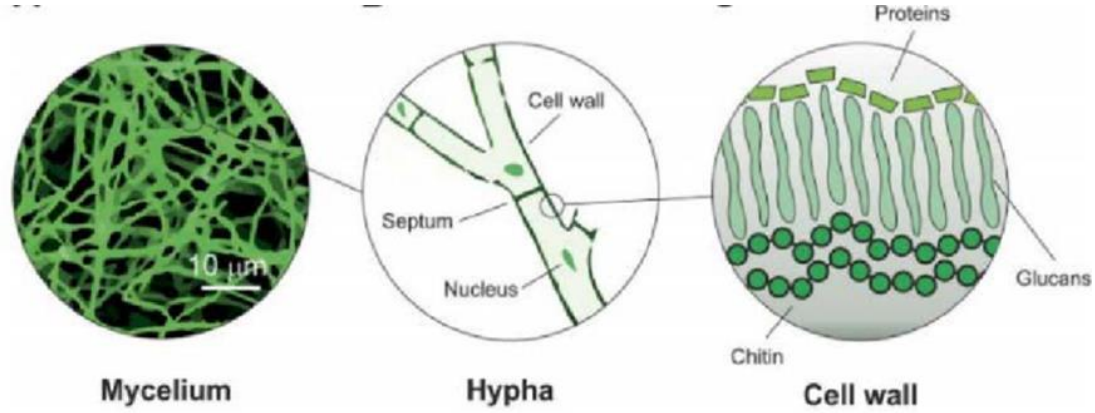

Figure 2.3 Structure of mycelium

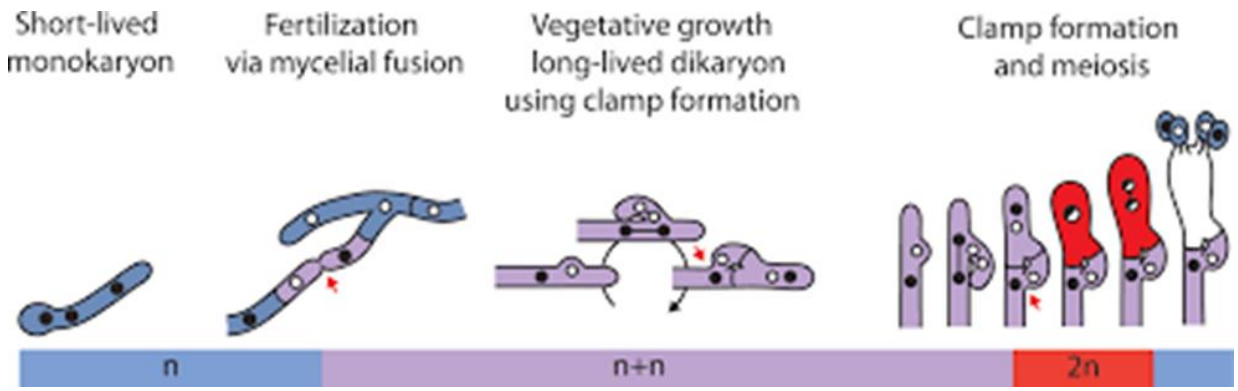

Figure 2.4 Formation of clamp

Mycelia can show a response to stress in different conditions and having different densities. [46)] Radiation is one type of stress that could affect the growth of mycelia, either making it faster or slower. If the effects of radiation induce the fast growth of mycelia, this will enhance the quality of the spawn directly, which in turn result in high production of the mushrooms.[47)]

In recent studies, the application of mutation techniques using gamma radiation on mushroom mycelia have been suggested for developing new varieties and enhancing their production.[48)] Gamma radiation has resulted in the transformation of mushroom mycelia of *P. sajor-caju, L. edodes*, *Volvariella volvacea* and *A. auricula-judae*, as reported. [41,42, 47, 49)]

From the study by Rosnani *et al*.,[48)] the growth rate of irradiated mycelium was lower from the control and decreased as the irradiation dose from 0.1 kGy to 0.6 kGy. The growth rate of *V. volvacea* mycelia increased from control (0 Gy) until 900 Gy gamma radiation before it started

showing a decreasing mycelial growth on PDA at doses of 1200 and 1500 Gy.[47] The study on the effects of UV radiation on mycelia growth of *V. volvacea* has also been reported with the results showing mycelial growth decreased with increasing doses of gamma radiation.[43]

2.4.3.2 High productivity

Extensive development and improvement of mushroom with high productivity varieties have been conducted by many researchers and breeders. The studies for variety improvement of mushroom by gamma and UV-rays have been reported since 1980.[35, 36] Among the important characteristics in pursuit are high productivity and also mention as high yield and high biological efficiency (BE) in many publication. The BE was calculated as follows:

$$\% \text{ BE} = \frac{\text{FWm}}{\text{DWs}} \times 100$$

where, BE is Biological Efficiency (%); FWm is total fresh weight (g) of mushroom yield for all flushes, and DWs is substrate dry weight (g).[50]

A study by Rosnani *et al*.,[41] has shown irradiation can induce more fruiting bodies with no significant difference on size of fruiting bodies (Figure 2.5). The more fruiting bodies were reflected the yield and productivity of mushroom. This result is similar recorded for *Pleurotus djamor*, where production of fruiting bodies at dose of 25 Gy was higher than from 20 Gy.[11] The difference in growth attributes of mutants may be due to the genetic changes caused by gamma radiation.

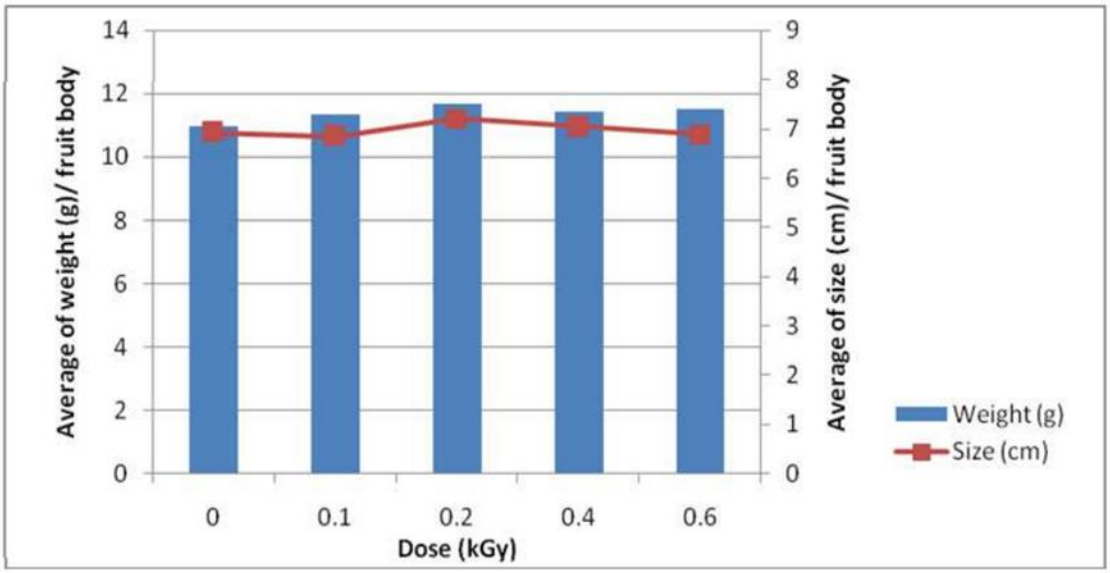

Figure 2.5 Graph of average weight and size of mushroom fruit bodies for each irradiation dose study by Rosnani *et al.,* [41].

Gamma irradiation on mycelia of *Plerotus florida* at 0.75 kGy with dose rate at 1.149 kGy per hour yielded varieties with higher productivity than the control, which was reflected by significantly higher number of fruit bodies, higher fresh weight, and dry weight yield of three successive flush periods.[36] Gamma irradiation at doses of 0, 100, 200, 300, 400, and 500 Gy per hour were used for induction of genetic diversity in *A. bisporus* white button mushrooms for achieving genotypes with desirable traits such as high yield and quality.[51] Results from this study showed that quality of *A. bisporus* was enhanced through gamma radiation induction, producing greater number of fruit bodies, greater weight, and yield than the control. Mutation induction using gamma irradiation has also been used for the development of *V. volvacea* variety with higher yield than the control.[39]

Meanwhile, UV radiation also has shown able to induce mushroom with high yield or BE. The exposure to UV radiation at 210 nm for 90 min resulted in the induction of the total weight of fruit body, BE and production rate of *Pleurotus pulmonarius.*[38] The mycelium of the *Plerotus tuber-regium* strain exposed to UV radiation at a distance of 45 cm generated mutants with characteristics of high yielding and capable of maintaining stability for more than twenty (20) generations of subculturing.[52]UV radiation also has been used in *Pleurotus* sp. to produce strains with high BE.[37, 40] In addition, a study on UV mutagenesis has also been conducted on *Ganoderma*

lucidum. A high yielding varieties of *G. lucidum* was obtained by UV mutagenesis. The yield was 21% higher than the control.[53] High yielding and fast-growing varieties of *V. volvacea* mushroom have been generated by UV-induced mutagenesis.[43]

2.4.3.3 High or low temperature tolerance

Mushrooms require low temperature and high level of relative humidity to produce mushroom fruiting bodies.[54] However, the ambient temperature has risen due to the global warming. Hot temperatures cause deformed fruiting bodies, and consequently low mushroom yield. Therefore, farmers cultivate mushrooms in shelters to obtain appropriate environmental condition for mushroom growth.[55] Generation of temperature tolerant mushroom mutants through mutation induction has also been initiated by mushroom breeders.

One of the reports on development of high temperature mushroom was on *Pleurotus ferulae*. *P. ferulae* is a low temperature edible mushroom. Therefore, this mushroom species cannot be cultivated throughout the seasons. At high temperatures, bud formation and fruiting body growth of this species are suppressed. From the ion beam radiation, the result showed the growth temperature of the fruiting body *P. ferulae* is increased at temperatures up to 30°C compared to control.[56]

2.4.3.4 Low-sporing

During the cultivation of mushrooms, enormous number of spores produced by the fruiting bodies can impart adverse effects on human health. Mushroom fruiting bodies produce billions of spores from their gills. These spores can cause health problems such as lung allergies and fever attacks.[33] According to Vereda *et al.*,[57] spore-related respiratory allergies have been reported in workers handling oyster mushrooms. Therefore, spore allergies can be one of the limitation factors for the large production of mushroom fruiting bodies.

The production of a low sporing varieties of *Pleurotus columbinus, Pleurotus erynjii, P. florida*, and *P. sajor-caju* through UV radiation mutation induction has been reported. [58, 59, 60] The study by El-Fallal *et al.*,[59] showed that UV exposed for 30 min was sufficient to induce low sporing characteristic in *P. columbinus* for. Mutation induction of *P. sajor-caju* yielded an extremely low-sporing mutant after 75 min of exposure to UV radiation.

2.4.3.5 Shelf-life prolongation during storage

Mushrooms fruiting bodies are fresh products and can lose quality immediately after harvest. The short shelf-life of mushrooms is a major constraint for distribution and marketing of fresh produces. Their short shelf-life is due to postharvest changes such as browning, cap opening, stipe elongation, weight loss and texture changes. These changes are due to the high respiration rate and lack of physical protection to avoid water loss or microbial attack.[61, 62)]

Prolonging postharvest storage with preserving their quality would benefit to the mushroom industry and the consumers. Extended shelf-life is a key factor for making any food commodity more profitable and commercially available for prolonged periods of time at the best possible quality. In food technology, preservation technique is the one of extensive key research for developing and establishing methods that are less severe and less damaging to food products. Several techniques have been used for food preservation, including chemical treatments, refrigeration, washing, coating, modified or controlled atmosphere and ozone treatment. Moreover, most food preservation techniques aimed to slow down or inhibit growth of microorganisms causing spoilage. In contrast, heat and ionizing irradiation processing can inhibit or inactivate microbial growth completely, resulting in commercially sterile and shelf-stable food products.

Radiation preservation offers a method of "cold sterilization" where the mushrooms may be preserved without any changes in their natural characters. Low doses of gamma radiation can be used to reduce the microbial contamination and extend the shelf-life of mushrooms. However, irradiation should be applied immediately after harvest for optimum benefits. Many beneficial effects of radiation have been observed in preserving the button mushrooms and oyster mushrooms.[28)]

Radiation can be applied for mushroom shelf-life prolongation for up to 21 days by preventing vital activity and growth of microorganism.[63)] Irradiation has been found to delay the maturation, or development of caps, stalks, gills, spores, and reduce the loss of water, colour, flavour, and texture. The amino acid content in fresh mushroom has been reported to be better preserved by low doses of gamma irradiation.[28)] The study of storage life on *P. sajor-caju* and *P. pulmonarius* has shown gamma irradiation is able to extend the shelf life without adversely affecting key nutritional components. Koorapati *et al.*,[31)] evaluated the effect of electron-beam

irradiation on quality of white button mushroom and observed that irradiation levels above 0.5 kGy prevented microbe-induced browning.

2.4.3.6 High bioactive compounds

Mushrooms are well recognized as organic food containing high protein, low fat, and low energy contents. They have high contents of minerals such as iron and phosphorus, and also in vitamins like riboflavin, thiamine, ergosterol, niacin, and ascorbic acid. Mushroom also contain bioactive constituents like secondary metabolites such as polyphenolic compound, terpenoids, acids, alkaloids, sesquiterpenes, lactones, sterols, vitamins and polysaccharides in the main β-glucans and glycoproteins. These bioactive compounds have properties including immune-potentiating, anti-cancer, antiviral, hypocholesterolemic agents and natural antioxidants which is helpful in reducing cell damages.

The effects of gamma radiation on *Agaricus bisporus* for bioactive components such as vitamin C, vitamin D, folic acid and total antioxidant activity has been reported. [29] From this report, gamma radiation at dose 0.75 kGy could enhance the vitamin C, vitamin D and folic acid contents as well as antioxidant activity. Other study by Pelcaru *et al.*,[64] reported that gamma radiation on mycelium of *Fomes fomentarius* increased total phenolic compounds and total flavonoids in methanolic extract. The present study will discuss further the effects of ionizing radiation on the chemical characteristics as well as bioactive compounds of *P. sajor-caju*. These bioactive compounds are investigated as radioprotective agents.

2.5 References

1) Market Analysis report. Mushroom Market Size, Share & Trends Analysis Report by Product (Button, Shiitake, Oyster), By Form, By Distribution Channel, By Application (Food, Pharmaceuticals, Cosmetics), By Region and Segment Forecasts, 2022 – 2030.
2) J. R. Daniel. A global perspective on the high five: *Agaricus*, *Pleurotus*, *Lentinula*, *Auricularia* & *Flammulina*. *Proceedings of the 8th International Conference on Mushroom Biology and Mushroom Products* (ICMBMP8): 1-6 (2014).
3) A. R. Rosnani, M. Mohd Meswan, S. Shuhaimi, A. N. Mohd Hafiz and H.M.H. Hamdani Hassan Mutaat. Biological effects of LD_{50} gamma radiation on fruiting bodies of *Pleurotus* sp. mushroom. *Konvensyen Teknikal Nuklear Malaysia* (2021).

4) Z. Ahmad, A. Abu Hassan, S. Salleh, S. Ariffin, S. Shamsudin and M. N. Basiran. Pertanika *Journal of Tropical Agricultural Science*. 35 (3): 631-636 (2012).

5) S. P. Chandran and Y.K. Lee, Y.K. Mutation Breeding of Mushroom by Radiation. *Journal of Radiation Industry*. 5 (4): 285-295 (2011).

6) L. Ma, F. Kong, K. Sun, T. Wang and T. Guo. From classical radiation to modern radiation: past, present and future of radiation mutation breeding. *Frontier in Public Health*. 9: 1-11 (2021).

7) B. S. Ahloowalia and M. Maluszynski. Induced mutations - A new paradigm in plant breeding. *Euphytica*. 118(2): 167-173 (2001).

8) F. I. S. Medina, S. Tano and E. Amano. Mutation Breeding Manual. *Forum for Nuclear Cooperation in Asia, Tokyo* (2004). Retrieved from http://www.fnca.mext.

9) S. M. Jain. Mutation-assisted breeding for improving ornamental plants. *Acta Horticulturae (ISHS)*. 714: 85-98 (2007).

10) H Nakagawa. Induced mutations in plant breeding and biological researches in Japan. In: Q. Y. Shu (ed.), Induced Plant Mutations in the Genomics Era. *Food and Agriculture Organization of the United Nations, Rome*. 51-58 (2009).

11) K. R. Jyothi and S. S. Thara. Development of improved strain in species of Pleurotus by gamma irradiation. *Journal Food Science Technology*. 58 (9): 3540–3547 (2021).

12) M. Z. Rosmiza, W. P. Davies, C. R. Rosniza Aznie, M. J. Jabil and M. Mazdi. Prospects for Increasing Commercial Mushroom Production in Malaysia: Challenges and Opportunities. *Mediterranean Journal of Social Sciences*. 7 (1 S1): 406-415 (2016).

13) H. Mohd Tarmizi, R. Hairazi and A.D. Rozhan. Understanding the mushroom industry and its marketing strategies for fresh produce in Malaysia. *Economic and Technology Management Review*. 8: 27-37 (2013).

14) M. T. Islam. Z. Zakaria, N. Hamidin, M. A M. Ishak and C. S. Fern., Selection and Cultivation of Oyster Mushroom for the Indoor Controlled Environment in Malaysia and Similar Ecological Region. *Journal of Agricultural Science*. 1: 14-22 (2017).

15) P. Mattila, K. Konko, M. Eurola, J. M., Pihlava, J. Astola and L. Vahteristo. Contents of vitamins, mineral elements, and phenolic compounds in cultivated mushrooms. *Journal Agriculture. Food Chemistry*. 49: 2343-2348 (2001).

16) I. Md Shaiful. Cultivation Techniques of Edible Mushrooms: *Agaricus bisporus*, *Pleurotus* spp., *Lentinula edodes* and *Volvariella volvocea*. *Wageningen Ur*: 1-33 (2013).

17) M. A. Mohd Zaffrie, H. Azahar, M.A.W. Mohd Amirul, R. Hairazi and H. Mohd Tarmizi, H. A study of consumer behaviour towards mushroom-based products in Malaysia. *Economic and Technology Management Review*. 12: 55-63 (2017).

18) K. Manikandan. Nutritional and Medicinal values of Mushrooms. In. Mushrooms: Production, consumption and Marketing. *Directorate of Mushroom Research*. M. Singh, B. Vijai, S. Kama and G. C. Wakchaurae (eds) (2011).

19) K. Mori. Cultivated Mushrooms in Japan. *Development in Crop Science*. 10: 455-459 (1987).

20) Y. Amano. Radiation technology for a more prosperous and sustainable future. IEAE Bulletin: Radiation Technology the industrial revolution behind the scenes. *Vienna, Austria* (2017).

21) M. D. Khairul Zaman and K. A. M. R. Nahrul. 30 Status and Prospect of Radiation Processing Technology in Malaysia. *JAERI-Conf 2001-005* : 255-265 (2001).

22) V. K. Garg and R. Gupta. Vermicomposting of agro-industrial processing waste. In. Biotechnology for Agro-Industrial Residues Utilization. Springer, *Netherlands:* 431–456 (2009).

23) A. Mat Rasol, W.H. Wan Badrin and O. Tajuddin. Oil palm empty fruit bunch as media for mushroom cultivation. *Nuclear Science Journal of Malaysia*. 16 (1):81-89 (1998).

24) J. Kaur, H.S. Sodhi, S. Kapoor, P. K. Khanna and R. K. Jaswal. Strain improvement of specialty mushroom, *Calocybe indica*, through mutagenesis. *Applied Biological Research*. 13:62-69 (2011).

25) V. Barshteyn and T. Krupodorova. Utilization of agro-industrial waste by higher mushrooms: modern view and trends. *Journal of Microbiology Biotechnology and Food Sciences*. 5(6): 563-577 (2016).

26) N. K. Kortei, G. T Odamtten, M. Obodai, M. W. Kwagyan and M. Dzomeku. Comparative bioconversion of gamma irradiated and steam sterilized 'wawa' sawdust (Triplochiton scleroxylon L.) by mycelia of oyster mushrooms (*Pleurotus ostreatus* Jacq. Ex.Fr. Kummer). *International Food Research Journal*. 25(3): 943-950 (2018).

27) A. Kashif and J. H. Kwon. Food Irradiation for Mushrooms: A Review. *Journal of the Korean Society for Applied Biological Chemistry*. 53(3): 257-265 (2010).

28) R. D. Rai T. Arumuganathan. Post-harvest technology of mushrooms. *Technical Bulletin, National Research Centre for Mushroom (Indian Council of Agricultural Research)*. 1-92 (2008).

29) K. Bandi and M. Kola. Gamma Irradiation of Mushrooms and its Impact on Bioactive Components. *Annals. Food Science and Technology*. 21 (2): 266-273 (2020).

30) A. Minnaar, J.R.N. Taylor, N.N. Dersley and A.E.J. McGill. Technological feasibility of heat-irradiation combination treatments for low-acid food products. *Radiation Physic Chemistry*. 48: 371-372 (1996).

31) A. Koorapati, D. Foley, R. Pilling and A. Prakash. Electron beam irradiation preserves the quality of white button mushroom (*Agaricus bisporus*) slices. *Journal Food Science*. 69:25-29 (2004).

32) A. Q. Fahad and K. Salim. Mutagenesis effects of sodium azide and its application in crop improvement. *World Applied Sciences Journal*. 6: 1589-1601 (2009).

33) B. Chakravarty. Trends in Mushroom cultivation and breeding. *Australian Journal of Agricultural Engineering*. 2 (4): 102-109 (2011).

34) T. Wang and T. Guo. From classical radiation to modern radiation: past, present and future of radiation mutation breeding. *Frontier in Public Health*. 9: 1-11 (2021).

35) T. J. Elliott and F.A. Langton. Strain improvement in the cultivated mushroom *Agaricus bisporus*. *Euphytica*. 30 (1):175–182 (1981).

36) I. Djajanegara and A. Harsoyo. Mutation study on white oyster mushroom (*Pleurotus floridae*) using gamma (60CO) irradiation. *Journal of Chemical and Natural Resources Engineering*. 4(1): 12-21 (2000).

37) P. H. F. Beejan and R. Nowbuth, Use of Irradiation for the Induction of Mutations in Oyster Mushrooms for Improvement of Strains. In. Induced Plant Mutations in the Genomics Era. Q. Y. Shu (ed.). *Food and Agriculture Organization of the United Nations*, Rome: 289-292 (2009).

38) E. A. Adebayo, J. K. Oloke, A.K. Bordoloi, M. Barooah and T. C. Bora. Improvement of *Pleurotus pulmonarius* Lau 09 through mutation for yield performance. *Research Journal of Mutagenesis*. 4 (1): 1-13 (2014).

39) N. Sermkiattipong and S. Charoen. Development of straw mushroom strain for high yield by gamma radiation. *Journal of Agricultural Technology*. 10 (5): 1151-1164 (2014)

40) R. Sharma and B.M. Sharma. Strain improvement in *Pleurotus ostreatus* using UV light and ethyl methyl sulfonate as mutagens. *African Journal of Microbiology Research*. 8 (5): 432-436 (2014).

41) A. R. Rosnani, D. Fauzi, A. Mat Rasol, M. Hassan Hamdani, S. Sahidan, M. Azhar, M. Mohd Meswan and K. Abdul Rahim. Evaluation of Mycelia Growth, Morphology and Yield for Low Dose Gamma Irradiated Grey Oyster Mushroom *Pleurotus sajor-caju. International Journal of Innovation and Scientific Research*. 24 (2): 332-336 (2016).

42) I. Mahmood, A. Mohamad, F. Daud, Z. A. Kadir, H. J. Nie, H. H. Hussain, W. S. W. A. Razak, A. O. B. Airianah and S. Fazry. Variants production of *Lentinula edodes* spores, monokaryon mycelium and dikaryon mycelium by γ-irradiation. *Malaysian Applied Biology*. 46 (1): 77–82 (2017).

43) B. Eakaphun, S. Korapan and R. Urarux. UV-induced mutagenesis in *Volvariella volvacea* to improve mushroom yield. Songklanakarin *Journal. Science Technology*. 42 (4): 910-916 (2020).

44) E. Kothe. Mating-type genes for basidiomycete strain improvement in mushroom farming. *Applied microbiology and biotechnology*. 56 (5 & 6): 602-612 (2001).

45) A. G. Rosnina, S. T. Yee, A. Noorlidah and S. Vikineswary. Morphological and Molecular Characterization of Yellow Oyster Mushroom, *Pleurotus Citrinopileatus*, hybrids obtained by interspecies mating. *World Journal of Microbiology and Biotechnology*. 32 (2): 1-9 (2016).

46) M. R. Islam, G. Tudryn, R. Bucinell, L. Schadler and R. C. Picu. Morphology and mechanics of fungal mycelium. *Scientific Reports*. 7:13070:1-12 (2017).

47) F. N. Fisol, S. Abdullah, A. Mohamad, M. T. M. Mohamed, A. Misran, S. Salim and B. Azhar3. Effect of Different Doses of Acute Gamma Radiation on the Cultivation of *Volvariella volvacea. International Journal of Agriculture & Biology*. 27:53–59 (2022).

48) A. R. Rosnani, D. Fauzi, S. Sahidan, A. Mat Rasol, M. Azhar, M. Hassan Hamdani and M. Mohd Meswan. Radiosensitivity study and radiation effects on morphology characterization of grey oyster mushroom *Pleurotus sajor-caju. AIP Conference Proceeding*. 1614: 570-574 (2014).

49) H. J. Nie, A. Mohamad, Z. A. Kadir, W. S. W. A. Razak, I. Mahmood and H. H. Hussain. Dose response for *Auricularia auricula-judae* against acute gamma irradiation. *Malaysian Applied Biology*. 46 (1): 71–75 (2017).

50) Z. Girmay, W. Gorems, G. Birhanu and S. Zewdie. Growth and yield performance of *Pleurotus ostreatus* (Jacq. Fr.) Kumm (oyster mushroom) on different substrates. *AMB Express*. 6: 87: 1-7 (2016).

51) T. Harfi, A. M. Azar, F. Rasouli and F. Z. Nahandi. Genetic Improvement of White Button Mushrooms for Qualitative and Quantitative Traits. *Progress in Nutrition*. 23 (4): 1-11 (2021).

52) O. B. Comfort, K. O. Julius and F. D. Joanna. Development of high yielding strain of *Pleurotus tuber-regium*: fructification, nutritional and phylogenetic studies. *Journal Food Science Technology*. 56 (8):3597–3608 (2019).

53) L. Lixin, Z. Shaoqi and Y. Ruhua Y. Mutation breeding and submerged culture of *Ganoderma lucidum*. Industry Microbiology. 3: DOI: CNKI1. ISSN:001-6678.0.1998-03-002 (1998).

54) M. T. Islam. Z. Zakaria, N. Hamidin, M. A. M. Ishak and C. S. Fern. The Management of Humidifying Treatment for Low Contamination Risks During Indoor Cultivation of Grey Oyster Mushroom (*Pleurotus pulmonarius*). *MATEC Web of Conferences*. 9:1-7 (2017).

55) B. I. Setiawan. Designing temperature control system for mushroom cultivation. *IFAC Proceedings*. 158-161 (2001).

56) H. Chen, H. Wan, J. Zhang and X. Zeng. Mutation-screening of *Pleurotus ferulae* with high temperature tolerance by nitrogen ion implantation. *Plasma Science Technology*. 10:503-505 (2008).

57) A. Vereda, S. Quirce, M. Fernandez-Nieto, B. Bartolome and J. Sastre. Occupational asthma due to spores of *Pleurotus ostreatus. Allergy*. 62: 211–212 (2007).

58) Y. Obatake, S. Murakami, T. Matsumoto and Y. Fukumasai-Nakai). Isolation and characterization of a sporeless mutant in *Pleurotus eryngii*. *Mycoscience*. 44: 33-40 (2003).

59) A. A. El-Fallal, A. K. A. El-Sayed and H. M. El-Gharabawy. Induction of low sporulating-UV mutant of oyster mushroom with high content of vitamin D2. *3rd International conference on Biotechnology and its Application in Botany and Microbiology*: 1-12 (2013).

60) S. Ravishankar, M. Pandey, R. P. Tewari and V. Krishna. Development of sporeless/low sporing strains of Pleurotus through mutation. *World Journal of Microbiology and Biotechnology*. 22: 1021-1025 (2016).

61) K. Akram and J. H. Kwon. Food irradiation for mushrooms: A review. *Journal of Korean Society for Applied Biological Chemistry.* 53: 257-265 (2010).

62) P. Singh, H. C. Langowski, A. A. Wanib and S. Saengerlaub. Recent advances in extending the shelf life of fresh Agaricus mushrooms: A review. *Journal of Science and Food Agriculture.* 90: 1393–1402 (2010).

63) A. A. Dril, L. A. Mayurnikova and L. N. Rozhdestvenskaya. The influence of ionizing radiation on free protein content and microorganisms' growth in oyster mushrooms. *IOP Conf. Series: Earth and Environmental Science*. 640: 1-10 (2020).

64) F. Pelcaru, M. Ene, A. M. Petrache and D. C. Negut. Low Doses of Gamma Irradiation Stimulate Synthesis of Bioactive Compounds with Antioxidant Activity in *Fomes fomentarius* Living Mycelium. *Applied Sciences*. 11 (4236) :1-12 (2021).

Chapter 3: Radiation Protection Studies Involving Radioprotective Agents

Chapter 3 gives a review on studies or works on radiation protection utilising radioprotective agents. This chapter includes introduction of radiobiology, effects of radiation on biological samples, types of biological samples, free radicals, radiation protection, natural radioprotectors and natural sources of radioprotectors.

Abstract

Radiation has been used in many fields as well as in medicine. However, radiation exposed to the human body may result in risks. Therefore, study on radiation protection is one of the scopes to focus, to enable good understanding on radiation risk management. This chapter will provide an introduction of radioprotection, radiobiology, effects of radiation to human body, types of free radicals, mechanism of radioprotection, types, and mechanism of radioprotector agents. In this review, the status on radioprotection studies and research using radioprotector agents are discussed. The radioprotection by natural radioprotector agent will be elaborated in detail and applied in subsequent chapters of the present study.

3.1 Introduction

Radiation has proven to provide many benefits to humans and various industries, despite being associated with risks. While harnessing its beneficial application in sectors such as medicine manufacturing, agriculture and power generation, the risks to workers, patients, public and the surrounding environment that may arise from these uses need to be assessed, controlled and aligned with safety standards. Although radiation risk cannot be eliminated, but can be effectively managed.

For humans, the risks of radiation are related to the radiation dose, which may affect human health. The higher the radiation dose received the more will be the risk to human health. High doses received can lead to radiation sickness and in the severe cases can cause death (deterministic effects). However, in some people even small radiation dose received will also can have late effects such as cancer formation and hereditary effects (stochastic effects).

A well-known radiation protection principle, namely time, distance and shielding, can be applied to minimize the dose received. However, there will always be some remaining radiation

dose absorbed in the human body. The another way of protection against radiation is the use of radioprotectors. Radioprotectors protect the human body by minimizing the damage created from exposure to radiation. The human body has natural radioprotectors inside the cells. However, the quantity of natural radioprotectors is not sufficient to overcome the harmful effects of natural radiation received in our body every day. When a nuclear accident happens, the situation can be completely different.

Natural radioprotectors in the human body can be increased by consuming healthy foods with high antioxidant content. Mushrooms are one of the option healthy foods to be consumed for this purpose. Many studies have been conducted to evaluate, determine and investigate bioactive compounds in mushrooms. Studies on the effects of bioactive compounds using extract from mushroom on cancer patients have also been reported. Therefore, incorporating mushrooms into our daily life diet can help to boost our immune system and protect our body from external factors, including irradiation.

Many studies have been conducted in the field of radioprotector in order to investigated the radical-scavenging effects of various chemical compounds, either naturally and artificially using many methods such as DPPH analysis, colony assay using living cells and electrophoresis. From the published reports, many researchers worldwide have investigated how the compounds provide protection to the human body from chronic diseases such as cancer. Cancer is one of the effects of radiation exposure.

3.2 Radiobiology

Radiobiology is a combination of two disciplines which are radiation physics and biology. Radiobiology is also known as radiation biology. Radiobiology is a branch of science study about the effects of ionizing radiation on living organisms. Living organism are important of biomolecules such as DNA, proteins, lipids, and carbohydrates, which could dissolve or be suspended in water. Ionizing radiation can cause change or damage to the structure of biomolecules. Depending on the number of damages and the rate of repairing mechanism to fix the damages, ionizing radiation could be harmful to and have potentially lethal effects on living organisms.

3.2.1 Ionizing radiation

Ionizing radiation is when radiation carries enough energy to knock an electron out from the atom or molecules. Radiation cannot be detected by human senses therefore, humans were largely unaware of radiation existence. Before the 1890s, there were only natural sources of the ionizing radiation such as radiation from cosmic or terrestrial sources, radioactive materials inside rocks, air, and soil. Ionizing radiation also can occur from manmade in an accelerator or reactor facilities, which radiation sources such uranium, radium and plutonium is used.

The ionizing radiation was first discovered in 1895 by Wilhelm Conrad Roentgen of unknown rays or X-rays. Since then, many are aware of these invisible rays that could allow us to see inside the human body. In 1896, Henri Becquerel discovered radioactivity from uranium. Since that many types of natural ionizing radiation were discovered, such as beta and alpha particles were discovered by Rutherford in 1896 and 1899, respectively. Gamma rays were discovered by Villard in 1900 and neutrons were discovered by James Chadwick in 1932. The present study will focus on gamma radiation; hence we will discuss the gamma radiation characteristics.

Gamma radiation consist of energy photon which do not have a rest mass or electrical charge and move at the speed of light. Photons particles can be naturally expelled from the nucleus of the unstable isotopes (through gamma decay) in the form of gamma radiation. Gamma rays are forms of high-frequency (high-energy) ionizing radiation, which means they have enough energy to remove an electron from (ionize) an atom or molecule. Gamma radiation can travel 10 meters or longer compared to alpha and beta particles. However, gamma rays deposited less energy along of the paths.

3.2.2 Effects of radiation on living organisms

Ionizing radiation can interact with the substances it passes through. There are two types of radiation interaction that lead to biological responses in the living organism. Firstly, radiation can directly damage DNA molecules, and secondly, radiation can interacts with other molecules to form free radicals that can damage DNA molecules. From these interaction mechanisms, ionizing radiation causes harm or damage to biological materials directly or indirectly. However, living organisms have mechanisms for repairing the damages from radiation. If the repair mechanism is successful, it could lead to no damage. However, if the repair mechanism is unsuccessful, the living organism could die, become inactive, or mutated. The details and

schematic diagram of the damage and repair mechanism of ionizing radiation is shown in Figure 3.1.

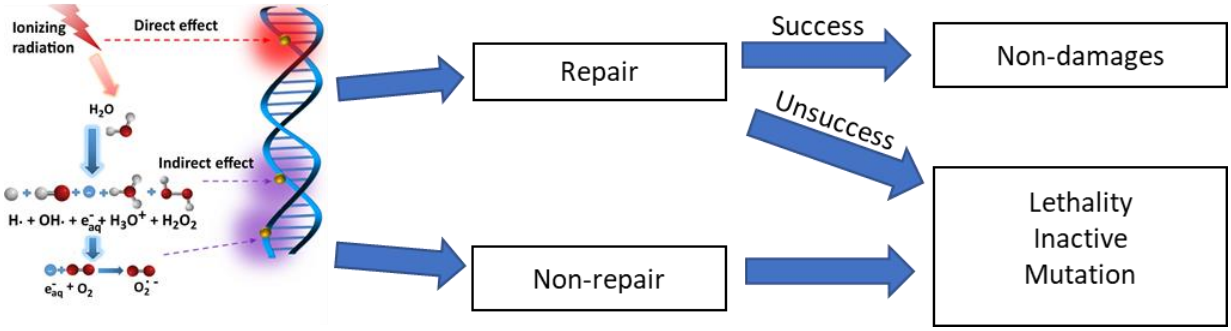

Figure 3.1 Schematic diagram of the effects of and damages from ionizing radiation

3.2.3 Direct and indirect effects

Direct effects of ionizing radiation happen when the ionizing radiation energy directly interacts with biological target structure, which causes ionization. For example, in DNA if the incoming radiation energy is sufficient enough to remove an electron from the DNA molecule (via photoelectric effects and Compton interaction), the bond will be broken on one or both DNA strands.

The absorption of energy depends on the abundance of material in the path of the radiation. Water is the most predominant molecule in living organisms which can make up to about 80% of the mass of living cells. Indirect effects of ionizing radiation happen when the incoming ionizing radiation energy interacts with the water within the cell before damaging the cell. Interaction of ionizing radiation and water will cause water hydrolysis or decomposition of water, which could produce many free radicals. Free radicals eventually damage the critical biological target within the cell by breaking the chemical bonds and produce chemical changes that lead to biological damage.

The occurrence for how many direct or indirect effects of ionizing radiation is dependent on the Linear Energy Transfer (LET) from incoming ionizing radiation. The LET depends on the type of radiation and the energy of radiation. Table 3.1 shows the value of LET for different types of radiation.

Table 3.1 LET value for different type of radiation.

Types of radiation	LET (KeV/μm)
Cobalt-60 gamma radiation	0.3
250kVp X-ray	2
10MeV protons	4.7
150Mev protons	0.5
Recoil proton from fission neutrons	45.
14MeV neutrons	12.
2.5MeV alpha particles	166.
2GeV Fe nuclei	1000.

High LET radiation has more energy per unit length of material, the probability of DNA damage in a short period of time is higher compared to low LET. Therefore, high LET radiation causes most of the damages through direct effects, while low LET radiation mostly causes damages through indirect effects.

3.2.4 Free radical and oxidizing species

The first free radical reaction was reported by Fenton in 1894. However, at that time, free radicals were not known. In 1900, Moses Gomberg, a chemistry professor at the University of Michigan, USA, discovered an organic free radical identified as triphenylmethyl radical. The term radical in radical theory was also used for bound parts of the molecule, especially when they remain unchanged in reactions.

A free radical can be defined as an atom, molecule or chemical compound which has unpaired electron on the boundary (atomic and molecular) orbitals. The presence of an unpaired electron seeks to pair with another electron by a form of bond and this makes free radicals highly chemically reactive. Free radicals can either donate an electron to or accept an electron from other molecules, therefore could be behaving as oxidants or reductants.[1)] On the other hand, oxidizing species is relatively stable, but their existence could also create damages and lead to free radical formation by some reaction mechanism.

Free radical and oxidizing species can be grouped into Reactive Oxygen Species (ROS) group and Reactive Nitrogen Species (RNS) group. Both of these could be created within the cell naturally, which is considered as a waste of the cell metabolism and respiration., or from external sources such as pollution and ionizing radiation. Both the ROS and RNS can be classified into two groups of compounds namely, radicals and non-radicals. Radicals are species that contain at least one unpaired electron in the atomic orbitals and are capable of independent existence. The non-radical species are not free radicals but can easily lead to free radical reactions in living organisms. [2)] The name of ROS and RNS free radical species, oxidizing species, characteristics, and half-life are shown in Table 3.2.

Table 3.2 The name of free radical species, oxidizing species, and its characteristics.[3, 4)]

Name		Formula/ Symbol	Characteristics	Half-life (s)
Reactive Oxygen Species (ROS)				
Radicals	Hydroxyl radical	$^{\cdot}OH$	Free radical, highly unstable, very reactive agent	10^{-10}s
	Hyperoxide/ superoxide	$^{\cdot}O_2^-$	Highly unstable, signaling function, synaptic plasticity	10^{-6}s
	Hydroperoxyl	$HO_2^{\cdot}$		s
	Alkoxyl	$RO^{\cdot}$	Free radical, reaction product of lipids, can be generated by the decomposition of alkyl peroxides (ROOH)	10^{-6}s
	Peroxyl	$ROO^{\cdot}$	Free radical, reaction product of lipids, can be generated by the decomposition of alkyl peroxides (ROOH)	17s
Non-radical	Hydrogen peroxide	H_2O_2	Cell toxicity, signaling function generation of other ROS	Stable
	Ozone	O_3	Environmental toxin	s

	Singlet oxygen	$^{l}O_2Dg$	Induced/excited oxygen molecule, radical and nonradical form	10^{-6}s
	Hypochlorous acid	HOCI		Stable (min)
	Hypochlorite anion	OCI^-	Reactive chlorine species, enzymatically generated by	
	Peroxynitrite	$ONOO^-$		10^{-3}s
Reactive Nitrogen Species (RNS)				
Radicals	Nitric oxide	$^{\cdot}NO$	Environmental toxin, endogenous signal molecule	s
	Nitrogen dioxide	$^{\cdot}NO_2$	Highly reactive reaction, Environmental toxin	s
	Nitrate radical	$^{\cdot}NO_3$		s
Non-radical				
	Peroxynitrite	$ONOO^-$	Highly reactive reaction, intermediate of $^{\cdot}O_2$ and $^{\cdot}NO$	10^{-3}s
	Nitrous acid	HNO_2		s
	Nitrosonium cation	NO^+		s
	Nitroxyl anion	NO^-		s
	Dinitrogen trioxide	N_2O_3		s
	Dinitrogen tetroxide	N_2O_4		s
	Peroxynitrous acid	ONOOH		Fairly stable
	Nitryl chloride	NO_2Cl		s
	Nitrogen oxides	NO_X	Environmental toxin, derived from the combustion process	

The most important oxygen-containing free radicals in many disease states are hydroxyl radical, superoxide anion radical, hydrogen peroxide, oxygen singlet, hypochlorite, nitric oxide radical, and peroxynitrite radical. These are highly reactive species, capable in the nucleus, and in the membranes of cells of damaging biologically relevant molecules such as DNA, proteins, carbohydrates, and lipids.

This research involves ionizing radiation, the scope of the review and discussion will focus on the free radical and oxidizing species created from water radiolysis, which is indirect effects of ionizing radiation. The schematic diagram of water radiolysis that leads to the formation of free radicals and oxidizing species is shown in Figure 3.2.

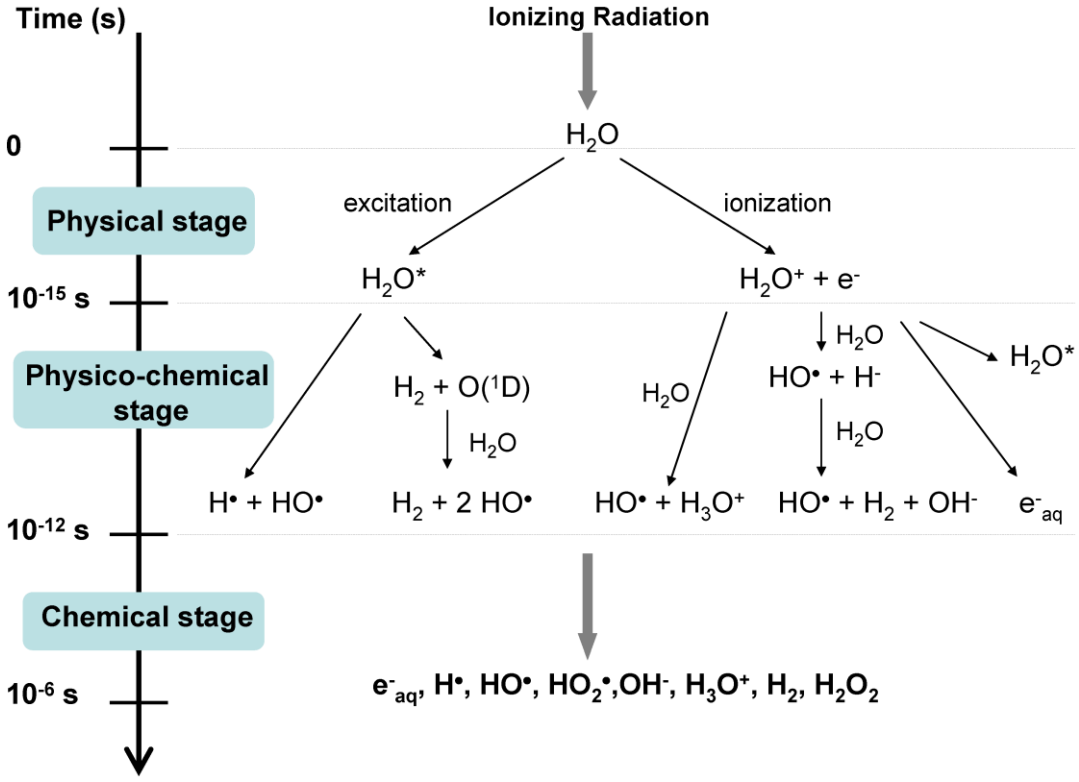

Figure 3.2 Schematic diagram of water radiolysis

When water is exposed to radiation, either excitation or ionization will occur in the physical stage 0 to ~10^{-15}s. In this stage, three products will be produced H_2O^*, H_2O^+ and e^-. Water molecules in an excited state (H_2O^*), as a product of excitation, could release its excess energy to make it stable again. If the energy is sufficient it could also lead to secondary ionization. Free

monoenergetic electron (e^-) as a product of ionization besides water ion oxoniumyl (H_2O^+) could also hit and transfer energy to another H_2O, this could take H_2O molecule into excited state (H_2O^*).

At the end of physico-chemical stage 10^{-15}s to 10^{-12}s, three initial products will be replaced by hydronium ion (H_3O^+), hydroxyl radical ($^{\cdot}OH$), hydrogen radical ($H^{\cdot}$), hydrated electron (e^-_{aq}) and molecular hydrogen (H_2). However, H_2 does not react further. Of these five species created in this stage, three species are free radicals because they have unpaired electrons. Those three species are $^{\cdot}OH$, $H^{\cdot}$ and e^-aq.

In the chemical stage 10^{-12}s to 10^{-6}s, the end products of pre-chemical stage begin to migrate randomly from their initial positions in thermal motion. As their diffusion in the water proceeds, individual pairs can come close enough to react chemically. In this stage, many new species or products were produced. The formation of hydrogen peroxide (H_2O_2), which is not a free radical species although it belongs to the ROS group. H_2O_2 is considered an oxidizing species and harmful because it is toxic to cells or molecules and it can generate free radicals through some reactions.[5)]

The number of each product or species created by water radiolysis can be described as G-value. The G-value refers to the number of molecules, atoms or free radicals created or destroyed per 100eV of energy deposited in the water. In addition, G-value provides an estimate of product or species generated along the paths of an incident primary charged particle (He ion beam) in water irradiated by ionizing radiation, as shown in Table 3.3.

Table 3.3 G-value for product/ species for γ-radiation and fast electron (Low LET)

Product/ Species	γ-radiation and fast electron (Low LET)	
	μmol/J	Molecules / 100eV
H_2	0.047	0.45
H_2O_2	0.073	0.70
e^-aq	0.28	2.70
$H\cdot$	0.062	0.60
$OH\cdot$	0.28	2.70
$HO_2\cdot$	0.0027	0.03

From Table 3.3, OH·has the strongest and the most reactive free radical produced in water radiolysis, which could react very rapidly with anything, either organic or inorganic molecules. [4] This makes OH· the most damaging radical species to living organisms because it could react with most biomolecules and change its structure. [6] The formation OH·in water radiolysis is also dominant compared to other free radicals; thus, making OH· the most dangerous of the free radical. Most damages in cells exposed to low-LET radiation is caused by OH·radical. From these facts, it is necessary to protect human body from indirect effects of ionizing radiation. One of the methods will be discussed in this present study, is by using radioprotector to scavenge of OH and others free radical species.

3.2.5 Damages by free radical

Free radicals can be defined as molecular entities or molecular fragments which contain one or more unpaired electron in atomic or molecular orbital. This unpaired electron will donate or accept electron. General interaction of free radical species with biomolecules can be described in the reaction as below.

$$CH + R\cdot \longrightarrow C\cdot + RH$$

When hydrocarbon base biomolecules (represented by C) react with the free radical species (represented by R·), free radical will be neutralized or detoxified (represented by RH) due to accept one electron or hydrogen atom from biomolecule. A biomolecule could be changed to biomolecule radical (represented by C·) due to lose one of its electrons or hydrogen. The illustration of this interaction is shown in Figure 3.3.

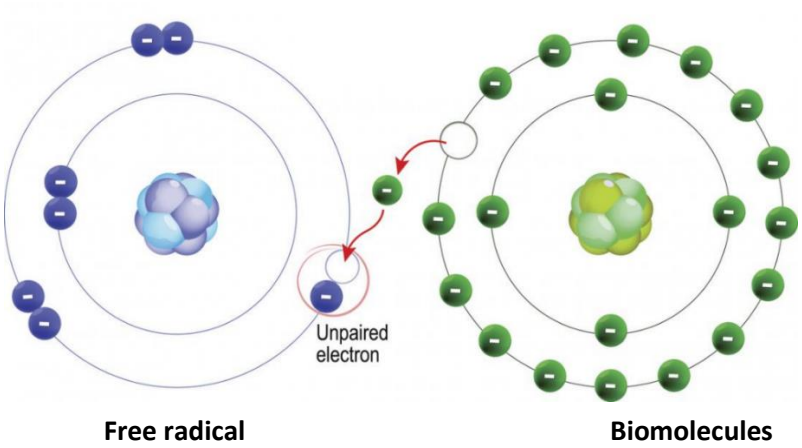

Figure 3.3 The illustration of interaction free radical with biomolecules

Cells contain inorganic compounds such as water and minerals, and also contain organic compounds such as proteins, carbohydrates, lipids and nucleic acid. Typically, mammalian cells consist of ~70% to 85% of water, ~10-20% of proteins, ~10% carbohydrates, ~2-3% of lipids and the rest is nucleic acid and others. Due to these compositions in the cell, biomolecules such as proteins, carbohydrates, lipids and nucleic acid are easy targets to be attacked by free radicals. The mechanism of damage for each biomolecule is described below.

i. Protein oxidation by free radical

Proteins are large dan complex biomolecules that play many critical roles in the body. Protein consists of one or more amino acids. Amino acids are coded by combinations of three DNA building blocks (nucleotides). Each protein is assembled using a unique amino acid sequence encoded in genes. Therefore, each protein must have a specific sequence and specific shape for its specific function. When the free radical interacts with protein, free radicals will oxidize the proteins. This will cause the sequence and shape of protein to be modified and resulting in protein loss its function, for example enzyme activity. The illustration of protein oxidation is shown in Figure 3.4.

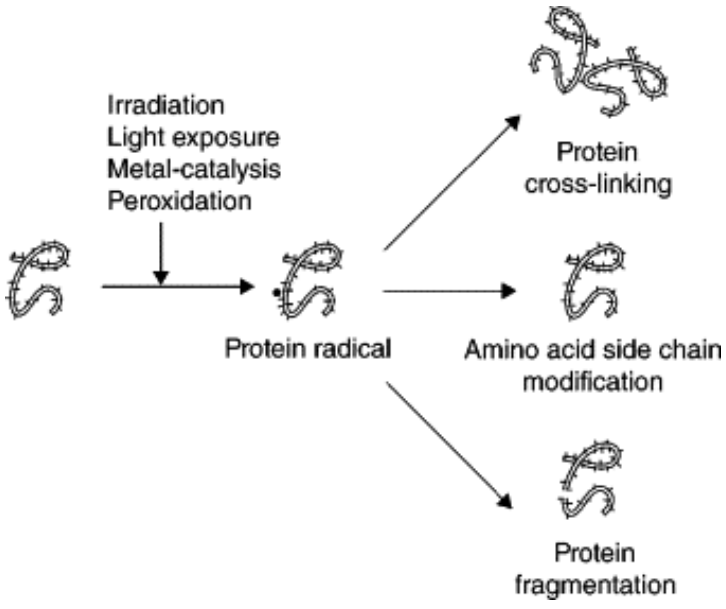

Figure 3.4 The illustration of protein oxidation by free radical

They do most of the work in cells and are required for the structure, function, and regulation of the body's tissues and organs. Proteins are made up of hundreds or thousands of smaller units called amino acids, which are attached to one another in long chains. There are 20 different types of amino acids that can be combined to make a protein. The sequence of amino acids determines each protein's unique 3-dimensional structure and its specific function.

ii. Carbohydrates breakage by free radical

Carbohydrates are sugar molecules. Carbohydrates are components of deoxyribonucleic acid (DNA) due to nucleotides' base attached to a sugar molecule in the form of deoxyribose. The DNA molecule consists of two parallel long chains of deoxyribose (containing nucleotides base) linked by phosphate groups, coil around each other every 10 base pairs. The three dimensions of coil shape (double helix structure) DNA is illustrated in Figure 3.5.

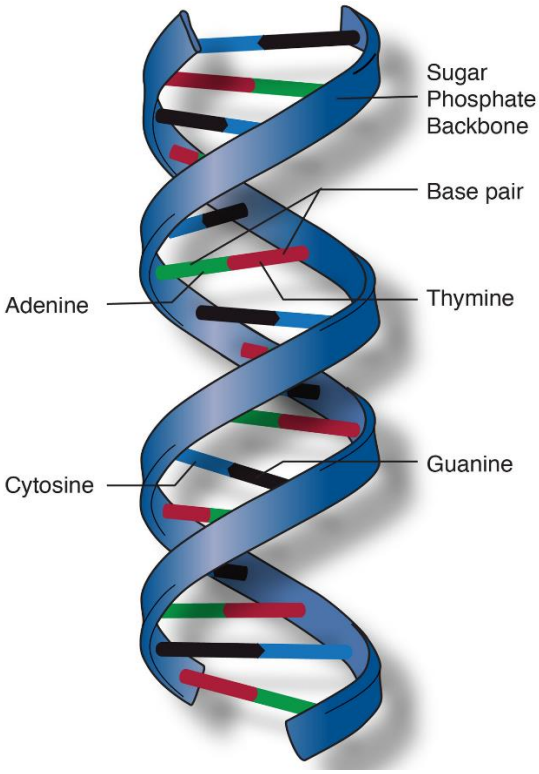

Figure 3.5 The coil shape (double helix structure) of DNA

Interaction by direct effect of radiation or free radical (indirect effects) will localize breakage of the hydrogen in deoxyribose-phosphate bonds, which are deleterious to the structural integrity of the DNA molecules. If the localized breakage in deoxyribose-phosphate is limited to only one in the DNA helix structure, it is referred to as a single strand break (SSB). Meanwhile, when the localized breakage in deoxyribose-phosphate is on both strands of the DNA helix it is referred to as double strand break (DSB). Both SSB and DSB as shown in Figure 3.6.

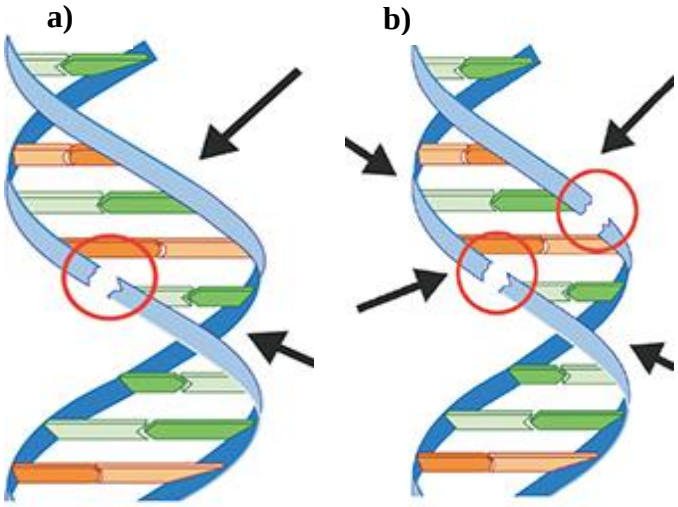

Figure 3.6 Breakage of deoxyribose-phosphate in DNA; a) SSB and b) DSB

iii. Lipids peroxidation by free radical

Lipid peroxidation is the reaction of oxidative degradation of lipids. In this reaction, free radicals will steal electrons from the lipids in cell membranes and resulting in cell damage. Cell membrane gives the cell its structure, keeps important substances inside the cell and regulates only certain materials and molecules can enter and leave the cell (selective permeable). Therefore, cell membranes can protect the cell by preventing poisons or dangerous substances from entering the cell. Oxygen is needed for cell respiration, and carbon dioxide is a byproduct of respiration that can easily enter and exit through the membrane. Water can cross the membrane freely at a slower rate.

The main component of lipids in the cell membrane is phospholipids. When free radical attacks lipids in the cell membrane, it could lead the formation of lipid alkyl radical ($L^{\cdot}$) (E°=2300mV) and can also react with oxygen (O_2) to form lipid peroxyl radical ($L^{\cdot}$) (E°=1000mV) which has even higher value of reduction potential. This reaction could lead to a chain reaction and resulting in the rupture or leakage of the cell membrane. The rupture or leakage of cell membrane could make any molecules can enter easily to the cell and important substances to cell for survive could leave away from the cell. The reaction as shown as below.

$$LH + \cdot OH \longrightarrow \cdot L + H_2O$$

$$\cdot L + O_2 \longrightarrow LOO\cdot$$

$$LH + LOO\cdot \longrightarrow \cdot L + LOOH$$

iv. Nucleic acid (DNA) oxidation by free radical

DNA is the molecule that carries genetic information for the development and functioning of an organism. The information in DNA is stored as a code of four chemical bases also known as nucleotides base. The nucleotides base is adenine (A), cytosine (C), guanine (G) or thymine (T). The nucleotides base is paired with each other, which A with T and C with G to form units called base pairs. The order or sequence of the base pairs in DNA will determine the information in a living organism.

An important property of DNA is that can replicate or make a copy itself. This is critical when the cells divide due to each new cell needs to have an exact copy of the DNA present in the old cell. When free radicals, for example OH encounter DNA bases, it could modify the base by binding the base. A modified base could behave like a different base in the DNA replication process and will lead to a cell mutation due to failure in recognizing the original base with the addition of OH. DNA base damage is the most dominant type of DNA damage followed by SSB and DSB.

3.3 Repair mechanism

When the human body is exposed to radiation many damages will occur due to free radical formation and its reaction with biomolecules. However, the human body has internal defense systems that are able to recognize, repair or replace the damages by biomolecules. The repair mechanism will be depending on the type of damages, level or number of damage and location of damages. The repair mechanisms of damages by each biomolecule are described as below.

For protein oxidation, an enzyme called protease will recognize the oxidized proteins and degrade completely to amino acid. Then, new replacement protein molecules are synthesized and appears probable that most of the amino acid from an oxidized and degraded protein are reutilized for protein synthesis. Lipid peroxidation involves the production of a variety of breakdown products including alcohols, ketones, alkanes, aldehydes, and ethers.[4)] For lipids peroxidation, enzymatic repair mechanisms are available to cells to countering lipid peroxidation and also patching and sealing the rupture or the leakage in cell membrane.

For DNA damages either Single Strand Break (SSB), Double Strand Break (DSB) or base damages have specific repair mechanisms. Base damage can be easily repaired through Base Excision Repair (BER) repair pathway. Meanwhile, SSB can be repaired through Single Strand Break Repair (SSBR) repair pathway. In general, both pathways have same steps including detection, removal and cleaning the damage site, fill the right base based on opposite undamaged strand as template, and its sugar-phosphate backbone and sealed.

DSB can be repaired by Homologous Recombination (HR) or Non-Homologous End Joining (NHEJ). However, DSB is more difficult to be repaired compared to SSB. In SSB, one undamaged strand of DNA can be used as a template, while the breaks in DSB occur in both strands of DNA and separated by only a few base pairs. HR and NHEJ are different in the genes involved, the position in the cell where they primarily act, in the speed and accuracy of repair. HR and NHEJ are described as below.

- Homologous Recombination (HR)

  HR joins two DSB ends together with requiring homologous undamaged DNA template to repair the break, leading to the reconstitution of the original sequence.[7)] In general, the step of HR repair pathway is detection and modifying DNA end, exchange with homologous DNA, DNA synthesis and DNA ligation.

- Non-Homologous End Joining (NHEJ)

  NHEJ joins two DSB end together ends without requiring homologous DNA sequences.[8] In general, the step of NHEJ repair pathway are detection and binding at both end of DSB, modifying DNA ends.

The pathway HR repair and NHEJ repair as shown in Figure 3.7. HR repair pathway has more steps but its error free repair mechanism due to use of the other DNA strand with the same sequence as a basis for repairing. Meanwhile, NHEJ pathways have a simple step so the repair is more rapid than HR but less accurate with small deletion or insertions often resulting at the repaired break site and this could lead to a mutation.

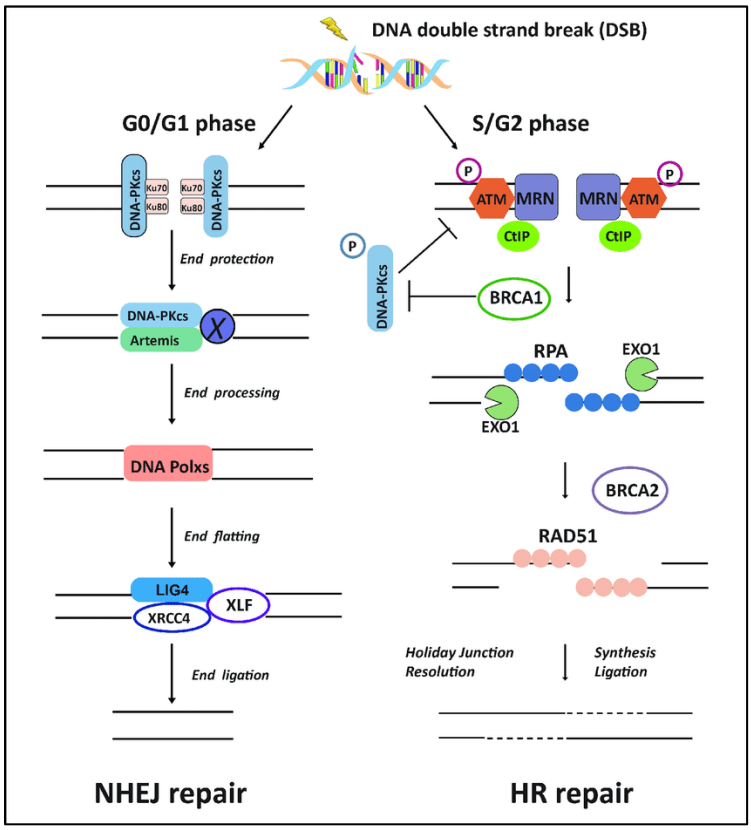

Figure 3.7 Schematic pathway of HR repair and NHEJ repair

3.3.1 Radiation effect evaluation using biological samples.

Ionizing radiation could cause harm and damage to living organisms. Many researchers in this field use an organism as a model for the experiments or studies in order to understand the

mechanism of interaction between living thing and physical factor such as ionizing radiation.[9] The models normally used ranges from unicellular organisms s bacteria, yeast and algae to multicellular organisms such as mice, rats, and primates. Multicellular organisms are more complex organisms compared to unicellular organisms.

Humans are multicellular due to the cells of organism have specialized into many different types of cells such as muscle cells and nerve cells; has and have many different functions. Multicellular organisms are used as model organisms, but the complexity of these organisms is one of the problems to evaluate the biological effects from ionizing radiation. Therefore, to avoid these problems unicellular organisms such as yeast cells, could be used as biological samples. The use of yeast cells as model in radiobiology studies is described below.

3.3.2 Yeast as model organism

Saccharomyces cerevisiae, is also known as yeast. Yeast are eukaryotic single-celled microorganisms and classified as members of the Kingdom Fungi. The first yeast originated hundreds of millions of years ago and at least 1,500 species are currently recognized. Yeast contains as same organelles of mature eukaryotic cell such as nucleus, mitochondria, and Golgi bodies. The structure of yeast cell is shown in Figure 3.8. The taxonomy of yeast is as in Table 3.4.

Typically, the size of yeast cell is 5 µm x 10 µm, which is bigger than the size of a bacterium, at 0.5 µm x 5 µm. Yeast cells have a resistance to antibiotics, sulfamides and other anti-bacterial agents. This resistance is genetically, naturally, and not liable to be modified or transmitted to other microbes. Yeast has a genome of nearly 6000 genes in 12 mega base pair of DNAs on 16 linear chromosomes in the nucleus. This genome is considered very compact for a eukaryote, where the number and size of gene are relatively small, but the density is high.

Yeast is widely dispersed in a variety of habitats. Yeast is commonly found on plants such as leaves, flowers, and fruits, soil, and in water either fresh or salt water. Yeast can also be found on the surface of the skin and in the intestinal tracts of warm-blooded animals, which may live symbiotically or as parasites. However, several species of yeast may be isolated from extreme environments such as high sugar or salt concentration, low temperature, and low oxygen availability.

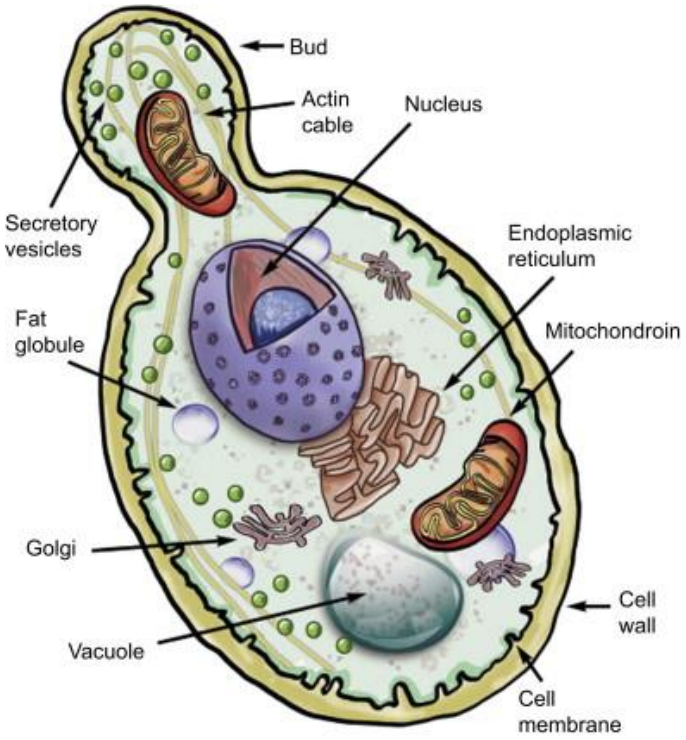

Figure 3.8 The structure of a yeast cell

Table 3.4 Taxonomy of yeast cell, *S. cerevisiae*

Domain	Eukarya
Kingdom	Fungi
Division	Ascomycota
Class	Saccharomycetes
Order	Saccharomycetales
Family	Saccharomycetaceae
Genus	Saccharomyces

In liquid culture, yeast cells will continue dividing as long as the conditions such as temperature is favorable and plentiful with nutrients. The completing a round of the cell cycle for yeast cells approximately is 90 minutes. *S. cerevisiae* can reproduce sexually or asexually. For sexual reproduction, haploid yeast came in two mating types a cell and α cells. Each mating type secretes its corresponding pheromone, a small peptide that tells neighboring cells which mating

type. When a particular haploid cell senses a pheromone from the opposite mating type, it will prepare to mate by forming a mating projection.

For asexual, new daughter cells arise mitotically as buds that grow in size and eventually split from the mother cell. The cell cycle consists of four distinct phases (G1, S, G2 and M) and is regulated similar to that of the cell cycle in larger eukaryotes. During S phase of the cell cycle, a small bud (diameter of 1-7µm) emerges from the ovoid mother cell (diameter 5-50µm). This bud will continue to grow as the cell prepares for cell division (mitosis in M phase). During mitosis, the nucleus divides, and full complement of DNA is packaged into the large bud before being pinched off. Both mother and daughter cells will go to G1 phase, where they grow and subsequently embark on a new round of the cell cycle. The asexual reproduction of *S. cerevisiae* is shown in Figure 3.9.

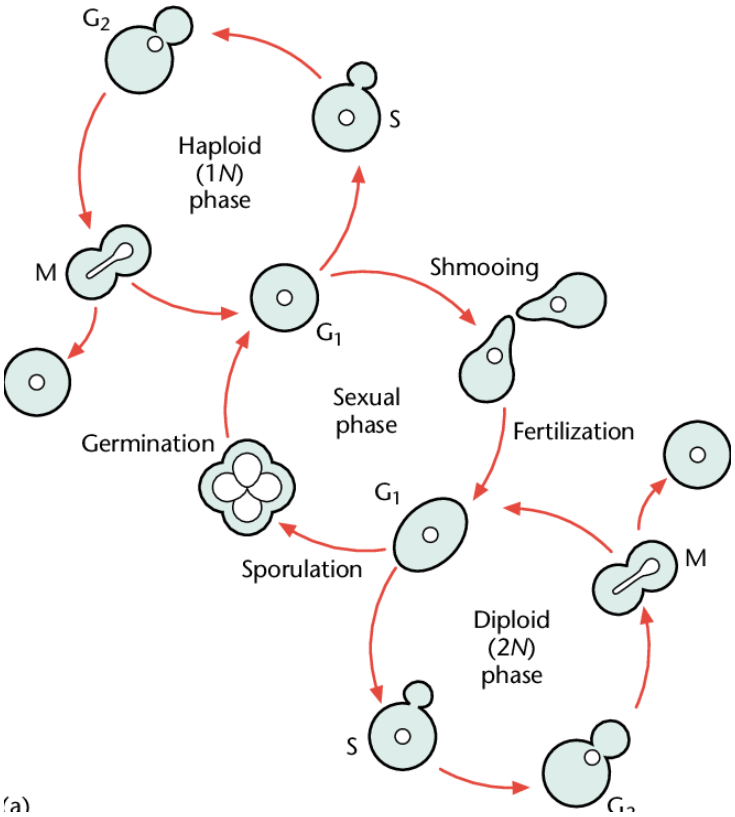

Figure 3.9 The asexual reproduction of *S. cerevisiae*

A model organism is a biological term that has been used on an organism to understand particular biological processes or biological phenomenon. In general, this organism will be

representative of a larger class of living beings for whatever biological process or phenomenon is interested. It can also be represented for human to explore potential causes, effects, and treatment for human disease when human experimentation would be unfeasible and unethical. As example, in laboratory mice and rat are used since decades to test drugs, fruit flies and tiny worms for scientists to understand new findings.

Selected species for a model organism should meet several requirements. Usually, many aspects of these organisms are similar to the larger class of living beings including humans. The similarity can be in the repair pathway, behaviour, morphology, genetic, and metabolism. Furthermore, the genome is well known, able to trace the gene and which part of gene are involved and gave the reaction. The organism is also chosen as model due to it being easy to maintain and propagate or breed in laboratory setting, have a short life cycle and genetic manipulation can be performed.

In this present study, yeast was used as a model organism to evaluate the ionizing radiation effects with added radioprotector agent. Yeast is the most well studied and widely used as model organism to study the life process or eukaryotic organisms at different levels either for genetic or molecular.[10)] A lot of similarities with other eukaryotic organisms especially with human cells is one of many reasons whereby yeast is widely accepted as a model system for studying eukaryotic organisms.[11)]

3. 4 Radiation protection

Radiation protection aims to reduce unnecessary radiation exposure with a goal to minimize the harmful effects on health and well-being of ionizing radiation.[12)] In March 1896, after 4 months X-ray was discovered, eye irritation due to use of X-rays was first reported.[13)] This report marked as initial recognition of the hazard of radiation. After this time, numerous injuries on dermatitis due to X-rays and more serious injuries have been reported and published in the manuscript or scientific literature. To date, the knowledge and awareness about the hazard of ionizing radiation is better and radiation workers know how to protect themselves. There are three methods that can be applied to reduce exposure to ionizing radiation: (1) distance (2) minimizing the time of exposure, (3) using shielding and 4) setting acceptable exposure limits and by radioprotective agents.

3.4.1 Distance

A greater distance from the radiation source can reduce radiation exposure. The concept of distance is easy to understand by using principle when see the light bulb. Shorten distance from the light bulb will give brighter light. When we move away, the brightness of the light bulb will be reduced. This concept also can be applied for radiation, the more distance we a from the radiation source, the less exposure will be detected. This concept can be explained using the inverse square law mechanism. The details of this concept as in Figure 3.10.

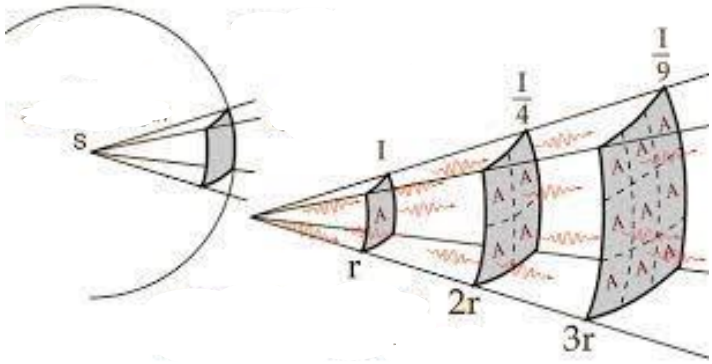

Figure 3.10 Inverse square law mechanism

3.4.2 Time

Radiation exposure can be accumulated over the time of exposure. The longer the exposure time, the more radiation exposure to the personnel in charge. The formula can be written as below.

Dose= Dose rate x time

From the formula, the radiation dose received to the personnel in charge is equal to the time spent in the area (e.g., minute, hour) multiply by the dose rate within the area (e.g., gray per hour, mrem per hour). Reducing the time spent in the radiation area can reduce radiation dose that will exposure. As example, when we enter the radiation area with survey meter reading is 50 gray per

hour (dose rate) in particular position, if we reduce spend time at radiation area only to ½ hour, that means the dose will be ½ of 50 gray or 25 gray at the same position.

3.4.3 Shielding

Reducing the time of radiation exposure and increasing the distance from radiation sources with the use of shielding devices for radiation protection are important. Basically, from the methods to reduce exposure of ionizing radiation based on time and distance, the radiation received is dependent on the dose rate. The dose rate depends on the radioactive source. The dose rate from the radioactive source will be reduced by placing a shielding in between radioactive source and survey instrument. The shield will attenuate or absorb some of the radiation coming from the radioactive source and remaining radiation will be able to penetrate or pass through the shielding. The types of shields depend on the types of radiation. Figure 3.11 illustrates types of shields for each radiation type.

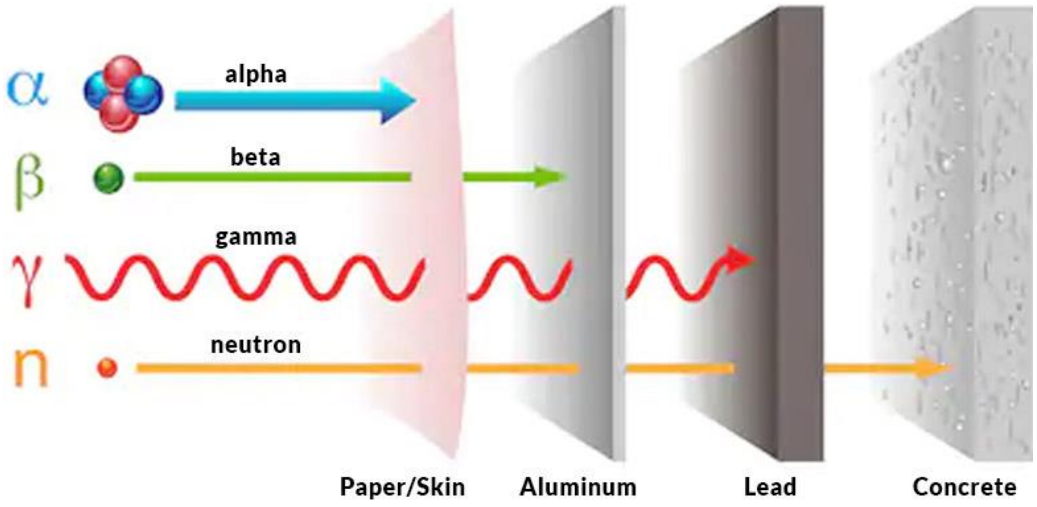

Figure 3.11 Types of shields for each radiation types

From Figure 3.11, alpha particles can be stopped easily. A piece of paper is sufficient to stop alpha particle. Shields for beta particle are usually made from aluminum, brass, plastic, or

other materials with low atomic number. For x-rays or gamma rays collimated into a narrow beam are attenuated exponentially through a shield according to the following equation.

$$I = I_0 e^{-\mu x}$$

Where is; I = the intensity outside of as shield of thickness x

I_0= the unshielded intensity (intensity before hitting the shield)

μ= the linear attenuation coefficient of the shielding material (1/cm), and

x = the thickness of shielding material (cm)

Value of μ is dependent of the types of materials. A denser atom in the shielding material will give the greater value of μ, which means more protection. For example, with the same of thickness the μvalue of lead (Pb) is greater than aluminum (Al). This means Pb give better and more protection of ionizing radiation compared to Al.

3.4.4 Radioprotective agent

The ability of potent protective agents to provide protection against the damaging effects of ionizing radiation was first reported in 1949.[14)] Radioprotective agents are substances or chemicals with the ability to reduce the biological effects of ionizing radiation by the scavenging of free radicals or by repairing radiation injury. Radioprotective agents can be divided into three groups, 1) radioprotector, 2) adaptogens and 3) adsorbents. Radioprotectors have the ability rapidly (within minutes) to give the protective effects by scavenging free radical created from ionizing radiation. Adaptogens will give the protection with increase the resistance to radiation by activating the antioxidant system, the repair system, and other protective systems. Meanwhile, adsorbents protect the organism from internal radiation and chemicals by binding radionuclides to accelerate their excretion.

The term radioprotector is also recognized as antioxidant due to have same protection mechanism by scavenging free radical. The term antioxidant originally was used to refer specifically to a chemical with properties that has the ability to prevent consumption of oxygen. The radioprotector can be classified into several types, which are 1) natural and synthetic

radioprotector, 2) endogenous and exogeneous radioprotector and 3) enzymatic and non-enzymatic radioprotector. Natural radioprotectors are plant compounds that have protective effects on the cells from the damage caused by radiation such as for radiation therapy.[15] This study will investigate natural radioprotective from natural sources, which is mushroom. The information on natural radioprotective from natural sources will be described below.

3.4.4.1 Antioxidant as natural radioprotector from natural sources

The antioxidant and radioprotector term used for chemicals or compounds that has a protective effect from any kind of free radicals. Specifically, antioxidant term used for chemical has protective effects by scavenging of free radical from internally such cell normal metabolism etc. or externally such as UV radiation, and ionizing radiation. Meanwhile, radioprotector term used for chemicals that have protective effects by scavenging of free radical from ionizing radiation. On the other hand, antioxidants can be used as radioprotective agents.

The human body can produce antioxidants naturally during normal metabolism. However, some antioxidants cannot be produced by human body. Therefore, these types of antioxidants must be supplied or get in the diet, which is human need to take these from their foods source. Mushrooms are natural sources food with high content of antioxidant.[16] Natural sources are nontoxic with proven therapeutic benefits and have been utilized since ancient period for curing various ailments. About 60% of the 1,184 new drugs developed over the past 25 years are from natural sources.[15] Some studies also have reported associations between mushroom consumption and low risks of chronic disease such as cancer, and metabolic syndromes.

3.4.4 2 Mushroom as natural sources for natural radioprotector

P. sajor-caju is commonly known as grey oyster mushroom. This mushroom is a species of saprophytic and classified as members of the Kingdom Fungi. The species was named by Professor S. T. Chang, a great mycologist who introduced this species to the mushroom industry.[17] The consumption of mushrooms is increasing due the nutritional and medicinal benefit to the human. Mushrooms contain high protein, fiber, vitamin, and minerals, but with low-fat levels. [18] As a result of these properties, some mushroom extracts are used to promote human health and are found as dietary supplements.

In recent years, radiation has gained tremendous use in the diagnosis and treatment for a wide range of malignant conditions such as cancer. However, this will also cause damage and death to the normal cells. Some studies have been reported to use mushroom as a protective agent and its potential to the radiation, such as extract from *Phellinus rimosus*[19] *Cordyceps militaris*[120] and *Agaricus bisporus*[21] In the present study, protective effects of extract from *P. sajor-caju* as natural radioprotector agents to ionizing radiation will be investigated. Yeast is used as the model living organism to investigate this extract as potential natural radioprotector agents against ionizing radiation.

3.5 References

1) K. H. Cheeseman and T. F. Slater. An introduction to free radical chemistry. *British Medical Bulletin*. 49 (3):481-493 (1993).
2) M. Genestra. Oxyl radicals, redox-sensitive signalling cascades and antioxidants. *Review Cell Signal.* 19 (9):1807–19 (2007).
3) A. Phaniendra, D. B. Jestadi and L. Periyasamy. Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases. *Indian Journal of Clinical Biochemistry*. 30 (1):11–26 (2015).
4) G. Martemucci, C. Costagliola, M. Mariano, L. D'andrea, P. Napolitano and A. G. D'Alessandro. Free Radical Properties, Source and Targets, Antioxidant Consumption and Health. *Oxygen*. 2: 48–78 (2022).
5) O. Desouky, N. Ding and G. Zhou. Targeted and non-targeted effects of ionizing radiation. *Journal of Radiation Research and Applied Sciences*. 8: 247-254 (2015).
6) A. Maradi. Master thesis: radiation protection effects on living organism by addition of ECGC and ascorbic acid. *RINE, University of Fukui*. (2019).
7) L. H. Thompson and D. Schild. Homologous recombinational repair of DNA ensures mammalian chromosome stability. *Mutation Research*. 477 (1-2):131–153 (2001).
8) M.R. Lieber. The mechanism of human nonhomologous DNA end joining. *Journal Biology Chemistry.* 4 (283-1): 1-5 (2008).

9) E. F. Garmana and M. Weikb. Radiation damage to biological samples: still a pertinent issue. *Journal of Synchrotron Radiation*. 28: 1278–1283 (2021).

10) D. Botstein, S. A. Chervitz, and J. M. Cherry. Yeast as a Model Organism. *Science*. 277 (5330): 1259–1260 (1997).

11) J. C. Mell and S. M Burgess. Yeast as a model genetic organism. *Encyclopedia of Life Sciences*. Macmillan Publishers Ltd, Nature Publishing Group (2002).

12) V. Tsapaki, S. Balter, C. Cousins, O. Holmberg, D.L Miller, P. Miranda, M. Rehani, E. Vano. The International Atomic Energy Agency action plan on radiation protection of patients and staff in interventional procedures: Achieving change in practice. *Physic Medical*. 52:56-64 (2018).

13) Y. Rojavin, M. J. Seamon, R. S. Tripathi, T. J. Papadimos, S. Galwankar, N. Kman, J. Cipolla, M. D. Grossman, R. Marchigiani, and S. P. A. Stawicki. Civilian nuclear incidents: An overview of historical, medical, and scientific aspects. *Journal of Emergencies, Trauma and Shock*. 4 (2): 260-272 (2011).

14) A. Shirazi, E. Mihandoost, S. R. Mahdavi and M. Mohseni. Radio-protective role of antioxidant agents. *Oncology Reviews*. 6(16):1-5 (2012).

15) V. Kuruba and P. Gollapalli. Natural radioprotectors and their impact on cancer drug Discovery. *Radiation oncology Journal*. 36(4):265-275 (2018).

16) D. M. Ba, X. Gao, J. Muscat, L. Al-Shaar, V. Chinchilli, X. Zhang, P. Ssentongo, R. B. Beelman and J. P. Richie Jr. Association of mushroom consumption with all-cause and cause-specific mortality among American adults: prospective cohort study findings from NHANES III. *Nutrition Journal*.20 (38): 1-11 (2021).

17) S.T. Chang and J.A. Buswell. Mushroom nutraceuticals. *World Journal Microbiology Biotechnology*. 12: 473-476 (1996).

18) M. E. Valverde, T. H. Pérez and O. P. López. Edible Mushrooms: Improving Human Health and Promoting Quality Life. *International Journal of Microbiology*. 1-14 (2015).

19) J. Joseph, S. N. Panicker and K. K. Janardhanan. Protective effect of polysaccharide-protein complex from a polypore mushroom, *Phellinus rimosus* against radiation-induced oxidative stress. *Redox Report*. 17 (1): 22-27 (2012).

20) M. H. Jeong, Y. S. Park, D.H Jeong, C. G. Lee, J. S. Kim, S. J. Oh, S. K. Jeong, K. Yang and W. S. Jo. In vitro evaluation of *Cordyceps militaris* as a potential radioprotective agent. *International Journal of Molecular Medicine*. 34: 1349-1357 (2014).

21) C. Y. Hwang, Y. Halim, M. Sugata, D. Rosa, S. P. Wijaya and E. Steven. Assessment of *Agaricus bisporus* Mushroom as Protective Agent Against Ultraviolet Exposure. *Emmerich Research Center, Jakarta, Indonesia*. 1-11 (2021).

Chapter 4: The Biological Effects of Lethal Dose 50 (LD_{50}) 2.2 kGy Gamma Radiation on Morphology Characteristics of *Pleurotus sajor-caju* Mushroom

In **Chapter 4**, a study on radiation mutagenesis at Lethal Dose 50 (LD_{50}) 2.2 kGy by gamma radiation on *Pleurotus sajor-caju* mushroom is investigated and reported. This chapter discusses morphology effects of gamma radiation at LD_{50} on mycelium growth, morphology and productivity of fruiting bodies *P. sajor-caju*.

Abstract

Mutagenesis is a valuable tool in crop improvement, resulting from high probability of selecting mutant varieties with desired traits, including high productivity or yield, in addition to superior quality. Mushrooms have been consumed and are high in demand due to their exceptional nutritional values. *Pleurotus* spp. mushrooms are the most commercially cultivated and stand second place in world production amongst edible mushrooms, with 19% contribution to total world mushroom production. However, the production is still insufficient for consumption, and meeting the high demand. Therefore, works on strain improvement with selected characteristic such as fast growth, high productivity, better quality fruiting bodies and disease resistance are crucial in the development for maximising high productivity for the sustainability of the mushroom industry. Radiation mutagenesis is a technique that can be applied for mushroom strain improvement and breeding. This chapter will discuss the biological effects of gamma radiation at dose lethal dose 50 (LD_{50}) on mycelium growth, morphology and productivity of fruiting bodies *P. sajor-caju*.

4.1 Introduction

The mushroom industry does have problems such as senescence induced browning during storage, disease and low productivity.[1)] Mutation has long been exploited in crops breeding programmes to improve both quality and productivity.[2)] Therefore, using radiation mutation technique, obtaining improved mushroom strains with desired characteristics such as fast growth, high productivity, disease resistance, sporeless, resistance or tolerance against biotic and abiotic stresses, better shelf life and colour, nutritional of fruiting bodies are crucial to support the development and sustainability of the mushroom industry. [1, 3)]

Radiation mutagenesis has been used for nearly 100 years and has been successful for generating high quality crops by increasing genetic variations.[4)] Compared to other breeding

methods such as cross-breeding and chemical mutagenesis, radiation mutation breeding has incomparable advantages with a wide mutation spectrum and high mutation efficiency.[5])

According to International Atomic Energy Agency (IAEA), more than 60 countries in the world have used radiation, including fast neutrons, X-rays and gamma radiation, for the improvement and development of crops. More than 64% of the above use gamma radiation. Gamma radiation is an effective ionizing radiation due to its energy level, which can penetrate cell wall of mycelium and targeted cells effectively.[1)]

Mushrooms are fruit bodies of macrofungi, and considered as natural and healthy food products. Mushrooms can be classified into four major groups, namely, edible, medicinal, poisonous and wild.[1]) Mushrooms have been widely consumed and highly in demand due to their high nutritional contents. There are five main genera of mushrooms, namely, *Lentinula, Pleurotus, Auricularia, Agaricus* and *Flammulina,* constituting about 85% of the world's mushroom supply. Mushrooms have also been increasingly utilised in the pharmaceutical, nutraceutical and cosmeceutical industries.

Pleurotus spp. are commonly known as oyster mushrooms. There have been several studies on mutagenesis induction in many species of *Pleurotus*.[2)] The studies of radiation mutation breeding on *Pleurotus* spp. such *as Pleurotus pulmonarius, P. ostreatus, P. djamor, P. florida,* and *P. sajor-caju,* has been reported. [3, 6, 7, 8, 9)] *Pleurotus* spp. constitute about 30% and ranks third among the cultivated mushrooms grown widely in the temperate, subtropical and tropical regions of the world.[9,10)]

Cultivated mushrooms including *P. sajor-caju* (Figure 4.1) face the problems of loss of genetic diversity and strain degeneration.[11)] Therefore, development and improvement of mushrooms strain *P. sajor-caju* are crucial to ensure the demand by consumer and to support the sustainability of the mushroom industry. Radiation mutagenesis has been exploited in breeding programmes to improve both quality and productivity of mushroom species. This chapter discusses the biological effects of gamma radiation at LD_{50} on mycelium growth, morphology and productivity of fruiting bodies *P. sajor-caju*. From our earlier study, the LD_{50} of *P. sajor-caju* was determined at 2.2 kGy.[12)]

Figure 4.1 Fruiting bodies of *P.sajor-caju* mushroom

4.2 Experimental

4.2.1 Preparation of Mycelium Culture

The mycelia of *P. sajor-caju* used in this study were obtained from culture collection of Block 46, Agrotechnology and Biosciences Division, Malaysian Nuclear Agency. Selected mycelia of *P. sajor-caju* were cultured onto potato dextrose agar (PDA) in Petri dishes and incubated at ambient temperature. After 10 days, the mycelial cultures were irradiated at dose $LD_{50,}$ which is 2.2 kGy.

4.2.2 Gamma Irradiation at Dose LD_{50}

The determination of dose LD_{50} for *P. sajor-caju* as mentioned in paper Rosnani *et al.,*[12)] Irradiation was conducted at Malaysian Nuclear Agency using Biobeam GM 800 gamma irradiation facility. After irradiation, the mycelia were cut using core borer size 0.7 cm and transferred onto new PDA in 9 cm Petri dishes. The growth of the irradiated mycelia was observed for 2 weeks with 3 days interval, and data were analysed. The irradiated mycelia with the fastest growth were selected for preparation of liquid seed for further cultivation. PDA Petri dishes with cultures that showed no growing activity during this period was considered as no surviving mycelia.

4.2.3 Preparation of Liquid Seeds of Irradiated *P. sajor-caju*

The selected irradiated mycelia of *P. sajor-caju* with the fastest growth at dose LD_{50} were subcultured onto new PDA Petri dishes. The fast growing and healthy mycelia were used for preparation of liquid seeds. Liquid seeds were prepared by inoculation of the selected mycelia in potato extract liquid medium supplemented with glucose. The inoculated mycelia in potato extract liquid were placed in a horizontal shaker and incubated at 27°C for a week.

4.2.4 Preparation for Mushroom Cultivation and Mushroom Fruiting

Matured and healthy liquid seeds without contamination were cultivated on baglog substrate. Baglogs substrates were prepared by mixing saw dust, rice bran and limestone powder with the ratio 100:10:1. The substrate was adjusted to 70% moisture content and packed into plastic bags. Each bag weighed around 600 g. Forty baglogs substrate were prepared for cultivation for each treatment. Packed baglogs substrate were sterilized using the autoclave at 121 °C for 1 h. The sterilized baglog substrates were inoculated with 20 ml of liquid seeds. The inoculated baglogs were incubated at ambient temperature of about 27 °C to allow growth for a certain period until the baglogs were fully covered with mycelia. The growth of mycelia on baglog substrate was monitored periodically and recorded every week. The fruiting bodies were harvested and weighed. Total mushroom productivity was calculated as biological efficiency (BE) as stated in Chapter 3. The morphology of fruit bodies such as diameter of cap, length of stipe and shape of cap were measured, observed, and recorded. The data were collected for three cycles of flushes to evaluate the trend of productivity, number and size of mushroom fruiting bodies. The schematic diagram from irradiation, preparation of liquid seeds and cultivation is shown in Figure 4.2.

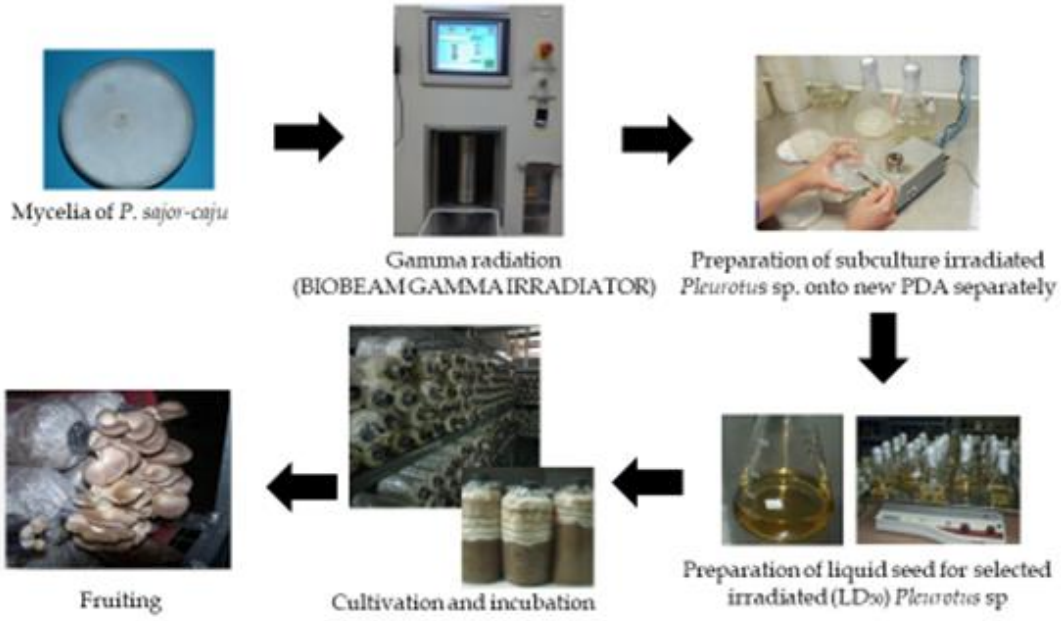

Figure 4.2 Schematic diagram from irradiation, preparation of liquid seeds and cultivation

4.3 Results and Discussion

4.3.1 Effects on mycelium growth and productivity

In this study, the LD_{50} of dose radiation was be obtained from our earlier study.[12] From this report, LD_{50} of mycelium *P. sajor-caju* was determined to be at 2.2 kGy as shown in Figure 4.3. According to Dewi *et al.*, [13] LD_{50} is different between species and varieties in a species as well as for different mushrooms. The LD_{50} gamma radiation for mycelium of *Auricularia auricula-judae* was at 1.5 kGy and dikaryon mycelium of *Lentinula edodes* was at 0.735kGy.[14,15] The mycelia of *P. sajor-caju* mushroom were comparatively radiation-resistant compared to those of *Auricularia auricula-judae* and *Lentinus edodes*. Determination of LD_{50} is particularly important to predict radiosensitivity level of irradiation and its effects prior to inducing mutation.[16] However, studies on LD_{50} value for mushroom species have not been extensively reported.

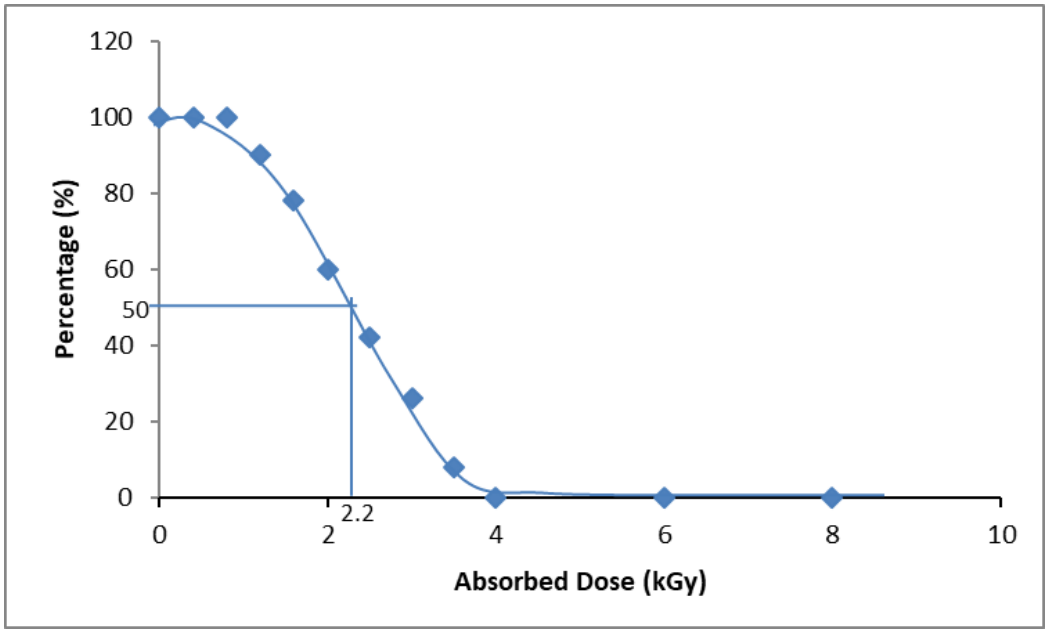

Figure 4.3 Survival curve of irradiated mycelium *P. sajor-caju* at 12 days of incubation [12)]

The results on growth rate of LD_{50} of irradiated *P. sajor-caju* mycelia on PDA Petri dishes are shown in Figure 4.4. The effect of radiation on growth rate of the irradiated mycelium was clearly observed in three (3) days of incubation. However, after three (3) days of incubation, the irradiated mycelia began to grow slower. In this period, the irradiated mycelia in repairing mechanism due to injured as effects to radiation. Cell has repair mechanism, whereby injured cell due to irradiation can survive and regrow after a certain repairing period.[17)] After nine (9) days of incubation, non-irradiated mycelia have fully grown and covered the PDA surface, while the irradiated mycelia took twelve (12) days of incubation.

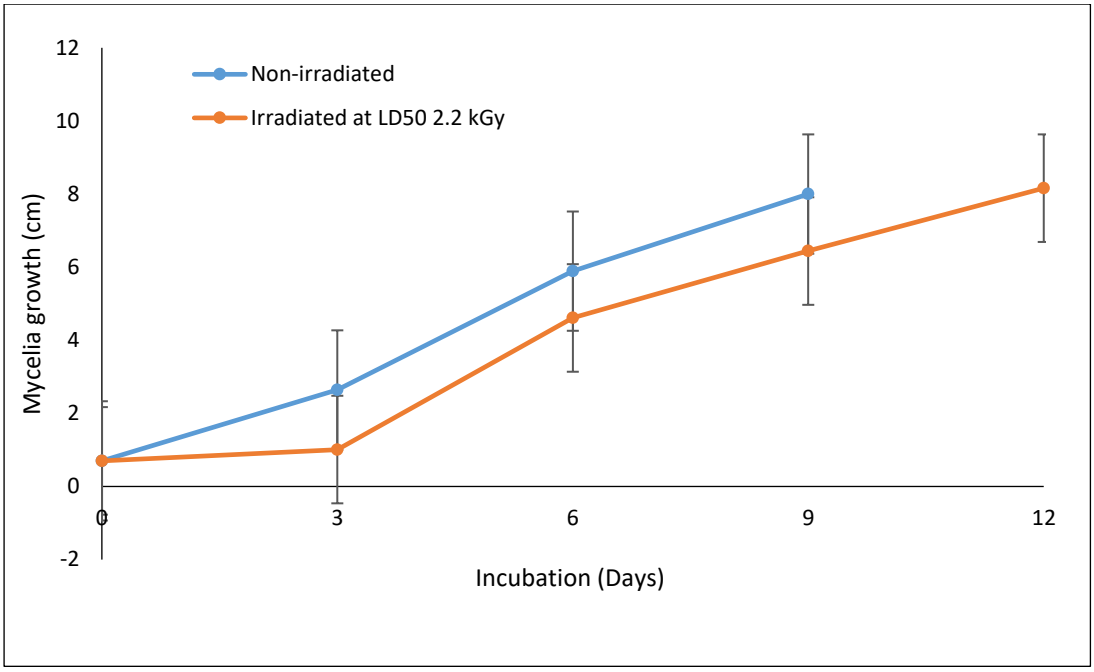

Figure 4.4 Average mycelium growth of LD_{50} irradiated *P. sajor-caju* mycelia on PDA petri dish

The results on growth rate of LD_{50} irradiated *P. sajor-caju* mycelia on baglog substrate are as shown in Figure 4.5. From Figure 4.2, growth of irradiated mycelia did not show much difference than the non-irradiated mycelia at first week of incubation. However, from the second week of incubation and onwards, growth for irradiated mycelia clearly slowed down as compared to the non-irradiated mycelia. Non-irradiated mycelia were fully grown in four (4) weeks, while irradiated mycelia at LD_{50} 2.2 kGy had full growth after five weeks. A study by Beejan and Nowbuth,[7)] showed that gamma irradiation on a different strain of *Pleurotus* species resulted in no definitive trend, as observed in mycelia colonization rates on media with the increasing doses of gamma radiation; however, the mushroom yield or productivity was enhanced.

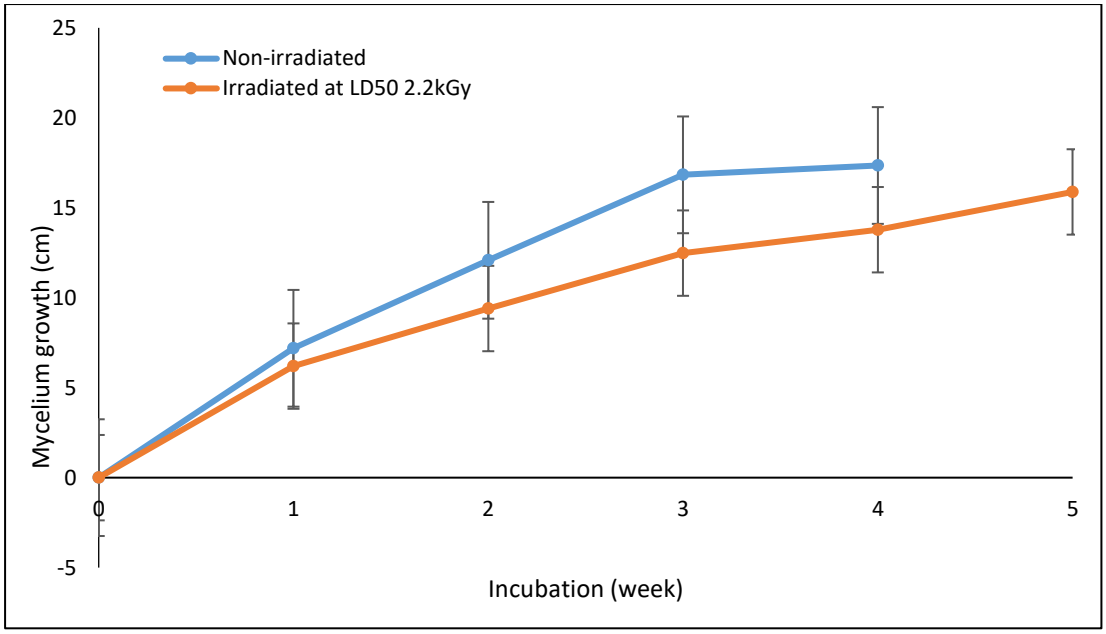

Figure 4.5 Average mycelium growth of LD_{50} of irradiated *P. sajor-caju* mycelia on baglog substrate

The results of mushroom yield, number, and size of fruiting bodies for *P. sajor-caju* are shown in Table 4.1 1, where the average of yield and number of fruiting bodies for irradiated is slightly higher compared to the non-irradiated. From the earlier study by Rosnani *et al*., [3] (2016) it has shown that irradiation can induce more fruiting bodies with no significant difference on size of fruiting bodies. However, results in this study have shown irradiation at LD_{50} 2.2 kGy have effects on the length of stipes. The fruiting bodies exhibited longer stipes compared to non-irradiated fruiting bodies. From Figures 4.6 and 4.7, yield have a good correlation coefficient for cap diameter, R^2= 0.9966 and stipe length, R^2=0. 9926. Studies by Kortei et al. (2018) also reported that yield have a correlation with size of fruiting bodies and large size of fruiting bodies are widely perceived to be of superior quality. The size of fruiting bodies is an important criterion for grading quality as well as for pricing of mushrooms.[18]

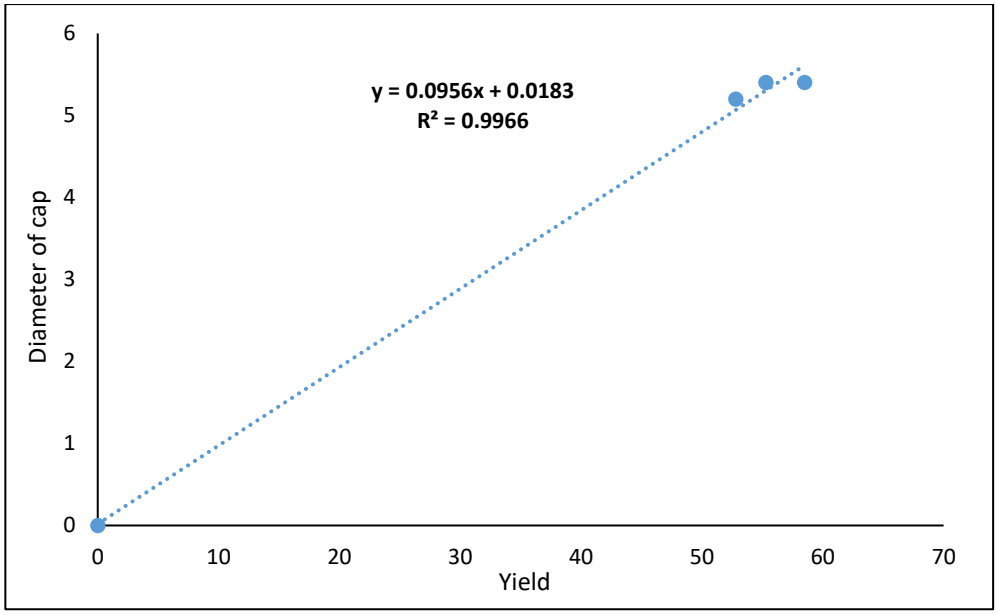

Figure 4.6 Correlation of yield and diameter of cap for fruiting bodies of *P. sajor-caju* irradiated at LD_{50} 2.2 kGy.

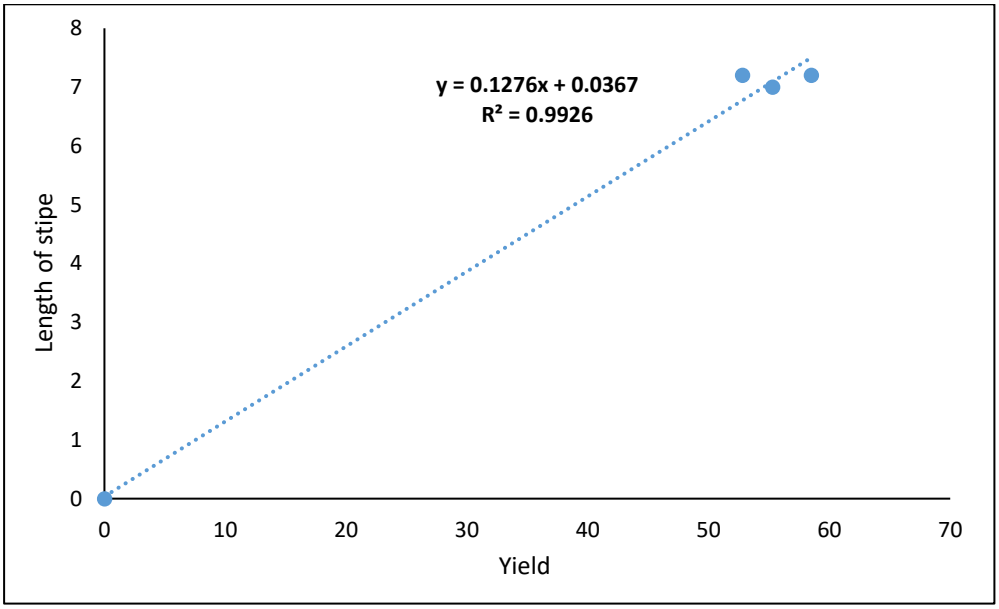

Figure 4.7 Correlation of yield and diameter of length of stipe for fruiting bodies of *P. sajor-caju* irradiated at LD_{50} 2.2 kGy.

Table 4.1 Average of mushroom yield, number of fruiting bodies per bag and diameter of cap and stipe

	Average												BE (%)
Sample	Mushroom yield (g)			Number of fruiting bodies			Diameter of cap (cm)			Length of stipe (cm)			
	Number of flushes												
	1	2	3	1	2	3	1	2	3	1	2	3	
Non-irradiated	45.1	49.4	52.4	7	6	5	5.3	5.4	5.3	3.7	4.0	4.4	81.61
Irradiated LD_{50} 2.2 kGy	52.8	55.3	58.5	5	8	8	5.2	5.4	5.4	7.2	7.0	7.2	92.56

4.3.2 Effects on morphology characteristics of fruiting bodies

Mushroom fruit bodies are classified into four (4) classes of morphology, i.e., straight, curly, mixed of straight and curly, and stunted, as reported by Rosnani *et al*.,.[3)] The photographs of four (4) classes morphology is shown in Figure 4.8. The "straight" morphology of fruit bodies of *P. sajor-caju* for non-irradiated shown was the highest in comparison to a mix of "straight and curly"; and "curly" morphologies. However, the irradiated at LD_{50} 2.2 kGy *P. sajor-caju* shown morphology of fruit body with characteristic of "mix of straight and curly"; and "curly" were higher than those with "straight" morphology (Figure 4.9). To date, there has been a lack of study on the effects of radiation on morphology characteristics of mushroom fruiting bodies. Hence, the information for comparison is limited.

Figure 4.8 Morphology of fruiting bodies *P. sajor-caju* a) curly, b) straight, c) mixed of straight and curly and d) stunted.

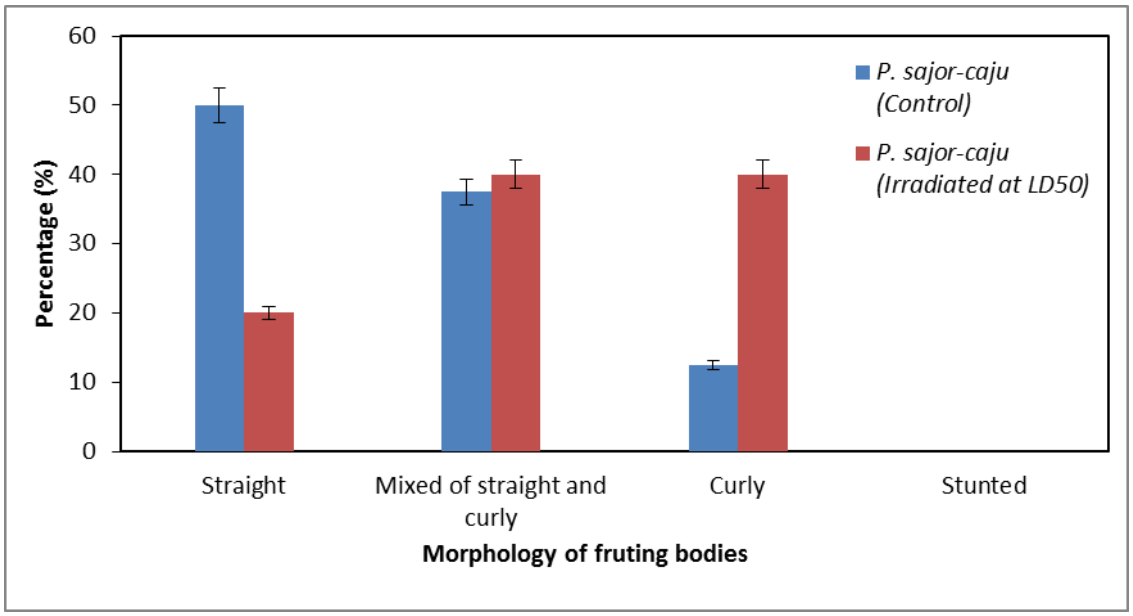

Figure 4.9 Percentage of morphology fruiting bodies for *P. sajor-caju* non-irradiated and irradiated at LD_{50} 2.2 kGy.

4.4 Conclusion

Consumption of mushrooms for the nutritional and medicinal properties is on the increase worldwide, irrespective of religion, culture, and food habits. Therefore, research has to be focused on breeding of high yield and superior quality mushroom strains to meet this requirement.

Radiation mutagenesis is a modern breeding method. Radiation mutagenesis is a powerful tool for creation of variability in genetically saturated species due to improvement of desirable traits. From this study, radiation mutagenesis by gamma radiation at LD_{50} 2.2 kGy has shown to impart effects on mycelia growth, whereby irradiated mycelia have slower growth rate; however, mushroom yield can be enhanced. Yield may increase due the higher number and size of fruiting bodies. These finding implied that radiation mutagenesis is capable of generating new strains of mushroom with high yield and improvement in size of fruiting bodies. This improvement and enhanced characteristics have great potential in the mushroom market and able to support and expand the mushroom industry in Malaysia.

4.5 References

1) S. P. Chandran and Y. K. Lee. Mutation Breeding of Mushroom by Radiation. *Journal of Radiation Industry*. 5 (4): 285-295 (2011).
2) B. T. Bahram, R. P. Hamid, J. M. Matin and J. Javad. Chemically and physically induced mutagenesis in basidiospores of oyster mushroom *Pleurotus ostreatus* var. florida. *International Journal of Advanced Research*. 2 (11): 915-921 (2014).
3) A. R. Rosnani, D. Fauzi, A. Mat Rasol, M. Hassan Hamdani, S. Sahidan, M. Azhar, M. Mohd Meswan, and A. R. Khairuddin. Evaluation of Mycelia Growth, Morphology and Yield for Low Dose Gamma Irradiated Grey Oyster Mushroom *Pleurotus sajor-caju*. *International Journal of Innovation and Scientific Research*. 24 (2): 332-336 (2016).
4) L. Ma, F. Kong, K. Sun, T. Wang and T. Guo. From classical radiation to modern radiation: past, present and future of radiation mutation breeding. *Frontier in Public Health*. 9: 1-11 (2021).
5) K. Shirasawa, H. Hirakawa, T. Nunome, S. Tabata and S. Isobe. Genome-wide survey of artificial mutations induced by ethyl methanesulfonate and gamma rays in tomato. *Plant Biotechnology Journal.* 14:51–60 (2016). doi: 10.1111/pbi.12348.
6) E. A. Adebayo, J. K. Oloke, A.K. Bordoloi, M. Barooah and T. C. Bora. Improvement of Pleurotus pulmonarius Lau 09 through mutation for yield performance. *Research Journal of Mutagenesis.* 4 (1): 1-13 (2014).
7) P. H. F. Beejan and R. Nowbuth. Use of radiation for the induction of mutation in oyster mushrooms for improvement of strains. In Q. Y. Shu (ed.). Induced plant mutations in the genomic era. *Food and Agricultural Organization of the United Nations, Rome, Italy*: 289-293 (2009).

8) I. Djajanegara and A. Harsoyo. Mutation study on white oyster mushroom (*Pleurotus floridae*) using gamma (60CO) irradiation. *Journal of Chemical and Natural Resources Engineering*. 4(1): 12-21 (2000).

9) R. Sharma and B. M. Sharma. Strain improvement in *Pleurotus ostreatus* using UV light and ethyl methyl sulfonate as mutagens. *African Journal of Microbiology Research*. 8 (5): 432-436 (2014).

10) K. R. Jyothi and S. S. Thara. Development of improved strain in species of *Pleurotus* by gamma irradiation. *Journal Food Science Technology*. 58 (9): 3540–3547 (2021).

11) S. Wang, Y. Yin, Y. Liu and F. Xu. Evaluation of genetic diversity among Chinese *Pleurotus eryngii* cultivars by combined RAPD/ ISSR marker. *Current Microbiology*. 65(4): 424–431 (2012).

12) A. R. Rosnani, D. Fauzi, S. Sahidan, A. Mat Rasol, M. Azhar, M. Hassan Hamdani and M. Mohd Meswan. Radiosensitivity Study and radiation effects on morphology characterization of grey oyster mushroom *Pleurotus sajor-caju*. *AIP Conference Proceeding,*.1614: 570-574 (2014).

13) I. R. Dewi, Herman and F. Isro. Lethal dose 50 (LD50) of mungbean (*Vigna radiata* L. Wilczek) cultivar kampar. *SABRAO Journal of Breeding and Genetics*. 47 (4): 510-516 (2015).

14) H. J. Nie, A. Mohamad, Z. A. Kadir, W. S. W. A. Razak, I. Mahmood and H. H. Hussain. Dose response for *Auricularia auricula-judae* against acute gamma irradiation. *Malaysian Applied Biology*. 46 (1): 71–75 (2017).

15) I. Mahmood, A. Mohamad, F. Daud, Z. A. Kadir, H. J. Nie, H. H. Hussain, W. S. W. A. Razak, A. O. B. Airianah and S. Fazry. Variants production of Lentinula edodes spores, monokaryon mycelium and dikaryon mycelium by γ-irradiation. *Malaysian Applied Biology*. 46 (1): 77–82 (2017).

16) A. A. G. Soloklui, M. Kordrostami and R. Karimi. Determination of optimum dose based of biological responses of lethal dose ($LD_{25,\ 50,\ 75}$) and growth reduction ($GR_{25,\ 50,\ 75}$) in 'Yaghouti' grape due to gamma radiation *Scientific Reports*: 13:2713 (2023).

17) K. Suzuki and S. Yamashita. Low dose radiation exposure and carcinogenesis. *Japan Journal of Clinical Oncology*. 42 (7): 563-568 (2012).

18) B. O. Onyango, V. A. Palapala, P. F. Arama, S. O. Wagai and B. M. Gichimu. Suitability of selected supplemented substrates for cultivation of Kenyan native wood ear mushrooms (*Auricularia auricula*). *American Journal of Food Technology*. 6: 395–403 (2011).

Chapter 5: The Effects of Lethal Dose 50 (LD_{50}) 2.2 kGy Gamma Radiation on Bioactive Compounds and Antioxidant Properties of Extract *Pleurotus sajor-caju* Mushroom

In **Chapter 5**, a study the effects of lethal dose where 50% of the population die (LD_{50}) 2.2 kGy gamma irradiation on total phenolic content (TPC), total flavonoid content (TFC) and antioxidant activities of *Pleurotus sajor-caju* is described. In this study, *P. sajor-caju* mushroom fruiting bodies from 2.2 kGy gamma radiation and non-irradiated as control sample were extracted. Three types of extraction solvent were used, which was using hot water, ethanol and azeotropic (80% water: 20% ethanol) solvent. The TPC of the extracts was determined by Folin–Ciocalteu assay and the TFC was determined by aluminum chloride assay. The antioxidant activities were analysed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP). The results from this chapter provide information for subsequent chapters.

Abstract

Pleurotus spp. are commonly known as oyster mushrooms and are the most cultivated worldwide. Mushrooms have been studied and found to accumulate a variety of secondary metabolites with antioxidant activities such as phenolic and flavonoid compounds. These bioactive components are generally associated with antioxidant activities. Phenolic compounds can neutralize free radicals which are responsible for oxidative damage. The formation of free radicals can be triggered by ionizing radiations such as gamma-rays and X-rays even at low doses. The results showed yield of extract by azeotropic solvent was higher compared to by other extractants. The total phenolic content (TPC) and total flavonoid content (TFC) were quantified. The results showed that the water extract contained the highest TPCs and TFCs among the extracts obtained from non-irradiated and irradiated *P. sajor-caju*. Antioxidant activities of the *P. sajor*-caju extracts were analyzed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ferric reducing antioxidant power (FRAP) analysis. The results showed that antioxidant activity from non-irradiated and irradiated *P. sajor-caju* extracts was highest from the water extract, followed by azeotropic and ethanol.

5.1 Introduction

Mushrooms are widely cultivated and consumed in many countries in the world due to the taste, economic and nutritional properties. *Pleurotus* spp. can grow on various lignocellulosic substrates and form shell shaped fruiting bodies of high nutritional value such

proteins, vitamins, and minerals.[1)] Mushrooms contain 90% water and 10% dry matter.[2)] Research on the medicinal properties of oyster mushrooms started in the late 20th century.[1)] Research on mushroom bioactive compounds became intensified in the early 21st century. These studies confirmed the health-promoting and medicinal properties of various oyster mushroom species such as *P. ostreatus, P. pulmonarius, P. sajor-caju* and *P. citrinopileatus*. [3,4,5)]

Studies on the biological activity of active compounds in mushroom derived from the fruiting bodies and mycelia are attractive due to the medicinal properties such as antioxidant, antitumor, antiviral, anti-inflammatory, anticoagulant, anticarcinogenic, antiviral, antibacterial, antifungal, antidiabetic and immunomodulating effects.[6,7,8)] Mushrooms were found to have medicinal properties from the richness of their bioactive compounds. The concentration and efficacy of the bioactive compounds are varied and depend on the type of mushrooms, substrate, fruiting conditions, stage of development, shelf life of the fresh mushroom, storage conditions and cooking procedures.[9)] Bioactive compounds of mushroom can be divided into four (4) groups, namely, i) Polysaccharide ii) Terpenes iii) Phenolic Compounds and iv) Peptides and Proteins.[8)] Mushroom phenolics have been found to be an excellent antioxidant and synergist.[10)] Mushrooms have been studied and found to accumulate a variety of secondary metabolites with antioxidant activities such as phenolic compounds.[11)]

Many studies have been reported that phenolic compounds exhibit antioxidant activity in biological systems and acting as free radical inhibitors, peroxide decomposers, metal inactivators, or oxygen scavengers. Studies the effects heat treatment on bioactive compounds such as phenol, flavonoid and ergothioneine of P. *ostreatus, P. sajor-caju* and *P. djamo*r has been reported.[12)] However, until now no study effects radiation on bioactive compounds on *P. sajor-caju* has been reported. The key role played by antioxidants is their ability to react with free radicals. A free radical is a chemical compound that contains one or more unpaired electrons. Reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl radical, hydroxyl ion, and nitric oxide are reactive molecules and free radicals derived from molecular oxygen. ROS are reactive, radicals search out ways of pairing up their electron, so radicals often attack nearby chemical compounds.[8)] The formation of free radicals can be trigger by ionizing radiations such as gamma-rays and X-rays even at a low dose. This ionization process will lead to production of short-lived free radicals, which will interact further with other biological molecules in the cell including DNA.[13)] Therefore, to protect the cells from damage and to support cell functions, antioxidants are needed to scavenge these free radicals. Many mushrooms have been reported to have antioxidant properties, which enable

them to neutralize free radicals.[14] The effects of gamma radiation at LD_{50} 2.2 kGy on phenolics and flavonoid content and its antioxidant activities of the extract *P. sajor-caju* by DPPH and FRAP are investigated and clarified in this chapter.

5.2 Experimental

5.2.1 Reagent

2,2-Diphenyl-1-Picrylhydrazyl (DPPH), Ferric Reducing Antioxidant Power (FRAP) kit, Folin Ciocalteu, gallic acid, quercetin and aluminium chloride and other reagents were purchased from Sigma-Aldrich, Germany. DPPH can simulate and estimate hydroxyl radical donation, while FRAP can simulate and estimate reduction activity.

5.2.2 Mushroom samples

The fresh fruiting bodies of *P. sajor-caju* were harvested from Block 46, Agrotechnology and Biosciences Division, Malaysian Nuclear Agency (Nuklear Malaysia). Fruiting bodies of non-irradiated and irradiated were cut into small pieces and dried using hot air dryer for 12 h. Dried fruiting bodies were ground into powder using a blender and sieved at size 125 µm. The powder was stored in airtight container and kept at 4 °C for later use.

5.2.3 Extraction

The powder of *P. sajor-caju* was extracted with water, ethanol and azeotropic solvents (ethanol: water), separately. Water extraction was conducted using reflux extractor with volume 200 mL for 2 h at 100 °C as shoen in Figure 5.1. Ethanol and azeotropic extraction were conducted with soaked powder *P. sajor-caju* in conical flask with 100 mL ethanol or azeotropic solvent and shaken at 100 rpm for overnight. Azeotropic solvent used in this study contains 80% water and 20% ethanol. For all extraction methods, 100 g of powder *P. sajor-caju* was transferred into each flask. Then, solvent with extract was filtered with a vacuum pump using Whatman filter paper no. 1. After filtration, the extracted was evaporated using a rotary evaporator at temperature of 65-70 °C to obtain concentrated extract. The concentrated extract was freeze dried for 24 h. The yield of extract was weighed and stored at 4 °C in tight container for further use. The extraction yield was calculated by the following equation:

$$\text{Extraction yield (\%)} = \frac{W_1}{W_2} \text{ x100}$$

Whereas; W_1 is the mass of extract and W_2 is the mass of the sample

Figure 5.1 Setup of reflux extractor for water extraction

5.2.4 Determination of total phenolic content (TPC) by Folin-Ciocalteu assay

The TPC was determined as described by Egra *et al*., [15)] with minor modification. The 1 mg extract was dissolved in l mL pure water. The mixture was vortexed and shaker incubated for 30 min at room temperature. Then 100 µL of the extract solution was pipetted into a new tube and 0.75 mL Folin Ciocalteu reagent was added. The Folin Ciocalteu reagent was diluted 10X in ionized water. The mixture was shaken and incubate for 5 min at room temperature under dark condition. Later, 0.75 mL 7.5 % of natrium carbonate was added, vortexed and incubated for 1.5 h. The absorbance was measured at 725 nm wavelength using a UV/VIS spectrophotometer (Hitachi UH5300, Japan). Gallic acid was used as a standard. The standard curve comprised of 5 dilutions: 0, 0.25,0.50, 0.75 and 0.10mg/ mL, starting from a 1 mg/mL stock solution.

5.2.5 Determination of total flavonoid content (TFC) by aluminium chloride assay

The TFC was determined as described by Egra *et al*.,[15)] with minor modification. The 5 mg sample was dissolved in 1 mL ethanol. The mixture was vortexed and shake incubated for 30 min at room temperature. Then 250 µL of the extract solution was pipetted into a new

tube and 75 µL 5% of sodium nitrite and 1.25 mL pure water were added. This mixture was incubated at room temperature for 5 min. After that 150 µL of 10 % AlCl3 was added, then incubated at room temperature for 6 min. Then 500 µL of 1 M NaOH and 275 µL of pure water was added and incubated at room temperature for 20 min. The absorbance was measured at 510 nm wavelength using a UV/VIS spectrophotometer (Hitachi UH5300, Japan). Quercetin was used as a standard. The standard curve comprised five (5) dilutions: 0, 0.01, 0.03, 0.05 and 0.07 mg/mL, starting from a stock solution of 1 mg/mL in ethanol.

5.2.6 Determination of antioxidant activity by 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) analysis

The antioxidant activity of mushroom *P. sajor-caju* extract was determined by DPPH as described by Makpol *et al*., [16] with minor modification. Extract of mushroom fruiting bodies *P. sajor-caju* from irradiated at LD_{50} and non-irradiated were used at concentration 1mg/ml (w/v). Approximately 0.1 mg/mL of DPPH (Figure 5.2) was prepared in ethanol, whereas various concentrations of *P. sajor-caju* extract were prepared using distilled water and were added into falcon tube containing 1.5 mL of DPPH reagent. The mixture was shaken vigorously and allowed to stand in dark at room temperature for 10 min. The absorbance was measured at 517 nm wavelength using a UV/VIS spectrophotometer (Hitachi UH5300, Japan) in comparison to a blank. The percentage of DPPH scavenging-activity was calculated using the following equation:

$$\text{Free radical-scavenging activity (\%)} = \frac{(\text{Abs}_{\text{control}} - \text{Abs}_{\text{sample}}) \times 100}{\text{Abs}_{\text{control}}}$$

Whereas; $\text{Abs}_{\text{control}}$ is the measurement taken from blank sample, without extract. $\text{Abs}_{\text{sample}}$ is the measurement taken from sample which contains P. *sajor-caju* extract.

Figure 5.2 DPPH in a bottle

5.2.7 Ferric reducing antioxidant power (FRAP) assay

The total antioxidant power in mushroom *P. sajor-caju* extract was measured based on the reduction of ferric (Fe^{3+}) complex to ferrous (Fe^{2+}), which has an intense blue colour. *P. sajor-caju* extract with concentration 1mg/ml (w/v) was prepared using distilled water. The FRAP kit with catalogue number MAK369 from Sigma, Japan (Figure 5.3) are used in this study. The method for standard and samples preparation used in this study as provided by Sigma with slight modifications. The absorbance of each solution was measured at 593 nm wavelength using UV/VIS spectrophotometer (Hitachi UH5300, Japan). The standard curve of FeSO4.7H2O with concentration 0,4, 8, 12, 16 and 20 nmol was plotted. Mean FRAP value for each sample was determined from the standard curve and Ferrous equivalent was calculated using the following equation:

$$\text{mM Ferrous equivalent} = \frac{B \times D}{V}$$

Whereas; B is ferrous ammonium sulphate amount from standard curve (nmol), D is sample dilution factor and V is sample volume added into the reaction well (µL)

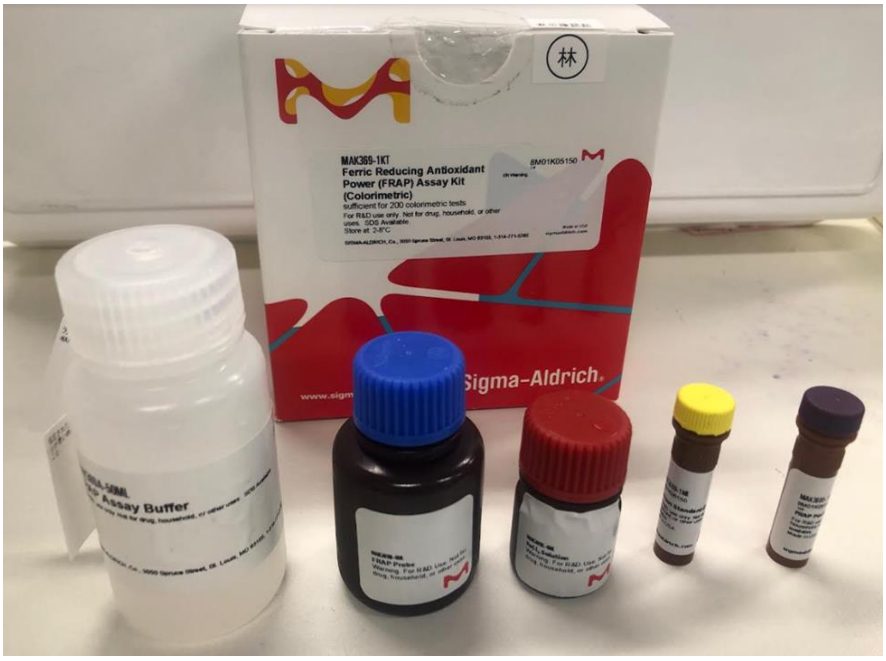

Figure 5.3 The FRAP kit with catalogue number MAK369 from Sigma, Japan

5.3 Results and Discussion

5.3.1 Yield of extraction

Extraction is a major and important step in the separation of bioactive compounds from raw materials and natural sources such as medicinal plants or fungi. Recently, advanced extraction methods have been developed for the extraction of bioactive compounds and its largely depends on the specific nature of the bioactive compound being targeted. The basic operation for extraction included steps such as pre-washing, drying of plant materials or freeze drying, grinding to obtain a homogenous sample and often improving the kinetics of analytic extraction and increasing the contact of sample surface with the solvent system. Proper actions must be taken to assure that potential active constituents are not lost, distorted, or destroyed during the preparation of the extract from samples.[17)]

In this study three types of solvent are used for extracting the bioactive compounds from *P. sajor-caju* as in Table 5. 1. The result showed, the highest yield of extract for *P. sajor-caju* non-irradiated and irradiated was with the azeotropic solvent, while the lowest yield with the ethanol. Water was use as solvent to dissolve water soluble compounds, while ethanol to dissolve compounds non-water-soluble compounds. The parameters in extraction process such as temperature, pressure, time, and solvent used can affect the efficiency of the extraction process.[18,19)] In this study, extraction with heat method was used. The amount of extract is also influenced by the polarity index in the solvent; the lowest to highest polarity index in this study

is, respectively water: ethanol, water, and ethanol. Study by Nawaz *et al*.,[20], suggests the use of a combination of polar and nonpolar solvents to increase efficiency of extraction with good antioxidant quality. The results have showed of *P. sajor-caju* extract contain more compounds soluble in water compared ethanol.

Table 5.1 Average of yield extraction *P. sajor-caju* mushroom in different solvents

Samples	Extraction solvent	Weight of sample (g)	Average extract yield (g)	Extract (%)
P. sajor-caju (non-irradiated)	water	100	1.46	1.46
	ethanol	100	0.22	0.22
	azeotropic (water: ethanol)	100	2.46	2.46
P. sajor-caju (irradiated)	water	100	2.12	2.12
	ethanol	100	0.69	0.69
	azeotropic (water: ethanol)	100	2.72	2.72

5.3.2 Total phenolic and flavonoid contents

The TPC was detected using the Folin–Ciocalteu method, which is based on electron transfer from phenolic compounds in the extracts to the reagent. Meanwhile, the TFC was detected using the aluminium chloride method. This method is based on the hydroxyl groups of flavonoids forming a yellow complex with aluminium chloride. Phenolic and flavonoid components develop the main components of the natural antioxidant that scavenges free radicals due to its ability to divide the hydrogen atoms or electron and the balance of radical compound.[21,22] The antioxidation capacities of extracts were often linked to their levels of phenolic and flavonoid contents, therefore in this study the TPC and TFC of *P. sajor-caju* mushroom extract are obtained. Phenolic compounds can neutralize free radicals which are responsible for oxidative damage.[23] Other than phenolics, flavonoids from mushrooms can also act as free radical scavengers to terminate the radical chain reactions that occur during the oxidation.

Table 5.2 shows the results of TPC and TFC for non-irradiated and irradiated *P. sajor-caju* extracts obtained using different solvents. The results showed that irradiated *P. sajor-caju* from water extraction gave the highest amount of TPC and TFC compared non irradiated *P. sajor-caju.* This finding consistent with the results reported by Boonsong et al., [24] who

compared water extract with extracts obtained using 50% ethanol and diethyl ether. Phenolic and flavonoid can be found in cell wall and high polarity compounds. Thus, these compounds were easily dissolved using water, which is more polar than ethanol. The high TPC and TFC values indicative of higher antioxidant activity.

Table 5.2 Total phenolic and flavonoid contents of *P. sajor-caju* extracts obtained using different extraction solvents

Sample	Extraction solvent	Phenolics (mg GAE/g)	Flavonoids (mg KE/g)
P. sajor-caju (non-irradiated)	water	23.49	10.75
	ethanol	13.02	2.52
	azeotropic (water:ethanol)	20.14	5.58
P. sajor-caju (irradiated)	water	25.73	15.31
	ethanol	12.88	5.26
	azeotropic (water:ethanol)	23.35	5.40

According Azieana *et al*.,[11] mushrooms have been studied and found to accumulate a variety of secondary metabolites with antioxidant activities such as phenolic and flavonoid compounds as well as in mushroom of the *Pleurotus* genus. These medicinal substances can be found in the mycelia, fruiting bodies, and extracts. Several studies have been reported such as from *Ganoderma lucidum, Schizophyllum commune* and *Hericium erinaceus, Pleurotus djamor P. florida* and *P. ostreatus.*[25, 26, 27, 28]

5.3.3 Antioxidant properties by DPPH and FRAP analysis

Antioxidant assay aimed to determine the ability and capacity of extract from *P. sajor-caju* to scavenge free radicals. Mechanism of scavenging free radicals can be used to calculate the activity of antioxidant. The DPPH analysis is useful in predicting antioxidant activities by inhibiting lipid oxidation. The DPPH is a rapid, simple, inexpensive, and widely used method to measure the ability of compounds to act as free radical scavengers or hydrogen donors and to evaluate antioxidant activity.[29]

Antioxidant analysis of *P. sajor-caju* non-irradiated and irradiated was done by scavenging free radicals which are measured by spectrophotometer with DPPH free radical agent. The value of free radical scavenging activity is presented as the percentage of the inhibition with an indication of colour changes of DPPH. The results of the antioxidant activity *P. sajor-caju* non-irradiated and irradiated mushroom extract is presented in Figure 5.4. For both samples, the highest activity has been shown by water extract, followed azeotrophic and ethanol only. These results showed that irradiation at LD_{50} 2.2 kGy increased the bioactive compound of *P. sajor-caju* extract as well as capacity of scavenging the DPPH free radical. However, this study displayed the antioxidants activity by scavenging the DPPH free radical is influenced by the type of extraction solvent. The *P. sajor-caju* irradiated extracted using water showed the highest antioxidant capacity compared to others.

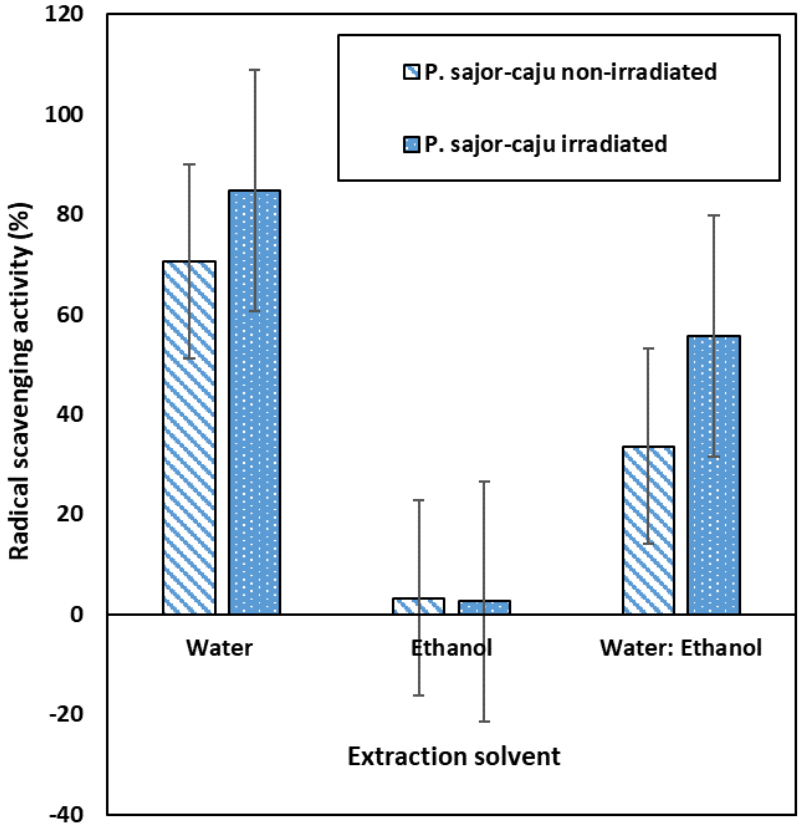

Figure 5.4 DPPH radical scavenging activities of *P. sajor-caju* mushroom extracts from different extraction solvent. Data are reported as mean ± SE (n = 3)

Similar results were observed for FRAP assay (Figure 5.5), which antioxidant power from *P. sajor-caju* mushroom extract irradiated are highest from non-irradiated for all different extraction solvent. Studies the effects or use of gamma irradiation to stimulate bioactive

compound synthesis in *Fomes fomentarius* living mycelium and *Inonotus obliquus* submerged cultures has been reported.[30, 31] *P. sajor-caju* is the species that is most cultivated in the world. However, from our knowledge no study on the effects of gamma irradiation on bioactive compound in *P. sajor-caju* has been reported to date. Hence, this study shows the importance to explore in depth the effects of radiation on this mushroom species, especially with respect to bioactive compounds.

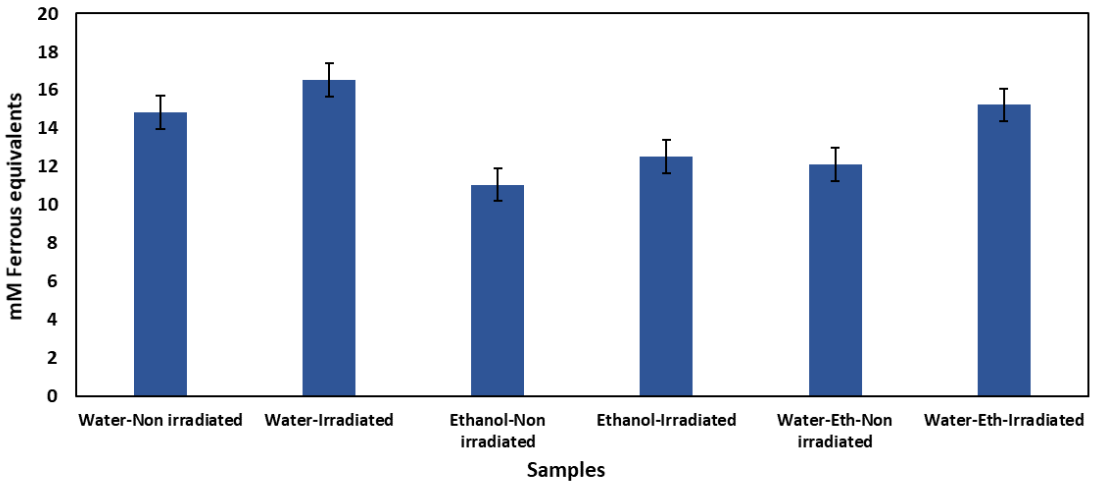

Figure 5.5 Amount of antioxidant power of *P. sajor-caju* from non-irradiated and irradiated mushroom extracts, through different extraction solvents. Data are reported as mean ± SE (n = 3)

5.4 Conclusions

The effects of gamma radiation on bioactive compounds of *P. sajor-caju* was evaluated by TPC, TFC and antioxidant activities. The results suggested the possibility of gamma radiation induced or increased the bioactive contents as well as antioxidant activity of *P. sajor-caju*. Antioxidant can also act as a radioprotective agent. This preliminary indicated that *P. sajor-caju* extract has great potential to be used as a radioprotectant agent against ionizing radiation. To elucidate this finding, an experiment on *P. sajor-caju* extract as a potential radioprotectant agent against ionizing radiation in living organism such as yeast cells was conducted. The results are discussed in a later chapter.

5.5 References

1) G. S. Iwona, K. Alina, S. Tomasz, S. Marek and S. Krzysztof, S. Bioactive compounds and medicinal properties of Oyster mushrooms (*Pleurotus* sp.). *Folia Horticulturae*. 30 (2): 191-201 (2018).

2) A. Anmut and A. Galana. The Effect of Edible Mushroom on Health and Their Biochemistry. *International Journal of Microbiology*: 1-7 (2022).

3) R. Cohen, L. Persky and Y. Hadar Y. Biotechnological applications and potential of wood-grading mushrooms of the genus *Pleurotus*. *Applied Microbiology Biotechnology*. 58: 582-594 (2002).

4) I. Lavi, D. Friesem, S. Geresh Y. Hadar and B. Schwartz. An aqueous polysaccharide extract from the edible mushroom *Pleurotus ostreatus* induces antiproliferative and pro-apoptotic effect on HT-29 colon cancer cells. *Cancer Letter*. 244: 61-70 (2006).

5) Y. L. Lee, G. W. Huang, Z. C. Liang and J. L. Mau. Antioxidant properties of three extracts from *Pleurotus citrinopileatus*. LWT. *Food Science Technology*. 40: 823-833 (2007).

6) S. T. Chang and J. A Buswell. Mushroom nutriceuticals. *World Journal Microbiology Biotechnology*.12: 473-476 (1996).

7) S. Badalyan, S. Medicinal aspects of edible ectomycorrhizal mushrooms. DDM 34. *Springer, Berlin*: 317–334 (2012).

8) S. Carmen. Bioactives from Mushroom and Their Application. Springer International Publishing AG 2017 M. Puri (ed.). *Food Bioactives*. 2: 23-57 (2017).

9) S. Guillamón, A. García-Lafuente and M. Lozano. Edible mushrooms: role in the prevention of cardiovascular diseases. *Fitoterapia*. 81 (7): 15–723 (2010).

10) B. Kalyani and K. Manjula. Irradiation of Mushrooms – An Overview. *International Journal of Science and Research*. 2 (12): 6-8 (2013).

11) J. Azieana, M. N. Zainon, A. Noriham and M. Nor Rohana. Total Phenolic and Flavonoid Content and Antioxidant Activities of Ten Malaysian Wild Mushrooms. *Open Access Library Journal*. 23: 1-9 (2017).

12) I. Insyirah, A. Farhat and R. Siva. Systematic Review: Heat Treatments on Phenolic Content, Antioxidant Activity, and Sensory Quality of Malaysian Mushroom: Oyster (*Pleurotus* spp.) and Black Jelly (*Auricularia* spp.). *Frontiers in Sustainable Food Systems*. 5: 1-16 (2022).

13) C. Borek. Antioxidants and Radiation Therapy. 2004 American Society for Nutritional Sciences. *Journal Nutritional*. 134: 3207S–3209S (2004).

14) C. Sánchez. Modern aspects of mushroom culture technology. *Applied Microbiology Biotechnology*. 64 (6): 756–762 (2004).

15) S. Egra, I. W. Kusuma, E. T. Arung and H. Kuspradini. The potential of white-oyster mushroom (*Pleurotus ostreatus*) as antimicrobial and natural antioxidant. *Asian Journal of Natural Product Biochemistry*. 17(1): 14-20 (2019).

16) S. Makpol, T. A. F.T. Ahmad, Z. Jubri, N. Rejab, N. Yusof and Y. A. M. Yusof. Gelam honey acting as a radioprotectant agent in gamma-irradiated human diploid fibroblasts. *Journal of Medicinal Plants Research*. 6(1): 129-138 (2012).

17) S. Sasidharan, Y. Chen, D. Shraavana, K.M. Sundram and L. Yoga Latha. Extraction, isolation and characterization of bioactive compounds from plants' extracts. African *Journal of Traditional, Complementary and Alternative Medicines*. 1-10 (2011).

18) M. M. Cowan, M. M. Plant products as antimicrobial agents. Clinical Microbiology Reviews. 12 (4): 564-582 (1999).

19) J. Azmir, I. S. M. Zaidul, M. M. Rahman, K. M., Sharif, A. Mohamed, F. Sahena, M. H. A. Jahurul, K. Ghafoor, N. A. N. Norulaini and A. K. M. Omar. (2013). Techniques for extraction of bioactive compounds from plant materials: a review. *Journal of Food Engineering.* 117(4): 426-436 (2013).

20) H. Nawaz, M. A. Shad, N. Rehman, H. Andaleeb and N. Ullah. Effect of solvent polarity on extraction yield and antioxidant properties of phytochemicals from bean (*Phaseolus vulgaris*) seeds. *Brazilian Journal of Pharmaceutical Sciences*. 56: e17129: 1-9 (2020).

21) F. Shahidi and P. K. Wanasundara. Phenolic Antioxidants. *Critical Reviews in Food Science and Nutrition.* 32: 67-103 (1992).

22) G. C. Yen and D. Y. Chuang. Antioxidant properties of water extracts from *Cassia tora* L. in relation to the degree of roasting. Journal of Agriculture and Food Chemistry. 48: 2760–2765 (2000).

23) H. S. Yim, F. Y. Chye, C.T. Tan, Y. C. Ng and C. W. Ho, C.W. Antioxidant Activities and Total Phenolic Content of Aqueous Extract of *Pleurotus ostreatus* (Cultivated Oyster Mushroom). *Malaysian Journal of Nutrition*. 16: 281-29 (2010).

24) S. Boonsong, W. Klaypradit and P. Wilaipun. Antioxidant activities of extracts from five edible mushrooms using different extractants. *Agriculture and Natural Resources*. 50: 89-97 (2016).

25) A. Noorlidah, I. Siti Marjiana, A. Norhaniza, S. Adawiyah Suriza and B. F. Lau. Evaluation of Selected Culinary-Medicinal Mushrooms for Antioxidant and ACE

Inhibitory Activities. *Evidence-Based Complementary and Alternative Medicine.* 464238: 1-12 (2012).

26) A. K. Saha, S. Acharya and A. Roy. Antioxidant level of wild edible mushroom: *Pleurotus djamor* (Fr.) Boedijn. *Journal of Agricultural Technology*. 8(4): 1343-1351 (2012).

27) A. Ganeshpurkar, P. Pardhi, S. S. Bhadoriya, N. Jain, G. Rai and A. P. Jain. Antioxidant Potential of White Oyster Culinary-Medicinal Mushroom, *Pleurotus florida* (Higher Basidiomycetes). *International Journal of Medicinal Mushrooms*. 17(5): 491-498 (2015).

28) Suseem S. R, Mary Saral. A. Analysis on total Anti-Oxidant activity of *Pleurotus eous* Mushroom Correlated to its Phenolic and Flavonoid Content. *International Journal of Drug Development & Research*. 5 (1):1-5 (2013).

29) B. Kedare & R. P. Singh. Genesis and development of DPPH method of antioxidant assay. *Journal Food Science Technology*. 48 (4): 412–422 (2011).

30) C. F. Pelcaru, M. Ene, A. M. Petrache and D. C. Negut. Low Doses of Gamma Irradiation Stimulate Synthesis of Bioactive Compounds with Antioxidant Activity in *Fomes fomentarius* Living Mycelium. *Applied Science*. 11(4236): 1-12 (2021).

31) A. Petre, M. Ene. D. C. Negut, F. Gatea and E. Vamanu. The use of gamma irradiation to stimulate bioactive compound synthesis in *Inonotus obliquus* submerged cultures. *NUKLEONIKA.* 66 (3):83–90 (2021).

Chapter 6: Radiation Protection Effects on Yeast Cells by Addition of Natural Compounds Extracted from *Pleurotus sajor-caju*

Abstract

Chapter 6 narrates a study on radiation protection effects on yeast cells by addition of natural compounds extracted from grey oyster mushroom, *Pleurotus sajor-caju*... This extract was obtained from the mushroom fruiting bodies. The extract is the mixture of compounds. However, as highlighted in Chapter 5, the compounds of *P. sajor-caju* focused are the phenolics and the flavonoids. The compounds from *P. sajor-caju* have the ability to acted as radioprotector. The experiment involving radiation was conducted using a gamma irradiation facility at Osaka University with radiation doses of 0, 50, 100 and 150 Gy. The results on survival rate (SR) and mutation frequency of no additive, with additive of extracts from non-irradiated and irradiated *P. sajor-caju* were analysed and discussed.

6.1 Introduction

Radiation has proven to give many benefits to the industries as well as to man. However, at the same time, radiation can be a risk to humans and other living organisms. While exploiting its beneficial applications under normal circumstances, such as in the medical industry, manufacturing industry, agriculture industry and power generation; radiation risks to workers, patients, the public and the environment that may arise from these utilizations have to be assessed, controlled and must be subjected to safety standards.

For human, the radiation risks are associated with the radiation dose, which affect human health. The higher the dose received the more dangerous to the human health. High dose received in human body can lead to radiation sickness, and in the worst case excessive radiation exposure may lead to death (deterministic effects). However, for some people small radiation doses received can cause adverse effects later on the persons health, which include cancer formation, and hereditary effects (stochastic effects).[1)]

Radiation protection principles include time, distance and shielding, and can be used to minimize the dose received as well as the effects of radiation. To minimize there will be remaining radiation dose absorbed in the human body. There is another way to protect a human body from radiation, which is by using a radioprotective agent or

radioprotector. A radioprotector protects the human body by minimizing the damages created from exposure to radiation. The human body has natural radioprotectors inside the cells. However, the amount of natural radioprotectors is not enough to overcome the harmful effects of natural radiation received in our body every day.

Natural radioprotectors in the human body can be increased by taking healthy foods with high antioxidant content. Mushrooms are examples of healthy food with high antioxidant content. Many studies have been conducted to evaluate, determine, and investigate bioactive compounds in mushrooms. Many studies have reported that bioactive compounds from mushrooms exhibit antioxidant activities in biological systems and act as inhibitors of free radicals, decomposers of peroxides, inactivators of toxic metals, or scavengers of oxygen.[2)] Studies on the effects of bioactive compounds from mushroom extract onto cancer patients has also been reported. Hence, consuming mushroom in our daily life diet is good to boost our immune system as well as protect our body from external causes, including irradiation.

Many studies have been conducted in the field of radioprotector to investigate the radical-scavenging effects from many chemical compounds, either naturally and artificially using different methods, including DPPH assay, colony assay using living cells, and electrophoresis. From the published reports, many researchers around the world tried to investigate how chemical compounds might give protection to the human body from chronic diseases such as cancer. Studies on the use of mushroom as dietary food for cancer patients and to minimize undesirable side effects after chemotherapy and radiation therapy has been reported.[3)]

Studies or research on radioprotective agents must continue to find novel radioprotectors that can protect against radiation induced damage in cells and tissues. Although radioprotectors may be able to minimize life-threatening effects of irradiation, few radioprotective agents are in clinical use due to their undesirable side effects, which include hypotension, vomiting, nausea, sneezing, and hot flashes.[4)] The discovery and development of antioxidant radioprotectors with less toxicity is also essential in radiobiological research, for example, establishing methods to improve the therapeutic effects in treatment of patients who have received radiotherapy or other kinds of radiation application.[4)] For example, the radioprotective effect of *Cordyceps militaris* mushroom has been reported.[5)]

Findings from other researchers have encouraged us to investigate the potential of bioactive function from *P. sajor-caju,* especially, as a source of radioprotective agent. In this present study aimed to clarify the antioxidant properties of natural bioactive compounds extracted *P. sajor-caju* mushroom and elucidate and investigate the protective effects of these natural compounds on yeast cells exposed to gamma radiation. The experiment on radiation for protective effects was conducted using ^{60}Co γ-rays at the Radiation Laboratory in the Institute of Scientific and Industrial Research, Osaka University at doses of up to 150 Gy with dose rates of 0.83-3.30Gy/min. The results on survival rate (SR) and mutant frequency are discussed in relation to the protective effects of *P. sajor-caju* extract on yeast cells irradiated by gamma radiation.

6.2 Experimental

6.2.1 Chemicals and reagents

The 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) kit, Folin–Ciocalteu reagent, gallic acid, quercetin, aluminum chloride, and other reagents were purchased from Sigma-Aldrich, Germany. DPPH can simulate and estimate hydroxyl radical donation, while FRAP can simulate and estimate reduction activity.

6.2.2 Materials

6.2.2.1 Yeast strain S288c

The yeast strain S288c (genotype: MATα SUC2 gal2 mal mel flo1 flo8-1 hap1 no bio1 bio6) used in this study were obtained from laboratory stock. This strain was isolated by Robert K. Mortimer through genetic cross. Strain S288c was derived primarily (~88% of its genome) from strain EM93, which was isolated by Emil Mark from a rotting fig in Central California in 1938. The remaining 12% of the S288c genome came from five different progenitors.

6.2.2.2 YPD media

YPD media is a type of rich media with all nutrients needed by yeast cells to grow. The initials YPD refer to what this media contains, namely, Yeast extract (a complex mixture of peptides and amino acid), Peptone (a water-soluble protein derivative) and

glucose (also known as Dextrose). Yeast cells can be cultured either in liquid or solid YPD. Fur culturing in solid YPD, this media has to be solidified by adding agar.

The composition of the YPD media used in this study is 5 g of dried yeast extract, 10 g of polypeptone, 10 g of glucose and 10 g of agar. All these compositions are put into Schott bottle and dissolved in 500 ml water. that the bottle was then shaken to mix the contents well. The preparation was then put into the autoclave for sterilization at 120˚C for 20 minutes. After sterilization, the media were cooled to 60˚C; then 10 ml of YPD media were poured into each 5 cm petri dishes.

6.2.2.3 5-Fluoroorotic Acid (5-FOA) media

5-FOA is widely used to study the molecular genetics of yeast cells. In this study, 5-FOA media was used for measuring the mutation frequency of yeast cells. 5-FOA is converted by gene involved in uracil metabolism (*URA3*) to 5-fluorouracil, which is toxic. The composition of 250 ml of 5-FOA media as shown in Table 6.1.

Table 6.1 The composition of 250 mL of 5-FOA media

Reagent		Amount to add
Distilled water		250 mL
Agar		8 g
20% Glucose		40 mL
Yeast nitrogen base		40 mL
10x HC-6aa D		40 mL
Amino acid	*URA*	7 mL
	ADE	8 mL
	LYS	4.8 mL
	TRP	3.6 mL
	LEU	1.6 mL
	HIS	0.8 mL
5-FOA		0.4 g

Firstly, 8 g of agar are put into Schott bottle and dissolved in 250 mL distilled water. The solution the put into the autoclave for sterilization at 120˚C for 20 minutes. After autoclaving was completed, the rest of reagent was added with the amount as in Table 6.1. The mixture was shaken and mixed well. Then 10 ml of 5-FOA solution were poured into 5 cm petri dishes.

6.2.2.4 Mushroom extract as a source of natural radioprotector

The water extract from *P. sajor-caju* mushroom fruiting bodies at a concentration of 1 mg/mL (based on w/w) was dispensed. that the extract was dissolved in YPD liquid, and then incubated at 37˚C for 24 hours. The solution was sterilized using a vacuum filtration unit as shown in Figure 6.1.

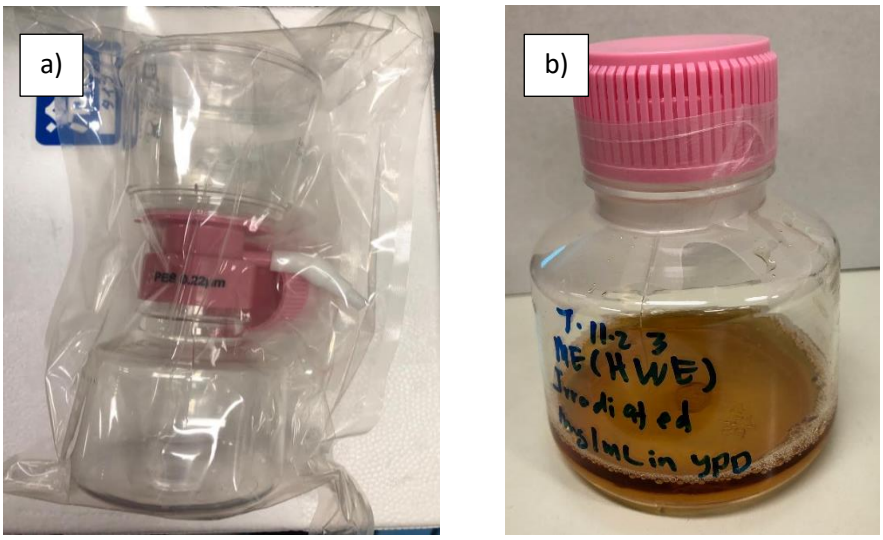

Figure 6.1 Vacuum filtration unit for sterilisation (a); and vacuum-filter sterilised mushroom extract inside bottle and sealed (b).

6.2.3 Methods

6.2.3.1 Toxicity evaluation of mushroom extract on yeast cell

Before conducting the experiment on irradiated yeast cells with mushroom extract as radioprotective agent, the toxicity of the extract on the yeast cells was evaluated using the colony assay method. The toxicity test was conducted with yeast cells grown in YPD liquid medium added with extract of *P. sajor-caju* mushroom at 30˚C for 24 hours. About

20 µL of the mixture was then spread on YPD agar medium in a petri dish. The petri dish was incubated at 30˚C for 2 days. After 2 days, the grown colonies were observed.

6.2.3.2 Preparation of samples for Survival Rate (SR)

Yeast cells were grown at 30˚C for 24 hours in YPD liquid medium. After 24 hours of incubation, yeast cell concentration was determined using a Neubauer chamber under the microscope with 40X magnification. After the initial concentration was known, yeast cell solutions were diluted at log phase to achieve a concentration of 40 cells/mL. After calculating the total yeast cells solutions are needed, 0.1 mg/mL of sterilized mushroom extract from *P. sajor-caju* non-irradiated and irradiated was added into final yeast cells solution for each falcon tube. Yeast cells solution with no addition of extract was considered as a control. The tube was then incubated at 30˚C for 2 hours. After incubation, 5 mL of yeast cells solution were filtered through a vacuum filtration unit (Sterifil aseptic system, Millipore®, Billerica, MA) (Figure 6.2) using nitrocellulose 47 mm 0.45 µm membrane filter (Millipore®, Billerica, MA) (Figure 6.3). Each membrane contained 200 yeast cells and was then put into a 5 cm petri dish. For each dose of radiation, 5 membranes were prepared for each group of natural radioprotector. After irradiation was completed, the membranes were transferred onto YPD solid media. Each membrane was transferred onto one YPD solid media. The petri dishes were incubated at 30˚C for 3 days. After 3 days, the surviving yeast colonies were counted.

Figure 6.2 Vacuum filtration unit

Figure 6.3 Nitrocellulose membrane filter

6.2.3.3 Preparation of samples for mutation frequency

Yeast cells were grown for 24 hours at 30˚C in YPD liquid medium. After 24 hours of growing, cell concentration was counted using Neubauer chamber under the microscope with 40X magnification. After the initial concentration was known, yeast cell solutions were diluted in log phase to get concentration of 2.0x 10^7cells/mL. After calculated the total yeast cells solutions are needed, 0.1mg/mL of sterilized mushroom extract from *P. sajor-caju* non-irradiated and irradiated was added into final yeast cells solution for each falcon tube. Yeast cells solution with no added of extract considered as a control. They were then incubated at 30˚C for 2 hours. After incubation, 5 mL of yeast cells solution were filtered through a vacuum filtration unit (Sterifil aseptic system, Millipore®, Billerica, MA) using nitrocellulose 47 mm, 0.45 µm membrane filter (Millipore®, Billerica, MA). Each membrane contained 2.0 x 10^7cells/membrane and put into 5 cm petri dish. For each dose of radiation, 3 membranes were prepared for each group of natural radioprotector. After irradiation procedure was completed, the membranes were transferred onto 5-FOA media in 5 cm petri dish, and then incubated at 30˚C for 10 days. After 10 days, the surviving colonies were counted.

6.2.3.4 Dose assessment and radiation

Samples were kept in low temperature to prevent cell growing. For gamma irradiation at the Radiation Laboratory in the Institute of Scientific and Industrial Research, Osaka University, all samples were kept in cooler box to maintain low temperature. At the

irradiation facility, the distance of samples for 50 Gy, 100 Gy and 150 Gy absorbed dose to the radiation source were computer calculated based on the current activity of the ^{60}Co radioactive source. All samples were taken out from cooler box and placed based on the calculated distance, facing the radiation source as shown in Figure 6.4.

Figure 6.4 The arrangement of samples for gamma irradiation

The relatively high doses used in this study was for research purpose only. Furthermore, at relatively high doses the radiation protection effects of radioprotector were clearer and easier to be analysed. After completion of the irradiation procedure, all membranes were transferred onto YPD solid medium and incubated at 30˚C for 2 days for samples of SR, and for 10 days for samples of mutation frequency.

6.2.3.5 Colony counting

During the incubation in YPD solid medium, yeast cells became active again due to nutrients in the medium and the ideal incubation temperature. Surviving yeast cells grew well, multiply and produce colonies. One single colony represented one single surviving yeast cell. After 2 days of incubation, the size of the yeast colonies formed were clearly visible by naked eye, as shown in Figure 6.5.

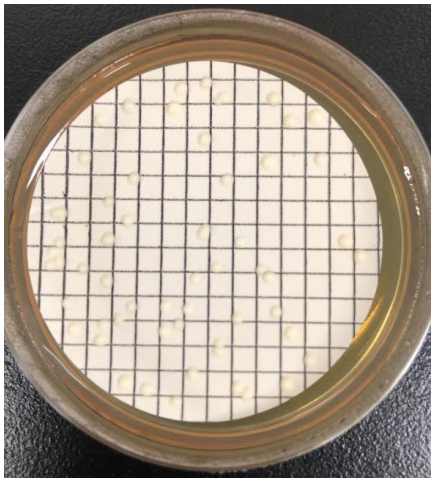

Figure 6.5 Yeast colonies on the nitrocellulose membrane

All visible yeast colonies on each membrane were counted. The SR rate was calculated using this equation [1)]: (Maradi, 2019):

$$\text{Survival rate (\%)} = \frac{\text{Number of colonies}_{\text{irradiated with X Gy}}}{\text{Number of colonies}_{\text{non-irradiated}}} \times 100$$

Number of colonies on each membrane irradiated by gamma radiation (50 Gy, 100 Gy and 150 Gy) were compared with number of colonies from non-irradiated membrane (0 Gy) to get SR.

Using the data from SR, we calculated the percentage (%) of radiation protection (PR) of the extract from non-irradiated and irradiated *P. sajor-caju* using the formula below:

$$\text{PR (\%)} = \frac{[\text{SR}_{\text{with additives at X Gy}} - \text{SR}_{\text{without additives at X Gy}}]}{\text{SR}_{\text{without additives at X Gy}}} \text{ X } 100\%$$

For mutation frequency, the viable or surviving colonies were counted after 10 days of incubation at 30ºC. The mutation frequency was determined using this equation:

$$\text{Mutation frequency (\%)} = \frac{\text{Number of matated colonies}_{\text{irradiated X Gy}}}{\text{Number of colonies}_{\text{non-irradiated}}} \times 100$$

Whereas; X Gy is absorbed dose in gray unit

The value of mutation frequency was plotted into a graph, with absorbed dose (Gy) represented by x-axis, and mutation frequency ($x10^{-7}$) represented by y-axis.

6.3 Results and Discussion

6.3.1 Toxicity evaluation of yeast cells given extract of *P. sajor-caju*

The toxicity of extract from *P. sajor-caju* (0.1 mg/mL) on the growth of yeast cells in YPD liquid medium, containing was evaluated. The cells grown were observed as in Figure 6.6. This was to evaluate whether the mushroom extract imparted toxicity effect on the yeast cells before pursuing to the next experiments.

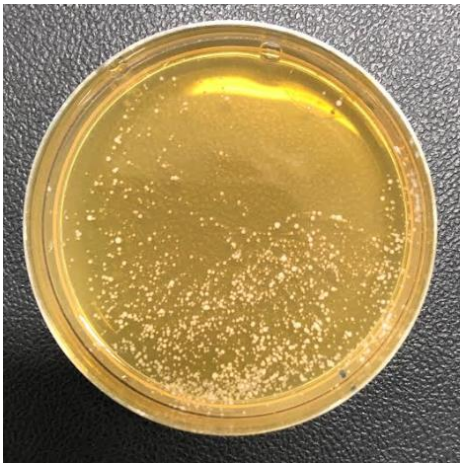

Figure 6.6 Yeast colonies on YPD agar medium after 24 hours of incubation with the presence of extract from *P. sajor-caju*.

6.3.2 SR of yeast cells

The results of SR of yeast cells after being exposed to gamma radiation at doses of 50 Gy, 100 Gy, 150 Gy, and 0 Gy as a control, with and without radioprotector from extract of *P. sajor-caju* are shown in Figure 6.7. The result showed the SR for yeast cells with the addition of extract *P. sajor- caju* mushroom is relatively higher in comparison

to the SR of yeast cells without extract *P. sajor- caju*. This was likely due to the higher antioxidant properties of extract obtained from irradiated *P. sajor- caju*.

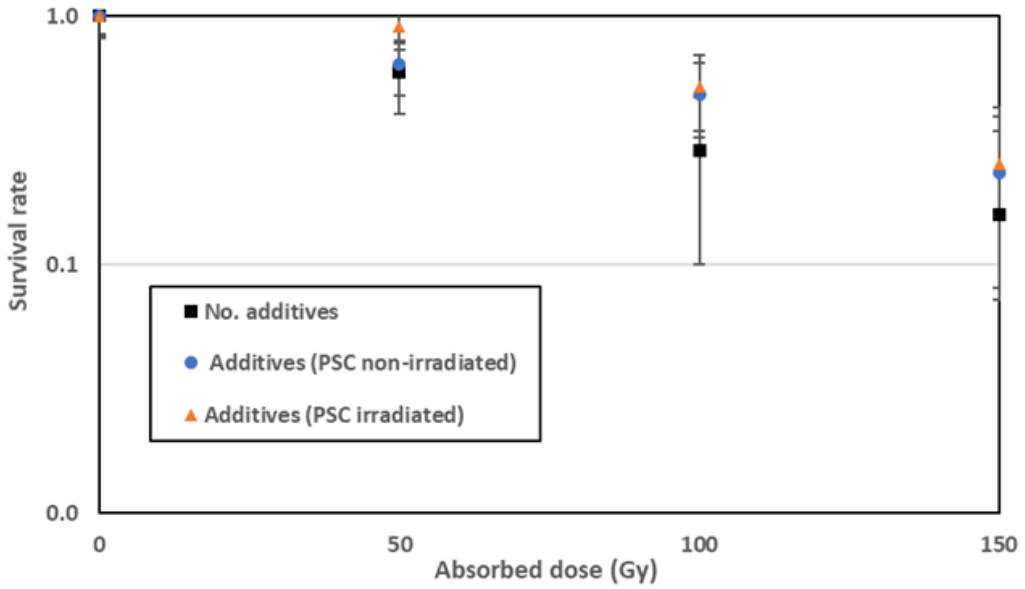

Figure 6.7 The SR of yeast cells irradiated with gamma rays and the effects of adding *P. sajor-caju* extracts. Values are shown as the mean ± SE (n = 5)

The SR of yeast cells at dose 50 Gy with the addition of radioprotective extract *P. sajor-caju* mushroom is approximately two times higher than the SR of yeast cells without radioprotective extract *P. sajor-caju*, which is 90.4%, in comparison to 59.2 %, respectively. Based on these finding, it can be inferred that the presence of extract from *P. sajor-caju* gives a protection to yeast cells against gamma radiation. Extract from *P. sajor-caju* contains a natural radioprotector, comprising a mixture of active compounds. This is different from studies by other researchers using single compounds, such as epigallocatechin gallate (EGCg), vitamin C and epicatechin (EC). However, we have not identified the specific compounds imparting the highest effect of radioprotection. However, results from this study, as shown in Figure 6.8, are similar to findings by Matuo *et al*.[6] (2008).

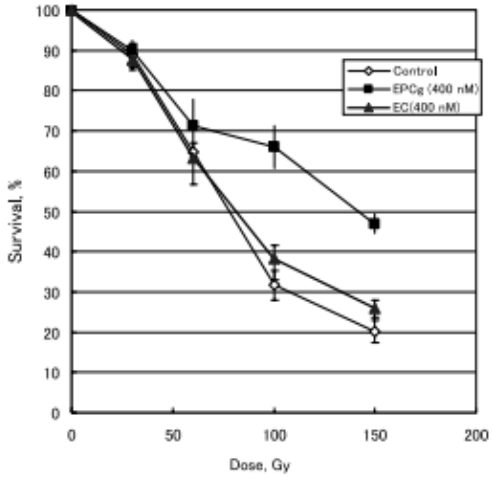

Figure 6.8 The SR of wild type strains exposed to gamma radiation in the presence EGCg and EC.[6)]

From this result, we can ask why yeast cells could survive after being exposed to ionizing radiation, and what is the main cause of cell death by radiation. According to Matuo *et al.*,[6)] for yeast cells without radioprotective additive, the cells survived due to its own repair mechanism. They used yeast strain S288c as a control, and strain *rad52*$^{-}$ that do not have repair gene especially double stand break through HR. Both strains were exposed to gamma radiation. The results are as in Figure 6.9.

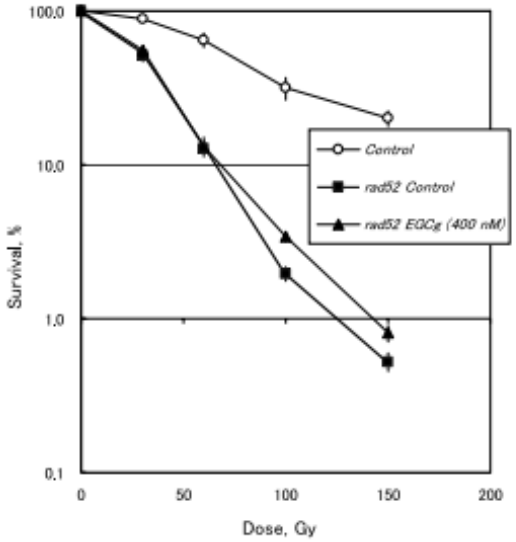

Figure 6.9 The SR of yeast strain S288c and strain *rad52*$^{-}$ exposed to gamma radiation. [6)]

From Figure 6.9, we can see that when the capability of the repair gene was removed from the yeast cells (strain *rad52*$^{-}$), higher lethality of cells was observed, compared to the control (strain S288c). This finding showed that yeast cells survived due to its efficient repair mechanism, where most of the DNA double strand break (DSB) damages induced by gamma radiation were repaired successfully. Furthermore, this data also showed the main cause of yeast cell death by the radiation was due to DSB in the DNA.

For the yeast cells with radioprotector (in the present study it refers to the extract from *P. sajor-caju*), besides due to its repair mechanism, the cell survived due to the presence of radioprotector by scavenging free radicals formed. Gamma radiation the type of radiation with low LET. For the radiation with low LET, the cells damages are dominant cause by indirect effects. However, a radioprotector could protect the cells by scavenging free radicals created from indirect effects of radiation through water radiolysis before they could damage and kill the yeast cells.

6.3.3 Mutation frequency of yeast cells

In the Chapter 3, we also mentioned that radiation could lead to cell mutation. Mutated cells could also survive and form a mutant colony after being exposed to radiation. It means, all observed and counted colonies to collate the SR data also included mutated cells. The results of mutation frequency of yeast cells exposed to gamma radiation at dose 50 Gy, 100 Gy, 150 Gy, and 0 Gy as a control with and without radioprotector from extract of *P. sajor-caju* are shown in Figure 6.10. The results showed mutation frequency for yeast cells with no addition of extract *P. sajor- caju* mushroom is highest for all doses of radiation compared to those given extract o *P. sajor- caju*. However, yeast cells with and without additives of extract from *P. sajor-caju* showed the highest at 100 Gy, and then decreased at dose of 150 Gy. The results from the current study is in concurrence with the findings by Matuo *et al*., [6] (2008), where yeast cells with EGCg showed low number of mutation frequency compared to those without EGCg (Figure 6.11).

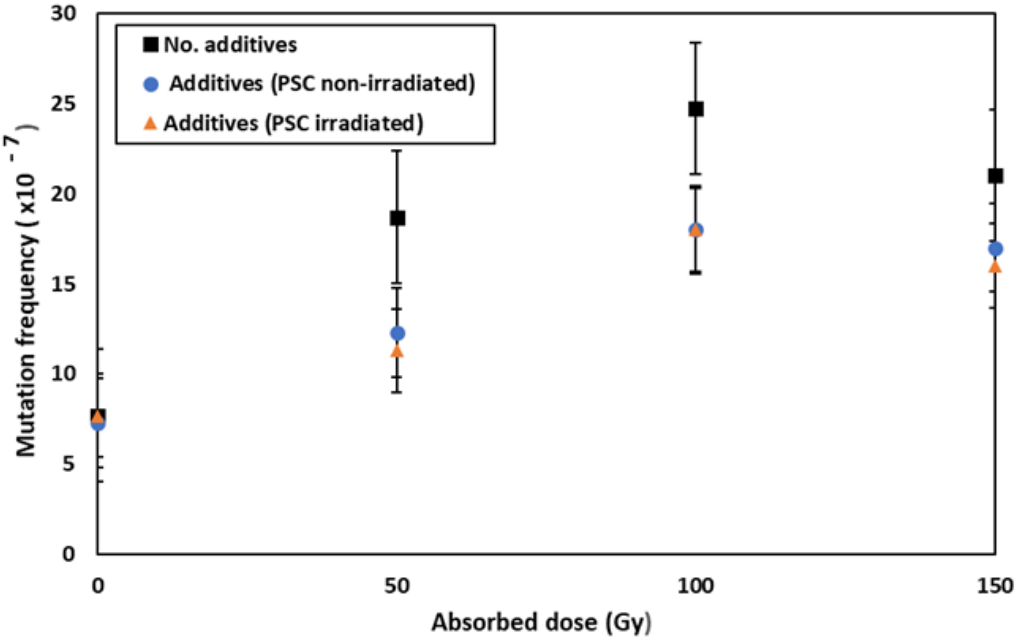

Figure 6.10 The mutation frequency of yeast cells exposed to gamma rays and the effects of adding *P. sajor-caju* extracts. Values are shown as the mean ± SE (n=3)

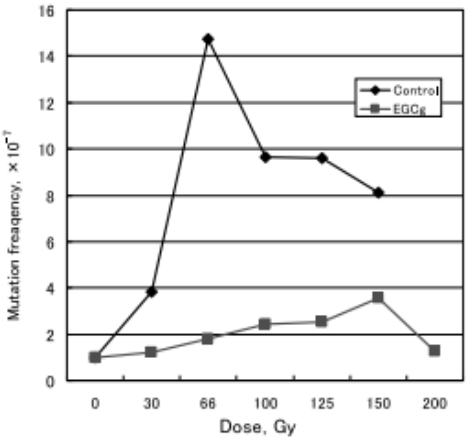

Figure 6.11 The mutation frequencies of yeast cells exposed to gamma radiation. [6)]

6.3.4 Protection ratio (PR) for additives from non-irradiated and irradiated *P. sajor-caju*

The results show the PR by the extracts from irradiated P. *sajor-caju* was higher than that form non-irradiated *P. sajor-caju*. The highest difference in PR between the

extracts from irradiated and non-irradiated *P. sajor-caju* was observed at an absorbed dose of 50Gy as shown in Figure 6.12.

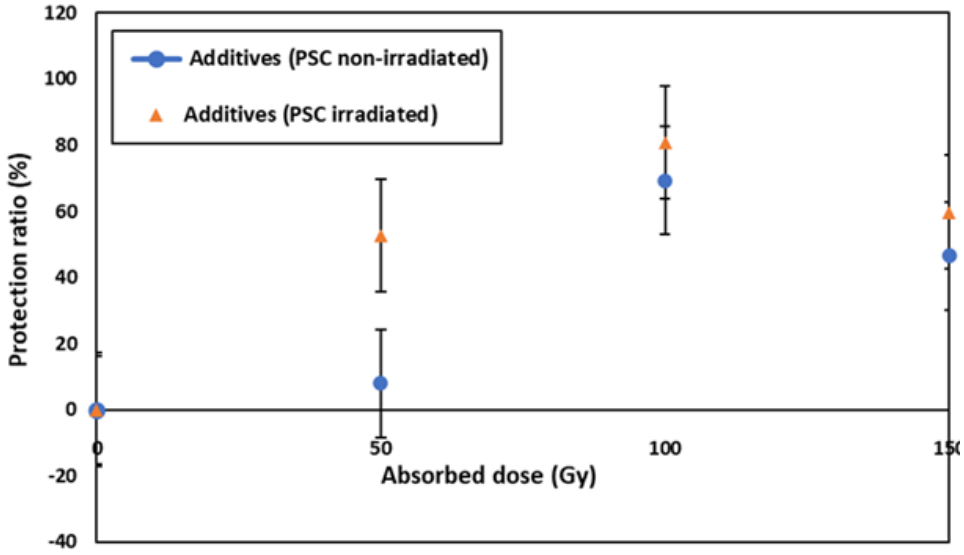

Figure 6.12 PR calculated for extracts obtained from non-irradiated and irradiated *P. sajor-caju*. Values are shown as the mean ± SE (n = 5)

6.4 Conclusions

The main cause of death of yeast cells induced by gamma radiation is through DNA DSB. When the yeast cells were being exposed to gamma radiation, yeast cells depended entirely on its repair mechanism to repair the damage on DNA, especially DSB. This means, a surviving yeast cell has succeeded to repair most of its DSB damages. Mutation also occurred as a surviving cell after being exposed to gamma radiation. However, the number of mutation frequency is small compared to death of cells by gamma radiation.

The results and findings from the present study showed that the bioactive extract from P. *sajor-caju* has the protective properties. The data showed SR yeast cells with addition of extract off *P. sajor- caju* was relatively high compared to SR of yeast cells without addition the mushroom extract. Data on mutation frequency showed that extract from *P. sajor- caju* can reduce the induction of mutation of yeast cells. This bioactive

extract from *P. sajor-caju* has the potential as a natural radioprotector to protect as well as minimize the effects of ionizing radiation.

6.5 References

1) A. Maradi. Master thesis: radiation protection effects on living organism by addition of EGCG and ascorbic acid. RINE, University of Fukui. (2019).
2) J. Azieana, M. N. Zainon, A. Noriham and M. Nor Rohana. Total Phenolic and Flavonoid Content and Antioxidant Activities of Ten Malaysian Wild Mushrooms. *Open Access Library Journal*. 23: 1-9 (2017).
3) M. Jayachandran, J. Xiao and B. Xu. A Critical Review on Health Promoting Benefits of Edible Mushrooms through Gut Microbiota. *International Journal of Molecular Sciences*. 18, 1934: 1-12 (2017).
4) A. Shirazi, E. Mihandoost, S. R. Mahdavi and M. Mohseni. Radio-protective role of antioxidant agents. *Oncology Reviews.* 6: e16: 130-134 (2012).
5) M. H. Jeong, Y. S. Park, D. H. Jeong, C. G. Lee, J. S. Kim, S. J. Oh, S. K. Jeong, K. Yang and W. S. Jo. In vitro evaluation of *Cordyceps militaris* as a potential radioprotective agent. *International Journal of Molecular Medicine*. 34: 1349-1357 (2014).
6) Y. Matuo, S. Nishijima, T. Ikeda and K. Shimizu. Radical scavenging effects of naturally-occurring epigallocatechin gallate against oxidative damage caused by gamma-ray radiation. *Radioisotopes*. 57: 723-731 (2008).

Chapter 7: Summary

7.1 Summary

In this study, the effects of gamma radiation on induced mutation of *Pleurotus sajor-caju* were investigated and discussed. The gamma radiation induced mutation were focused on the improvement of morphologies characteristics and bioactive compounds and its application as natural radiaoprotective agents on yeast cells wild type S288c.

In **Chapter 1**, a background of radiation application in mushroom study and reasons why the authors were interested to study the LD_{50} gamma radiation of *P. sajor-caju* for the improvement on morphology characteristic such as mycelium growth, productivity of fruiting bodies and bioactive compounds and its potential as radioprotectant agent on yeast cell, represented in this thesis are described.

Chapter 2, provides a background of the mushrooms industry and radiation application in studies of mushroom such as for sterilization mushroom substrate for cultivation, sterilization fruiting bodies after postharvest and mutation induction.

Chapter 3, gives a review on studies or works on radiation protection against with addition of radioprotective agent was discussed and summarized. This chapter also includes introduction of radiobiology study, effects of radiation on biological samples, types of biological samples, free radical, radiation protection, natural radioprotector and natural sources for natural radioprotector.

In **Chapter 4**, the effects of LD_{50} gamma irradiation on mycelium growth, yield, and fruiting bodies *P. sajor-caju* are investigated and described. The study was conducted at the Biobeam GM 800 radiation facility, Malaysian Nuclear Agency using caesium-137 as the gamma source at LD50 dose of 2.2 kGy, with dose rate of 0.227 Gy s-1. Non-irradiated mycelia were used as control. The results showed that gamma radiation can reduce the growth rate of mycelia, while inducing the size of fruiting bodies up to 7.13 cm and productivity up to 92.56 % by high number and size of fruiting bodies.

In **Chapter 5**, the effects of LD_{50} gamma irradiation on bioactive compounds as well as total phenolics content (TPC), total flavonoid content (TFC) and antioxidant properties from *P. sajor-caju* are investigated and discussed. The bioactive compounds from dried powder of fruiting bodies non-irradiated and irradiated *P. sajor-caju* was extracted by water, ethanol and azeotropic

(80 % ethanol: 20% water) solvents separately. The yield of extract from irradiated *P. sajor-caju* mushroom is higher compared to non-irradiated *P. sajor-caju* for all types of extraction solvent. The azeotropic solvent gave the highest yield of extract. The TPC was determined by Folin-Ciocalteu assay and TFC by aluminium chloride assay. The antioxidant properties of *P. sajor-caju* was determined by 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) and Ferric Reducing Antioxidant Power (FRAP) assay. The results shown the antioxidant activity from non-irradiated and irradiated *P. sajor-caju* extracts was highest from the water extract, followed by the azeotrophic and ethanol solvent.

Chapter 6, discusses the protective effect of bioactive compounds extracted from *P. sajor-caju* against the oxidative stress induced by gamma radiation on yeast cells are described. This study was conducted to investigate the potential of bioactive compounds from *P. sajor-caju* as radioprotectant on yeast cells. The yeast cells were exposed to gamma radiation at doses up to150 Gy. The effects were evaluated and determined based on survival rate and mutation frequency of yeast cells. The results shown the survival rate of yeast cells increased while those undergoing mutation decreased in presence of extracts *P. sajor-caju.* These indicated the extract of gamma irradiated *P. sajor-caju* has a higher radioprotective effect than the extract of non-irradiated P. sajor-caju by reduce the damage induced by γ -rays on yeast cells.

7.2 Conclusion

The studies the effects of gamma radiation on induced mutation for improvement morphologies characteristics of *Pleurotus sajor-caju* evaluated by increasing the size and productivity of fruiting bodies, while the effects gamma radiation on the bioactive compounds evaluated by ability of the extracts to protect yeast cells from radiation damage. The results from these studies suggested that the possibility of gamma radiation increased the size and productivity of fruiting bodies, as well as bioactive contents of *P. sajor-caju* with radioprotective properties by protecting yeast cells against damage from ionizing radiation such as γ-rays.

Acknowledgement

For above all, Thanks to Almighty ALLAH SWT for the strengths and the ability to understand that had been given to me so this study can be completed on time,

Thanks to RONPAKU-JSPS Scholarship program, Malaysian Nuclear Agency (NUKLEAR MALAYSIA), Malaysia and Research Institute of Nuclear Engineering (RINE), University of Fukui, Japan, so this research and study can be supported and conducted,

Deepest thanks, gratitude and appreciation to my supervisor, Prof. Yoshinobu IZUMI for his supervision, advices, assistance and wisdom; and also, to my sensei, Asst. Prof Youichirou MATUO for the advices, assistance and kind endless helps,

I would like to thank all the staff of RINE for their assistance and hospitality especially dealing with all the administration requirements during my stay,

Deepest thanks to all my friends in IZUMI/ MATUO lab for their kind help and friendship,

And

Last but not least, thank you for my family especially to my parent, my siblings, my husband, my relatives and my friends for their support and understanding the situation.

Malaysia-Japan

Rosnani binti Abdul Rashid