FNCA Guideline for

Biofertilizer Quality Assurance and Control

Vol. 2 Production of Biofertilizer Carriers using Radiation Technology

Biofertilizer Project

Forum for Nuclear Cooperation in Asia (FNCA)

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Preface

The publication of FNCA Guidelines for Biofertilizer Quality Assurance and Control, Volume 2 on "Production of Biofertilizer Carriers using Radiation Technology" is timely indeed, after the publication of FNCA Guidelines for Biofertilizer Quality Assurance and Control, Volume 1 on "Quantification of Beneficial Microbes in Biofertilizer" in 2014. The publication, also available online, is very well received by students, researchers and practitioners in the biofertilizer industry.

The FNCA Biofertilizer Project advocates the use of radiation sterilization in the preparation of biofertilizer carriers; we encounter various biofertilizer carriers being used in the region, for instance, mixed soils, peat, sand, charcoal and plant-based composts. Gamma irradiation of these carriers has the added advantage over heat treatment such as autoclaving, due to its high efficiency – in addition to its high throughput, radiation sterilization imparts negligible physical and chemical changes, and the provides possibility of inoculation of biofertilizer microorganisms into their final form of packaging. Like the previous publications, information and first-hand experience from among many project leaders and members enrich the present publication. We also acknowledge the contribution from colleagues who have little or no access to irradiation facilities. The excellent collaboration in the FNCA Biofertilizer Project is exemplary. We are confident that this publication too, would be very useful for biofertilizer researchers and practitioners in the biofertilizer industry, and hope the output enhances extension of quality biofertilizer products to farmers and the agriculture industry in general, for their respective sustainable development goals.

Having quality standards for products related to the agriculture industry, including on biofertilizer is inevitable, especially if we aspire to be competitive in the global market. A few countries have developed standards for biofertilizer products, and more will move in that direction, including Malaysia. We hope that these standards will complement each other and help boost the agriculture industry, enhance food security and safety, and hence a vibrant economy and healthy populations.

This publication is especially dedicated in memory of the late FNCA Coordinator of Japan, HE Dr. Sueo Machi, whose dedication to the FNCA vision and mission is legendary, and also to all founder members, counterparts and collaborators of FNCA Biofertilizer Project for their outstanding contributions throughout the years, from 2001 until the present.

Thank you.

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Part 1. Sterilization of carrier for biofertilizer

1.1 Carrier of biofertilizer (Malaysia and Vietnam)

Biofertilizer is a substance containing living microorganisms which, when applied to seed, plant surfaces, or soil, colonizes the rhizosphere or the interior of the plant and promotes growth by increasing the supply or availability of primary nutrients to the host plant (Vessey, 2003). Biofertilizers are generally applied to soil, seeds or seedlings, without or with some carrier medium for the microorganisms.

Carrier is a vehicle to bring biofertilizer inoculum to the field. A good biofertilizer product needs a good carrier or substrate, which should be free from microbial contamination, and can optimise the growth of the biofertilizer microorganisms. Selection of carrier depends on nature of biofertilizer inoculums, cost, available of carrier, sterilization methods and agriculture practises.

There are four categories of carrier characteristic (Bashan, 1998; Vee *et al*., 1997).

i) Chemical characteristic: non-toxic, suitable C:N ratio for organic carrier, near neutral pH, high water-holding capacity and biodegradable.
ii) Physical characteristic: tolerance to sterilization by heat or gamma irradiation, without changing of physical form; preferably small sized particles and uniform.
iii) Manufacturing characteristic: easily available carriers, low cost and easy to handle.
iv) Agricultural practise characteristic: carrier can hold high population of viable cells (10^{6} to 10^{8} cfu g^{-1}) for long shelf life storage until application in farm; at least 6 months shelf life; preferably no cool storage is needed; biofertilizer products suitable for use with agricultural machinery.

Carrier for plant growth-promoting microorganisms (PGPM) is the major portion, by volume or weight, of the inoculant that helps to deliver a suitable amount of PGPM in good physiological condition. The characteristics should be ensuring chemical and physical stability, suitability to incorporate as many PGPM species and strains as possible, the possibility of mixing with other compounds (i.e. nutrients or adjuvants) and being composed of biodegradable and non-polluting compounds. The standard sizes of the powder material may vary from 75 μm to 0.25 mm.

According to Keyser *et al*. (1993), the carrier should be designed to provide a suitable microenvironment for the PGPM and should assure a sufficient shelf life of the product, at least 2-3

months for commercial purposes, and preferably at room temperature. The formulation should allow an easy dispersion or dissolution in the volume of soil near the root system. A good carrier should, therefore, possess as much as the following properties: good moisture absorption capacity, easy to process and free of lump-forming materials, near-sterile or easy to sterilize by autoclaving or by other methods (e.g. gamma-irradiation), low cost and availability in adequate amounts and good pH buffering capacity.

For carriers used for seed coating, a good adhesion to seeds is important (Hegde and Brahmaprakash, 1992) and to assure the survival of the PGPM on the seed since seeds are normally not immediately sown after seed coating (Muresu *et al*., 2003).

Carrier materials containing available nutrients and/or habitable microspore to the PGPM are desirable. According to Somasegaran and Hoben (1994), the properties of a good carrier material for seed inoculation are:

(1) Non-toxic to inoculant bacterial strain,

(2) Good moisture absorption capacity,

(3) Easy to process and free of lump-forming materials,

(4) Easy to sterilize by autoclaving or gamma-irradiation,

(5) Available in adequate amounts,

(6) Inexpensive,

(7) Good adhesion to seeds,

(8) Good pH buffering capacity, and

(9) Non-toxic to plant.

Other essential criteria for carrier selection relating to survival of the inoculant bacteria should be considered.

a) Survival of the inoculant bacteria on seed. Seeds are not always sown immediately after seed coating with the inoculant bacteria. The bacteria have to survive on seed surface against drying condition until placed into soil.

b) Survival of the inoculant bacteria during the storage period.

c) Survival of the inoculant bacteria in soil. After being introduced into the soil, the inoculant bacteria have to compete with native soil microorganisms for the nutrient and habitable niche, and have to survive against grazing protozoa. Such carrier materials that offer the available nutrient and/or habitable micro-pore to the inoculant bacteria will be desirable. In this sense, materials with micro-porous structure, such as soil aggregate and charcoal, will be good carrier for soil inoculant.

1.2 Types of carrier (Indonesia , Malaysia, The Philippines and Thailand)

Types of carrier as below:

Wood charcoal	Compost	Zeolite
Peat	Clay	Rice husk
Rice bran	Vermiculite	Organic pellet
Kaoline clay	Bentonite clay	Mixture of soil and charcoal

(Source: Julieta, 2018; Nurrobifahmi, 2016; Phatchayaphon; 2008; Phua, 2018 and Sasmita, 2017)

1.3 Comparison of gamma irradiation and autoclaving for carrier sterilization

In this chapter, comparison of two methods for carrier sterilization, viz. gamma irradiation and autoclaving, are described by a) Bangladesh, b) China, c) Indonesia, d) Japan, e) Malaysia, f) The Philippines and g) Thailand.

a) Bangladesh

A carrier is a supporting material which acts as a vehicle for the microorganism to be used as biofertilizer inoculum for the convenience of application. Carrier-based inoculumis said to be more effective than carrier-free inoculum because it can increase the effectiveness of biofertilizer, and enable easy handling of the biofertilizer product. Generally, biofertilizer with carrier material promoted plant growth more efficiently than did the carrier-free biofertilizer, since the carrier protects functional microbes from soil and climatic stress (Daza *et al*., 2000) and thus may enhance the survival of inoculant providing a protective environment. There are many instances where different carrier material has improved biofertilizer growth and survival (Hong-yuan *et al*., 2014).

It has been shown that the survival of inoculants in biofertilizer may be affected by physical and chemical properties of the carrier material and by competition with native microbes. To eliminate contamination from native bacteria or grazing protozoa, it is essential to sterilize the carrier material before inoculation with biofertilizer. This step is critical, to secure the quality of biofertilizer (Tejima *et al*., 2012). Sterilization of carrier is also helpful to prevent undesirable dispersion of pathogenic bacteria to agricultural fields that might occur through contaminated carrier (FNCA, 2006; Tejima *et al*., 2012). Sterilization of carrier material is essential to keep high or sufficient number of inoculants bacteria on carrier for long storage period before use.

Quality of carrier material has a positive influence on the quality of biofertilizer or inoculant. This quality not only depends on the physico-chemical and biological properties of carrier materials but also on the sterilization method on the carrier (Tittabutr *et al*., 2012). It was reported that methods used to sterilize various carrier can substantially affect the survival of inoculant microorganisms (Strijdom *et al*., 1981; Khavazi *et al*., 2007). Therefore, carrier materials must be sterilized with suitable techniques to produce quality inoculants.

Although it is now generally accepted that sterilized carrier is superior to a non-sterilized one, there are some disagreement about the most suitable method of carrier sterilization (Strijdom *et al*., 1981). Among several sterilization methods, autoclaving (steam sterilization) and gamma-irradiation are practically used in the different countries subject to availability of good facilities for sterilization.

Although many carriers can be sterilized by autoclaving at 121˚C for 1 h or more, autoclaving at 121˚C for 45 min may result in non-sterile carriers (Phua *et al*., 2009). Autoclaving of local peat carrier at 124˚C for 3.5 h produced carrier for high quality inoculant (Barend and Henri, 1981). However, carriers from Mushroom Spent Compost (MSC) can be sterilized at 121˚C for 30 min (Rosnani *et al.*, 2016). Double autoclaving in a row at 121˚C for 60 min with 18 h waiting could also efficiently sterilize peat and compost carrier with 10% moisture (Tittabutr *et al*., 2012).

Steam sterilization method has advantages and disadvantages. Steam sterilization has been applied to sterilize carrier in many industries for producing inoculants with high quality because of its easy availability in the market. Sterilization by autoclaving may result in changes in the chemical compositions of carrier during the autoclaving process, including imparting toxicity to the inoculants (Strijdom *et al*., 1981). Excessive heat during autoclaving renders the peat unfavourable for subsequent growth and survival of biofertilizer. Moreover, there is a risk of contamination of steam sterilized carrier when bags are removed from the autoclave before being sealed off and this drawback makes autoclave sterilization method less attractive (Strijdom *et al*., 1981).

Through γ-irradiation, carriers can be sterilized at a dose of 50 kGy to produce sterile carrier for biofertilizer products (Phua *et al*., 2009). However, some carriers including MSC can be sterilized at 30 kGy (Rosnani *et al*., 2016). It was also reported that lower doses, of gamma irradiationat 10-20 kGy, can efficiently sterilize the carriers from peat and compost, used for rhizobial inoculant production (Tittabutr *et al*., 2012).

Like steam sterilization, γ-irradiated sterilization method has some advantages and disadvantages too. This sterilization method is very promising for sterilizing carrier without changing its physical and chemical properties but the availability of gamma irradiation facilities is limited (Tejima *et al*., 2012). It was reported by Khavazi (2007) that rhizobial population were higher in materials

pre-sterilized by gamma radiation than those pre-sterilized by autoclaving. Strijdom (1981) reported that gamma irradiation is the most suitable way for carrier sterilization because the process result in almost no change in physical and chemical properties of the carrier material and the final products can be considered of high quality (Abd El-Fattah *et al*., 2013). Sterilization with radiation may not cause considerable physical or chemical changes that are toxic to the inoculants (Parker and Vincent, 1981; Daza *et al*., 2000). Moreover, a large volume of carrier products can be sterilized with high throughputs and least or less undesirable changes occurs in the carrier and its packaging materials as compared to autoclaving process.

In a study, viable cell count of biofertilizer inoculant mixed with gamma-irradiated carrier was compared with those of inoculant mixed with autoclaved-sterilized carrier and non-sterilized carrier to observe the effect of method used for carrier sterilization. Three sets of peat carrier samples were prepared for the study as follows: (a) Carrier sterilized with gamma irradiation at dose of 50 kGy from a cobalt-60 (^{60}Co) gamma source; b) Carrier sample sterilized by autoclaving at 121˚C for 60 min, and (c) Control carrier (without any treatment). After mixing the inoculants (*Rhizobium* sp.) with the carriers sterilized by the different methods, viable cell count was determined at particular intervals for 6 months by spread plate technique on YMA medium. Result showed that biofertilizer prepared with gamma-irradiated carrier could maintain higher number of viable cell than that of autoclave-sterilized carrier. Autoclave sterilized carrier could maintain higher number of viable cell than that of non-sterilized carrier to a greater extent.

Considering above literature review and our finding, it can be concluded that the sterilization method of different carrier materials with gamma-irradiation is superior to autoclave or steam sterilization method for producing quality biofertilizer in different aspects. Some comparative features of both methods are outlined in the Table 1.

Table 1: Comparison of methods (Ionizing Radiation vs. Autoclave) for carrier sterilization

Parameters	**Steam sterilization (Autoclave)**	**Ionizing radiation (γ-irradiation)**
Property changes	Some physical and chemical changes likely to occur	No significant changes occur in the physical or chemical properties of the carrier materials due to irradiation
Outcome of the property changes	Property changes may exert detrimental effect on inoculant viability, e.g. some substance may be produced that are toxic to inoculants	No significant detrimental effect of irradiation exerted on inoculant viability
Costing/ Price	Costly and time consuming	Relatively cheaper and time saving (0.6 $USDkg^{-1}$)
Ease of Handling	Easy to handle	Handling by competent radiation workers
Availability	Available in the market	Limited availability in the market
Regulation stringency on irradiation facility	No	Very strict
Post operative chance of contamination	There is a risk of contamination when bags are removed from the autoclave before being sealed off	Chance of contamination is nil because sealed packs are irradiated and therefore, no need for re-packing
Distortion of packing materials	Occurs if the packing material is heat labile	Unlikely
Extent of throughputs	-Comparatively less-Only small volume can be sterilized at a time	-Comparatively High -Large volume of carrier materials can be sterilized in each lot
Mode of action	-Heating under moist condition-Moist heat denatures cell proteins rendering the microbial cells inactive or dead	-Non-heating process (also called cold sterilization) under dry condition -Breakdown the DNA single and double stands; also reacts with nucleotide base of DNA rendering the cell inactive
Output	Maintains relatively less number of cell in the inoculants over long storage time	Maintains relatively higher number of cells in the inoculant over long storage time

b) China

(1) Efficient of γ-irradiation of peat

Cobalt-60 (^{60}Co) γ-irradiation sterilization of peat carrier was conducted. Six plastic bags of 300 L peat each were prepared for irradiation with doses of 20 kGy, 35 kGy, 50 kGy, 75 kGy, 100 kGy, 200 kGy, and 300 kGy, respectively. The ^{60}Co irradiation facility is located in the Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences. After radiation sterilization, bacteria, fungi and actinomycetes in peat were enumerated with plate counting method using beef extract medium, potato dextrose yeast extract agar (PDYA) medium and Gauserime synthetic agar medium, respectively (Kucey, 1983).

(1) Beef extract medium (or beef-protein medium) for bacteria enumeration
Beef extract 4 g, Peptone 6 g, glucose 5 g, NaCl 5 g, add distilled water to 1000 ml, pH 7.2 and 2% agar.
(2) Gauserime synthetic agar medium for actinomycetes enumeration
KNO_3 1.0 g, K_2HPO_4 0.5 g, NaCl 0.5 g, $FeSO_4.7H_2O$ 0.01 g, soluble starch (amylogen) 20 g, a $MgSO_4.7H_2O$ 0.5 g, add distilled water to 1000 ml, pH 7.2-7.4 and 2% agar.
(3) PDYA medium for fungi enumeration
Potato dextrose yeast extract agar (PDYA) (0.5% w/v).

The results showed the population of indigenous microorganisms in peat decreased with increasing γ-irradiation doses. At 20 kGy, the population of indigenous microorganisms in peat was the highest with the fungal population averaged 2.05×10^6 cfu g^{-1} and bacteria at 4.00×10^6cfu g^{-1}, actinomycetes were not detected. At 35 and 50 kGy treatments, the fungal populations averaged 3.77×10^5 and 1.0×10^4 cfu g^{-1}, bacterial populations averaged 7.27×10^5 and 3×10^4 cfu g^{-1}, respectively, while actinomycetes were only 6.3×10^3 cfu g^{-1} at 35 kGy treatment. After receiving 75-100 kGy irradiation, there were only very low number of fungi and bacteria in the peat. At irradiation doses of 200-300 kGy, no indigenous microbes survived in the peat carrier (Table 2).

Table 2: Effect of gamma irradiation doses on population of indigenous microorganisms in peat

^{60}Co γ-irradiation doses (kGy)	PDYA ($\times 10^4$ cfu g^{-1})	Bacteria ($\times 10^4$ cfu g^{-1})	Actinomycetes ($\times 10^3$ cfu g^{-1})
20	205.3a	400.0 a	0.0 c
35	37.7b	72.7 b	6.3a
50	0.0d	0.0 d	0.0 c
75	1.0c	3.3 c	3.3b
100	3.7c	4.0 c	0.0 c
200	0.0d	0.0 d	0.0 c
300	0.0d	0.0 d	0.0c

(2) Survival of *Aspergillus niger* strain 1107 in various carriers sterilized by autoclaving and γ-irradiation (Wang *et al.*, 2015)

The objectives of this study were to select a suitable carrier for inoculating P-solubilizer, to confirm the effect of γ-irradiation for sterilization of biofertilizer carrier, and finally to develop an inexpensive and simple P-solubilizing biofertilizers, which could be adapted by the industries in developing countries. Various potential carrier materials, including peat, perlite, corn cobs, cattle manure fertilizer (CMF), and mixture of 1:1 corn cobs and perlite, which were widely available in many developing countries, were investigated. The shelf-life of P-solubilizer inoculants and their survival in soil when the carriers were sterilized by autoclaving and γ-irradiation was compared. Since inoculants are usually stored at room temperature, survival at 25˚C was evaluated in this study. At the same time, survival at 4˚C was also tested as control.

Carrier preparation: Five kinds of carriers including peat, corn cobs, perlite, CMF and 1:1 corn cobs/ perlite mixture were used in the present study. Carrier materials were first powdered and passed through a 100-mesh sieve for physico-chemical characterization before being evaluated (Table 3). The major criteria for the carrier selection are the possibility to adjust the pH to neutral value, maximum water holding capacity, cost, and availability. All the materials were thoroughly mixed with certain amount of $CaCO_3$ powder to adjust the pH to 7.0, the mixtures were completely shaken before the pH value measurement.

Carrier sterilization procedure: 500 g of each of the five carriers were put into 10 cotton bags with a size of 50 cm×16 cm, separately. The thickness of the cotton bags was about 1.0 mm. Packages were sterilized by γ-irradiation or by autoclaving.

For γ-irradiation, these packages were put into the polypropylene plastic bags with the thickness of 0.08 mm.γ-irradiation was carried out in the ^{60}Co gamma cell source installed at Institute of Food Science and Technology, Chinese Academy of Agricultural Sciences, Beijing, China. Irradiation was done at dose of 50 kGy with a dose rate of 15 Gy min^{-1}. Autoclaving was done for 40 min at 121˚C. Bags were put into another sterile big cotton bag after overnight cooling in the autoclave. After sterilization, these packages were dried for 12 h at 60˚C in the blowing-type oven.

Inoculant preparation and incubation: In the present study the P-solubilizing fungal strain 1107 was isolated from corn crop rhizospheric soil. The isolate was identified as *Aspergillus niger* based on their colony morphology and microscopic studies. Fresh conidia were generated by plating 10^8 spores ml^{-1} on potato dextrose agar (PDA) and incubated at 30°C for 3-5 days. Conidia were harvested from plates in 5 ml of sterile 0.2% Tween-20 collected by gentle scrapping. Conidia concentration was determined by microscopic counts using a haemocytometer and conidial densities were adjusted to a final concentration of 10^9 spores ml^{-1}.

Determination of shelf-life: For shelf-life evaluation, the formulated products were transferred into sterilized screw-top tubes (50 ml volume) and stored in the dark at 4 and 25˚C. The P-solubilizing inoculants were turned over every three to four days for 7 months. Non-inoculated raw material was used as the control. After 7, 14, 30, 60, 90, 120, 150, 180, 210 days storage, the numbers of surviving fungi (conidia) were evaluated by plate count. Aseptic conditions were maintained throughout the process.

Table 3: Chemical and physical characteristics of materials used as carriers

Material	N(%)	P(%)	K(%)	EC(ms·ml^{-1})	pH	OM(%)	WHC(%)
Peat	1.73	0.21	0.32	0.24	6.14	65.4	100
Corn cob	0.38	0.07	0.92	1.47	4.54	96.4	480
Perlite	0.02	0.01	0.05	0.06	6.16	0.1	200
Cattle manure fertilizer	1.63	1.03	1.78	24.5	5.50	46.2	120

EC: electrical conductivity; OM: organic matter; WHC: water holding capacity.

The first set of experiments investigated the survival of *Aspergillus niger* strain 1107 in various carriers sterilized by autoclaving or γ-irradiation and stored at 4˚C or 25˚C for 7 months. Figure 1 showed that the survival conidia of the P-solubilizing biofertilizer microorganisms stored at 25˚C were higher than those stored at 4˚C during the beginning 1-3 months, and at the following time the survival of conidia stored at 4˚C were higher than those stored at 25˚C. Thus, storage at room temperature is suitable for a short period of time, while storage at 4˚C can prolong the shelf-life. Figure 2 showed the survival of *A. niger* strain 1107 in various carriers sterilized using autoclaving or γ-irradiation and stored at 4˚C. Most carriers, with the exception for CMF sterilized by γ-irradiation, resulted in better performance of the biofertilizer inocula for up to 5-6 months storage than those sterilized by autoclaving.

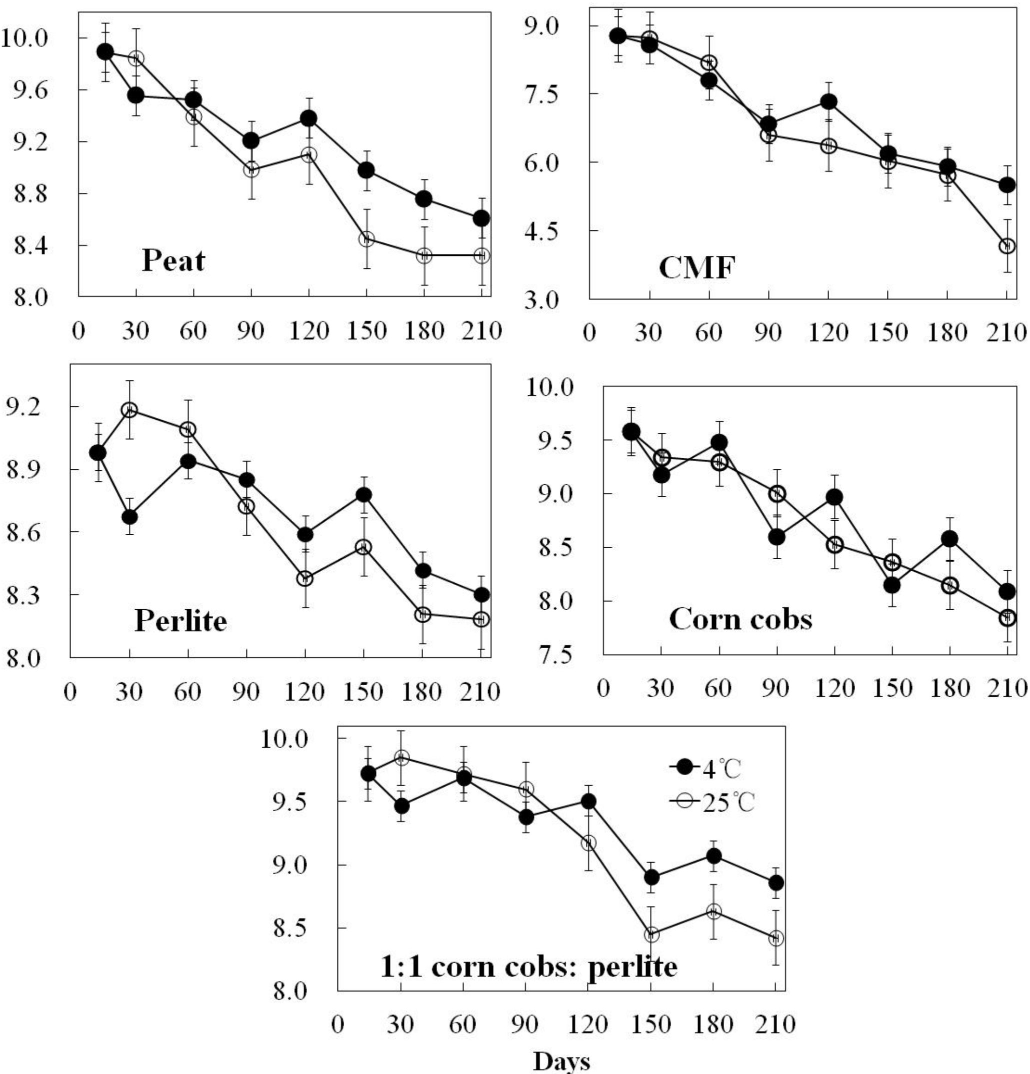


Figure 1: Survival of *A. niger* strain 1107 in various carriers sterilized using γ-irradiation when stored at 4˚C and 25˚C. Each point represents the mean of three replicates. Vertical bars represent ±1 S.D.

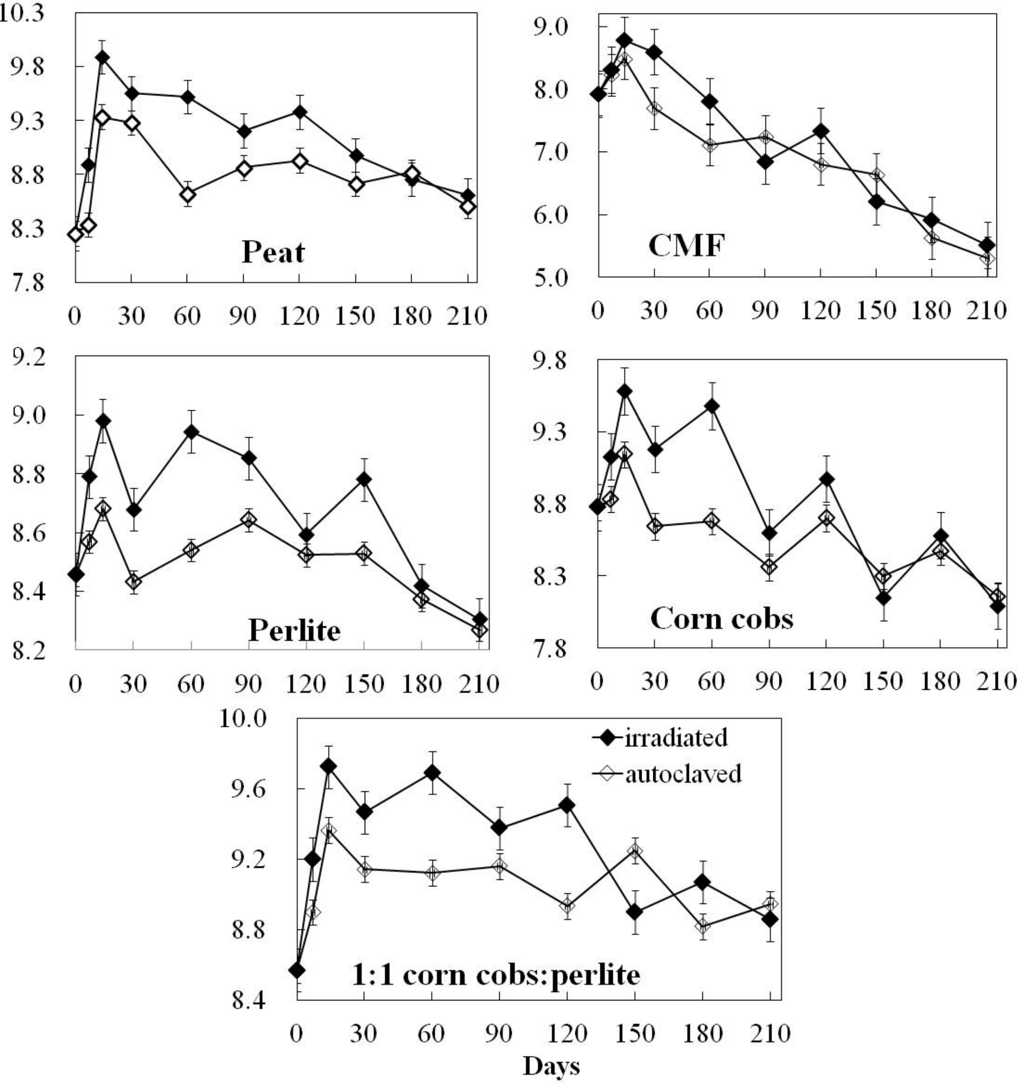


Figure 2: Survival of *A. niger* strain 1107 in various carriers sterilized by autoclaving or γ-irradiation prior storage at 4˚C. Each point represents the mean of three replicates. Vertical bars represent ±1 S.D.

The suitable carriers sterilized using γ-irradiation for *A. niger* strain 1107 population are presented in Figure 3. The irradiated carriers can maintain *A. niger* strain 1107 populations higher than 10^8 cfu g^{-1} for at least 7 months, which meets the standards set by most countries, that is, ranging between 10^7 and 10^9 cfu g^{-1} inoculant (Ogbo, 2010). The only exception was the *A. niger* conidia in the CMF inoculants, which degreased rapidly in 7 months. Compared to corn cobs and perlite alone, the 1:1 corn cobs/perlite mixture maintained slightly higher *A. niger* conidia with log cfu g^{-1} inoculant more than 8.8, which was similar to peat.

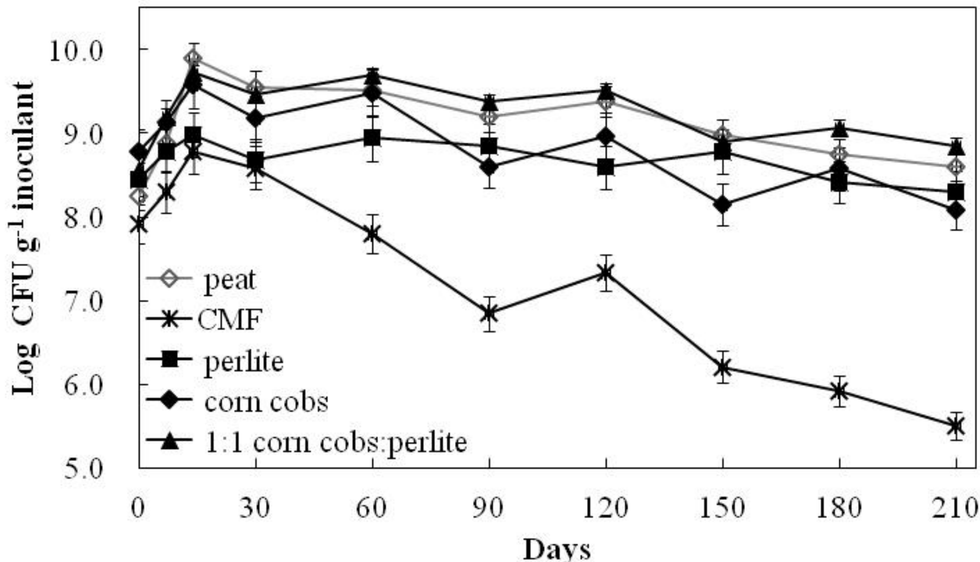


Figure 3: Survival of *A. niger* strain 1107 in various carriers sterilized usingγ-irradiation when stored at 4˚C. Each point represents the mean of three replicates. Vertical bars represent ±1 S.D.

c) Indonesia

Zeolite and compost were used as carriers with *Gigaspora margarita* mycorrhizal biofertilizer. Sterilization methods of carriers using gamma irradiation and autoclaving were compared. Zeolite (1-2 mm) and compost were sterilized by autoclaving at 121˚C for 60 min or by gamma irradiation at 10, 20, 30, 40 and 50 kGy with Irradiator Gamma Chamber 4000 A with ^{60}Co as source of gamma radiation (Nurrobifahmi *et al.*, 2017).

Gamma irradiation at 40 and 50 kGy and autoclaving eliminated microbes in zeolite but gamma irradiation at lower than 40 kGy was less effective in elimination of microbes in zeolite or compost.

Table 4: Total number of microbial contaminants on the zeolite and compost (cfu g^{-1})

Type of sterilization	Zeolite	Compost
Without sterilization	39.0×10^3	179.0×10^5
Autoclave	0	38.0×10^2
Gamma irradiation at 10 kGy	79.0×10^2	108.0×10^5
Gamma irradiation at 20 kGy	71.0×10^2	87.5×10^5
Gamma irradiation at 30 kGy	67.5×10^1	55.5×10^2
Gamma irradiation at 40 kGy	0	20.5×10^1
Gamma irradiation at 50 kGy	0	5.00×10^1

(1) Application of Radiation Sterilization of Carrier for Production of Better Quality Biofertilizer

(Taufik Bachtiar, Iswandi Anas, Atang Sutandi, Ishak)

Aim

This study was conducted to evaluate the effectiveness and the effect of sterilization methods (autoclave and radiation with ^{60}Co) on characteristics of carrier materials (two mineral soils, two peats and one compost).

Materials and Methods

Sterilization methods that consists of without sterilization, autoclave, and gamma radiation ^{60}Co at the rate of 10 to 50 kGy. Carriers were: mineral soils Latosol Pasar Jumat West Java and Latosol Jasinga West Java; peat from Rawa Pening Lake, Central Java and peat from South Kalimantan; and compost.

The characteristics (properties) of carrier materials evaluated were pH, soluble P, nitrate, soluble Mn, Fe, Zn, and the total number of microbes.

Results

Table 5: Effect of sterilization methods (autoclave and gamma irradiation) on total number of microbes in five carriers (cfug^{-1})

Dose (kGy)	Latosol Jasinga	Latosol Pasar Jumat	Compost	Rawa Pening Peat	Kalimantan Peat
D0	1.33×10^{6}	2.39×10^{7}	1.34×10^{7}	1.25×10^{7}	2.73×10^{7}
D10	4.83×10^{4}	2.88×10^{5}	1.41×10^{4}	4.87×10^{4}	5.13×10^{4}
D20	6.10×10^{4}	6.47×10^{4}	0	0	1.13×10^{4}
D30	0	0	0	0	0
D40	0	0	0	0	0
D50	0	0	0	0	0
Autoclave	0	0	0	0	0

Note: The number followed by the same letter in the same column shows the results were not significantly different based DMRT at the 5% significance level

Table 6: Effect of sterilization methods (autoclave and gamma irradiation) on the NO_3^- (me L^{-1})

Dose (kGy)	Latosol Jasinga	Latosol Pasar Jumat	Compost	Rawa Pening Peat	Kalimantan Peat
D0	0.12 a	0.41 a	11.43 a	2.57 a	0.35 a
D10	0.11 a	0.41 a	11,86 a	3.08 c	1.10 b
D20	0.14 b	0.40 a	12.49 a	3.43 b	1.22 b
D30	0.12 a	0.41 a	12.44 a	2.78 ab	1.84 c
D40	0.14 b	0.41 a	12.09 a	2.88 bc	2.79 de
D50	0.15 b	0.41 a	11.62 a	3.15 c	2.53 d
Autoclave	0.18 c	0.41 a	12.13 a	3.14 c	2.85 e

Note: The number followed by the same letter in the same column shows the results were not significantly different based DMRT at the 5% significance level

Table 7: Effect of sterilization methods (autoclave and gamma irradiation) on soluble P (ppm)

Dose (kGy)	Latosol Jasinga	Latosol Pasar Jumat	Compost	Rawa Pening Peat	Kalimantan Peat
D0	0.50 a	2.16 a	67.33 a	10.82 a	2.14 a
D10	0.37 a	4.19 a	72.64 b	16.52 c	4.00 b
D20	1.29 a	5.26 ab	71.79 ab	16.13 c	4.98 c
D30	3.61 ab	5.19 ab	74.21 bc	16.32 c	5.38 bc
D40	3.60 ab	5.50 ab	72.51 b	16.59 c	6.49 e
D50	4.94 b	9.51 ab	74.74 bc	15.01 bc	5.90 d
Autoclave	6.16 b	3.53 a	78.01 c	13.70 b	6.62 e

Note: The number followed by the same letter in the same column shows the results were not significantly different based DMRT at the 5% significance level

Table8: Effect of sterilization methods (autoclave and gamma irradiation) on soluble Mn (ppm)

Dose (kGy)	Latosol Jasinga	Latosol Pasar Jumat	Compost	Rawa Pening Peat	Kalimantan Peat
D0	55.38 a	61.57 a	55.80 a	60.7 a	9.85 a
D10	55.78 a	61.58 a	56.53 a	61.65 b	9.99 a
D20	55.61 a	61.48 a	61.85 b	62.27 bc	10.41 ab
D30	56.05 a	63.50 c	59.23 ab	62.57 c	10.71 ab
D40	57.1 ab	63.05 bc	61.50 b	62.83c	10.81 ab
D50	57.77 b	62.32 ab	59.42 ab	62.18 bc	11.48 b
Autoclave	114.22 c	127.00 d	121,32 c	62.67 c	16.06 c

Note: The number followed by the same letter in the same column shows the results were not significantly different based DMRT at the 5% significance level

Table 9: Effect of sterilization methods (autoclave and gamma irradiation) on soluble Fe (ppm)

Dose (kGy)	Latosol Jasinga	Latosol Pasar Jumat	Compost	Rawa Pening Peat	Kalimantan Peat
D0	47.38 a	12.13 a	55.95 a	314.00 c	584.38 a
D10	59.98 ab	24.57 c	62.75 b	302.08 b	643.98 b
D20	72.40 bc	24.85c	64.68 b	305.77 b	667.09 bc
D30	72.42 bc	19.81 b	72.18 c	297.45 a	677.18 c
D40	83.65 cd	21.30 b	64.70 b	295.3 a	680.81 c
D50	96.58 de	25.28 c	71.93 c	293.77 a	670.96 bc
Autoclave	116.40 e	47.19 d	96.90 d	310.85 c	688.32 c

Note: The number followed by the same letter in the same column shows the results were not significantly different based DMRT at the 5% significance level

Table 10: Effect of sterilization methods (autoclave and gamma irradiation) on soluble Zn (ppm)

Dose (kGy)	Latosol Jasinga	Latosol Pasar Jumat	Compost	Rawa Pening Peat	Kalimantan Peat
D0	17.83 a	34.55 a	73.92 a	25.29 a	3.20a
D10	27.99 a	83.90 b	76.18 a	60.71 c	4.40 ab
D20	19.08 a	91.14 bc	113.18 cd	44.15 b	7.34 c
D30	28.12 a	96.33 bc	99.91 b	48.30 b	7.20 c
D40	42.01 b	93.87 bc	119.35 d	84.30 d	6.61 b c
D50	44.17 b	99.81 c	102.87 bc	85.35 d	9.25 c
Autoclave	64.17 c	89.67 bc	120.72 d	89,82 d	9.36 c

Note: The number followed by the same letter in the same column shows the results were not significantly different based DMRT at the 5% significance level

Table 11: Effect of sterilization methods (autoclave and gamma irradiation) on the pH of carrier

Dose (kGy)	Latosol Jasinga	Latosol Pasar Jumat	Compost	Rawa Pening Peatland	Kalimantan Peatland
D0	4.61 d	4.97 a	7.90 f	5.55 a	3.92 a
D10	4.57 cd	5.02 b	7.87 e	5.64 b	3.88 b
D20	4.35 a	5.17 d	7.69 d	5.86 e	3.88 b
D30	4.46 b	5.12 c	7.64 c	5.74 d	3.86 c
D40	4.52 c	5.13 c	7.62 bc	5.87 e	3.86 c
D50	4.53 c	5.17 d	7.56 a	5.86 e	3.86 c
Autoclave	4.42 b	5.35 e	7.60 b	5.71 c	3.78 d

Note: The number followed by the same letter in the same column shows the results were not significantly different based DMRT at the 5% significance level.

Conclusions

1. Gamma irradiation at dose of 30 kGy and autoclaving are effective to sterilize mineral soils, peat and compost.
2. Gamma irradiation change properties of carriers less than autoclaving.
3. Soluble N (NO_3^-) and P in mineral soils, peats and compost may increase when sterilized with gamma irradiation or autoclave.
4. Soluble Mn in mineral soils and compost slightly increase by gamma irradiation, however autoclaving significantly increased soluble Mn. Gamma irradiation and autoclave increased slightly soluble Mn in peats.
5. Gamma irradiation increased slightly soluble Fe in mineral soils and compost but less in peats but autoclaving increased soluble Fe in mineral soils and compost, less in peats.
6. Gamma irradiation and autoclaving increased soluble Zn in mineral soils, compost and peats
7. Gamma irradiation and autoclaving may change the pH of mineral soil, compost and peats.

(2) Application of Radiation Sterilization of Carrier for Production of Better Quality Biofertilizer

(Nurrobifahmi, Iswandi Anas, Yadi Setiadi and Ishak)

Aims

1. To evaluate the effectiveness of sterilization methods by gamma irradiation and autoclaving.
2. To evaluate the effect of sterilization by gamma irradiation and autoclaving on solubility of Fe, Mn, Zn in carriers (zeolite, compost and mineral soil).
3. To study the effect of sterilization methods on the viability and germination of spores of *Gigaspora margarita* in carriers (zeolite and compost).

Results

Table 12: Effect of sterilization methods on microbial population (cfu g^{-1})

Dose (kGy)	Zeolite	Compost
D0	39.0 x 10^3	179.0 x 10^5
D10	79.0 x 10^2	108.0 x 10^5
D20	71.0 x 10^2	87.5 x 10^5
D30	67.5 x 10^1	55.5 x 10^2
D40	0	20.5 x 10^1
D50	0	5.00 x 10^1
Autoclave	0	38.0 x 10^2

Table 13: Effect of sterilization methods (autoclave and gamma irradiation) on soluble Fe (ppm)

Dose (kGy)	Soluble Fe (ppm)		
	Zeolite	Compost	Latosol
D0	6.34 a	106.87 a	28.10 a
D10	7.44 a	81.60 c	37.03 a
D20	6.86 a	115.24 a	25.64 a
D30	5.98 a	112.53 a	32.47 a
D40	6.45 a	111.88 a	28.18 a
D50	4.39 a	95.59 b	29.10 a
Autoclave	4.86 a	50.03 d	28.12 a

Note: The number followed by the same letter in the same column shows the results were not significantly different based DMRT at the 5% significance level

Table14: Effect of sterilization methods (autoclave and gamma irradiation) on available Mn (ppm)

Dose (kGy)	Soluble Mn (ppm)		
	Zeolite	Compost	Latosol
D0	15.43 b	73.85 a	115.54 d
D10	13.49 b	56.35 a	227.25 c
D20	13.56 b	61.94 a	218.75 c
D30	14.49 b	58.98 a	208.68 c
D40	16.22 b	65.20 a	207.98 c
D50	37.52 a	63.59 a	302.92 b
Autoclave	13.13 b	58.62 a	534.56 a

Note: The number followed by the same letter in the same column shows the results were not significantly different based DMRT at the 5% significance level

Table15: Effect of sterilization methods (autoclave and gamma irradiation) on soluble Zn (ppm)

Dosa (kGy)	Soluble Zn (ppm)		
	Zeolite	Compost	Latosol
D0	0.57 f	9.05 a	3.33 a
D10	5.70 ed	6.91 b	3.41 a
D20	6.06 cd	8.76 a	3.47 a
D30	6.66 bc	7.04 b	3.20 a
D40	7.15 b	9.20 a	3.45 a
D50	7.98 a	7.60 b	3.50 a
Autoclave	5.13 e	5.49 c	3.36 a

Note: The number followed by the same letter in the same column shows the results were not significantly different based DMRT at the 5% significance level

Table 16: Effect of sterilization methods (autoclave and gamma irradiation) on germination of spores after three months storage

Dose (kGy)	No. of spores after 3 months storage of 50 spores added		No. of germinated spores		Percentage of germinated spores	
	Zeolite	Compost	Zeolite	Compost	Zeolite	Compost
D0	42.0	7.3	14.7	0	35.4ab	0
D10	38.3	7.3	18.0	0	45.8 a	0
D20	45.0	6.3	10.0	0	21.3bc	0
D30	36.3	7.3	13.7	0	36.8ab	0
D40	47.3	11.7	5.3	0	11.3 c	0
D50	40.0	13.3	6.7	0	17.3bc	0
Autoclave	40.7	11.7	9.3	0	21.6bc	0

Note: The number followed by the same letter in the same column shows the results were not significantly different based DMRT at the 5% significance level

Conclusions

1. Gamma irradiation using ^{60}Co at 30 kGy was effective to sterilize zeolite but not completely sterilize compost. Autoclaving was effective in sterilizing zeolite but did not completely sterilize compost.
2. Gamma irradiation may increase solubility of Fe, Mn and Zn, depending on the kind of carriers but autoclave significantly increase solubility of Mn in soil.
3. Zeolite sterilized either by using gamma irradiation or autoclaving can be used to store and germinate spores of *Gigaspora margarita* but compost can not.

d) Japan

To demonstrate the effect of γ-sterilization, the survival of the N_2-fixing bacterium *Bradyrhizobium japonicum* was monitored to assess the shelf life of biofertilizers (Tejima *et al*., 2012). As biofertilizer carriers, five kinds of typical Japanese soil-based materials were used in this study. Following the sterilization of carrier materials by γ-irradiation (50 kGy) or autoclaving (121˚C for 40 min), *B. japonicum* strain USDA110 was inoculated into each material. The biofertilizer was packed into polyethylene bags and stored for 12 months at 4˚C and 30˚C. After storage, viable inoculants in the biofertilizer were counted.

Results indicated that inoculant density (cfu g^{-1}) after storage was greater than the initial density in

biofertilizers made from sterilized carriers, whereas inoculant density (cfu g^{-1}) decreased significantly in biofertilizers from non-sterilized carriers. γ-sterilization was superior to sterilization by autoclaving in enhancing inoculant survival, in some cases. Due to the stability of supply, the high sterilization effect with lower radiation doses, and the high performance in maintaining a suitable inoculant density, Japanese peat soil (Keto-tsuchi) seems best suited as a biofertilizer carrier for *B. japonicum* among the tested soil materials.

e) Malaysia

Gamma irradiation and heat treatment by autoclaving are commonly used for sterilization of biofertilizer carriers. Gamma irradiation is commonly used to sterilize pharmaceutical products, medical products, food and herbs (FNCA, 2006). Autoclave is commonly used to sterilize media and laboratory equipment. There are some advantages and disadvantages for both gamma irradiation and autoclaving. An advantage of autoclaving is its availability in the market as compared to a gamma irradiation system. A gamma irradiation facility can sterilize objects of large size and volume, with high throughputs as compared to the autoclave. Autoclaving uses steam and heat to kill the microbes, whereby the moist end product may enhance the growth of microbes. Gamma irradiation produces a dry product. Autoclave cannot sterilize materials that are not tolerant to heat.

Sterile carrier is essential to optimise the growth of the biofertilizer microorganisms on carrier for long storage period. Carriers autoclaved at 121˚C for 60 min were observed to be sterile. However, autoclaving for 45 min and below resulted in non-sterility Phua *et al*. (2009). Result showed in Figure 4. Longer time is needed for heat to reach the centre of solid materials Themophilic bacteria with heat-resistant endospores may survive the heat treatment (Ho, 2000). Tittabutr *et al*. (2012) also reported sterilized peat and compost carriers with 10% moisture content by using autoclaving with tyndallization approach (autoclaving two times in a row at 121˚C for 60 min, with waiting period of 18 h after each time of autoclaving) could efficiently produce sterilized carriers. Steam sterilization of peat carrier for 3.5 h at 124˚C had resulted in high quality inoculants but there was high risk of contamination when bags were removed from the autoclave before being sealed off. This makes steam sterilization less attractive (Barend and Henri, 1981).

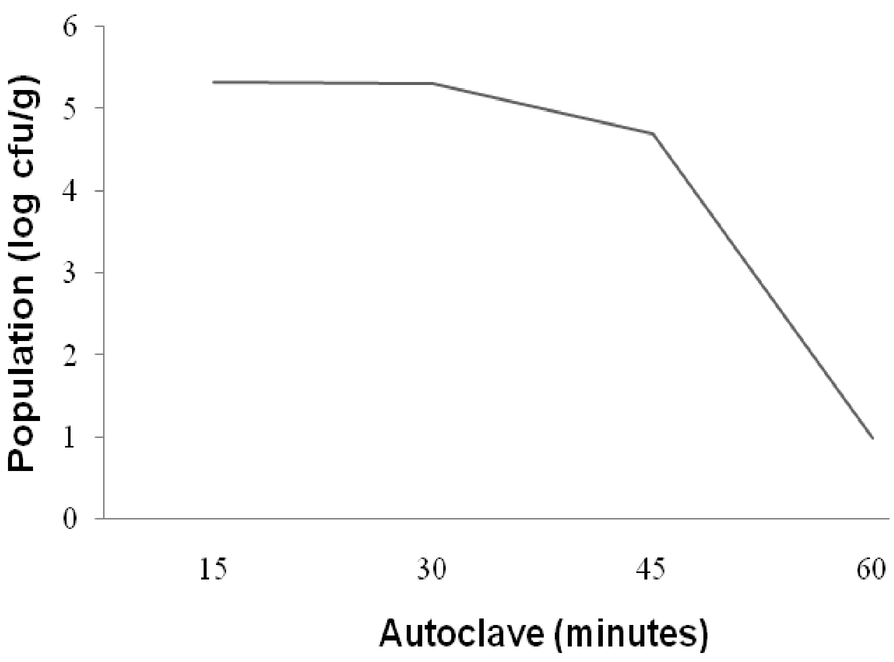


Figure 4: Effect of autoclaving on microorganism population in Natural Farming compost carrier

Barend and Henri (1981), Shamsuddin (2005) and Phua *et al*. (2009) reported that gamma irradiation at 50 kGy produced sterile carriers. However, there were some reports that showed low irradiation dose (10-20 kGy) can produce sterile carrier. Tittabutr *et al*., 2012 showed irradiation at 10-20 kGy effectively sterilized peat and compost carriers with 10% moisture content. According to Rosnani *et al*., 2016, Mushroom Spent Compost (MSC) is also suitable as a carrier or substrate to hold beneficial microorganisms in biofertilizer products. MSC was completely sterilized by gamma irradiation at the dose of 30 kGy (Figure 5). Six different formulations carrier were prepared using different materials, namely, peat moss, mixture of peat moss plus vermiculite 1:2 (w/w), wheat bran, rice husk, clay and sodium alginate were irradiated at dose rate 4 kGy for 1 h. *Azotobacter chroococcum* (A101) was mixed with carriers and stored at 8˚C and 30˚C for 6 months. Irradiated carriers were free from contamination for 6 months storage (Abd El-Fattah *et al*., 2013). Peat carriers for rhizobia were irradiated at 59.6 and 65.9 kGy and stored at 4˚C and 28˚C for eight weeks. Carrier incubated for eight weeks showed high viable cells for rhizobia (Kaljeet *et al*., 2011).

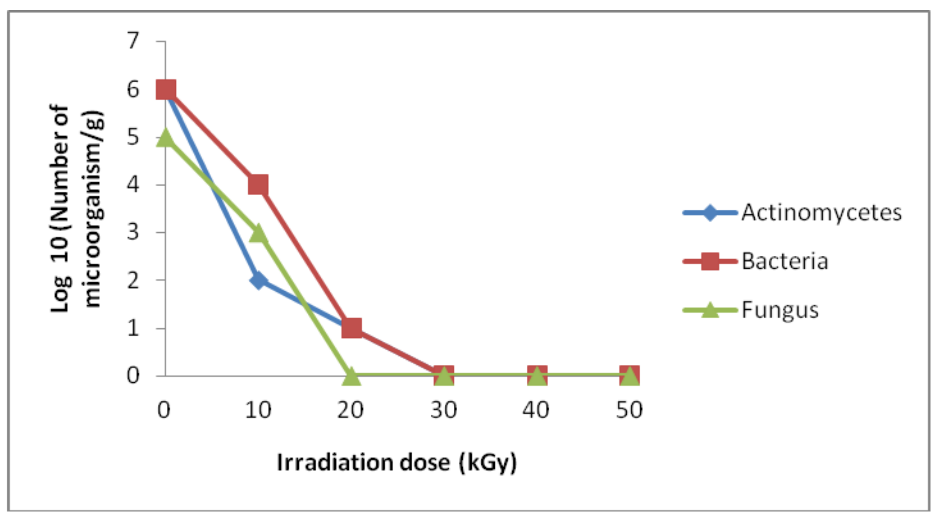


Figure 5: Effect of gamma irradiation on populations of microorganisms in MSC

Phua et al. (2009) reported compost carriers (Natural Farming Compost) irradiated at 10 and 20 kGy did not completely sterilize the carrier (Figure 6). This may due to the presence some radiation resistant microorganisms or spores. One of the reasons for this resistance is that cell nucleus (target of radiation) of microorganisms is very small. Another reason is DNA protection and repair capacity of the microorganisms. Some spore forming bacteria such as *Bacillus* sp. and *Clostridium* sp. are reported to be resistant to irradiation (FNCA, 2006). At 30 kGy and above, the carriers were observed to be sterile. This could be due to high penetrating activity of gamma irradiation that damaged DNA and caused cell death. Figure 7 also shows that commercial compost and peat has low population of microbes (1x10 cfu g^{-1}) after irradiated at 30 kGy. Carrier materials gamma irradiated at 50 kGy was observed to be sterile even after storage for 6 months, while autoclaved carriers showed microbial contamination after 6 month storage. Gamma irradiated carriers also had better physical properties compared to those autoclaved (Figure 8). The irradiated carriers were observed to be dry and the packaging materials did not undergo physical change. Autoclaved carriers were observed to be wet and the packing materials a little distorted (Figure8).

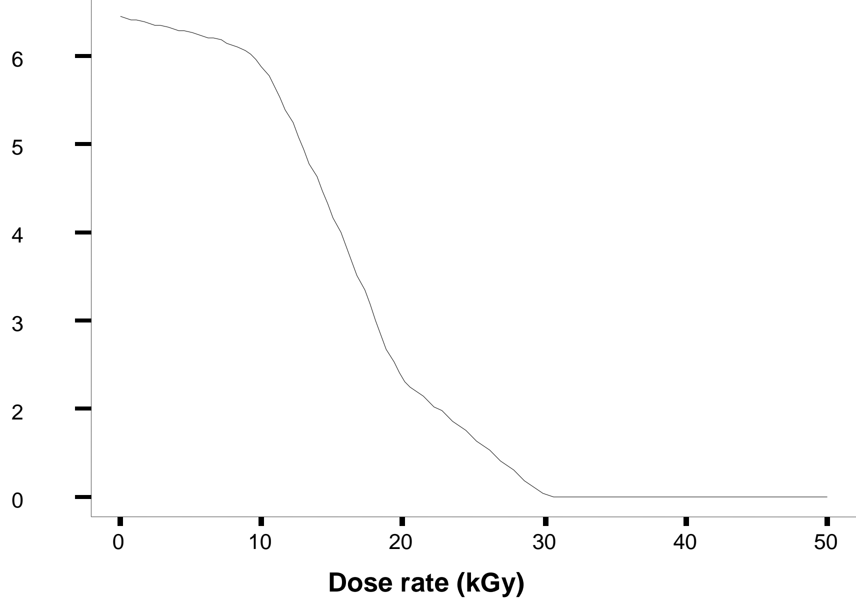


Figure 6: Effect of gamma irradiation on microorganism population in Natural Farming compost carrier

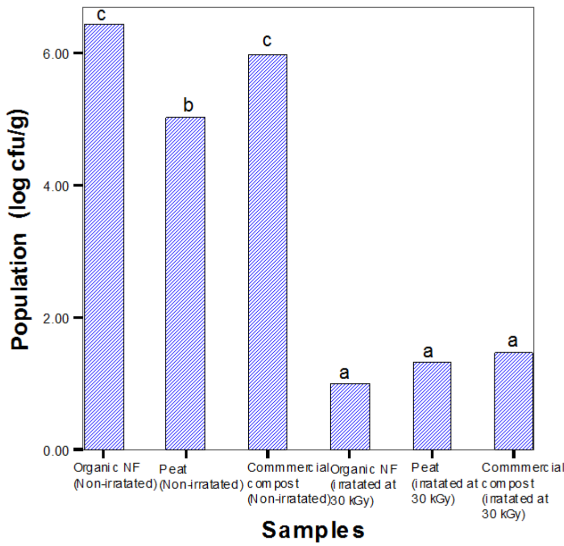


Figure 7: Comparison of microorganism population in various carriers without gamma irradiation and irradiation at 30 kGy

Figure 8: (a) Autoclave bag for packing, (b) carriers sterilized by autoclaving and (c) gamma sterilized carrier

A shelf life study of biofertilizers with autoclaved and gamma irradiated carriers was conducted in Malaysian Nuclear Agency (Nuclear Malaysia). Natural Farming organic fertilizer (compost) was used as carrier. Three biofertilizer inoculants (Figure 9), each mixed into carrier (100 g each) and sterilized by autoclaving at 121˚C for 60 min and sterilized with 50 kGy gamma irradiation. The samples were stored at room temperature for 8 months. Periodic population counts in samples were made using serial dilution and plate count method. Biofertilizer inoculants in carriers sterilized by autoclaving at 121˚C for 60 min had shorter shelf life compared to those in carriers sterilized by gamma irradiation at 50 kGy. Shelf life of inoculants AP2 and AP3 in gamma irradiated carriers was 7 months, but those in autoclaved carriers survived for only 3 months. Autoclaved carriers were contaminated with fungi at 4 months of storage. Compared to AP2 and AP3, shelf life for AP1 was shorter. Inoculant AP1 had 2 months shelf life in autoclaved carrier and 5 months in gamma irradiated carrier.

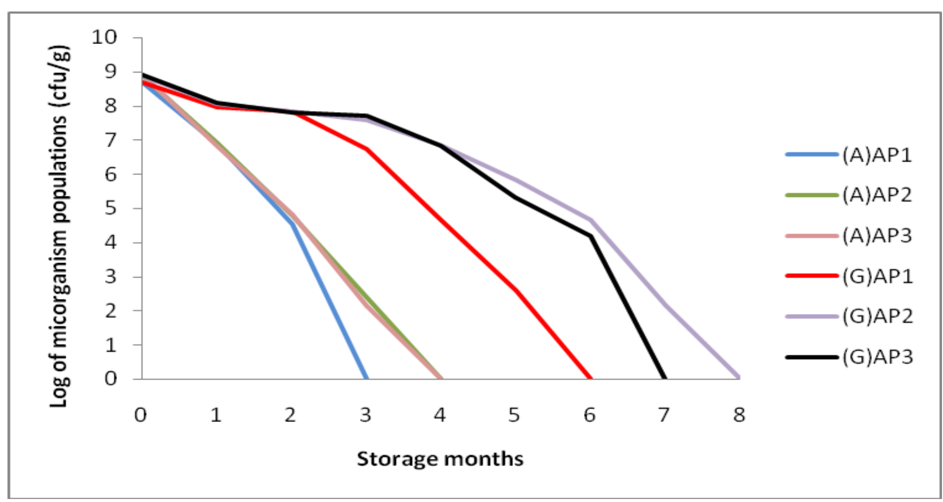


Figure 9: Shelf life of biofertilizer stored at room temperature
Key: (A) AP1, AP2 AP3 – Carrier sterilization by autoclaving at 121˚C for 60 min;
(G)AP1, AP2 AP3 – Carrier sterilization by gamma irradiation at 50 kGy

Biofertilizer inoculants used:
AP 1 – Phosphate solubilising bacteria and antagonistic against bacterial wilt disease
AP 2 – Phosphate and potassium solubilising bacteria and plant growth promoter
AP3 – Phosphate and potassium solubilising bacteria

In conclusion, gamma irradiation has more advantages to produce sterile carrier and result in better shelf life of biofertilizer. The success of biofertilizer may also depend on the selection and preparation a suitable carrier. Thus, this manual will discuss on selection and determination characteristic of carrier such as pH, moisture content, C: N ratio, and quality control. Advantage and disadvantage of two sterilization methods were summarized in Table 17.

Table 17: Comparison of carriers sterilized by gamma irradiation and autoclaving

Gamma Irradiation	Autoclaving
No changes in physical and chemical properties of the vermiculite*	May be some changes in physical and chemical properties* May be produce toxic substance to some bacterial strain* Some of the vermiculite lumping
Cheaper: RM 400 t^{-1}	Cost and time consuming: -the autoclave consume high electricity and it is limited. One time autoclave for 5 kg is equivalent to RM 5.00 current used. -60 kg of vermiculite can be sterilized/week/1 unit of autoclave
Easy to handle	-Need re-packaging into double layer of HDPE plastic bag.

f) The Philippines

Comparison of sterilization of carrier material using gamma irradiation at 20 kGy with heat treatment using autoclave has shown in Table 18 and Figure 10.

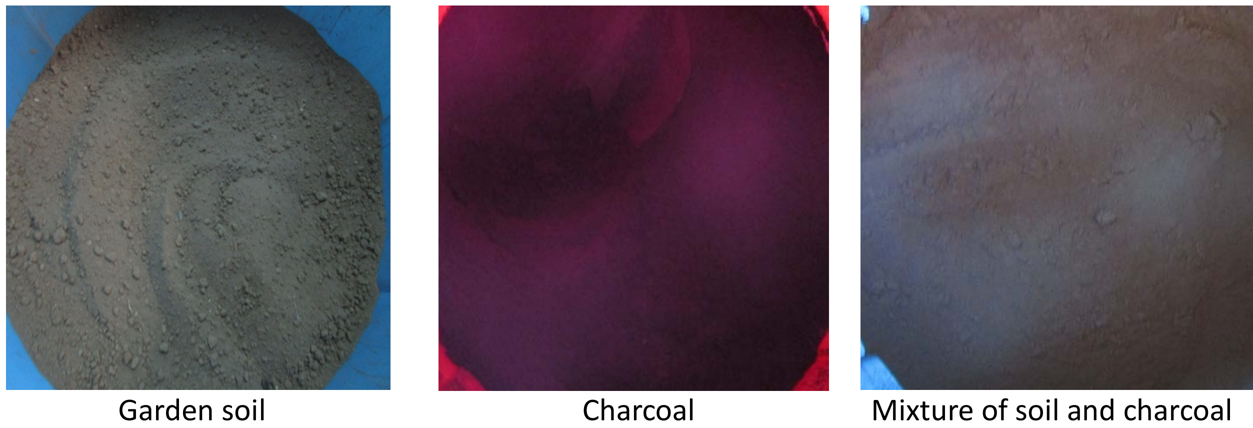
Table 18: Comparison of sterilization of carrier material using gamma irradiation and autoclave

Particulars	Heat autoclave carrier	Irradiated carrier
Efficiency use	1 t per week (5,000 packets)	1.7 t per week (10,303 packets)
Texture of the Carrier	Wet and laborious	Dry and ready to use
Expenses per packet	Php 1.03 ($0.02)	Php 1.30 ($0.03)
Lifespan	6 months	10 months

Figure 10: Carrier sterilized through steam sterilization (left) and gamma irradiation (right)

Comparison of the Survival of *Azospirillum* Population in Gamma Irradiated Carrier

The characteristic of the carrier is very essential to keep the microorganisms for longer life span and deliver to the end users with good quality. Carrier used in the Philippines for Bio N production is mixture of garden soil and charcoal (Figure 11). Bio N^{TM} microbial inoculant had sufficient numbers of viable nitrogen-fixing strains of at least 10^6 colony forming unit (cfu) using the conventional method of sterilization (steam sterilization) and had a life span of only six (6) months. Sterilization of carrier using the gamma irradiation technique was conducted. The radiation sterilization technique eliminates contaminating microorganisms and prolong the sterility of the carrier compared to steam sterilization technique. Gamma irradiation sterilization of Bio N carrier also enhanced the life span/storage of Bio N biofertilizer for up to 10 months as shown in the table 19.



Garden soil | Charcoal | Mixture of soil and charcoal

Figure 11: Carrier used in the Philippines for Bio N production

Table 19: Gamma irradiation sterilization and life span/storage of Bio N biofertilizer

Incubation Period	No. of colonies
1st month	**1 X10^8**
2nd month	**1x10^8**
3rd month	**1 X 10^8**
4th month	**1.38 X 10^8**
5th month	**1.50 X 10^8**
6th month	**5.65 X 10^7**
7th month	**5.55 X 10^7**
8th month	**4.5 X 10^7**
9th month	**1 X 10^6**
10th month	**1 X 10^6**

g) Thailand

To demonstrate the effect of γ-sterilization, the survival of the plant growth promoting bacteria (PGPR) biofertilizer, *Azospirillum brasilense* TS29, was monitored to assess the shelf life (Meunchang *et al*., 2015). As biofertilizer carriers, wood bark compost (Prongjunthuek *et al*., 2014) was used in this study. Following the sterilization of carrier materials by γ-irradiation (25 kGy) or autoclaving (121˚C for 60 min), *Azospirillum brasilense* TS29 was inoculated into each type of carriers. The biofertilizer was packed into polyethylene bags. In each bag contained carrier for 150 g and broth inoculant for 50 ml and stored at 25˚C or room temperature (30-35˚C) for 12 months. After storage, viable inoculants in the biofertilizer were counted.

Results indicated that inoculant density (cfu g^{-1}) after storage was greater than the initial density in

biofertilizers made from sterilized carriers, whereas inoculant density (cfu g^{-1}) decreased significantly in biofertilizers from non-sterilized carriers. γ-sterilization was superior to sterilization by autoclaving in enhancing inoculant survival. The γ-sterilized carrier and kept at temperature 25˚C seems best suited a biofertilizer carrier and kept condition for extend survival period of *Azospirillum* biofertilizer (Figure 12).

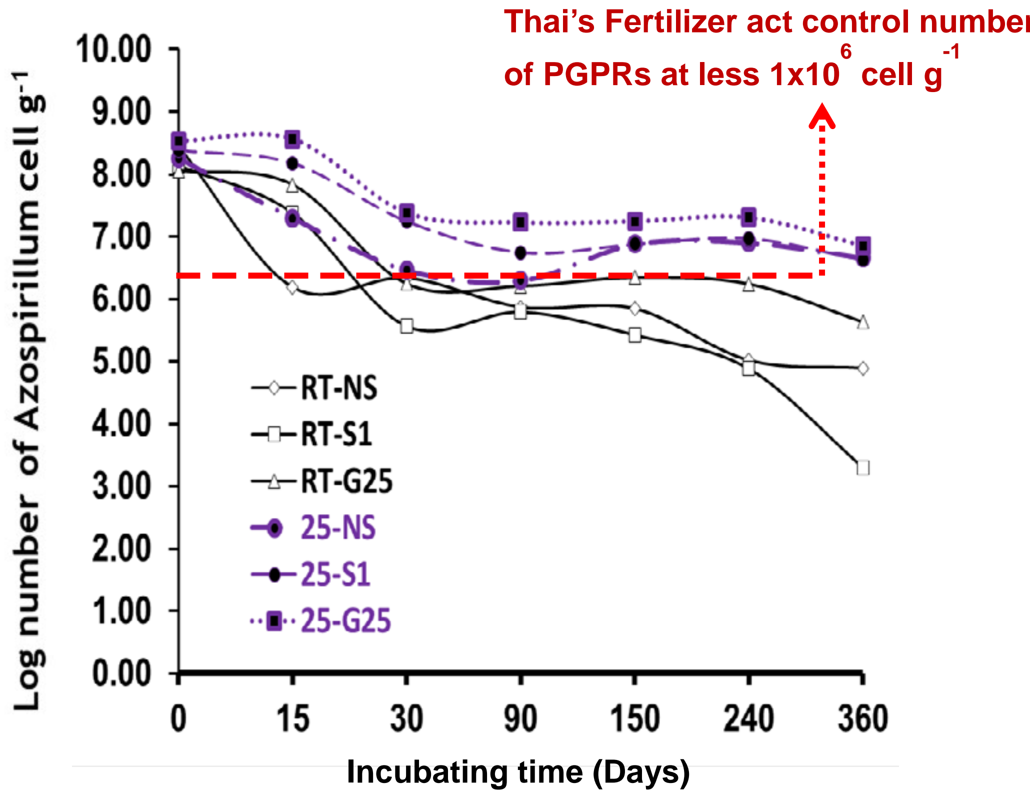


Figure 12: Survival of *Azospirillum* in gamma sterile carriers and effective incubation temperature on *Azospirillum* survival.

1.4 Commercial products of biofertilizer with gamma irradiated carrier

Country members of FNCA Biofertilizer Project have developed several commercial biofertilizer products using gamma irradiated carrier since 2001 until the present. Example of commercial biofertilizer products from Indonesia (Figure 13) and Malaysia (Figure 14) are as below:

a) Indonesia

Figure 13. Inoculant carriers for briofertilizer, Organic Fertilizer (Compost)
(Source: Nurrobofahmi, 2016)

b) Malaysia

Figure 14. AgriCare®ORGANIC-N

Malaysian Agri Hi-Tech Sdn.Bhd. (MYAGRI), the bioproducts company with BioNexus status, utilizing *Bacillus megatarium*, a biofertilizer inoculum generated through R&D of Nuclear Malaysia is one of its products, AgriCare®ORGANIC-N.

Part 2. Methods for selection and preparation of carrier

2.1 Toxicity test

a) Malaysia

1. Principle

The most important characteristic of a carrier is non-toxic. Carrier should be non-toxic to inoculums and planting materials. Some carrier such as peat may produce toxic compounds after heat sterilization. Therefore, toxicity test should be carried out before and after sterilization.

2. Equipment

Incubator

Laminar air flow cabinet / Biosafety cabinet

Petri dish

Pipette

Filter paper

Conical flask

Test tube

Forceps

Membrane filter (0.2 *u*m)

Shaker

3. Materials and chemicals

1) Carrier (before sterilization and after sterilization)
2) Planting material (such as seed)
3) Agar media (such as Nutrient agar)
4) Sodium hypochlorite 0.25%
5) 70% ethanol

4. Procedures

Toxicity test for seed

1) Mix 10 g carriers with 90 ml sterile distilled water in 250 ml conical flask. Shake mixture on shaker for 30 min.

2) Soak seeds in 0.25% sodium hypochlorite for 5 min and rinse with sterile distilled water. Sterilize suspension by using membrane filter. Soak seeds with filtered carrier suspension for 1 h. Seeds soaked in sterile distilled water were used as control treatment.
3) Place seeds on petri dish that in overlaid with moisture filter paper. Incubate seeds at room temperature. Record seeds germination rate daily.
4) Seeds germination rates should be more than 85%.

Toxicity test for inocula

1) Mix 10 g carriers with 90 ml sterile distilled water in 250 ml conical flask. Shake mixture on shaker for 30 min. Sterilize carrier suspensions by using membrane filter.
2) Mix inoculums with filtered carrier suspension in a ratio 1:1. Mixtures are used for counting of colonies. Inocula mixed with sterile distilled water were used as control treatment. Serial dilution and plate count are carried out (Appendix A).
3) Colonies counted should be more than 10^8 cfu g^{-1}.

b) Thailand

Phytotoxicity of microbes and carrier can be tested through germination index, a method to test for remaining phytotoxic substances in the carrier, e.g. ammonia gas and other forms of organic acids. Measurement unit is percentage (%).

1. Equipment and materials

Chinese cabbage seeds of a germination rate of not less than 75%.

Distilled water

A germination plate (9 cm in diameter)

Filter paper No. 42 (9 cm in diameter)

Carrier samples

2. Methodology

1) Extracting a carrier solution by mixing a carrier sample with distilled water at the ratio (carrier: distilled water) of 1:10. Shake the mixture at about 180 times per minutes for one hour before filtering it with filter paper.
2) Draw a 10-boxes table onto the filter paper.

3) Place a Chinese seed to each box on the filter paper, which is placed on a germination plate (10 seeds each), make at least 4 replications.
4) Drop 3 ml of carrier solution onto each germination plates.
5) Drop 3 ml of distilled water onto a control plate.
6) Keep the plates incubated in 2.4 and 2.5 in a dark room at the temperature of 28°C to 30°C for 48 hours.
7) Collect the following data:
   a) An average germination rate per plate (unit: %)
   b) Root lengths of all germinated seeds and average them.
8) Calculate the germination index of plant seeds by using the following formula

   Germination Index (%) =

$$\frac{\text{\% of germination in compost solution X root length in compost solution X 100}}{\text{\% of germination in distilled water X root length in distilled water}}$$

2.2 Determination of organic carbon (Walkley and Black Method) (Malaysia)

1. Principle

Determination of organic carbon in compost or soil should be carried out before using this material as carrier. This is due to some countries having minimum C:N ratio requirement for organic fertilizer. Walkley and Black chromic acid wet oxidation method is use to determination of organic carbon. This method measures the amount of carbon in plant and animal remains, including soil humus but not charcoal or coal.

2. Equipment

Hot plate
Fume cupboard
Pipette
Measuring cylinder (20 ml)
Conical flasks (250 ml)
Thermometer (200˚C)
Burette (50 ml)
Volumetric flask (100 and 1000 ml)

3. Materials and chemicals

1) 1 N Potassium dichromate. Dissolve 49.04g $K_2Cr_2O_7$ (dried at 105˚C) in sterile distilled water and transfer to a 1 L volumetric flask. Make volume by adding sterile distilled water.
2) Sulphuric acid H_2SO_4, concentrated
3) Phosphoric acid H_3PO_4, concentrated
4) 0.5N Ferrous sulphate. Dissolve 140 g $FeSO_4.7H_2O$ in 800 ml deionised water containing 40 ml concentrated H_2SO_4 and diluted to 1 L with sterile distilled water. Store in a dark bottle.
5) Diphenylamine indicator

4. Procedures

1) Mix 1 g sample with 10 ml 1 N $K_2Cr_2O_7$ and immediately add 20 ml H_2SO_4 in conical flask. Mix gently and stand the mixture for 30 minutes.
2) Dilute reaction mixtures with 200ml distilled water and add 10 ml H_3PO_4. Add 1 ml Diphenylamine indicator.
3) Titrate with standard 0.5 N $FeSO_4$ solutions to a blackish green colour. Run a blank simultaneously.

5. Calculation

$$N \text{ of } FeSO_4 = \frac{10 \text{ ml} \times 1 \text{ N}}{\text{Vol of FeSO4 used}}$$

$$\% \text{ Organic C} = \frac{(V_{blank} - V_{sample}) \times N_{FeSO4} \times 0.399}{\text{Weight of sample}}$$

% Organic matter = % Organic C x 1.72

2.3 Determination of Nitrogen (Kjeldahl Method) (Malaysia)

1. Principle

Determination of nitrogen is carried out if an organic carrier such as compost or soil is used. This is due to some country has minimum C:N ratio requirement for organic fertilizer. The Kjeldahl method is based on the principle that the organic matter is oxidised by treating the samples with concentrated sulphuric acid. Nitrogen in the organic nitrogenous compounds is converted into

ammonium sulphate $(NH_4)_2SO_4$ during oxidation. NH_4^+ ions in the samples are trapped by the acid that is liberated by distilling with NaOH. The liberated NH_4^+ is absorbed in boric acid and back titrated with standard H_2SO_4. Boiling point is raised by adding potassium sulphate during digestion. Catalyst such as copper sulphate and selenium powder mixture is added.

2. Equipment

Digestion block

Distillation unit

Burette

Volumetric pipette (10 ml)

Erlenmeyer flasks (250 ml)

Magnetic stirrers

Kjeldahl digestion tubes (200 ml)

3. Materials and chemicals

1) Concentrated sulphuric acid H_2SO_4
2) Catalyst mixture. Mix by grinding in a mortar 100 parts Na_2SO_4 or K_2SO_4 with 10 parts of Copper Sulphate ($CuSO_4.H_2O$) and 1 part selenium powder; mix thoroughly.
3) Sodium hydroxide NaOH, 40%. Dissolve 400 g NaOH in 800 ml distilled water. Cool the solution then add distilled water to 1 L volume.
4) Mix indicator. Mix 0.5 g bromocresol green and 0.1 g methyl red. Dissolve in 100 ml 95% ethanol.
5) Boric acid solution, 2%. Dissolve 20 g boric acid in 600 ml freshly boiled distilled water. Add 10 ml mix indicator. Make volume to 1 L.
6) Sulphuric acid solution, 0.1N. Pipette 2.82 ml concentrated H_2SO_4 and make to 1 L volume.
7) Oxalic acid, 0.1 N. Dissolve 6.3035 g Oxalic acid in distilled water and make to 1 L volume.
8) Phenolphtalein indicator, 0.1%. Dissolve 100 mg phenolphthalein in 100 ml 95% ethanol.
9) Sodium hydroxide, 0.1 N. Dissolve 4 g NaOH distilled water. Cool and make volume to 1 L.

4. Procedures

1) Weigh 1 g sample and transfer into digestion tube. Add 2 g of catalyst mixture.
2) Add 7 ml of concentrated H_2SO_4 and mix by swirling.
3) Place the digestion tube stand with the samples beside the block digester and fit the exhaust manifold on top of it.
4) Place the tubes with rack and exhaust manifold on the preheated digestion block in the fume-hood.
5) Digest for 3 h or until the digest is clear.
6) Allow to cool, and cautiously add 50 ml of distilled water and allow to cool.
7) Measure 10 ml boric acid into Erlenmeyer flask corresponding to the number of samples. Add 2 drops indicator solution. Place the flask under condenser of the steam-distillation unit.
8) Start distillation process. Add 3 stokes of NaOH (40%), wait 30 s and 2.5 min steam distillation.

Titrate the flask solution from green over colourless to a pink end point with 0.1 N H_2SO_4. Record the amount of H_2SO_4 used.

5. Calculation and data sheet

$[mg]N_{sample} = (A-B)\ N_{sample}*t_{HCl}*C_{HCl}*14$

$[mg]\ N_{blank} = (A-B)\ N_{blank}*t_{HCl}*C_{HCl}*14$

$\%N = 100*([mg]\ N_{sample} - [mg]\ N_{blank}) / [mg]\ W$

Sample			W	A	t_{HCl}	B	(A-B)	N_{sample}	N_{blank}	% N in oven dry sample
Code	Repl.	Weight, air dry (mg)	Weight, Oven dry (mg)	[ml] HCl C_{HCl} = 0.1 N	Titer HCl	[ml] NaOH C_{NaOH} = o.1 N	[ml] HCl consumed	[mg] N in sampl e	mg] N in blank	

2.4 Determination of moisture content (Malaysia)

1. Principle

Moisture content is an important factor for long shelf life of a biofertilizer. High moisture content enhances fungus growth. On the other hand, low moisture content decreases growth of biofertilizer. Sterilization method may change moisture content of carrier. Carrier sterilization using heat may increase moisture content. Moisture content for peat is 45%. Optimisation of moisture content of a carrier should be done before using a carrier.

2. Equipment

Analytical balance

Oven

Moisture dish

Desiccator

3. Procedures

1) Weigh moisture dish and record.
2) Weigh 10 g of carriers and put in moisture dish. Dry in oven at 105˚C until stable weight. (Normally 5 to 24 h drying period)
3) Cool samples in desiccators and weight. Weight loss is use as moisture content calculation.

4. Calculation

$$\% \text{ Moisture} = \frac{(\text{Wet weight} - \text{Dry weight}) \times 100}{\text{Dry weight}}$$

Note: Moisture content on the original sample should be determined. Determine sample before and after sterilization.

2.5 Determination of pH (Malaysia)

1. Principle

Different carriers have different pH. Biofertilizer needs optimum pH for high viability of cells. The carrier is acidic if pH is less than neutral pH 7. Otherwise it is alkaline if more than neutral pH 7. Most bacteria prefer neutral pH in the range of 6.5 to 7.0. Moulds and yeast prefer a slightly narrow range of pH 5 to 6. Peat carrier's pH is 6.5 to 7.5. Adjusted pH is done by adding $CaCO_3$ or

$Ca(OH)_2$.

2. Equipment

pH meter or pH paper

3. Procedures

1) Weigh 10 g carriers. Mix with 90 ml sterile distilled water. Shake for 30 min.
2) Measure blank (pH 4,7and 10). Measure mixture pH. Repeat 3 times.
3) Measure carrier before and after sterilization.
4) Adjust pH by adding Ca

Note: If no pH meter is not available pH paper can be use as alternative. Compare the change of colour to check pH.

Part 3. Methods for sterilization of carrier using gamma irradiation

3.1 Procedure to determine optimal dose of gamma irradiation (Malaysia)

1. Principle

Gamma irradiation is widely used for sterilization of pharmaceutical products, medical products, food and herbs. Gamma irradiation causes direct breakdown of double strand DNA, or disrupts biological system in the cell by producing free radicals formed through ionized water molecules. Spore forming microorganisms tolerant to heat sterilization maybe killed by gamma irradiation. Various dose rates have been used for carrier sterilization. Different types of carriers or packaging sizes require different dose rates. Although a dose of more than 50 kGy is used for efficient sterilization of carrier, lower doses are required for reducing energy and time of sterilization. On top of that, lower dose also reduce the toxic substances that may be produced when using high dose of gamma irradiation. Smaller size of packaging may reduce the dose rate and also cost of sterilization. Light and small size carriers such as vermiculite and rice bran have been used as carriers by biofertilizer companies. Determination of suitable dose and dose rate, type of carriers and packaging sizes are the key of success to produce good quality carriers and long shelf life of biofertilizer products.

2. Equipment

Gamma irradiation facility

3. Procedures

1) Add water to carrier to adjust moisture content.
2) Measure and adjust pH of carrier.
3) Weigh carrier and pack into polyethylene (PE) or polypropylene (PP) bags with a thickness of 0.88 mm. Seal the bags by using sealer. Keep in a box.
4) Irradiate carrier with a range of doses (e.g. 10, 20, 30, 40 and 50 kGy).
5) Carry out serial dilution and plate count (4.2) to check sterility. Population should be less than 10^2 cfu g^{-1} or no microbe.
6) Plot graph of log cfu g^{-1} over dose rates.

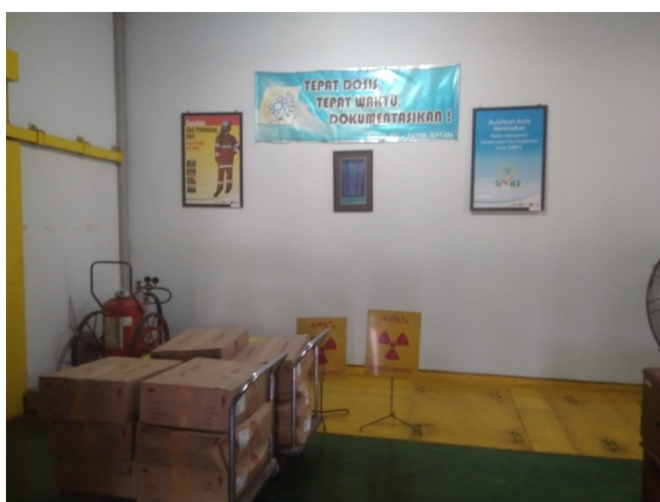
3.2 Flow chart of carrier sterilization using irradiation

a) Indonesia

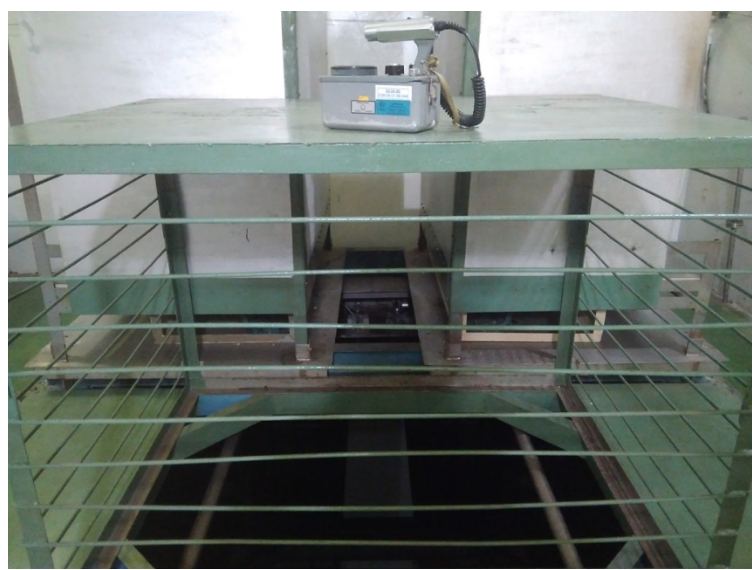
<u>Flow chart for gamma irradiation at BATAN, Indonesia</u>
(Iswandi Anas and Nurrobifahmi)

Control panel of gamma irradiation

Sample to be sterilized by gamma irradiation



Samples ready to be irradiated



Pool for ^{60}Co source storage

batan	BADAN TENAGA NUKLIR NASIONAL PUSAT APLIKASI ISOTOP DAN RADIASI	Nomor	: SOP 002.002/IN 00 06/AIR 8
	LAYANAN IRADIASI	No. Revisi/ Terbitan	: 0 / 1
		Tgl. Berlaku	: 15 Oktober 2015
		Halaman	: 1 dari 10

Kepada : Yth. Ka. Balai Iradiasi, Elektromekanik, dan Instrumentasi
Perihal : Layanan Iradiasi

Dengan hormat,

Mohon layanan iradiasi pada fasilitas : IRKA/ IRPASENA/ Gamma Chamber 4000A/ Gamma Cell 220-Upgraded/MBE GJ-2/ Mesin Ultra Violet (UV)*

Hari/tanggal : ………………………………………………………

Nama Pelanggan : ………………………………………………………

Penanggung jawab : ………………………………………………………

Bidang/ Instansi/ Perguruan Tinggi : ………………………………………………………

Bahan/sample : ………………………………………………………

Bentuk : cair/ padat/ lainnya*: ………………………………….

Sifat : berbahaya/ beracun/ lainnya*: ………………………….

Kemasan / ukuran / jumlah : ………………/…………………/…………………

Dosis radiasi : ………………………………… kGy / Gy*

Laju dosis radiasi (khusus G. Cell) : 7,90/ 4,03/ 2,92/ 0,93* kGy/jam

Tujuan iradiasi : ………………………………………………………

Atas layanan yang diberikan, kami ucapkan terima kasih.

Mengetahui, Jakarta, ……………………2016

Kepala Bidang …………………….. Pemesan,

……………………………………… ……………………………………

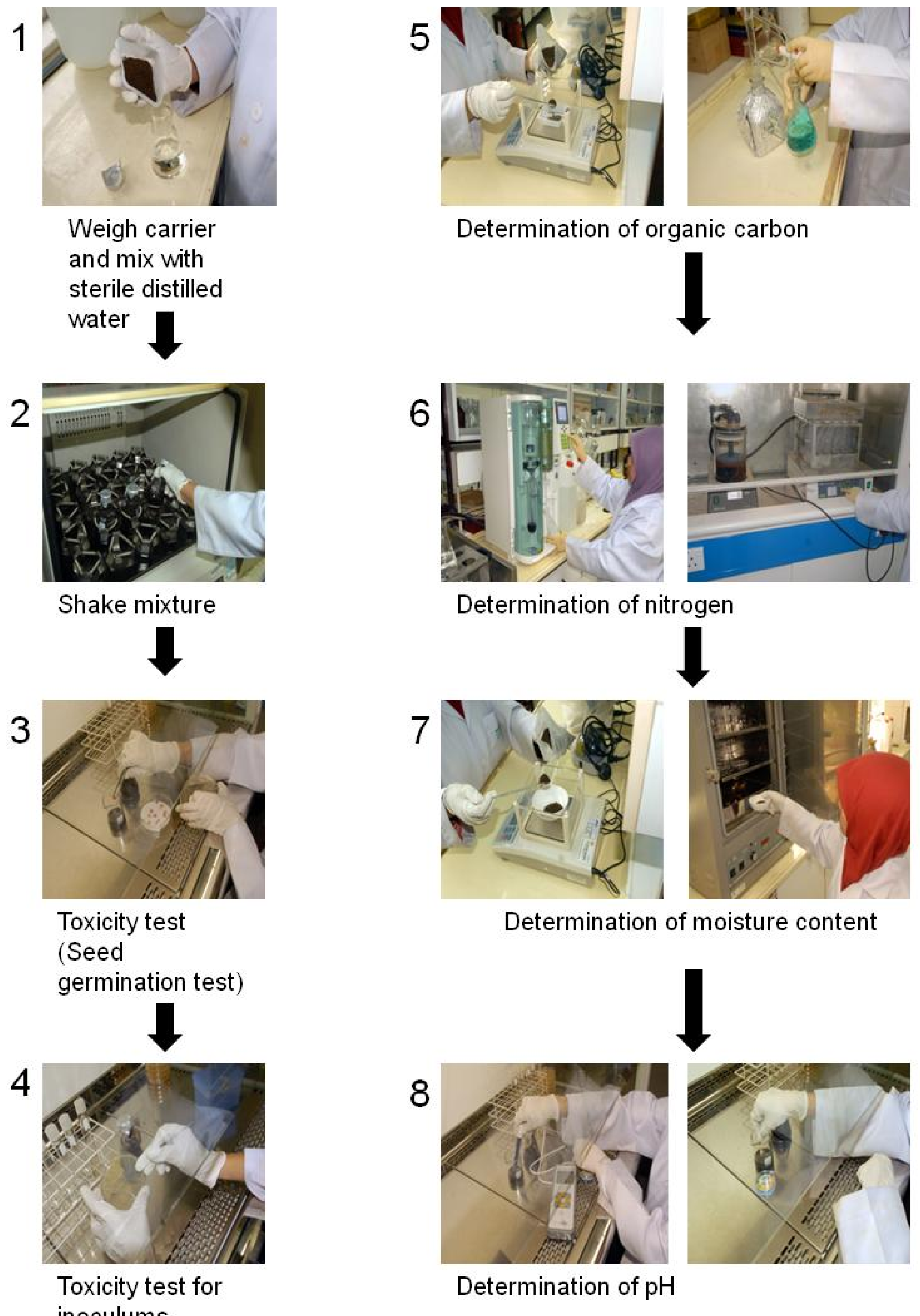
Ka. Balai IEI :	Operator Sampel diterima tgl.: Nama: Tanda Tangan: Pelaksanaan iradiasi:
Ka. Pok. Layanan Iradiasi :	Pelanggan Sampel diambil tgl.: Nama: Tanda Tangan:

Keterangan *: Coret yang tidak perlu

Form for irradiation service

b) Malaysia

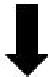
<u>**Laboratory test for carrier**</u>



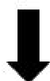
Gamma sterilisation for carrier

1

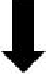
Weigh, pack and seal the carrier



Pack carrier in box and sent to gamma irradiation facility for sterilisation process



Receive, register, weight and label the carrier

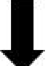


Preparation of dosimeter in QC laboratory

Gamma sterilisation for carrier

5

Put dosimeter and load carrier for sterilisation



Loading carrier into irradiation container

Gamma irradiation at specified doses. Load sterilised carrier

8

Sterilised carrier

3.3 Example of gamma irradiation facility

a) Bangladesh

Cobalt-60 Gamma Source Facility

of

Institute of Food and Radiation Biology (IFRB),

Atomic Energy Research Establishment (AERE),

Bangladesh Atomic Energy Commission, Bangladesh

A cobalt-60 (^{60}Co) batch type gamma irradiator was installed at Gamma Source Division of Institute of Food and Radiation Biology by Board of Radiation and Isotope Technology (BRIT), India in June, 2000. Initial load activity of the gamma source was 50.0 kCi and when it was decreased below the working level, the strength was replenished by BRIT to 65.0 kCi in 2009 and 95.0 kCi in 2014.

This gamma source is housed in a 260 mm thick lead shielded flask consisting of a biological shield housing, source cage and source pencils. A total of eight source pencils are contained in individual holders of the source cage. Each pencil is doubly encapsulated in stainless steel tube with outer diameter of 14 mm and length of 450 mm. To maintain high level of safety, this source flask is placed within reinforced concrete walls of 2.0 m thickness, and it is raised from 1.0 m depth of lead shielded flask while irradiation starts. Control devices operate to ensure that the source is raised only when the chamber is locked and it is immediately lowered if any malfunction occurs. Radiation monitoring is continuously employed to detect any radiation leakage during operation or source storage.

Materials to be irradiated are placed or loaded on irradiation platform when the source is lowered and moved around the raised source during irradiation. Irradiation dose uniformity is maintained by auto or manual shuffling. The recommended or required irradiation dose is calculated from exposure time and distance of the materials from the source. Different kinds of dosimetry (e.g. Fricke dosimeter for low dose level and Amber perspex or Ceric-Cerous dosimeter for high dose level) are applied to ensure the absorbed doses recommended for particular samples or products. Gamma Source Division is rendering R&D services and limited scale of commercial services to different research organizations and private companies.

Control panel of ^{60}Co gamma source

Product loaded at 8-tote box position

^{60}Co gamma source flask

b) Malaysia

Malaysian Nuclear Agency began developing radiation processing of various products in 1989 at the MINTec-Sinagama Irradiation Plant. Currently known as Sinagama, the plant is a certified MS ISO 9001:2008 and ISO 13485:2003 facility and registered under USFDA's Current Good Manufacturing Practice (GMP). The plant has been a tax-exempted area since 1999. A new plant, JS10000 (IR-219) has been installed and commissioned on 1 April 2004 for further improvement in which it is able to simultaneously irradiate various products requiring different doses.

Initially designed as a multi-purpose pilot facility for research and development purposes, the plant activities later diversified to offer services to the public for the sterilization of medical products and packaging materials, decontamination of food, pharmaceuticals, herbs and animal feeds, and the disinfestations of insects in agricultural commodities, including for quarantine purposes. The plant also provides tissue and bone sterilization services by gamma irradiation for tissue banking purposes to relevant authorities such as hospitals and National Tissue Bank.

As part a research institute, Sinagama is often approached for technical advice on the use of radiation technology from various interested parties with potential application of irradiation for their products. Apart from that, the plant is assisting research institute in sterilization of agrowaste for further alternative utilization such as biofertilizer or as a substrate for growing mushroom and subsequently converted into nutrient-rich animal feed. Furthermore, the plant has an independent support arm, the Research Loop, which allows irradiation of sample to be carried out without interfering with the regular commercial irradiation service of the main plant.

The customer-friendly dealings with the nation's small and medium industries help to promote further understanding of the technology and its possible application to this sector. As a result, Sinagama has enjoyed invaluable support from the local business community and also from neighboring countries like Singapore. Products treated at the plant have been traded both domestically and for overseas market. This has helped Sinagama to gain recognition as a premier service provider in irradiation and make its contribution to the country's economy

MINTec-Sinagama, Malaysian Nuclear Agency

RSP-1
Issue#: 4
Date: 15 Nov 2011

MINTec-Sinagama
MALAYSIAN NUCLEAR AGENCY
(NUCLEAR MALAYSIA)

CUSTOMER ORDER FORM FOR GAMMA IRRADIATION SERVICES

To:
Plant Manager,
MINTec-Sinagama,
Malaysian Nuclear Agency (Nuclear Malaysia),
Kompleks Jalan Dengkil,
Bangi, 43000 KAJANG, Selangor.

Dear Sir,
Please irradiate the following products with gamma radiation with a **minimum dose** of _______ kGy and **maximum dose** of __________ kGy.
OR
In accordance with **Product Specification No:**________________

The purpose of the irradiation is **for sterilization/pathogenic control/shelf life extension/phytosanitary treatment/ R& D (cancel where not applicable)**

Is this a first-time shipment for this product? Yes ☐ No ☐

No.	Product Code/Description	Manufacturing Batch/Lot	No. of Product Unit	Product Unit Weight (kg)	Product Unit Size Length X Width X Height (cm)

Do you need your product to be **plastic wrapped** after irradiation treatment **(RM 8/pallet)**?
Yes ☐ No ☐

Any special instruction? If yes, please specify:

____

____

Signature: ________________ Name: __________________________
Date: ______________

Company's Name/Address/Contact No: ___________________________________

____

____

____

For Official Use Only

Medical Product		Food & Herbs		Sample	

Dose Mapping Yes ☐ No ☐

Verified By: ______________________

RSP-15

AGENSI NUKLEAR MALAYSIA
(NUKLEAR MALAYSIA)
SINAGAMA
Bangi, 43000 Kajang,
Selangor Darul Ehsan.
Tel : 89112000 Fax : 89201099

SHIPPING ORDER

Date : ______________

Deliver to :			
Customer's Order No.			
Irradiation Lot No.			
Item No.	Product Code	Description of Product	Qty. Delivered
		Total	

Remarks:

Receives in good condition the product/ good mentioned above.	**Authorized signature**
NAME : **I/C NO. :** **VEHICLE NO. :** **SIGNATURE :**	(______________) **for Director General** **NUKLEAR MALAYSIA**

P.K.N., K.L

MINTec-SINAGAMA
AGENSI NUKLEAR MALAYSIA
BANGI, 43000 KAJANG, SELANGOR DARUL EHSAN.
Tel: (03) 89202097 : Fax. No: (03) 89201099 : e-mail:sgama@nuclearmalaysia.gov.my

Ruj. Tuan **:**
Your Ref. **:**

Ruj. Kami **:** **QC/KP/09 ( )**
Our Ref. **:**

KEPUTUSAN ANALISIS PENYINARAN
Results of Irradiation Analysis

Nama Dan Alamat Pelanggan
Customer Name and Address

Keterangan Bahan **:**
Product Description

Ukuran Bungkusan/Kuantiti **:**
Product Dimension/Quantity

Dose Permohonan **:**
Requested Irradiation Dose

Tarikh Penyinaran **:**
Irradiation Date

Keputusan **:**
Results

Catatan **:**
Remarks

Disahkan :- **Tarikh :**
-
(*Approved by* **)** *Date*

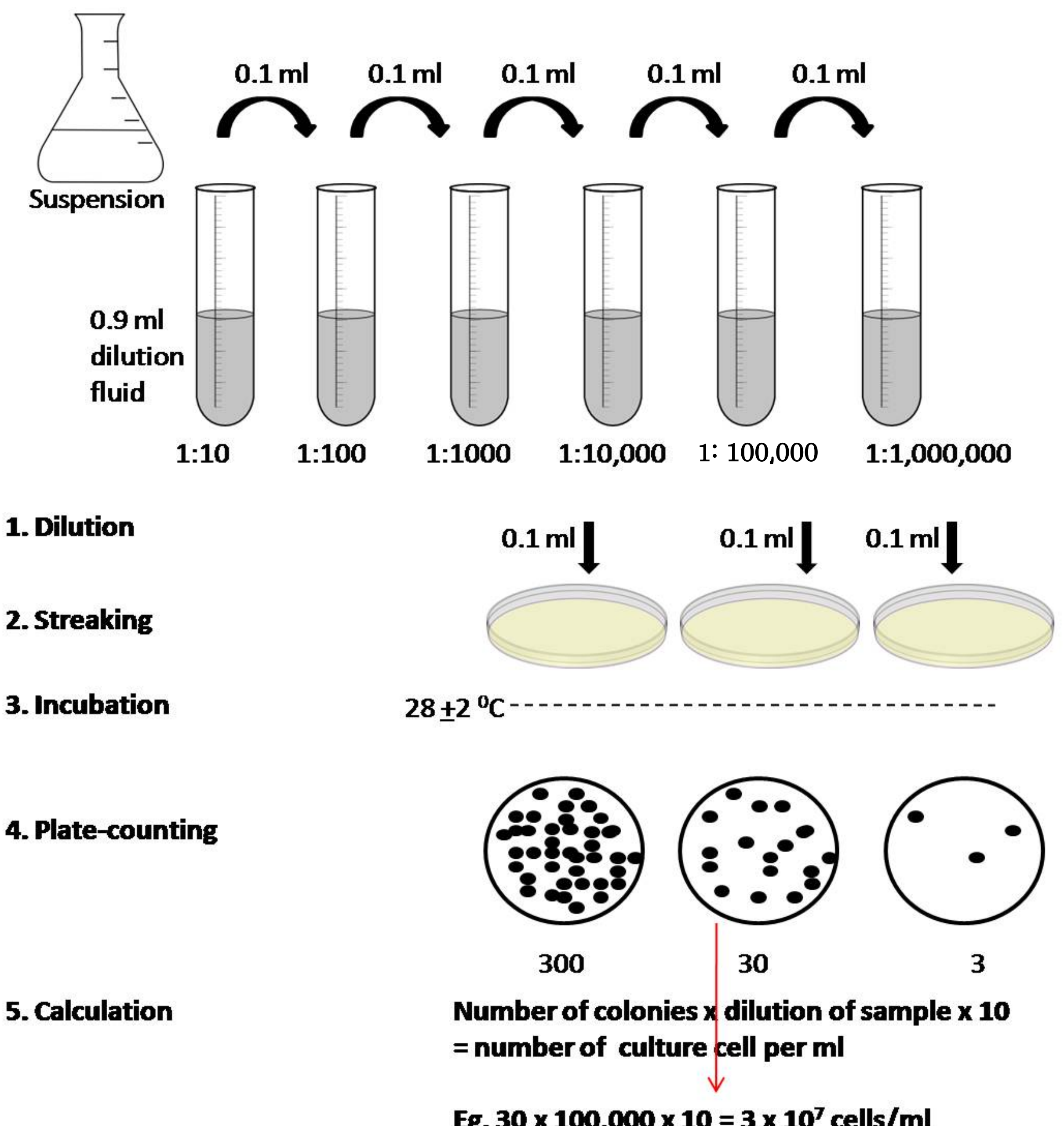
..

Part 4. Methods for inoculation of biofertilizer

4.1 Inoculation to sterilized carrier (Malaysia)

The gamma sterilized carriers, already packed in sealed polyethylene bags of specified size and weight are taken out of the box (Step 8 in the flow diagram on Gamma Sterilization of Carrier) in preparation for inoculation process. Each bag is placed in a laminar flow cabinet. A predetermined volume of biofertilizer inoculum (e.g. at population density of 1 x 10^{9}cfu ml^{-1}) were introduced aseptically into the carrier using a sterile syringe. The syringe punctured bag is then sealed at the puncture using a sticky tape. The bag is kneaded slowly to allow an even spread of the inoculant in the carrier and left overnight before repacking. The process is repeated for other sterilized bags of carriers.

4.2 Serial dilution diagram (Malaysia)



(Source: Ho, 2000)

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