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Organic fertilizer amended with immobilized bacterial cells for extended shelf-life

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ABSTRACT

One of the major constrains in the development of microbially-amended agricultural products is the incapability of upholding high microbial count in the commercial products and field soil. The efficiency of immobilization technique to retain high microbial population and extend the shelf-life in bio-organic fertilizer was corroborated in this study. Four Gram negative bacterial strains with beneficial traits such as phosphorus solubilization, indole acetic acid production, siderophore production and nitrogen fixation were immobilized in a modified alginate solution and incorporated with compost, organic fertilizer and mineral fertilizer. The bacterial count in compost and organic fertilizer (5:5:5) were 10^7 cfu/g and 10^6 cfu/g respectively after one year of shelf-life. Mineral fertilizer (15:15:15) and immobilized bacteria that were mixed in 1:50 ratio exhibited 10^5 cfu/g after 210 days. Pelleting was found to be more suitable for liquid bacterial cells + organic fertilizer mixture than immobilized bacterial cells + organic fertilizer mixture. Sterilization technique by autoclaving was proven to be more destructive in eliminating indigenous microbes and effective in retaining nutrient content and immobilized bacterial cells as compared to gamma irradiation technique. One part of immobilized bacterial cells was mixed to fifty parts of organic fertilizer to sustain at least 10^6 cfu/g for 348 days. Field study results exhibited the efficiency of immobilized bio-organic fertilizer in increasing cabbage head weight as compared to organic fertilizer. This is also a pioneer report on the shelf-life of immobilized bacterial cells incorporated with organic and mineral fertilizers.

1. Introduction

Bio-fertilizer is a substance that contains living or dormant microbial cells that promotes growth of the plant by increasing the availability of plant nutrients in the rhizosphere via various biological processes. The demand for bio-fertilizer is thriving due to its wide advantages to both human and environment. There is always a great support for bio-fertilizer products especially among a concern community on human health and environment. Bio-fertilizers may enhance nutrient availability to plants and increase crop yield (Minaxi, 2011; Pereg and McMillan, 2015). Microbial cell were developed to improve plant growth, suppress plant diseases and reduce reliance on chemical fertilizers and pesticides as well (Pereg and McMillan, 2015; Vassilev et al., 2015). The beneficial microorganisms facilitate dissolution of bound or unavailable nutrients in the organic fertilizers, compost or soil and make them accessible for plant absorption. Beneficial microorganisms also produce plant hormones that can enhance root growth and control the spread of pathogens through the production of organic acids, antibiotics, enzymes etc. In general, beneficial microorganisms have the ability to make insoluble nutrients available for plant uptake. Isolation and specific screening of microorganisms for beneficial traits is very crucial in the development of bio-fertilizer. It is well-known that total phosphorus in soils are not accessible to plants as most of the P fertilizers are fixed either in the form of iron or aluminium phosphate in acidic soils or in the form of calcium phosphate in neutral to alkaline soils (Sharma et al., 2013). Thus, microbes play an important role in mediating P availability to plants by directly solubilizing and mineralizing inorganic P (Mundra et al., 2011; Pereg and McMillan, 2015). On the other hand, siderophores have high affinity to chelate and solubilize iron from minerals or organic compounds (Rashid, 2015). It improves nutrition or inhibits the establishment of fungal pathogens through the sequestration of Fe from the environment (Souza et al., 2015). Indole acetic acid is also known for improving plant growth and root development (Arruda et al., 2013) and exhibiting antifungal activity (Jha et al., 2009). Meanwhile, atmospheric nitrogen is converted into ammonia, a plant-utilizable form by nitrogen fixing

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microorganisms via a complex nitrogenase system (Ahemad and Kibret, 2014).

The major hindrances in the production of bio-fertilizers are low microbial density and short shelf-life of microorganisms in the product. Thus, it is essential to develop long-lasting bio-fertilizers that can gain public confidence based on its efficiency. Most of the bio-fertilizer products in the market were produced in liquid form. Bacterial survival rates in liquid formulations decreases over time because this technique does not provide a protective environment for microorganisms (Schoebitz et al., 2013; Reetha et al., 2014). It has been a great challenge for the bio-fertilizer industries to sustain the efficiency and retain microbial concentration in the product. The microbial population declines over time as the nutrients deplete in the medium and may result in poor survival in soil (Bashan et al., 2014; Ivanova et al., 2006). This phenomenon is very common especially in bio-liquid cultures. Low microbial population in bio-fertilizers leads to abatement and inconsistency in the efficiency of the bio-fertilizer product after field applications. Microbially-amended fertilizers have not been widely accepted by farmers due to the failure of reproducing the beneficial effects (Vassilev et al., 2015). The failure of bio-fertilizer products to exert their beneficial effect reflects problems related to formulations and productions.

Formulations of bio-fertilizer are highly dependent on the carrier materials. Solid form of bio-fertilizers could be produced using various carriers and formulations such as peat, clay, talc, vermiculite, perlite, polyacrylamide, carrageenan, alginate, alginate-starch, alginate-humic acid and powder formulations (Schoebitz et al., 2013; Vassilev et al., 2015). Research on carriers to improve the efficiency of bio-fertilizer has been done since 1980's. Among all the tested carriers, alginate is considered as one of the efficient carrier of microorganisms through immobilization technique (Ivanova et al., 2006; Schoebitz et al., 2013). Immobilization involves entrapment of microbial cells in alginates that can be released slowly and degraded completely over time. Alginate also acts as a protective layer of microbial cells from abiotic stresses. Microbial survival can be improved by immobilization in alginate polymers compared to conventional liquid bacterial cells which do not provide adequate protection for microorganisms.

Soil is an unpredicted and heterogeneous environment where the inoculated microorganisms often fails to establish a niche due to plant-microbe interaction, inoculum density, soil type, competitors, predators etc. (Young et al., 2006). The initial microbial population in bio-fertilizer products needs to be copious to retain high microbial count throughout several stages such as manufacturing process, transportation, storing period and soil application. Slow and continuous release of the immobilized microorganisms shall maintain a high density of the microbial cells at the target site of application in rhizosphere. Furthermore, immobilization is a suitable technique to extend shelf-life, which may be challenging, particularly for Gram negative bacteria that do not form spores (Pereg and McMillan, 2015).

This technique remains at infant stage in the production of bio-fertilizers although it has recorded a great achievement in food and pharmaceutical industries. The number of research conducted to study the feasibility of immobilization technique to produce bio-fertilizers is scanty. According to Vassilev et al. (2015) very little research attention is paid to develop formulation procedures which affect the efficacy of bio-fertilizer products. There is still lack of innovative processes for the delivery of microbial cells for soil fertilization purposes. Formulation should include suitable carrier and additive that will aid in the stabilization and protection of the microbial cells during transportation, storage and delivery to the target zone (Young et al., 2006). Therefore, an effort was taken to study the efficiency of using alginate-immobilization technique to produce bio-organic fertilizer with high microbial population and extended shelf-life of bacterial cells.

Organic fertilizer (OF) could be enriched with either one or a combination of beneficial bacteria such as phosphorus solubilizing bacteria, potassium and silicate solubilizing bacteria, nitrogen fixing bacteria, phytohormone producing bacteria, chitinase producing bacteria, siderophore producing bacteria etc. There is no standard definition for the combination of organic fertilizer and bio-fertilizer which is formulated in a carrier. The mixture of bio-fertilizer with organic fertilizer can be termed as bio-organic fertilizer. In this study, the term IB refers to the beneficial bacteria that are prepared in alginate carriers as immobilized bio-fertilizer whereas the mixture of immobilized bio-fertilizer in organic fertilizer designated as IB + OF.

Table 1
Beneficial traits of bacteria.

Bacterial code	Superior Beneficial traits
P2-2	Phosphorus solubilization
CB36	Indole acetic production
P1-8	Siderohore production
8G	Nitrogen fixation

2. Materials and methods

2.1. Identification of beneficial bacteria

The bacteria were isolated from Malaysian soil and screened for beneficial traits such as phosphorus and potassium solubilization, phytohormone production (indole acetic acetic), siderophore production and nitrogen fixation using agar plate assays as listed in Table 1 (Stella and Sashikala, 2016). The genomic DNA of bacteria was extracted using DNA, RNA and protein purification kit (NucleoSpin, Macherey-nagel). Universal primers 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′ TAC GGT TAC CTT GTT ACG ACT T-3′) were used to amplify 16 S rDNA genes of bacteria using polymerase chain reaction (PCR). The 50 µl of reaction mixture consisted of 50 ng of genomic DNA, 1 µM of each primer, 1X REDiant PCR Master Mix and nuclease free water. The following thermal conditions were used. Initial denaturation of 95 °C for 3 min, followed by 35 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 2 min and a final extension step at 72 °C for 3 min. PCR products were purified using Gel and PCR Cleanup kit (NucleoSpin, Macherey-nagel) and resolved on a 1% (w/v) agarose gel mixed with DNA stain (Midori Green Adance, Europe) at 70 V for 60 min in 1X TAE buffer. The size of the PCR products was about 1500 bp. Sequencing of 16 S rDNA was carried out by Apical Scientific Sdn. Bhd. (Malaysia). The sequenced data was compared with available gene sequences of bacteria in the National Centre for Biotechnology Information GenBank using BLAST Search. The sequences were aligned using MEGA X software and phlylogenetic trees were constructed by maximum likelihood method.

These four strains of bacteria were tested for antagonistic activity and found to be compatible to exist in a consortium. The bacterial cells were prepared in the form of liquid culture and immobilized cells prior to combining in organic fertilizer. Both liquid culture and immobilized cells were prepared as single culture prior to form a consortium namely, liquid bacterial consortium and immobilized bacterial consortium.

2.2. Immobilization of bacteria

Sodium alginate (2%) and calcium chloride 0.1 M were autoclaved separately. Bacterial culture was grown overnight. One plate full of pure colony was transferred into 200 ml tryptic soy broth and incubated for two days. Later the cells were harvested by centrifugation at 9000 rpm for 5 min and cell sediments were washed with sterile water. Then, collected cell sediments were diluted to 1: 10 using sterile distilled water prior to adding skim milk (10 % w/v), glucose (10 % w/v) and glycerol (30 % w/v). Later an equal amount of 2 % of pre-sterilized sodium alginate was mixed to the bacterial solution. The experiment was done on a work bench cleaned with 90 % ethanol. The bacterial

cells-alginate mixture was stirred continuously on a magnetic stirrer throughout the experiment. The mixture was infused into a 60-ml syringe with the aid of a syringe pump and extruded drops through a needle (21 $^{G\ x1\ ½"}$) at the rate of 35 ml/min into sterile $CaCl_2$ 0.1 M. The droplets that formed a bead-like structure in $CaCl_2$ solution was left for 30 min prior to washing thrice in sterile distilled water.

2.3. Drying of immobilized bacterial cells

The immobilized bacterial cells were then collected using a sieve and dried (20-30% moisture content) on sterile filter paper at room temperature for 24 – 48 h. The moist filter papers were replaced with new filter papers for efficient drying when necessary. The moisture content of immobilized cells was 10-20%. The dried immobilized cells were used for the production of IB + OF. An equal weight of the immobilized cells of each bacteria was mixed together to be combined with pre-sterilized organic fertilizer.

2.4. Organic fertilizer production

The main component of the organic fertilizer was chicken manure (N: 2.4%, P: 1.8%) used at the rate of 60%. Rice husk biochar (RHC) was added into the substrates at 35% (by volume) and co-composted with the help of effective microbes (5%) EM.1® (Source: Pertubuhan Peladang Negeri Johor containing fermentation microbes).The composting process was carried out for approximately 60 days. Composting procedure as well as the determination of compost maturity was conducted according to Aini et al. (2005). After the decomposition period, the finished compost was enriched with other nutrient substrates such as fish meal (fresh weight: 15%) (N: 8%, P: 4%), Oil Palm Bunch Ash (fresh weight: 6%) (K: 28%) and Christmas Island Rock Phosphate (fresh weight: 20%) (P: 60%) and let to stabilize for 2 weeks prior to application in the field.

The prepared organic fertilizer substrates were mixed thoroughly using fertilizer mixing machine before pelleting. The moisture content of final organic fertilizer at < 10% was just sufficient to help the pelleting process. As to increase the strength of pellet and to enhance the pellet formation, bentonite clay was added at the rate of 2% in the organic fertilizer mixture. In later stage, organic fertilizer mixture was compressed using flat die pellet mill (ZLSP-D 150 B), electric driven (5.5 KW) with production capacity of 50 kg/h using D-type (Die-Turning-Type). The operating temperature in the pelleting machine is around 75 C and compressed to a cylindrical shape. The average pellet size achieved from the process was (0.5 x 2) cm, dried using blow drier, packed and kept at room temperature prior to application in the field for experiment.

2.5. Bio-organic fertilizer production

Organic fertilizer was sterilized via autoclaving twice at 121 °C for 30 min at an interval of 2 days prior to mixing with immobilized bacterial cells and liquid bacterial consortium in a mixer to ensure a homogenous distribution of the bacterial cells in organic fertilizer. The preparation of immobilized bacterial cell was described in section 2.2 and 2.3. Liquid culture was prepared by culturing one plateful bacteria into 1 L of Tryptic Soy Broth. The liquid culture was placed in a shaking incubator with 150 rpm for 48 h. Each type of bacteria was grown separately. An equal volume of single liquid culture was mixed together to prepare a consortium of liquid culture. Likewise, an equal weight of single immobilized cell was mixed together to form a consortium of immobilized cells. The liquid culture consortium was sprayed manually on organic fertilizer and mixed homogenously to prepare liquid bio-organic fertilizer. Meanwhile, organic fertilizers were mixed with immobilized cells according to a specified ratio to prepare immobilized bio-organic fertilizer which is known as IB + OF. For pelleted bio-organic fertilizer, liquid culture consortium was sprayed manually after the pelleting process whereas immobilized cells were mixed manually before the pelleting process.

2.6. SEM analysis

One immobilized bacterial cell bead was observed under Scanning Electron Microscope (Hitachi E-1010 Ion Sputter & Hitachi SU1510 SEM). The sample was observed at x100, ×5000 and ×10000 magnification at SE mode.

2.7. Shelf-life of bio-organic fertilizer

Bio-organic fertilizers were prepared according to various formulations and kept at room temperature to evaluate the shelf-life of the bacterial population in the product using pour plate method. One gram of each sample was mixed with 9 ml of sterile distilled water and was allowed to mix thoroughly in a shaker for 1-2 h prior to serial dilution until 10^{12}. Nutrient agar plates were incubated at 35 °C for 24 h. The pour plate method was done periodically for each sample.

Two organic fertilizers with NPK content of 3:3:3 and 5:5:5 were tested using the similar method to determine the compatibility of immobilized cells and organic fertilizers. Immobilized cells were mixed with organic fertilizers with a ratio of 1:5 and kept at room temperature. Samples were taken every week for microbial analysis using pour plate method.

Immobilized cells were mixed with NPK blue fertilizer (15:15:15) at a ratio of 1:10 and 1:50. Plate count was done on monthly basis to determine the compatibility of immobilized bacteria in mineral fertilizers.

2.7.1. Nutrient content of bio-fertilizer

Bio-organic fertilizer was prepared as described in 2.5. The effects of pelleted liquid bio-organic fertilizer, non-pelleted bio-organic fertilizer, pelleted IB + OF and non-pelleted IB + OF on nutrient content were also evaluated to determine the most stable form of bio-organic fertilizer. Nutrient content analysis such as phosphorus, potassium, nitrogen, total magnesium and total calcium were done based on accredited laboratory standards for fertilizer testing in Malaysia. The test was conducted by Food Agriculture Analysis Laboratory, MARDI (MS.ISOMEC 1025 TESTING SAMM No. 224).

2.7.2. Ratio of immobilized cells and organic fertilizers

Three ratios of immobilized cells and organic fertilizers namely 1:20, 1:30 and 1:50 were tested to determine the best formulation to produce bio-organic fertilizer with high bacterial population. Ratios 1:10 and 1:30 were used to determine the effect of ratio on nutrient content of bio-organic fertilizer.

2.8. The effect of sterilization method on the longevity and nutrient content of bio-organic fertilizer

Two methods of sterilization were used namely gamma irradiation and steam sterilization. The organic fertilizers (4:3:3) were treated with 50 kGy of gamma irradiation prior to the mixing of immobilized cells. Similarly, organic fertilizers were sterilized via autoclaving twice at 121 °C for 30 min at an interval of 2 days prior to mixing with immobilized cells. The shelf-life and nutrient content of bio-organic fertilizers were evaluated.

2.9. Field test

Two field tests were done to evaluate the efficiency of bio-organic fertilizers in the same plot. Four treatments with four replications were introduced in an open field test. Each treatment consists of 10 plants per bed. The treatments were as stated below.

T1: Liquid bio-organic fertilizer
T2: IB + OF (1:30 ratio)
T3: Organic fertilizer
T4: Control (without fertilizer)

The size of each bed was 5 m x 0.5 m. Each bed was transplanted with 10 healthy plants. Prior to transplanting, hybrid cabbage seeds (KIRIN 3007) were grown in nursery for one to two months. Cocoa peat was used during soil preparation and rice straw was used for mulching during planting. Neem spray and *B. thuriengiensis* spray were used to control pesticides. Besides, scouting method, pitfall and yellow sticker were used to trap pests. Organic fertilizer (4:3:3) was used to prepare immobilized bio-fertilizer and liquid bio-fertilizer as described in 2.2 – 2.5. Manual irrigation was done throughout the experiment. Each plant was applied with 200 g of fertilizer at 14 and 45 days after transplanting. Cabbage heads were harvested 90 days after transplanting.

3. Results and discussion

3.1. Molecular and phylogenetic analysis

The four isolates of bacteria that attributed various beneficial traits were identified as *Serratia marcescens* (P2-2 and 8G), *Enterobacter cloacae* (P1-8) and *Klebsiella pneumoniae* (CB36) based on phylogenetic analysis of 16 S rDNA sequences using maximum likelihood method (Fig. 1). *Serratia marcescens* was reported as an efficient phosphate solubilizer (Misra et al., 2012) and plant growth promoting endophytes (Devi et al., 2016). Lin et al. (2012) on the other hand, has reported *Enterobacter* sp. to increase the biomass production of sugarcane and oil palm. *Klebsiella* sp. was also reported to hold a great potential to be used as bio-fertilizer in saline soils as it protects the plants against adverse effects of salt and temperature (Singh et al., 2015). The aforementioned bacteria are also commonly classified as human pathogens. Rhizosphere can harbor these opportunistic bacteria that can only cause diseases to patients with a strong vulnerability to illness. Several studies have provided evidences that similar genomic functions are responsible for both beneficial effects on plants and virulence in humans (Preston et al., 2001). Plant and human associated bacteria may harbor similar ribosomal and housekeeping genes but their mere occurrence in genome is not an evidence of pathogenicity. It is impossible to draw conclusions about potential pathogenicity based on 16 S rDNA sequence information. Neutral bacterial strains can be dangerous or pathogenic bacteria can be harmless due to the presence or absence of any pathogenicity factor. Therefore proteomics and interaction studies seem to be necessary to assess the potential risk of opportunistic bacteria on human (Berg et al., 2013). An environmental and human safety index (EHSI) was recommended by Vicchez et al. (2016) to assess the potential risk of beneficial bacteria that released into the environment. However, at present there are no internationally harmonized reliable protocols to evaluate the safety of these opportunistic bacteria. Therefore, the current study exhibited these bacteria only as a model for evaluating immobilization technique to produce bio-fertilizer. Immobilized cells are protected by alginate layer. Thus, it could reduce the risk of direct contact with bacterial inoculants that are regarded as human pathogenic bacteria. However, it is recommended to do a thorough risk assessment to human, environment and animal prior to the registration and application of any microbial products regardless of its risk group classification.

3.2. Scanning Electron Microscope (SEM) analysis

The surface of one single bead contains numerous bacterial cells that can be easily released into the adjacent environment. The average diameter of one dried immobilized bead was 1.10 mm [Fig. 2 (a)]. Immobilized bead which is in sphere shape initially becomes an irregular size bead after the drying process. This was also reported by Young et al. (2006) that drying of the beads resulted in uneven surface. Fig. 2(b)–(c) clearly show that the distribution of bacterial cells is all around the bead and not at a specific region of the bead. Immobilization traps the cells within or throughout the matrix (Vidhyalakshmi et al., 2009). The shape and size of the immobilized cells are dependent on the formulation of immobilization technique. Reetha et al. (2014) reported that different formulation of dry alginate beads were 1.3 mm and 1.4 mm whereas the size of wet alginate beads ranged between 1.3 mm and 3.2 mm. Young et al., (2006) have stated that the average diameter of dry alginate bead was 1.2 mm and average diameter of wet alginate bead was 2.8 mm. However, this study did not emphasize on the shape or uniformity of alginate beads as it could increase the cost of production. A simple and practical way of production was adopted by not compromising with the fundamental principle of alginate immobilization technique to reduce the production cost and increase the shelf-life and microbial density. Furthermore, a compost-like feature of immobilized cells would be appropriate to be mixed with organic fertilizer compared with sphere shaped alginate beads that will not be suitable to be incorporated with organic fertilizer. Drying of beads shall improve the survival of microorganisms during storage. The water content in wet beads should be less than 10 % to stabilize microorganisms during storage (Reetha et al., 2014). Drying process is very important to determine specific moisture content in the alginate beads. Ivanova et al., 2006 has described two methods for drying alginate beads namely oven drying at 40 °C, 35 % RH and crossing the capsule bed with a dry air stream (5% RH). However, in this study, a simple and cost saving drying method at room temperature was proofed to be suitable for the production of bio-organic fertilizer.

3.3. Shelf-life of bio-organic fertilizer

3.3.1. Effect of various formulation of bio-organic fertilizer on bacterial shelf-life and nutrient content

According to Fig. 3, the initial bacterial count in four types of bio-organic fertilizers was in the range of 10^8-10^9 cfu/g. It was noticed that bacterial population started to decline after 64 days in non-pelleted liquid bio-organic fertilizer. Conversely, the population count was constant in pelleted liquid bio-organic fertilizer. Spraying of liquid culture after the pelleting process ensures proper and thorough mixing of microbes with pelleted bio-organic fertilizers. On the other hand, spraying the microbial culture onto non-pelleted organic fertilizer could not sustain high bacterial count. Microbial cells were introduced to a rich medium made of various organic components such as oil palm bunch ash, Christmas Island rock phosphate (CIRP), fish meal, chicken manure and rice husk biochar. Microbial cells are able to feed on these organic substances, multiply, involve in various biochemical activities, produce primary and secondary metabolites and undergo cell lysis eventually. During this period, the inoculated microorganisms may give way for other microorganisms to grow and multiply in the nutrient rich organic fertilizer. An uninvited competition might happen in between the inoculated and natural microorganisms that originate from the organic substances. If the sterilization process is inefficient, it might cause cross contamination of the bio-organic fertilizer with indigenous microbes of the organic material. In contrast, non-pelleted IB + OF could sustain high microbial population even though the results were fluctuating. This could be related to the protection provided by alginate in immobilized cells. The bacterial cells are not directly in contact with organic materials. However, inconsistency in microbial population in non-pelleted IB + OF could be due to improper mixing procedure. A mixer was used for the pelleting process whereas manual mixing was done for non-pelleted fertilizers. This result reveals that pelleting method is more suitable for liquid culture as the bacterial cells can be sprayed homogenously after the pelleting process. Pelleting is not recommended for immobilized cells in this study due to inconsistent distribution of bacterial cells in each pellet.

Fig. 4 indicates the effect of pelleting process on the nutrient

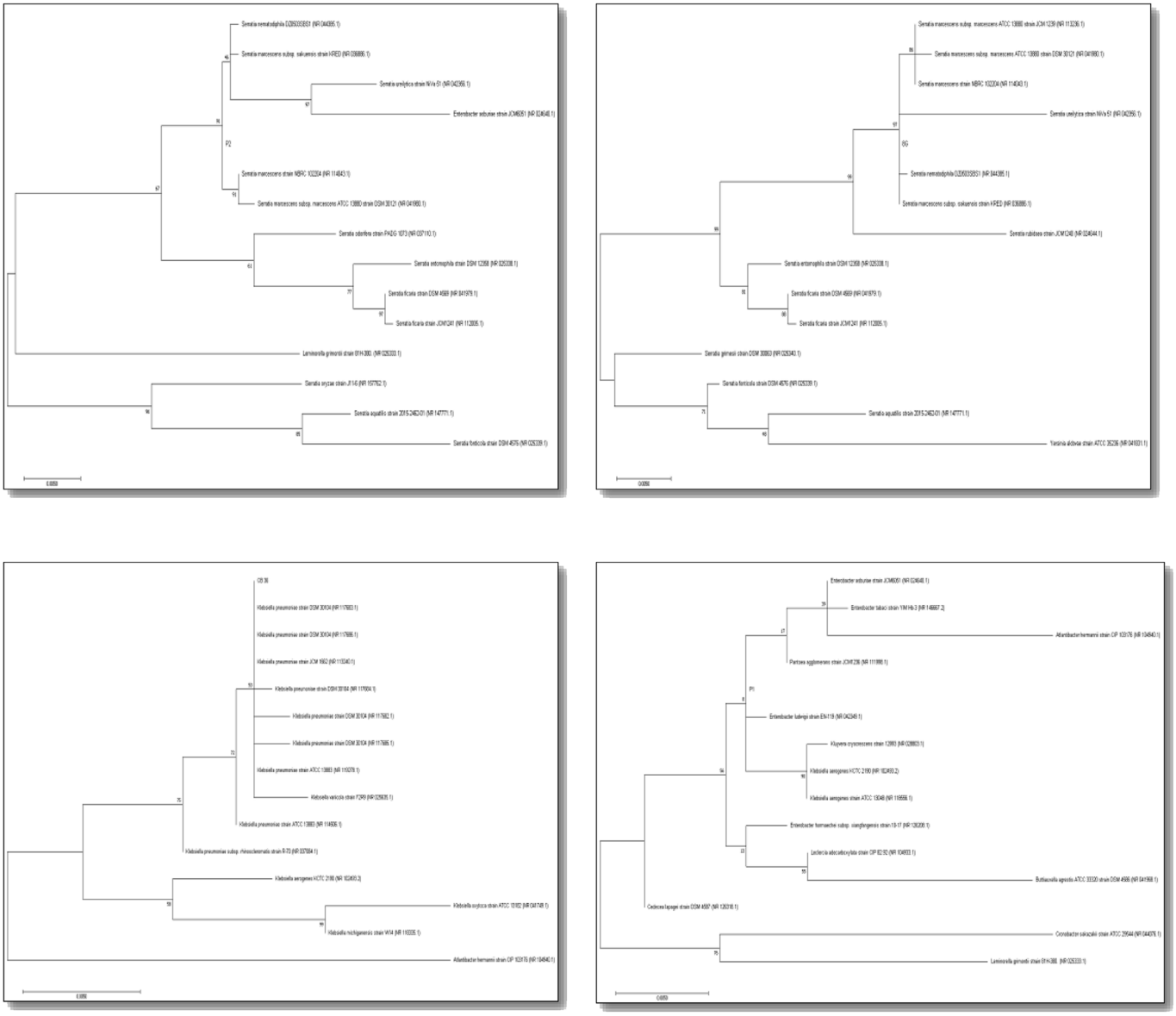


Fig. 1. Phylogenetic analysis of 16 S rDNA sequences of P2-2, 8G, CB 36 and P1-8 using maximum likelihood method.

content of bio-organic fertilizer. The analysis of nutrient content was done to determine the percentage of loss in the amount of nutrients in two weeks. In general, organic fertilizer contain higher amount of nutrients compared with liquid or immobilized bio-organic fertilizer. Nitrogen, phosphorus and potassium contents were reduced in 14 days for all types of fertilizers except for phosphorus content in liquid bio-organic fertilizer pellets that showed an increase after two weeks. Total calcium and magnesium did not change much in all three types of fertilizers. Nitrogen, phosphorus, potassium, total calcium and total magnesium were reduced to 11%, 3.9%, 5%, 2.4% and 3.6% respectively in organic fertilizer. In liquid bio-organic fertilizer no difference was observed in total magnesium and an increase of 13% in phosphorus content while nitrogen, potassium and total calcium were reduced to 8.2%, 9.5% and 4.3% respectively. In IB + OF, no difference was observed in the amount of total calcium whereas total magnesium increased to 4.3% while nitrogen, phosphorus and potassium content

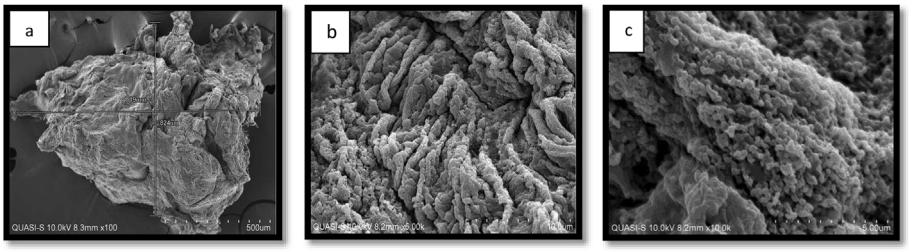
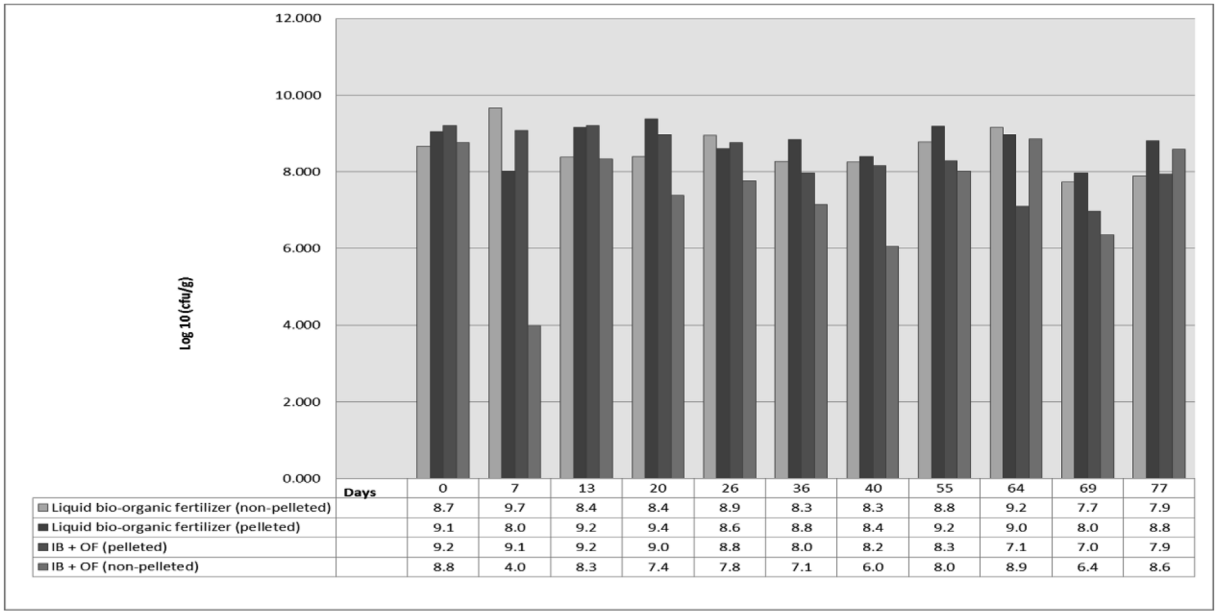


Fig. 2. Scanning electron microscope of immobilized bacterial cells in alginate bead 2(a) One irregular sized bead of immobilized bacteria at X100 magnification. 2(b) Magnification of immobilized bacteria at X 5 k resolution. 2(c) Magnification of immobilized bacteria at X10k resolution.



Days	0	7	13	20	26	36	40	55	64	69	77
Liquid bio-organic fertilizer (non-pelleted)	8.7	9.7	8.4	8.4	8.9	8.3	8.3	8.8	9.2	7.7	7.9
Liquid bio-organic fertilizer (pelleted)	9.1	8.0	9.2	9.4	8.6	8.8	8.4	9.2	9.0	8.0	8.8
IB + OF (pelleted)	9.2	9.1	9.2	9.0	8.8	8.0	8.2	8.3	7.1	7.0	7.9
IB + OF (non-pelleted)	8.8	4.0	8.3	7.4	7.8	7.1	6.0	8.0	8.9	6.4	8.6

Fig. 3. Shelf-life of bacterial cells in various formulations of bio-organic fertilizer. Liquid bio-organic fertilizer refers to organic fertilizer mixed with liquid bacterial consortium by spraying method. IB + OF refers to immobilized bacterial cells mixed with organic fertilizer.

showed a decline of 8.1%, 12% and 5.7% respectively.

It is quite common for nitrogen mineralization in organic fertilizers due to the indigenous microorganisms present in the organic materials. Organic fertilizer mixed with microorganism has exhibited lesser loss of nitrogen compared with organic fertilizer itself. Organic fertilizers that were incorporated with microbial cells were pre-sterilized before pelleting process while non-inoculated organic fertilizer was not sterilized at all. Chicken manure, one of the ingredients in the organic fertilizer contains more natural microorganisms that involves in mineralization of nitrogenous compound compared with liquid and immobilized bacterial cells. Nitrogen is the key building block of the protein molecules in microorganisms. Various forms of nitrogen such as ammonium, nitrate or organic N compounds are utilized by microorganisms for the metabolic activity and growth. Organic nitrogen is converted to ammonium and nitrate via mineralization process by microorganisms. Ammonia is assimilated by microorganisms for their growth and released through mineralization after the death of microbial cells. It is also known as nitrogen loss. This study has proofed that nitrogen could be retained in pelleted bio-organic fertilizers probably because the inoculated microorganisms were not specifically involved in extensive mineralization as in organic fertilizer that contains indigenous microorganisms.

Phosphorus loss was very minimal in organic fertilizer. Phosphorus content has dropped in immobilized bio-organic fertilizer but increased in liquid bio-organic fertilizer. Decrease in P content could be related to the use by microorganisms. Phosphorus mineralization occurs with the help of microorganisms to convert insoluble organic P into available P. Phosphorus mineralization is mediated by phosphatases. P is assimilated into microbial nucleic acids, phospholipids or other protoplasmic substances. Some microorganisms utilize phosphite (HPO3 =) as a sole

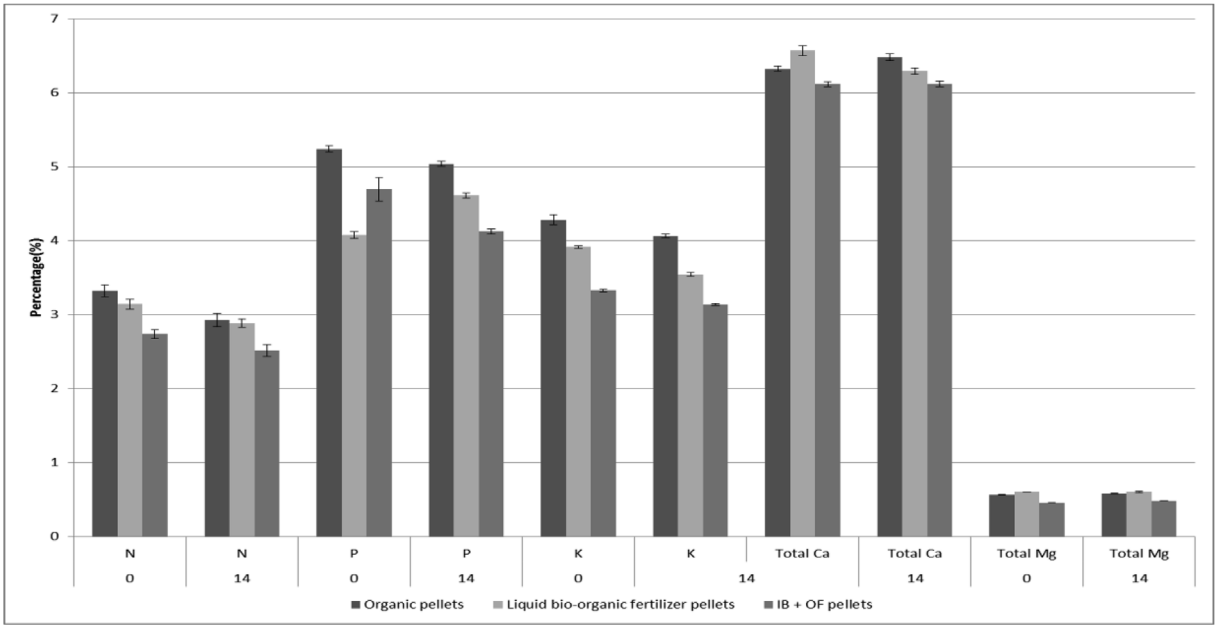


Fig. 4. The effect of pelleting process on nutrient content in organic fertilizer, immobilized bio-organic fertilizer and liquid bio-organic fertilizer after two weeks.

P source. Phosphorus that exists in an organic form in the protoplasm of the death or living organism, is changed to inorganic phosphoric acid. This is soon converted into insoluble salts of Ca, Fe, Mg and Al. Phosphorus, thus alternates between organic and inorganic, and soluble and insoluble forms. Insoluble P is solubilized by various acids produced by microorganisms.

Based on the changes in nutrient content, organic fertilizers could retain the nutrients with minimal loss in the pellet form compared with bio-organic fertilizer pellets. This result also suggests that, microbial incorporation with organic fertilizers during production could lead to nutrient loss through mineralization and microbial utilization for growth and development. However, lysed microbial cells may increase N and P level in the organic fertilizer. This factor needs to be taken into account when preparing bio-organic fertilizer to maintain the specified amount of nutrients in the fertilizer. The final nutrient content might not be the same as loss of nutrient is prominent during storage due to microbial activity either in organic fertilizer or bio-organic fertilizer in pellet form.

3.3.2. The effect of bacterial cells and organic fertilizer ratio on nutrient content and bacterial population

Three ratios of immobilized bacterial cells and organic fertilizers were analyzed for more than 400 days to determine the shelf-life of immobilized cells in an organic fertilizer with NPK value of 4:3:3 (Fig. 5). In general, the results show that there is a drop in the microbial population after mixing with organic fertilizer. The initial count before mixing was 10^{12} cfu/g but the count reduced to about 10^{8}-10^{9} cfu/g after mixing with organic fertilizer. This could be due to the microbial cell death during production and incompatibility with the substances in organic fertilizer. The microbial count was 10^{12} cfu/g initially but decreased to 10^{8} cfu/g, 10^{7} cfu/g and 10^{6} cfu/g after 100, 200 and 300 days respectively. The microbial population was stable at 10^{6} cfu/g after 413 days. A fluctuating trend was observed in microbial population that could be related to natural microbial cell lysis. It is quite regular that microbial population will be involved in minimal metabolic activity in the immobilization form. The moisture content of the organic fertilizer is about 18-20% where it is enough for the minimal activity of microorganisms. Gradual release of microorganisms from immobilized beads could have contributed to the cell lysis and reduction in microbial population. This result also indicates the ability of immobilized technique to uphold the living cells for more than a year in the presence of organic fertilizer. The formulation can be improved to increase microbial concentration by mixing microbial strains that are compatible with every ingredient in the organic fertilizer. Ratio of immobilized cells to organic fertilizer of 1:50 is recommended for solid bio-organic fertilizers as it shows microbial population of more than to10^{6} cfu/g after 348 days.

One part of immobilized bacteria mixed with 10 parts (1:10) and thirty parts (1:30) of organic fertilizer were selected to evaluate the nutrient content until 240 days (Fig. 6). IB + OF with a ratio of 1:30 showed higher nutrient loss as compared to IB + OF with a ratio of 1:10. All nutrients exhibited decline in value except for nitrogen. IB + OF with 1:10 ratio showed a decrease of about 18.9%, 11.4%, 19.3%, 20.6% and an increase of 20.8% for total calcium, potassium, total magnesium, phosphorus and nitrogen respectively. A reduction of 23.4%, 25.9%, 24.3%, 25.8% and a rise of 4.5% for total calcium, potassium, total magnesium, phosphorus and nitrogen was observed respectively in IB + OF with 1:30 ratio. This result reveals that IB + OF with a ratio of 1: 30 could not retain high nutrient content after 240 days. Microorganisms have the capability of recycling nutrients in organic fertilizers. Large microbial population could keep the nutrients in balance compared with low microbial population.

3.3.3. The effect of sterilization method on nutrient content and microbial population of non-pelleted IB + OF

The efficiency of immobilized bacteria in sustaining the nutrient content of organic fertilizer that was pre-sterilized by two common techniques namely, autoclaving and gamma-irradiation was investigated in this study for about 120 days and 240 days respectively (Table 2).

Sterilization by gamma irradiation has caused an increase of 4.6% nitrogen and a decrease of 25.9% phosphorus, 24.8% potassium, 23.4% total calcium and 24.3% total magnesium respectively. All the nutrients were reduced about one quarter from the total nutrient value over 240 days except for nitrogen. Accumulation of ammonium due to the decomposition of dead microorganisms could have caused an increase in nitrogen (Berns et al., 2008). This is not comparable to the sterilization method by autoclaving as the analysis was done till 120 days only.

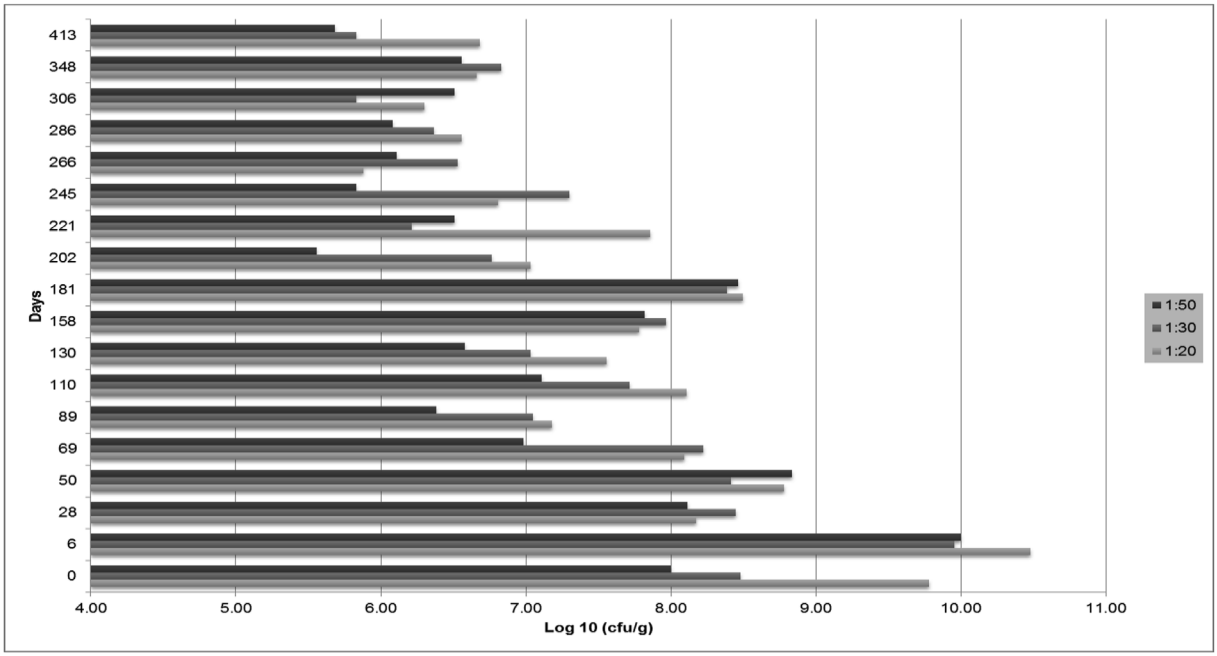


Fig. 5. Bacterial population in various ratios of immobilized bacteria and organic fertilizer.

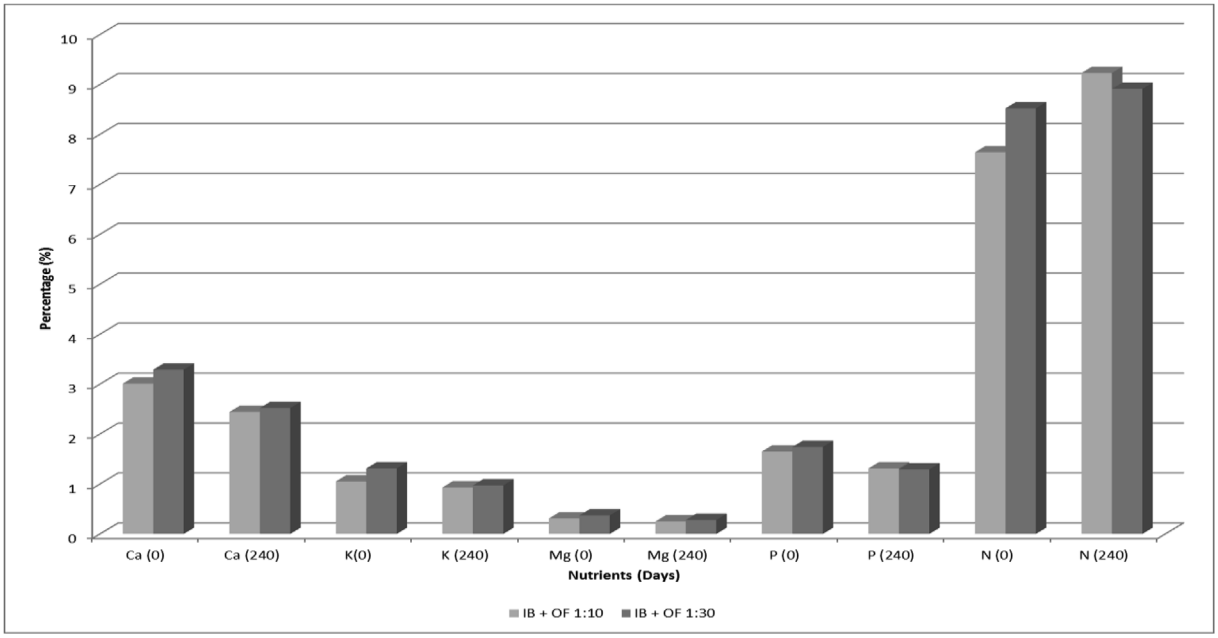


Fig. 6. Effect of 1:10 and 1:30 ratios of immobilized bacteria and organic fertilizer formulation on nutrient content after 240 days.

However, nutrient loss after gamma irradiation is more than the nutrient loss after autoclaving. A similar results was also reported by Wen et al. (1997), indicating that the effects of gamma irradiation is not consistent and have increased the mineralization of carbon and nitrogen when the high dos treatment (12 kGy) was used on sludge. High energy radiation interacts with fertilizer and causes chemical, biological and physical changes in the system.

Gamma irradiation is meant to sterilize the organic fertilizer to ensure it is free from contamination prior to mixing with bacterial cells. The loss of nutrients in gamma irradiated organic fertilizer shows that the indigenous microorganisms were not completely destroyed and actively involved in nutrient mineralization. The natural microorganisms originates from the organic materials have the ability to actively participate in nutrient recycling as compared with exotic or inoculated microorganisms. Therefore, it is predicted that sterilization process by autoclaving is more effective in eliminating the indigenous microorganisms as it was done twice. A similar finding was reported by Berns et al. (2008) that cell destruction is more pronounced in autoclaved soil compared to gamma sterilized soil.

Pre-sterilized organic fertilizer mixed with liquid culture and immobilized cells were analyzed for microbial population until 411 days (Fig. 7). Both fertilizers showed fluctuating trend of bacterial population till 280 days. The fluctuating trend could be related to cell division and cell lysis. The population deemed to be stable after 280 days. After 411 days, bacterial population in liquid bio-organic fertilizer was reduced from log 9.06 to log 6.85 whereas population in IB + OF was reduced from log 9.02 to log 7.83. The purpose of sterilization of organic fertilizer is to eliminate indigenous microorganisms in organic fertilizer. Sterilization allows the inoculated microorganisms to dominate the organic fertilizer and avoid interference or competition of indigenous microorganisms. This can be clearly seen as the initial bacterial density was log 9 in both bio-organic fertilizers. However, IB + OF exhibited high and stable bacterial population as compared to liquid bio-organic fertilizer. The bacterial population was always more than log 7 cfu/g except for one reading that fell at the border of log 7. The bacterial population in liquid bio-organic fertilizer achieved log 6 cfu/g after one year. In this study, bacterial count was maintained in liquid bio-organic fertilizer due to the pre-sterilization of organic fertilizer which is not a common practice in the majority of bio-organic fertilizer production companies.

The effect of gamma-irradiated organic fertilizer on the bacterial population in liquid bio-organic fertilizer and IB + OF was analyzed for about 233 days (Fig. 8). The initial count was 10^{10} cfu/g and dropped to log 4.9 cfu/g in liquid bio-organic fertilizer and log 6.01 cfu/g in IB + OF. In general, bacterial population in gamma irradiated organic fertilizer is lower than steam sterilized (autoclaved) organic fertilizer.

Table 2
Nutrient content changes in non-pelleted IB + OF after sterilization by autoclaving and gamma irradiation.

Nutrients	Sterilization by autoclaving at 121°C (after 120 days)	Sterilization by γ irradiation (50 kGy) (after 240 days)
Nitrogen	13.2 % ↓	4.6 % ↑
Phosphorus	2.44 % ↑	25.9 % ↓
Potassium	1.86 % ↑	24.8 % ↓
Total calcium	5.34 % ↑	23.4 % ↓
Total magnesium	2.85 % ↑	24.3 % ↓

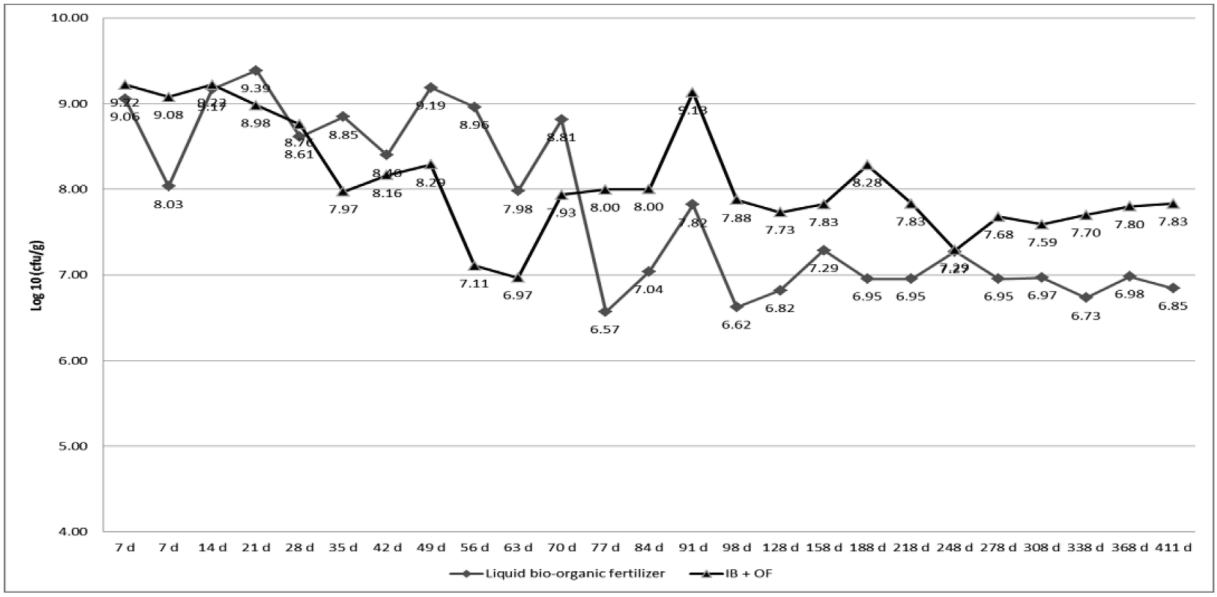


Fig. 7. Bacterial population in pre-sterilized organic fertilizer (autoclaving) mixed with liquid culture and immobilized cells.

The bacterial count in autoclaved fertilizers at 248 days were log 7.29 cfu/g which is higher than the gamma irradiated fertilizers at the same interval. It may also be affected by the nutrient content in gamma-irradiated organic fertilizers which showed a declining trend in 240 days. Gamma irradiation at 50 kGy could have caused degradation to the organic fertilizers. A drastic declining trend was observed in gamma irradiated organic fertilizer that is mixed with liquid culture. Gamma irradiation might not be effective in destroying the indigenous micro-organisms which could have led to a competition with inoculated bacteria in liquid bio-organic fertilizer. However, the microbial population difference of about one log between organic fertilizer treated by steam sterilization and gamma sterilization could be due to the unfavorable effect that caused by gamma irradiation to organic fertilizer. The degraded compounds in organic fertilizer could be harmful to microorganisms to a certain extent or high nutrient content in the organic fertilizer itself is not favorable for the liquid or immobilized bacterial cells. In this case, immobilized cells were protected in alginate membranes and therefore could withstand any adverse condition in the environment. In contrast, liquid microbial cells do not have a protection layer and thus are more exposed to the adverse condition that caused cell lysis. Based on the results, autoclaving is a preferred method of sterilization of organic fertilizer as it could support a higher microbial density and retain the nutrient content in the organic fertilizer.

3.3.4. *Shelf-life of immobilized bacteria in compost*

The microbial population of immobilized bacteria in compost with an initial count of 10^{11} cfu/g declines over time to10^{7} cfu/g in 351 days (Fig. 9). Compost is a common carrier material used in agriculture for the application of microorganisms to soil. The compost used in this study was made of vegetable wastes and paddy husk. Raw materials of compost have implication on the microbial density in it. A gradual decline indicates that microbial population is stable and could retain high count after almost a year. This finding justifies the compatibility of compost as a carrier for immobilized bacteria. A study conducted by Reetha et al. (2014) revealed that alginate beads contained up to10^{8} cfu/g of dry beads at the end of 360 days. This shows compost as

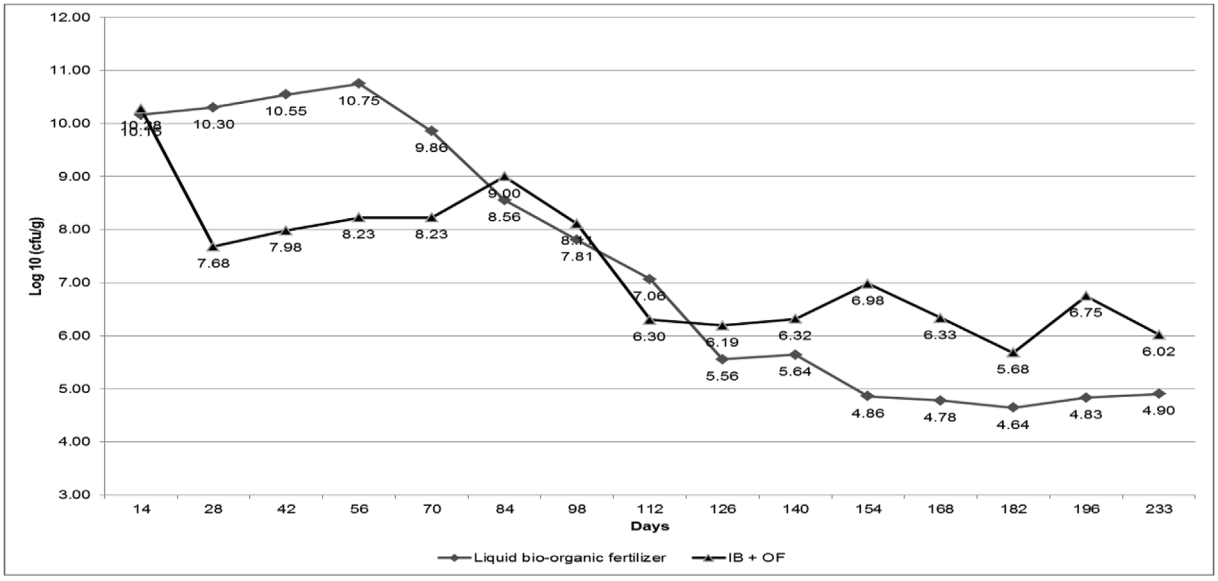


Fig. 8. Bacterial population in pre-sterilized organic fertilizer (gamma-irradiation at 50 kGy) mixed with liquid culture and immobilized cells.

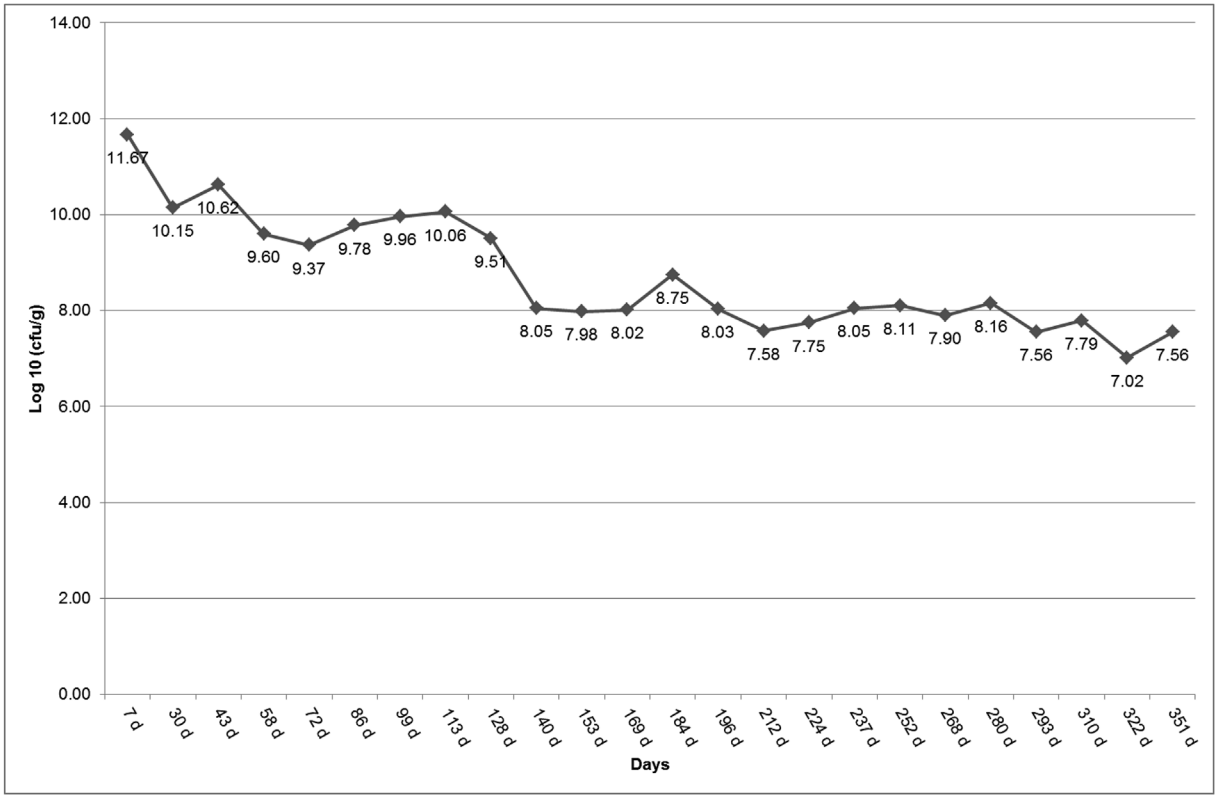


Fig. 9. Shelf-life of immobilized bacteria in compost.

a medium did not affect much on the population of bacteria in alginate beads. Compost holds moisture that enables the bacteria to involve in biological activities whereas in dry beads bacteria involve in minimal metabolic activities. Furthermore, the nutrient content in compost is very low compared to organic fertilizer and it is deemed to be more stable and non-toxic.

3.3.5. Shelf-life of immobilized bacteria in organic fertilizer

The compatibility of immobilized bacterial cells was tested in two organic fertilizers with NPK value of 3:3:3 and 5:5:5 (Fig. 10). The initial microbial population was 10^9 cfu/g and 10^{10} cfu/g in organic fertilizer with 5:5:5 and 3:3:3 NPK value respectively. After 433 days, the microbial count dropped to 10^6 cfu/g in 5:5:5 organic fertilizer and to10^8 cfu/g in 3:3:3 organic fertilizer. The reduction in microbial population was 100 times lesser in 3:3:3 organic fertilizer compared with 1000 times in 5:5:5 organic fertilizer. This result shows that the immobilized bacteria are more compatible with low nutrient content of organic fertilizers.

3.3.6. Shelf-life of immobilized bacteria in mineral fertilizer

The compatibility of encapsulated cells in high NPK content of 15:15:15 was analyzed (Fig. 11). The initial bacterial count was 10^{10} cfu/g but dropped drastically to10^5 cfu/g in 210 days for immobilized bacteria with 1: 50 ratio. Microbial population was quite stable, approximately 10^6 cfu/g at 210 days in immobilized bacteria with 1:10 ratio. This result shows that immobilized bacteria could survive in high concentration of mineral fertilizer due to the alginate layer that serves as a shield from harsh environment.

3.4. Field trial

Average weight of cabbage head in the first cycle of field test showed that liquid bio-organic fertilizer is more efficient than IB + OF and organic fertilizer (Fig. 12). The efficiency of IB + OF was not statistically significant as compared to organic fertilizer or liquid bio-organic fertilizer in the first cycle of field trial. However, liquid bio-organic fertilizer seems to be superior to both IB + OF and organic fertilizer. This is an expected result as freshly prepared liquid bio-organic fertilizer provides beneficial bacteria in a large amount to the

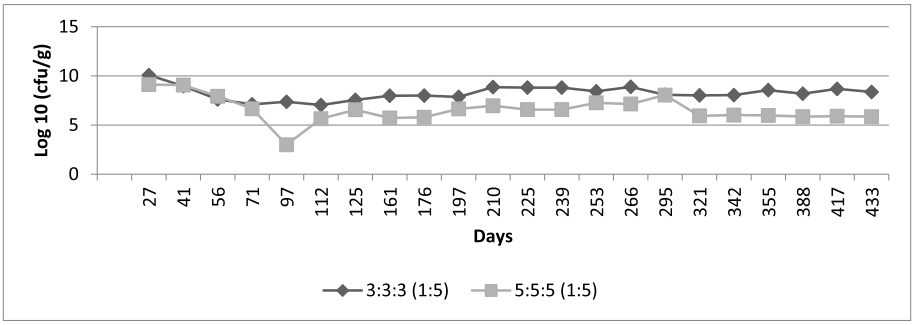


Fig. 10. Shelf-life of immobilized bacteria in organic fertilizer.

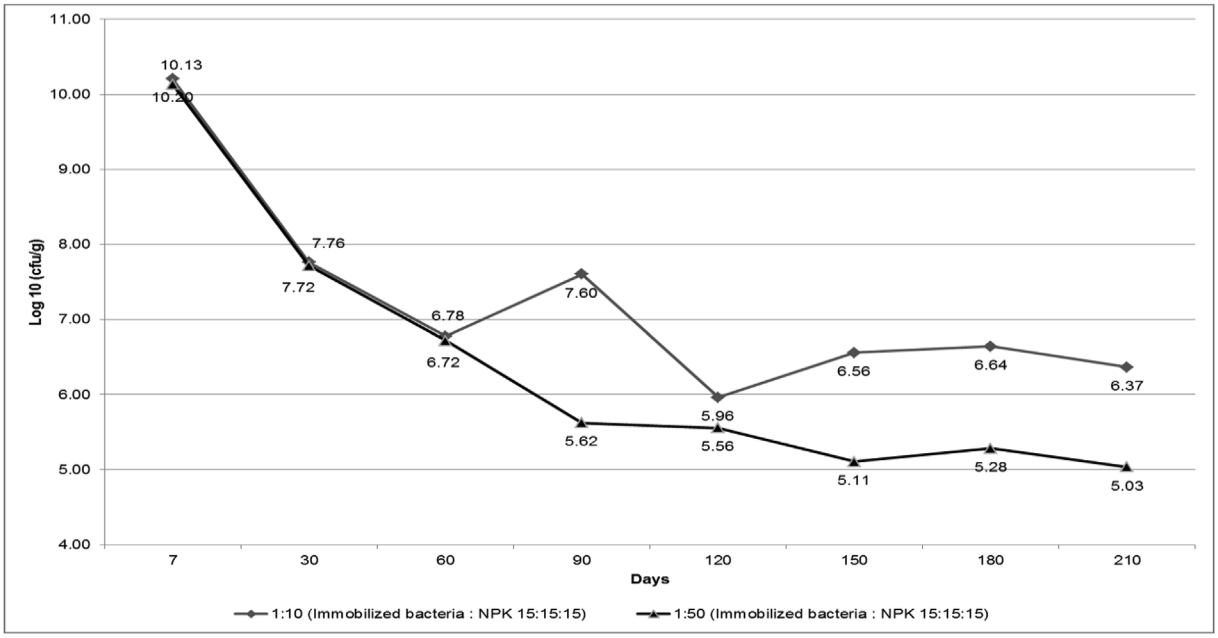


Fig. 11. Shelf-life of immobilized bacteria in mineral fertilizer.

target area. Cabbage, a short-term crop, needs immediate supply of available nutrients that can be only facilitated by active beneficial microorganisms. Immobilized bacteria mixed with organic fertilizer acts as a slow release fertilizer. Therefore, the efficiency of IB + OF was not as fast as liquid bio-organic fertilizer. IB + OF sustains the beneficial bacteria in the soil for long term use. This criterion is not attributed in liquid bio-organic fertilizer as the amount of beneficial bacteria will reduce periodically and soil need to be replenished with more liquid bio-organic fertilizer to sustain the beneficial effects of bacteria to revitalize the soil. Immobilized bio-fertilizer is proven to reinstate soil fertility in a sustainable and cost effective manner (Stella, 2018).

The second field trial showed statistically significant results at $p < 0.05$ (Fig. 13). It can be observed that IB + OF is superior to liquid bio-organic fertilizer and organic fertilizer in increasing plant yield when cabbage seedlings were planted in the same plot. Since the soils were previously applied with the same treatment of fertilizer, it is expected to restore the soil health. The results showed that only IB + OF treatment could enhance the plant productivity in spite of pesticide infestation and extreme weather (not shown) that have contributed to the decrease in cabbage yield as compared to the first cycle of field test. This could be due to the domination of beneficial bacteria that have been released slowly and constantly into soil. Therefore, immobilized beneficial bacteria shall be used to increase soil fertility especially for long-term crops.

3.5. Statistical analysis

Data were subjected to statistical analysis of one-way ANOVA. Differences of data at $p < 0.05$ level were considered significant.

4. Conclusion

Immobilization using alginate is a common method of entrapping active ingredient in food and pharmaceutical industries long ago. This technique is deemed to be very useful in agriculture industry especially as a method of microorganism delivery to field soil. Entrapment within

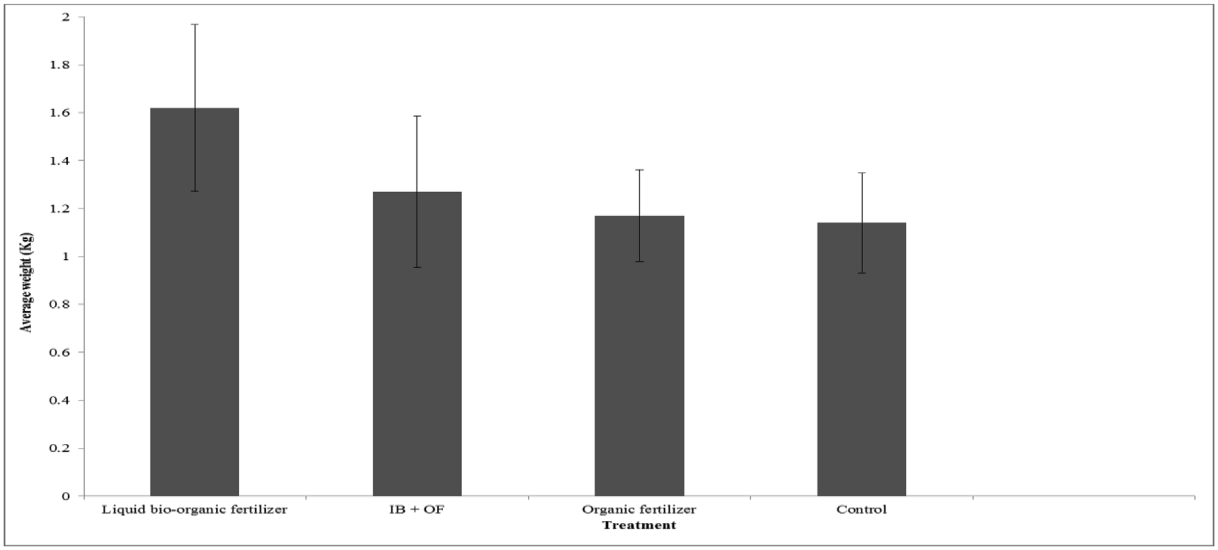


Fig. 12. The effect of liquid bio-organic fertilizer, immobilized bio-organic fertilizer and organic fertilizer on cabbage head weight (cycle 1).

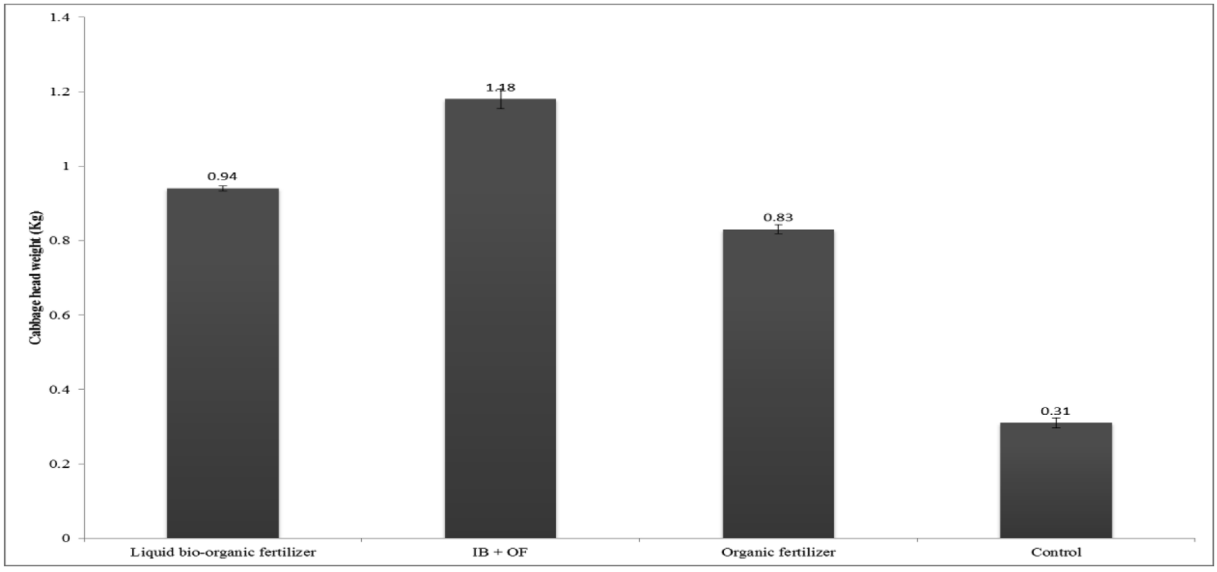


Fig. 13. The effect of liquid bio-organic fertilizer, immobilized bio-organic fertilizer and organic fertilizer on cabbage head weight (cycle 2).

gel carrier allows retention of cell viability and activity by supplying full growth media. Cells can multiply within the beads of gel to increase the cell density in each alginate bead.

This study is aimed to discover the efficiency of beneficial bacteria immobilized in alginates to retain high bacterial population and prolong the shelf-life when mixed in compost, organic fertilizer and mineral fertilizer. The influence of sterilization methods, bacterial inoculant: organic fertilizer ratio and pelleting of bio-organic fertilizer on nutrient content and bacterial count were investigated in this study.

Four Gram-negative bacteria that exhibited beneficial traits such as phosphorus solubilization, siderophore production, indole acetic acid production and nitrogen fixation were immobilized separately with an average size of 1.19 mm. These bacterial cells could survive and prolong their shelf-life up to 351 days in compost with a bacterial count of 10^7 cfu/g. The bacterial density was 10^6 cfu/g after 433 days, 10^8 cfu/g after 433 days and 10^5 cfu/g after 210 days in 5:5:5 organic fertilizer, 3:3:3 organic fertilizer and 15:15:15 mineral fertilizer respectively. Liquid culture is recommended to be used in pelleting process as it can ensure uniformity and homogeneity in bacterial cell distribution onto organic fertilizer. Immobilized cells and organic fertilizer shall be mixed homogenously as non-pelleted bio-organic fertilizer. Loss of nutrients is very minimal in organic fertilizer when compared to bio-organic fertilizer. Thus, it is advised to mix the immobilized cells and organic fertilizer during soil preparation as it will be more effective in retaining nutrient content and bacterial population. A ratio of one part of immobilized bacterial cells to 50 parts of organic fertilizer could retain bacterial count of 10^6 cfu/g in 348 days. Sterilization method by autoclaving showed more than 10^6 cfu/g in both liquid and immobilized bio-organic fertilizers and retained high nutrient content as compared to organic fertilizer sterilized by gamma irradiation. The adeptness of immobilized bio-organic fertilizer to boost plant productivity was also observed in the second cycle of field test. Immobilized bio-organic fertilizers are capable of restoring soil health and fertility.

This study clearly indicates the ability of immobilization technique to preserve bacterial density and reduce cell loss due to competition with indigenous microorganisms and adverse environmental condition. This technology has great implications in bringing advancement to agriculture practice. However, limited information is available on the cost of production and feasibility of this technology to be scaled up for the mass production of microbial cells for agriculture delivery. More studies need to be done to proof the technical and economic feasibility of this technique for the mass production and commercialization of bacterial inoculants for future research. Intensive research on the formulation of alginate-immobilization is very crucial to produce cost effective, efficient, long-lasting and high density bio-organic fertilizer products.

Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bcab.2019.101248.

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