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Effect of Various Carriers and Storage Temperatures on Survival of *Azotobacter vinelandii* NDD-CK-1 in Powder Inoculant

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Abstract

A study was carried out for determining the effect of various carriers and storage temperatures on survival of *Azotobacter vinelandii* NDD-CK-1. The experiment was laid out using a 4 x 5 factorial treatment arrangement in a Completely Randomized Design with three replications. The first factor is carrier with four kinds, viz. peat (Pt), peat mixed with corn stubble compost (PtCC), peat mixed with golden flamboyant leaf compost (PtLC), and Pt mixed with mushroom waste compost (PtMC). The second factor is storage temperature with five levels, viz. -16 ^{o}C, 5 ^{o}C, 25 ± 2 ^{o}C, 30 ± 2 ^{o}C and 37.5 ± 2.5 ^{o}C. Inoculum of *Azotobacter vinelandii* NDD-CK-1 was produced by a standard method using various carriers. The results revealed that types of carrier, storage temperatures and interaction between them showed significant effect on survival of azotobacter during 7 to 90 days. The survival rate was the highest in PtLC, followed by PtCC, PtMC, and Pt which gave the log number of bacterial viable cell of 6.41, 6.02, 5.67 and 5.50, respectively. The proliferation of azotobacter decreased with time and increasing temperature. The appropriate storage temperature at 7 to 15 days was -16 ^{o}C, while the most suitable temperatures for longer term (30 to 90 days) was 5 ^{o}C; followed by -16 ^{o}C, 25 ± 2 ^{o}C, 30 ± 2 ^{o}C and 37.5 ± 2.5 ^{o}C. The highest survival of azotobacter was found in PtCC at -16 ^{o}C (9.98 log cfu/g), similar to PtCC at 5 ^{o}C, PtLC at -16 ^{o}C, and PtLC at 5 ^{o}C (9.92, 9.85 and 9.77 log cfu/g, respectively).

Keywords: azotobacter, carrier, inoculum, temperature

1. Introduction

Application of biofertilizer for crop production is environmental friendly and sustainable for ecological system. Several types of biofertilizer have been developed from bacteria, particularly *Rhizobium* spp., *Azospirillum* spp., and *Azotobacter* spp., and used in production of various plants (Mala, 2003; Narula, 2000; Rai, 2006). *Azotobacter* spp., a free-living N_2-fixing bacteria is a beneficial biofertilizer which has profitable effects on plants and soil fertility (Holt, Novel, Peter, James, & Stanley, 2000). Azotobacter inoculant is normally produced in powder form for soil and seed inoculation (Burton, 1984). The inoculant can be prepared from several types of carriers such as peat, charcoal, farmyard manure, lignite, alginate, etc., in a standard method similar to rhizobial inoculum. Although peat is an ideal carrier for rhizobial inoculant, there are many disadvantages in using it. The quality of peat varies depending on its sources. It is rather expensive commodity and yet not widely available in some countries such as India (Sadasivam et al., 1986), and Thailand, thus causing significant drawback in using peat. Nowadays, several types of agricultural waste like maize stubble, plant compost, mushroom waste, rice straw, oil palm frond and bunch can be composted and used as bioinoculant carriers for rhizobial industry and

others. This system helps reducing the pollutants, saving energy, decreasing cost of production, and utilizing natural resources to the maximum benefit. In Thailand, agricultural waste from maize production is considered a major one. Annually, over 1.07 million hectares of maize were sown with the production of 4.25 Mt. Over 50 % of the plant parts is discarded and thrown away. Similarly, waste created from mushroom cultivation is also high due to increasing in production of various mushrooms in Thailand such as shitake, Indian oyster, oyster, yanagi mutsutake, golden needle mushroom, straw mushrooms, etc. This results cause a large amount of wastes each year. These agricultural wastes are recycled in various ways, including a raw material for production of organic fertilizer and bioinoculum carriers. Inoculum carriers serve as media in bioinoculant production, controlling quality and shelf life of bacterial inoculants by serving as microenvironment for microorganisms. Besides, types of carrier and storage temperatures are important factors determining shelf life of bioinoculants (Kremer & Peterson, 1983), and acceptance of agricultural products (Bashan, 1998). Storing such inoculants in a warehouse without refrigerator in the range of -5 to 30 ^{o}C often causes reduction in microbial longevity. Many researchers have evaluated for suitable carriers from agricultural wastes and investigated effect of temperatures on shelf life in packages at various temperatures (Thungtrakul, 1987; Rajakumar & Lakshmanan, 1995; Saleh, Nassar, & Yassen, 2001). The current study is designed to compare effect of carriers made of peat with agricultural waste composts as well as storage temperatures on longevity of azotobacter in powder inoculum.

2. Materials and Methods

This study was conducted during November 2009 to April 2010 in the Laboratory of Soil Microbiology, Department of Soil Science, Kasetsart University, Kamphaeng Saen Campus**,** Nakhon Pathom, Thailand. The procedures were described as followed.

2.1 Preparation and Analysis on Chemical Properties of Carriers

Four types of materials including peat (Pt), corn stubble compost (CC), golden flamboyant leaf compost (LC) and mushroom media compost (MC) were used in this study. The materials were prepared following the manual of Burton (1984). The raw materials were ground, sieved with 0.5 cm mesh screen and dried in a hot air oven at 60 ^{o}C for two days. Then, the materials were prepared into carriers of peat (Pt), Pt mixed with each agricultural waste compost at a ratio of 1:2 and named as peat with corn stubble compost (PtCC), peat with golden flamboyant leaf compost (PtLC), and peat with mushroom media compost (PtMC). The materials were autoclave at 121 ^{o}C at a pressure of 15 psi for 30 min. The carriers were analyzed for chemical properties, including total nitrogen (N_{tot}) by micro Kjedahl method, total phosphorous (P_{tot}) by vanadomolybdate yellow color, total potassium (K_{tot}) by turbidimetric techniques, organic matter (OM) by Walkley and Black, pH (1:10) and electrical conductivity (EC) by standard method (Soil Science Society of America, 1996).

2.2 Experimental Design

The experimental design was a 4 x 5 factorial arrangement in a Completely Randomized Design (CRD) with three replications. The first factor comprised four carriers, viz. Pt, PtCC, PtLC, and PtMC, while the second factor comprised five storage conditions, viz. deep freezing (-16 ^{o}C), refrigerating (5 ^{o}C), air conditioning, (25 ± 2 ^{o}C), ambient room temperature (30 ± 2 ^{o}C) and greenhouse temperature (37.5 ± 2.5 ^{o}C).

2.3 Production of Azotobacter Inoculum

Azotobacter vinelandii NDD-CK-1, a fast growing isolate screened from rhizosphere of Chinese kale at Nadindam Village, Loei Province, Thailand was chosen based on its high effectiveness. Inoculums were prepared in powder form under aseptic condition. A loopful of NDD-CK-1 pure culture was transferred into a 250 ml erlenmeyer flask containing 100 ml of Ashby's broth (20 g mannitol, 0.2 g K_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 0.2 g NaCl, 0.1 g K_2SO_4, 5 g $CaCO_3$, 0.05 g Na_2MoO_4, and 1 g NH_4Cl per liter of distilled water) and incubated at 28 ± 2 ^{o}C on 120 rpm rotary shaker for 72 hrs. Seventy-five milliliters of broth culture was mixed thoroughly with 100 g of each sterile carrier, adjusted the moisture content to 75 % water holding capacity, packed in polyethylene bags, sealed and incubated under room temperature for five days. The inoculums were repacked into sterile polyethylene bags.

2.4 Evaluation for Survival of the Azotobacter During Storage in Different Temperatures

The number of azotobacter was determined after the inoculum was subjected to different carriers and temperatures. Ten grams of each sample was taken for estimating viable cells at the initial date, 7, 15, 30, 60 and 90 days after storage using dilution plating method on Ashby's agar and incubated at 28 ± 2 ^{o}C for 5-7 days (Mala, 2003). The number of apparent azotobacter colonies were counted, calculated into viable cells and converted to log number per gram of dry inoculum.

2.5 Statistical Analysis

The collected data were statistically analyzed using R-program for Windows, version 2.15.1, and the treatment means were compared using Duncan's Multiple Range Test (DMRT) method.

3. Results

3.1 Chemical Properties of Inoculum Carriers

Some chemical properties of the carriers are shown in Table 1. There was a signigificantly difference among chemical properties of the carriers. N_{total} of the carriers were considered low. PtMC had the highest N_{total} of 1.40 %, while PtCC and PtLC were 0.83 % and 0.86 %, respectively. For P_{total} content, PtMC was the highest (0.47 %) while Pt was the lowest (0.07 %). K_{total} of PtMC was the highest at 2.21 % while those of PtCC and Pt were the lowest at 0.24 % and 0.27 %, respectively. The pH of all carriers showed high acidity (< 4.5). The highest pH was found in PtLC and PtMC (3.63 and 3.56, respectively), whereas that of Pt was the lowest at 2.80. EC of PtMC was the highest at 3.12 dS/cm, while that of PtLC was the lowest at 0.45 dS/cm. All materials were high in OM, with the highest in Pt (39.87 %) and the lowest in PtLC (31.44 %).

Table 1. Chemical properties of Pt, PtCC, PtLC and PtMC used as inoculum carriers for azotobacter

Carriers	Chemical properties					
	N_{tot} (%)	P_{tot} (%)	K_{tot} (%)	OM (%)	pH (1:10)	EC (dS/cm)
Pt	1.00b	0.07d	0.27c	39.87a	2.80c	1.19b
PtCC	0.83c	0.39b	0.24c	37.18ab	3.38b	0.60c
PtLC	0.86c	0.14c	0.98b	31.44c	3.63a	0.45d
PtMC	1.40a	0.47a	2.21a	34.26bc	3.56ab	3.12a
Mean	1.02	0.27	0.93	35.69	3.34	1.34
P-value	<.0001	<.0001	<.0001	0.0303	<.0001	<.0001
CV (%)	2.60	2.11	7.13	7.89	2.87	4.33

Remark: mean values in each column followed by the same letter are not significantly different by DMRT at P≤ .05.

3.2 Survival of Azotobacter in the Carriers During Storage in Different Temperatures

There were significant differences in survival of *Azotobacter vinelandii* NDD-CK-1 among carriers, storage temperatures at initial date to 90 days and interaction between carriers and temperatures during 7 to 90 days after storage (Figures 1 to 4). At the initial date, PtCC gave the highest bacterial number followed by Pt, PtLC and PtMC with 12.48 (Figure 2), 12.40 (Figure 1), 11.02 (Figure 3), and 10.96 log cfu/g (Figure 4), respectively. During 7, 15, 30, 60 and 90 days across five temperature regimes (Figure 5), PtLC had the highest bacterial count (10.04, 9.36, 8.88, 7.36 and 6.41 log cfu/g), followed by PtCC (9.60, 9.18, 8.86, 7.32 and 6.02 log cfu/g, PtMC (9.63, 9.00, 8.4, 6.78 and 5.67 log cfu/g) and Pt (8.20, 7.76, 7.56, 6.15 and 5.50 log cfu/g), respectively.

For storage temperatures, storing in deep freeze gave the highest growth rate of azotobacter at 7 to 15 days after preservation. During 7 days after storage, the survival of bacteria in deep freeze were 9.72 log cfu/g, while those stored in the refrigerator, green house, ambient temperature, and air-conditioned temperature had viable cells of 9.44, 9.33, 9.22 and 9.14 log cfu/g, respectively (Figure 6). At 15 days after storing, keeping the inoculants in deep freezer gave the highest number of azotobacter (9.35 log cfu/g) similar to that in refrigerator, but higher than those under air-conditioning temperature, ambient temperature and greenhouse condition which gave 8.59, 8.49 and 8.47 log cfu/g, respectively (Figure 6). While, at 30, 60 and 90 days after storage, preserving the inoculum in refrigerator gave the highest survival rate of 9.47, 8.03 and 7.52 log cfu/g, respectively, followed respectively by those kept in deep freezer (9.15, 7.97, and 7.30 log cfu/g), air conditioning temperature (8.17, 6.22 and 5.19 log cfu/g), ambient temperature (7.90, 6.21 and 5.17 log cfu/g) and greenhouse condition (7.46, 6.08 and 4.34 log cfu/g) as shown in Figure 6.

The interaction between carrier and storage temperature during 7 to 90 days showed that PtLC stored at 37.5 ± 2.5 ^{o}C gave the largest microbial population (10.37 cfu/g) (Figure 3), similar to that of PtCC at -16 ^{o}C (Figure 2), PtLC at 5 ^{o}C (Figure 3) and PtLC at -16 ^{o}C (Figure 3) with 10.32, 10.15 and 10.14 log cfu/g, respectively. While,

Pt at 5 ^{o}C and at 25 ± 2 ^{o}C had the lowest survival rates at one week after storage (Figure 1). At 15 days after storage, stored PtCC at -16 ^{o}C gave the highest survival rate (9.98 log cfu/g) (Figure 2), similar to PtCC at 5 ^{o}C (Figure 2), PtLC at -16 ^{o}C (Figure 3), and PtLC at 5 ^{o}C (Figure 3) (9.92, 9.85 and 9.77 log cfu/g, respectively). Whereas maintaining Pt carrier at 37.5 ± 2.5 ^{o}C gave the lowest number of azotobacter (7.26 log cfu/g) (Figure 1). At 30 days after preservation, maintaining the inoculant in PtCC at 5 ^{o}C showed the highest bacterial number (10.08 log cfu/g) (Figure 2), similar to those maintained in PtLC at 5 ^{o}C and PtLC at -16 ^{o}C (Figure 3) (10.03 and 10.00 log cfu/g, respectively). In contrast, Pt at 30 ± 2 ^{o}C had the lowest bacterial population (6.69 log cfu/g) (Figure 1), which was close to PtMC at 37.5 ± 2.5 ^{o}C (Figure 4), PtLC at 37.5 ± 2.5 ^{o}C (Figure 3), Pt at 25 ± 2 ^{o}C and Pt at 37.5 ± 2.5 ^{o}C (Figure 1) at 7.65, 7.48, 7.43 and 6.88 log cfu/g, respectively. Interaction between carrier and temperature had a significant effect on the survival of azotobacter stored for 60 days. The maximum number of bacteria was obtained from PtCC stored at 5 ^{o}C (Figure 2), similar to PtLC at 5 ^{o}C (Figure 3) and PtCC at -16 ^{o}C (Figure 2), gave 8.93, 8.88 and 8.70 log cfu/g, respectively. While, Pt at 37.5 ± 2.5 ^{o}C gave the lowest microbial population of 5.70 log cfu/g (Figure 1). At 90 days of preservation, the highest survival rate of azotobacter was found in PtLC at 5 ^{o}C (8.42 log cfu/g) (Figure 3) and PtCC at 5 ^{o}C (8.37 log cfu/g) (Figure 2) followed by PtCC at -16 ^{o}C (7.91 log cfu/g) (Figure 2). While PtCC at 37.5 ± 2.5 ^{o}C (Figure 2) and PtMC at 25 ± 2 ^{o}C (Figure 4) had the lowest survival cells at 3.69 and 3.71 log cfu/g, respectively.

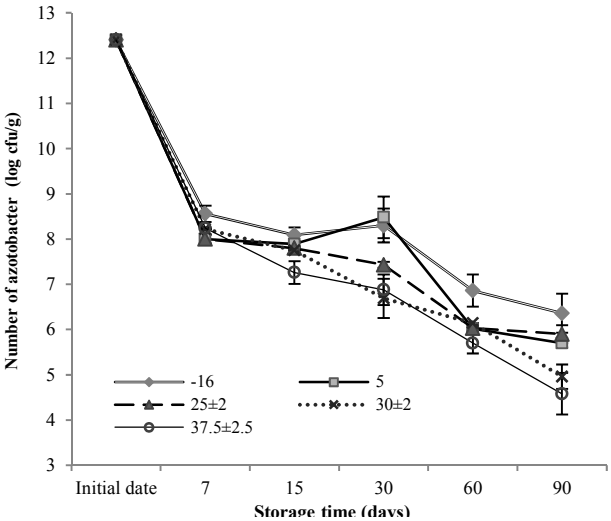

Figure 1. Population of *Azotobacter vinelandii* NDD-CK-1 existed in Pt carrier during initial date to 90 days after storage in different temperatures

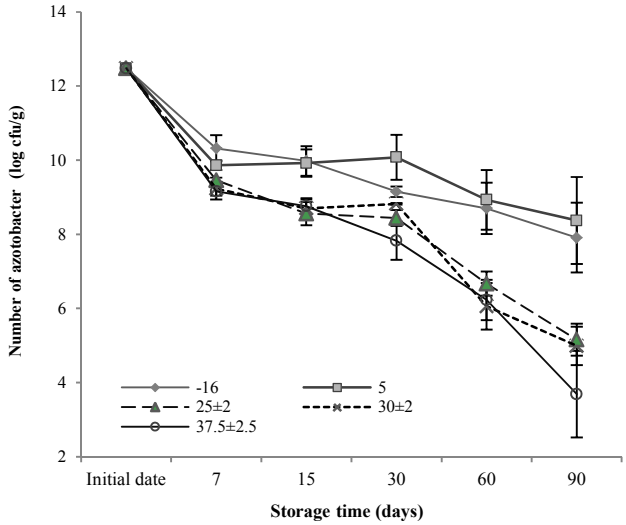

Figure 2. Population of *Azotobacter vinelandii* NDD-CK-1 existed in PtCC carrier during initial date to 90 days after storage in different temperatures

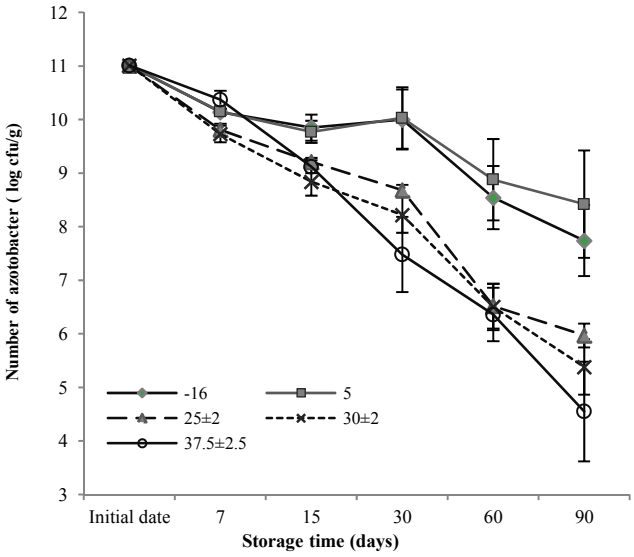

Figure 3. Population of *Azotobacter vinelandii* NDD-CK-1 existed in PtLC carrier during initial date to 90 days after storage in different temperatures

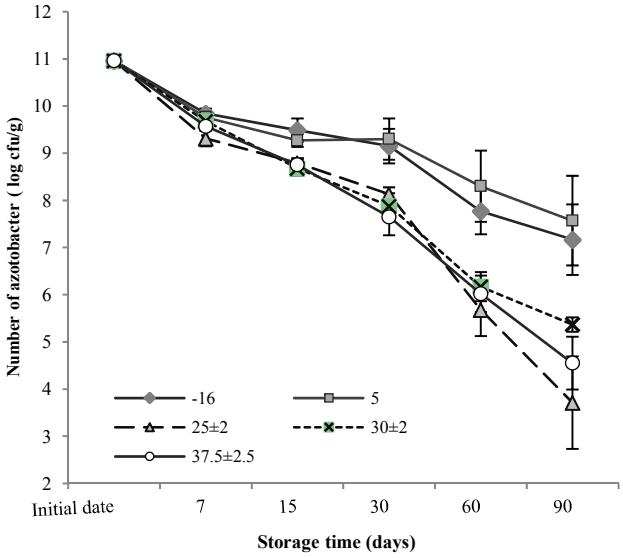

Figure 4. Population of *Azotobacter vinelandii* NDD-CK-1 existed in PtMC carrier during initial date to 90 days after storage in different temperatures

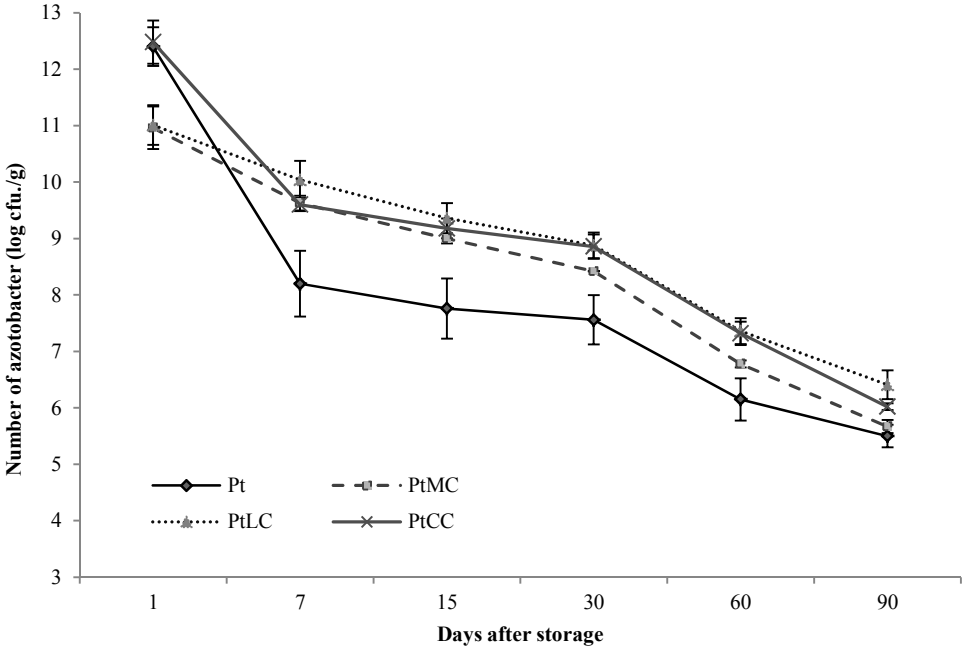

Figure 5. Effect of P, PMC, PtLC, and PCC carriers on mean number of *Azotobacter vinelandii* NDD-CK-1 averaged across temperatures

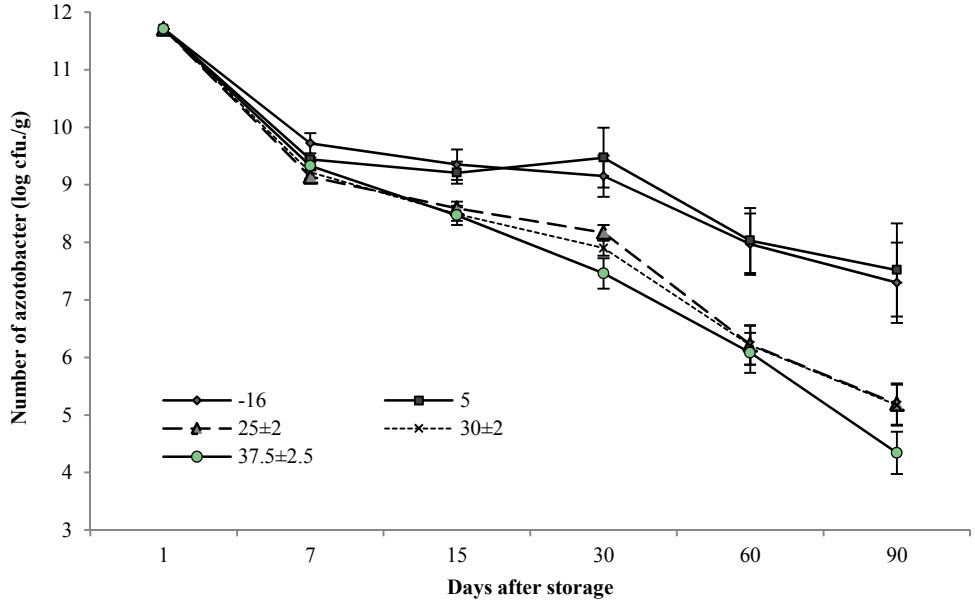

Figure 6. Effect of various storage temperature on mean number of *Azotobacter vinelandii* NDD-CK-1 averaged across carriers

4. Discussion

Population of *Azotobacter vinelandii* NDD-CK-1 decreased over time depending on types of carrier and storage temperatures. (Figure 5), similar to earlier reports (Youssef, Sedik, Fayez, & Hegazi, 1997; Saleh et al., 2001). Initial microbial populations of those materials were higher than those reported by Muthuselvam & Tholkappian (2008). The density of bacteria sharply dropped at 7 days due to lack of moisture and nutrients of the carriers. In this case, OM, N_{total}, P_{total} and K_{total} as well as moisture content of inoculum carriers were almost depleted by time due to bacterial activities and storage conditions while transitioning from logarithmic to stationary phase during incubation (Neidhardt, Ingraham, & Schaechter, 1990; Tate, 2000). The bacterial population slightly

declined until 15 days. During 7-30 days of storage the death rate was the smallest but greatly decreased from 30 to 60 days and leveled off at 90 days of storage (Figure 5). In this study, the survival of azotobacter in PtLC, PtCC and PtMC carriers were satisfactory in comparison with the biofertilizer standard, except in Pt carrier after storage for 90 days. However, the microbial population was less than those reported by many workers (Wangaruro, Karanja, Makatiani, Odee, & Woomer, 2000; Madan & Singh, 2010; Raja & Karmegam, 2010). They found that populations of *Azotobacter chroococcum* did not reduce below 10^8 cfu/g within three months.

4.1 Effect of Carriers on the Population of Azotobacter

The results indicated that PtLC was the most suitable carrier for production of azotobacter inoculum, followed by PtCC, PtMC and Pt (Figures 1-4). LC had more nutrients, especially N content than the others possibly from leaves of the legume yellow flamboyant. PtLC also had the highest pH but lowest EC (Table 1) that was suitable for promotiing microbial growth. PtLC may also release less toxic compounds during sterilization as compared to peat (Burton, 1984; Bashan, 1998; Anonymous, 2006). This compost also had high clay mineral derived from adding of clayey soil during composting process which played a critical function in promoting physical and biochemical environment for the microbial population. The increase in high specific surface area of PtLC can promote adsorption of organic and inorganic substances, cation exchange capacity, and water holding capacity. In addition, clay particles also encourage microbial catabolism by increasing adherence and tolerance capacity of azotobacter in the PtLC under hot condition (Tate, 2000). This finding is similar to the report of Bashan (1998) that survival of bacteria was increased by adding clay to the alginate beads as compared to alginate beads alone. Our finding showed that Pt gave poor survival of azotobacter during 90 days, similar to the report of Muthuselvam & Tholkappian (2008) and Thananusont (1993). Pt had lower quality and higher acidity than that of the others because over 50% of the peat was mixed with coconut husk which is considered a poor source of carrier. Thus, adding plant leaf compost and corn stubble compost to peat can improve the inoculum quality and longevity of azotobacter. Many researchers have suggested to use various combinations of carriers to prolong shelf life of bacterium inoculant (Jauhri, Bhatnagar, & Iswaran, 1979; Jauhri & Philip, 1984; Thungtrakul, 1987; Gaind & Gaur, 2004; Madan & Singh, 2010; Raja & Karmegam, 2010).

PtCC had the highest bacterial number at the initial date possibly due to its fine structure, which gave rise to higher specific surface area, more water content and nutrients spreading more thoroughly and easily adhered to microorganisms than those of the other carriers. This condition promoted bacterial growth within a few days after inoculation as compared to the condition in larger particle size carriers as in PtLC and PtMC. In contrast, PtMC had the lowest bacterial population due to the remaining of lignin and tannin from Para rubber saw dust, the major material of MC. The saw dust blocked bacterial respiration and reduced water absorption of MC resulting in less available water and inhibiting the bacterial population during incubation period.

4.2 Effect of Storage Temperature on Population of Azotobacter

The storage temperature affected proliferation of *Azotobacter vinelandii* NDD-CK-1in the carriers. Our study suggested that population of azotobacter was decreased by increasing temperatures (Figure 6). The survival of bacteria considerably dropped at 7 days and slightly declined during 15 to 30 days, similar to the result of Somasegaran (1985). After one month, number of azotobacter continuously declined until reaching the lowest number of viable cells at 60 and 90 days after storage. The suitable temperature for preservation of azotobacter until 90 days was 5 ^{o}C, followed by -16 ^{o}C which gave more viable cells than that stored at over 20 ^{o}C. The extreme temperatures reduced the survival rate of bacteria and its metabolism (Slonnezewski & Foster, 2010). Our finding is similar to the reports of Ben Rebah, Tyagi, & Prévost (2002) and Kibunja (1991). At the refrigerating temperature, the bacteria had lower metabolism and physiological activity which maintained high mineral contents and more available moisture than that stored over 20 ^{o}C, similar to the report of Bozida & Vladimir (1995). Inoculum stored at 5 ^{o}C had longer shelf life without formation of ice crystal as that stored at -16 ^{o}C. However, storing carriers at -16 ^{o}C gave the highest bacterial population at 7 to 15 days and sharply declined afterwards. Preservation at -16 ^{o}C for over a month caused water to become ice in the microbial cells and carriers, thus reduced the microbial density by initial killing at the time of freezing and afterwards (Neidhardt, Ingraham, & Schaechter, 1990).

4.3 Interaction between Carriers and Temperatures

The interaction between types of carrier and storage temperatures was similar to the report of Thungtrakul (1987), except at the initial storing period. At 7 days, the number of azotobacter in PtLC stored at 37.5 ± 2.5 ^{o}C was higher than those at other temperatures. It may be due to the increasing of temperature from 17-19 ^{o}C during incubation to 37.5 ± 2.5 ^{o}C in storage which promoted population of bacteria during the first week without limitation of water and nutrients, and then fell down sharply afterwards. A similar result was reported by Saleh et

al. (2001) that the population of *Azotobacter vinelandii* A1 in rice husk carrier rose up to 128 % from the initial population after storing at 30 ^{o}C. At 30 days after storage, the bacterial population increased slightly in Pt and PtLC stored at -16 ^{o}C, and Pt, PtCC, PtMC and PtLC at 5 ^{o}C.

During 7-15 days, PtCC stored at -16 ^{o}C gave the maximum survival rate. The explaination may be that PtCC had fine particle which acted as the protective insulation from the damage of ice crystal under sub-zero temperature. In the case of Pt carrier, population of azotobacter was maximum at -16 ^{o}C, followed by 5 ^{o}C (Figure 5). Among types of carrier, Pt showed a robust property in keeping high population after storage at 37.5 ± 2.5 ^{o}C, similar to PtLC and PtMC, but was higher than PtCC. Pt has lighter particle which protected bacterial cells from extreme temperatures better than the other carriers during 3 months. However, interaction between bacteria, carrier and temperature was a complicated mechanism limiting number of azotobacter under various environments. The survival ability of the bacteria varies depending on species and strains of azotobacter which are determined by gene expression under stressful conditions, habitat, and duration of preservation period in the medium.

5. Conclusion

PtLC is the most suitable inoculum carrier after storage for 90 days, followed by PtCC, PtMC, and Pt. PtLC and PtCC can be used as inoculum carriers of azotobacter with an advantage of utilizing natural resources to the maximum benefits, reducing the pollutant and inoculum cost. The usage of peat in combination with agricultural wastes resulted in high quality and survival of the bacteria. The inoculum can be kept for 90 days at 5 ^{o}C followed by -16 ^{o}C, 25 ± 2 ^{o}C, 30 ± 2 ^{o}C and 37.5 ± 2.5 ^{o}C. It can be stored up to the maximum of one month at an ambient temperature and up to three months in a refrigerator. PtLC, PtMC and PtCC inoculums can be stored at temperatures above 25 ^{o}C for a period of 1-2 months and still maintained a minimum of 10^{7}cells/g. Adding clayey soil in the inoculum or during composition process is a beneficial technique for improving the quality of bioinoculant and protecting target microorganisms by serving as a micro-environment. An appropriate proportion of clay is necessary for improving product quality.

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