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EVALUATION OF STORAGE TEMPERATURES TO MEET SHELF LIFE REQUIREMENT FOR *ACINETOBACTER BAUMANNII* BIOFERTILIZER PRODUCT

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ABSTRACT

Acinetobacter baumannii *(AP1) multifunctional biofertilizer bacterium has capabilities of atmospheric nitrogen (N_2) fixation, phosphate and potassium solubilisation and antagonism towards bacterial wilt disease of crops. AP1 is commercialised as GoGrow BioNPK. Shelf life study is essential for any commercial biofertilizer product. The minimum shelf life requirement for a biofertilizer product is six months. Optimum storage conditions, especially suitable storage temperatures are crucial, whereby the population of viable biofertilizer bacterial cells do not fall to below 10^8 colony forming unit per ml (cfu ml^{-1}). The objective of this study was to determine the shelf life of AP1 at cool and room temperatures. Two categories of AP1 concentration were prepared, viz. mother culture concentration (10^{11} cfu mL^{-1}) and commercial product concentration (10^9 cfu mL^{-1}) . The samples were kept at cool temperature (6 $\pm$ 2 ^oC) and room temperature (28 $\pm$ 2^oC) for six months. The viable population of AP1 was determined monthly by the serial dilution method. Results showed survival of AP1 cells and viable population of AP1 remained at around 10^8 cfu mL^{-1} at six months storage, indicating that AP1 met the product specification either stored at cool temperature or at room temperature. Thus, AP1 is considered as a good biofertilizer product due to its long shelf life, beyond the minimum requirement of 10^8cfu mL^{-1} as set by Department of Agriculture Malaysia, at 6 months of storage.*

INTRODUCTION

Biofertilizers, also called 'microbial inoculants' (Mohammadi & Sohrabi, 2012), are important components for sustainable agriculture and integrated nutrient management. Apart from being eco-friendly and cost effective, these beneficial microorganisms create a healthy rhizosphere by converting and making natural nutrients available through N_2 fixation, phosphate solubilisation and potassium solubilisation. These are the major characteristics of biofertilizers. Among important biofertilizer microorganisms are plant-growth promoters, which produce plant growth hormone or substances to enhance plant growth (Youssef and Eissa, 2014; Kaur and Kaur, 2018). Biofertilizers are generally applied to soil, seeds or seedlings, with or without a carrier medium.

Biofertilizer began in the 1970s in Malaysia with the production of *Rhizobium* inoculums. Since the 1980s, the isotopic tracer technique using ^{15}N stable isotope was used to quantify biological nitrogen fixation by microorganisms, including *Bradyrhizobium* and *Azolla* (Rahim, 2005). More studies on biofertilizers utilising ^{15}N isotope were subsequently conducted on various crops, including oil palm (Zakry et al., 2005); green stem mustard, white stem mustard, Chinese cabbages and tomatoes (Phua et al., 2009b and 2012); Chinese cabbage (Phua et al., 2012) and okra (Phua et al., 2013). Earlier works on biofertilizers in Malaysia mainly focused on nitrogen (N) and N-fixing bacteria (Zulkifli et al., 2004; Shamsuddin, 2005; Zakry et al., 2005). Thereafter, phosphate-solubilising microorganisms were given

more attention, and then followed by multifunctional biofertilizers or multi-strain biofertilizer inoculants (Naher et al., 2012; Othman et al., 2012; Phua et al., 2012). Currently, the market focus on products from single cultures with multifunctional activities such as *Bacillus salmalaya* strain 139SI, *Pseudomonas putida* M99 and *Acinetobacter* sp. (Nasarudin et al., 2018; Abdullah et al., 2019; Phua et al., 2021).

A carrier is a vehicle to bring the biofertilizer inoculum to the field (FNCA, 2018). 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. However, to get a carrier that meets the desired qualities is a big challenge. Liquid biofertilizer is the solution to the issue, where no solid carrier is needed to house the microbe (Brar et al., 2012). Therefore, Malaysian Nuclear Agency (Nuklear Malaysia) has embarked on the development of low-cost and long shelf-life liquid biofertilizers to be used in an innovative and high productivity cultivation system. A multifunctional biofertilizer that contains *Acinetobacter baumannii* (AP1) produced by Nuklear Malaysia, is commercialised as GoGrow BioNPK in a collaborative project between Nuklear Malaysia and Enviro Clean Energy Sdn. Bhd. AP1 has capabilities of atmospheric nitrogen (N_2) fixation, as quantified using ^{15}N stable isotope, and also capabilities of phosphate and potassium solubilisation, and being antagonistic towards bacterial wilt disease (Phua et al., 2009; 2012; 2016; 2017, 2019 and 2020).

Subsequent to the studies using isotopic tracer and determination of other functions of AP1, a shelf life or storage study ensued. Shelf life study is essential for any commercial biofertilizer product. The minimum shelf life requirement for a biofertilizer product is six months (FNCA, 2018). Optimum storage conditions, especially storage temperature is needed to improve shelf life quality of the products. The objective of this study was to evalute storage temperatures suitable to maintain the shelf life of AP1 through viable colony population counts. Two categories of concentration were prepared, viz. mother culture concentration at 10^{11} colony forming unit per ml (cfu ml^{-1}), and commercial product concentration at 10^9 cfu ml^{-1}.

MATERIALS AND METHODS

Acinetobacter baumannii was isolated in a previous study funded by MOSTI SciFund Project 02-03-01-SF0051. The isolate has capabilities of atmospheric nitrogen (N_2) fixation, phosphate and potassium solubilisation and antagonistic against bacterial wilt (Phua et al., 2009; 2012; 2016; 2017, 2019 and 2020). Nitrogen fixation was determined by using yeast extract–mannitol agar (YMA) containing 25 µg mL^{-1} bromothymol blue (BTB) (Swelim et al., 2010) and ^{15}N isotopic tracer technique (Phua et al., 2019). Phosphate and potassium solubilising activities were screened on phosphate agar plates (Freitas et al., 1997) and Aleksandrov's medium agar plates (Hu et al., 2006), respectively. A single colony of pure *A. baumannii* was selected from the nutrient agar (NA) plate and cultured in 100 mL of nutrient broth (NB) medium. The culture was grown in NB at 28 $\pm$ 2 °C in an orbital shaker (New Brunswick Scientific, Excella E24 Incubator Shaker Series, United Kingdom) at 125 rpm for 16 h. The culture was subsequently diluted into concentration of mother culture (10^{10} cfu mL^{-1}) and commercial culture (10^8 cfu mL^{-1} and kept in media bottles (Phua, 2020). Five replications of the biofertilizer were prepared for each storage temperature. The storage temperatures used were room temperature (28 $\pm$ 2 ^oC) and cool temperature (4 to 6 $\pm$ 2 ^oC). There were four treatments, viz. mother culture concentration at room temperature (T1); commercial concentration at room temperature (T2), mother culture concentration at cool temperature (T3) and commercial concentration at cool temperature (T4). Shelf life of AP1 was determined before storage, and at every one (1) month interval for a period of six (6) months by using serial dilution method.

The plating was carried out by spread plate technique where 0.1 mL of the suspension was spread over the surface of the NA plates by using a sterile L-shaped glass rod. Plates were incubated at 28 ^{o}C $\pm$2 ^{o}C for 24 h to determine population of AP1 and the graph was plotted by using log cfu mL^{-1} of the means of 5 replications results against months of storage.

RESULTS AND DISCUSSION

Fig.1 shows log number of viable cells recorded at monthly interval for four treatments, viz. mother culture concentration at room temperature (T1); commercial concentration at room temperature (T2), mother culture concentration at cool temperature (T3) and commercial concentration at cool temperature (T4) kept at room temperature (28 $\pm$ 2 ^{o}C) and low temperature (4 ^{o}C to 6 $\pm$ 2 ^{o}C). After six months, biofertilizer in mother culture concentration (T3) that were kept at low temperature (4 ^{o}C to 6 $\pm$ 2 ^{o}C.) had significantly higher viable cells than those kept at room temperature (28 $\pm$ 2 ^{o}C). The viable cells for T3 were 2.54 x 10^{12} cfu mL^{-1}. Viable cells for T1, T2 and T4 were 3.54 x 10^{8}, 4.12 x 10^{7} and 3.27 x 10^{7} cfu mL^{-1} respectively. Viable cells for the starter cultures were 2.81 x 10^{11} (T1 and T3) and 2.51 x 10^{9} (T2 and T4) cfu mL^{-1}, respectively (as shown in Table 1).

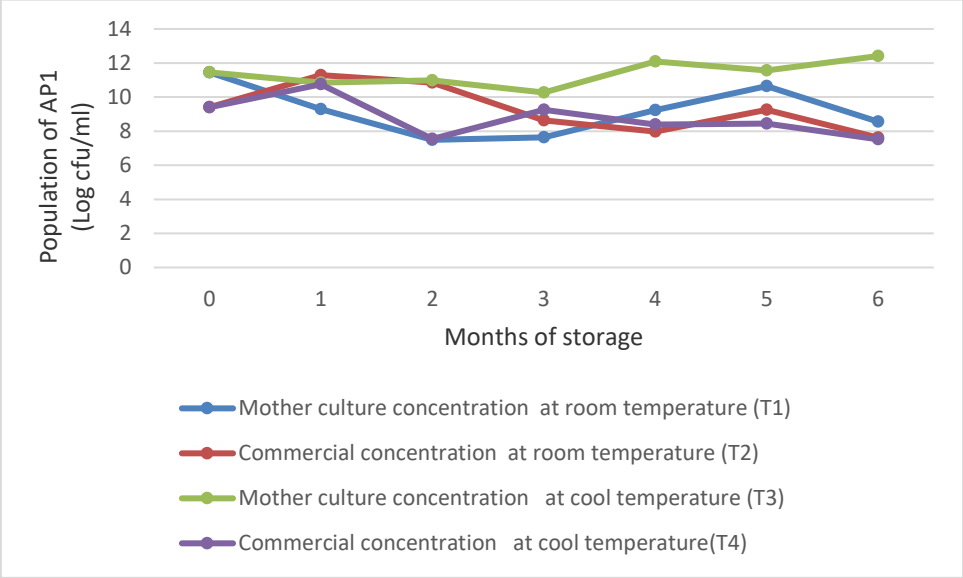

Fig. 1, Log number of viable cells in mother culture concentration and commercial concentration of liquid biofertilizer incubated at room and cool temperatures at monthly interval for six months storage

Table 1. Initial number of viable cell and after six months-storage (cfu mL^{-1}) in mother culture concentration and commercial concentration of liquid biofertilizer incubated at room and cool temperature

Treatment	Initial number of viable cell (cfu mL^{-1})	Viable cell after 6 months-storage (cfu mL^{-1})
T1	2.81 x 10^{11}	3.54 x 10^{8}
T2	2.51 x 10^{9}	4.12 x 10^{7}
T3	2.81 x 10^{11}	2.54 x 10^{12}
T2	2.51 x 10^{9}	3.27 x 10^{7}

Mother culture concentration was observed to increase after six months storage at cool temperature, indicating that the culture was still active at this temperature. The number of viable cells under room temperature storage condition maintained at 10^7 and 10^8 cfu mL^{-1} after 6 months storage, showing that the AP1 was good biofertilizer material. Higher viable count in low temperature allows no or little growth with less utilization of nutrients during storage, making them available to the organism in optimum concentration, and also protects the cell death in inoculums. Previous studies reported refrigerated conditions had higher viable count of phosphate-solubilising microorganisms. Velineni & Brahmaprakash (2011), reported *Bacillus megaterium* in liquid formulations supplemented with different cell protectants under the influence of high temperature and desiccation stress. *Pseudomonas* sp. strain P-36 amended with or without additives stored at refrigerator showed higher viable count as compared to inoculant stored to room conditions (Yadav et al., 2017). Phua & Rahim (2010) reported that nutrient broth liquid biofertilizer kept at low temperatures (9 ± 2 ^oC) showed significantly high survival rates after storage for six months as compared to other formulations and treatments, whilst Brar et al. (2012) reported liquid formulations can tolerate the temperature as high as 55 ^oC. Thus, it is suggested to carry out a further study on the effect of high temperature storage. Advantages of high temperature tolerance is no need specific cool storage then can reduce cost of storage.

CONCLUSIONS

Survival and shelf life of AP1, as indicated by viable population counts are maintained at around the initial populations at six months storage time at cool and room temperature. Thus, AP1 is considered as a good biofertilizer product since it meet the technical specification in terms of shelf life. This is crucial to demonstrate that GoGrow BioNPK Biofertilizer as good quality product where the viable cells of AP1 remained almost constant after storage for 6 months. It indicate there is not much requirement for cool storage at below 6 ^oC. Further studies for up to 12 months storage at high temperature are recommended. This will demonstrate suitability of storage under conditions normally found at users premises, while not compromising on product quality.

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