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Organic Carrier-Based Inoculant of Bacillus and Azotobacter Consortium

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Abstract

The use of biofertilizer is introduced for supporting soil health in sustainable agriculture. Spore-forming Bacillus and cyste-forming Azoto bacter are plant growt promoting rhizo bacteria which resist to dried soil. The purpose of the experiment was to select the particle size and water content of organic matter as well as concentration of zeolite for formulation of Bacillus-Azotobacter solid inoculant. The experiment consisted of two stages which set up in completely randomized design. Treatments of the first experiment were particle size and water content of composted cow manure; while the second one were the concentration of zeolite and liquid bacterial consortium. For both experiments, the formulated solid biofertilizer of Bacillus subtilis, B. megaterium, A. chroococcum and A. vinelandii then was analyzed for spore of Bacillus and vegetative cell of Azotobacter at 7, 14 and 28. Acidity and Electrical conductivity measurement of carrier-based multi-strain inoculant was performed at the end of second experiment. The results showed that no different bacterial growth up to 28 days in either 100 mesh or 200 mesh composted cow manure. Compost water content of 15% and 10% decreased population of Bacillus spore and Azotobacter. Solid carrier enrichment by use of 1% or 5% zeolite might be effective to enhance spore up to 1011 CFU/g and maintain Azotobacter cell viability up to 108 CFU/g. This study suggest that cow manure compost and zeolite may be used for Bacillus-Azotobacter solid biofertilizer.

Keywords: *Compost, bacterial population, carrier-based biofertilier, zeolite.*

I. INTRODUCTION

Biofertilizers that consist of single or mixed strain of beneficial microbes have been developed and commercialized. Commonly, there are two types of biofertilizers viz. liquid and solid carrier-based microbial inoculants. Commercial biofertilizers effectively increase soil fertility because they contain sufficient cells of efficient strains of specific microorganisms mainly plant growth promoting rhizobacteria (PGPR). These microorganisms live and proliferate in the rhizosphere and influence plant growth directly by among other fixing atmospheric nitrogen and solubilizing/mineralizing phosphorous [1]. PGPR also increase plant growth by phytohormones which affects the plant's hormonal balance and its response to stress [2].

Numerous commercial biofertilizer products contain *Bacillus* or *Azotobacter* as active ingredients are widely used. The genus of *Bacillus* is ubiquitous rhizobacteria in soil; and well known as phosphorous solubilizing bacteria while *Azotobacter* increase plant growth by non-symbiotic nitrogen fixation. The ability of

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both rhizobacteria to produce phytohormone was documented elsewhere [3],[4],[5]. In environmental stress condition, *Bacillus* and *Azotobacter* form resistant endo-spore and cyst respectively [6],[7] so that they can adopt to harsh soil condition mainly drought soil.

The formulation and commercial production of any biofertilizer requires the formulation leading to high active micororganism populations and long term survival of these organisms over time at less than optimum conditions. For commercial purpose, a good carrier material should be nontoxic, easy to sterilize and process, available in adequate amounts and inexpensive.

Moreover, particle size and moisture selection will relate to long-term bacterial survival in less optimum condition. Microbial absorption is occur in the carrier substance surface which relate to the particle size. Higher moisture content of carrier material may increase microbial cell proliferation but limited water induce spore and cyst formation.

Dry inoculants can be produced using different kind of inert material [8], vermiculite was the best inert material to enhance shelf life of multi-strain biofertilizer followed by zeolite, kaolin, perlite, talc, and bentonite [9].

Compost of cow manure is available in most agricultural region in Indonesia where animal cultivation taken place. Zeolite rocks are widely distributed in Java Island, Indonesia and has been commercialized. Zeolite powder as well as composted cow manure price is inexpensive so that it may be recommended to be used in carrier-based biofertilizer production.

In most cases, beneficial microbial inoculants consist of a single strain. Nowadays application of PGPR-rhizobia multi-strain biofertilizer to enhance growth and yield of food crops get more attention. Multi-strain mixture of biofertilizer have been developed due to better perform than single strains [10]. Co-inoculation of Pseudomonas, Bacillus, Stenotrophomonas, Serratia, Nocardia and Microbacterium as reported to increase plant growth compare with single bacterial inoculation [11].

The most limited factor of agricultural crops production in the tropic is low conent of Nitrogen and Phsophorous. Biofertilizer consist of N-fizing bacteria (NFB) as well as phosphate solubilizing bacteria PSB) might overcome that problem. Limited information is available about the carrier-based inoculant of PSB *Bacillus* and NFB *Azotobacter* consortium. The objective of the experiment was to select the particle size and water content of organic matter as well as concentration of zeolit for solid biofertilizer formulation contained multi-strain *Bacillus* and *Azotobacter*.

II. MATERIALS AND METHODS

A. Bacteria and Carrier Material

The experiments had been performed at November to December 2019 in the Soil Biology Laboratory, Faculty of Agriculture, Universitas Padjadjaran. Liquid microbial consortium for formulation of carrier-based biofertilizer was developed using *B. subtilis*, *B. megaterium*, *A. chroococcum* and *A. vinelandii* (Fig 1) in molasses-based media. The population of *Bacillus* spore in liquid inoculant was $>10^{10}$ CFU/mL enumerated by serial dilution plate method in Nutrient Agar [12]; and viable *Azotobacter* was $> 10^{7}$ CFU/mL counted by said method in N-free Ashby's mannitol agar [13]. Composted cow manure was used as biofertilizer carrier; the chemical properties of compost was neutral in pH contained 33.5% organic C, 1.8% total N, 22.3% water, 1.2% P_2O_5 and 3.7% K_2O.

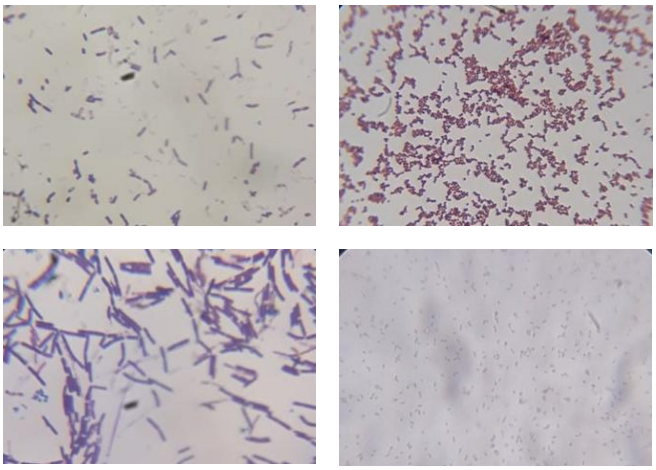

Figure 1. Gram staining of *B. subtilis, B. megaterium, A. vinelandii* and *A. chroococcum* (counterclockwise)

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B. Carrier Based-Biofertilizer Formulation

The formulation of solid biofertilizer consisted of two steps laboratory experiments. The first steps experiment was carried out using 100 mesh (149 µm) and 200 mesh (74 µm) composted cow manure with three level of water content, viz. 10%, 15% and 20%. The experiment was set up in Completely Randomized Block Design in which treatments was replicated 3 times. A total of 100 g compost was poured into aluminum foil bag, sterilized at 121 ^{0}C for 20 minutes and cooled overnight at room temperature. Bacterial consortium inoculation was performed by injecting 10% liquid inoculant and handshaking the bag evenly. All treated bags were stored at room temperature for 28 days. Population of *Bacillus* spore and *Azotobacter* vegetative cell was performed at 7, 14 and 28 days using serial dilution plate method with medium describe above.

Based on the first experiment results, the second trial was carried out using 200 mesh composted manure with 10% of water content. Manure was mixed with 0.1%, 1%, and 5% (w/w) zeolite with particle size of 100 mesh. The experiment was set up in Completely Randomized Block Design in which All treatments were replicated 3 times A total of 100 g carrier mixture was poured into sealed aluminum bag, sterilized at 121 ^{o}C for 20 minutes and keep overnight at room temperature. Bacterial consortium inoculation was done by injecting 10%, 15% or 20% (v/w) liquid inoculant into the bag and mixed thoroughly.

Bacterial population was counting from three replications for each incubation period. Counting of viable *Bacillus* spore were performed by serial dilution method on Nutrient Agar Plate after heating the bacterial suspension at 75 ^{0}C for 10 minutes in water bath [14]. Nitrogen-fixing *Azotobacter* count were carried out by said method on N-free Ashby's mannitol medium [13].

For both experiments, the dilution of sample was prepared by trasferring1 g of carrier-based biofertilizer in 9 mL sterilized 0.85% NaCl, shaking thoroughly for 5 minutes prior to further dilution. Final dilution was depended on the time of storage; from final dilution a total of 0.1 mL was taken and poured on nutrient agar or Ashby plates. The plate agar was incubated 30°C for 24-48 hours before the colony of *Bacillus* and *Azotobacter* were examined (Fig 2).

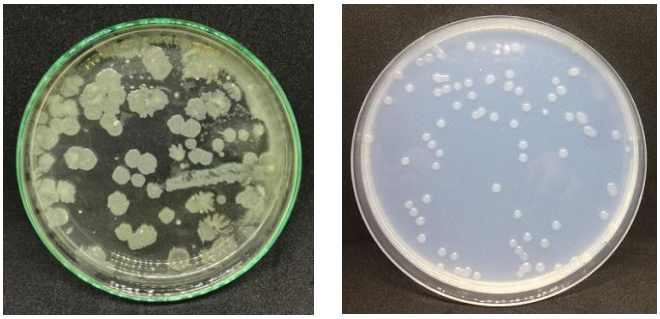

Figure 2. *Bacillus* and *Azotobacter* colonies appearance on Nutrient agar and Ashby's mannitol plate

C. Statistical Analysis

All data was subjected to analysis of variance with F test (p 0.05%). If the effect of treatments on bacterial parameter were significant the Duncan's Multiple Range Test (p 0.05%) was performed.

III. RESULTS AND DISCUSSION

Table 1 and Table 2 showed the survivability of *Bacillus* and *Azotobacter* in carrier based inoculant at different day of storage. Statistical analysis of first step formulation showed that effect of particle size and water content of compost on *Bacillus* spore population was significant at 7 days and 28 days after incubation but did not at 14 days. The lower size (200 mesh, 74 µm) of compost regardless of its water content significantly increased the colonies of *Bacillus* spore that grow on nutrient agar plate at 7-day storage (Table 1). At the end of storage, 10% water content of compost either with 100 mesh or 200 mesh particle size increased spore population.

There is an increase of spore in carrier-based inoculant between day 7 and day 28. Regardless the statistical analysis, the population of spore in every particle size and water content of compost reached 10^{10} CFU/g at day 28 when the experiment was terminated.

The effect of particle size and water content of the compost as bacterial carrier was significant on *Azotobacter* population only at day 14 (Table 2). The *Azotobacter* colonies on Ashby's plate was highest in the organic carrier with 200 mesh (74

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µm) in particle size and 10% in humidity. At the end of the storage, the population of *Azotobacter* in every treatment were similar, about 10^8 CFU/g. Based on the results of this first step formulation, 200 mesh compost with 10% water content will be used in the second step of the formulation.

Table 1. Effect of compost particle size and water content on the population of *Bacillus* spore in carrier-based inoculant

Compost particle size and water content	Spore population		
	Day 7 (10^6 CFU/g)	Day 14 (10^8 CFU/g)	Day 28 (10^{10} CFU/g)
100 mesh, 10%	11.32 b	13.00	3.63 b
100 mesh, 15%	11.16 b	12.62	2.78 a
100 mesh, 20%	7.68 a	12.70	1.05 a
200 mesh, 10%	15.14 c	12.65	4.54 b
200 mesh, 15%	14.24 c	13.03	2.99 a
200 mesh, 20%	14.17 c	12.98	2.97 a

Table 2. Effect of compost particle size and water content on *Azotobacter* population in carrier-based inoculant

Compost particle size and water content	*Azotobacter* population		
	Day 7 (10^5 CFU/g)	Day 14 (10^7 CFU/g)	Day 28 (10^8 CFU/g)
100 mesh, 10%	1.68	1.99 a	1.08
100 mesh, 15%	1.97	2.75 a	1.45
100 mesh, 20%	1.07	4.94 a	1.25
200 mesh, 10%	1.96	8.84 b	1.67
200 mesh, 15%	1.74	8.89 b	1.27
200 mesh, 20%	1.87	8.76 b	1. 57

At the 2nd experiment, count of spore colony forming unit in compost with different zeolite and initial mixed inoculant level was not differ except in the formulation with 1% zeolite with 15% mixed inoculant at day 7 (Fig 3).

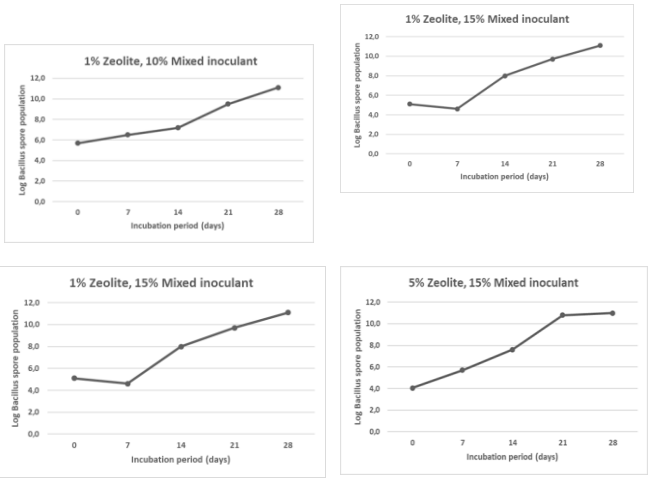

Figure 3. Effect of different zeolite concentration on *Bacillus* spore population in compost-based inoculant at two initial bacterial inoculant concentration during 28 days storage

In said formulation, spore population decreased up to 42,700 CFU/g which is clearly lower than other formulation. For all solid formulation, after 28-day storage the spore population was increased up to 10^{11} CFU/g. Higher final spore population was demonstrated by 5% zeolite with 10% initial mixed inoculant even though the value was not significant compared to other formulation.

Statistical analysis demonstrated that the effect of zeolite and initial mixed inoculant level on *Azotobacter* colonies in Ashby plate was not significant but did at day 7. The colony forming unit of compost-based solid biofertilizer with 5% zeolite and 15% initial inoculant was higher than other formulation (Fig 4). At the end of experiment, *Azotobacter* population of all formulation was similar (Fig 4).

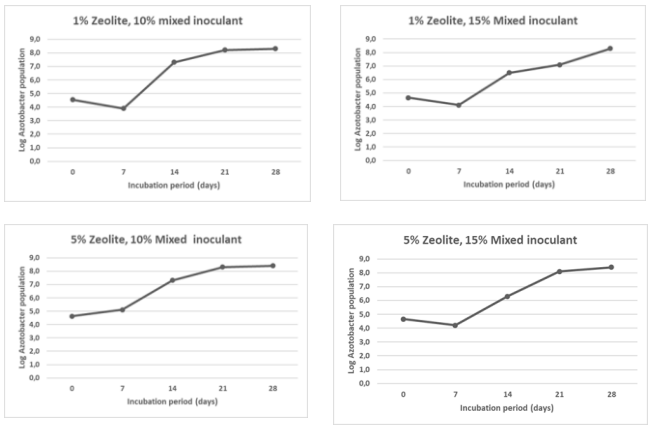

Fig 4. Effect of different zeolite concentration on

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Azotobacter population in compost-based inoculant at two initial bacterial mixed inoculant level during 28 day storage.

Population of active ingredient of solid biofertilizer depend on the quality of carrier [6] which in turn increase the shelf life. The prerequisite of carrier material is available throughout the year and not expensive. In Indonesia, mainly in Java Island, composted manure usually mixed with soil during soil tillage and land preparation. This agricultural practice makes solid biofertilizer application become easier. Even though shelf life of liquid biofertilizer is longer than carrier-based biofertilizer [15], it would be easier to incorporating during soil tillage and more acceptable by farmers due to similar physical properties with organic matter.

Table 1 and Table 2 demonstrated that lower size of compost may enhance bacterial population mainly the *Bacillus*. The said size is in accordance with the size of the powder carrier-material which is vary from 75 µm to 0.25 mm [8]. First step formulation showed that 74 µm compost size more supportive to bacterial proliferation during 28-day incubation. Particle size influence the surface area of solid material; in soils, most active bacteria are adhered on solid particles where they then attach and colonize the surface [16]. All of natural carriers absorb microbe in the substance/matrix of the carrier [17].

In second step formulation, effect of 1% of zeolite on both Bacillus spore and Azotobacter population at days 28 was similar to 5% zeolite (Fig 3 and Fig 4). Role of zeolite in solid biofertilizer formulation was to adsorb the water so that the water content of carrier material may be lower after enrichment with zeolite. Water adsorption capacity of zeolite was dictated by structural properties and particle size [18]. In this formulation, 5% zeolite enhanced the population of spore of Bacillus suggesting that low water content might induce spore formation and resistance to heat [19].

Indonesian Ministry of Agriculture strictly regulate the production and commercialization of either liquid or solid biofertilizer. Commercialized biofertilizer should have the acidity between 5-8, water content <35% and doesn't contaminated with Eschericia coli and Salmonella spp. All solid formulation of multi-strain *Bacillus* and *Azotobacter* was in accordance with that regulation. Electrical conductivity of all formula is moderate so it is safe to be used for crop production without salinity enhancement.

The experiment was terminated at day 28. Reduced in microbial count after several month shelf life of carrier based biofertilizer was reported. After five months, phosphate solubilizing bacteria (PSB) count in solid biofertilizer was reduced to 3.0×10^4 CFU/g [20]. At the end of six months, PSB and N-fixing bacteria in different carrier reduced sharply [21]. In this experiment bacterial population was monitored at weekly intervals up to one month. All bacterial population irrespective of carrier formulation were enhance from day7 to day 28. Further monitoring should be done to check whether the decreased of bacterial viability will retain the bacterial population up to 10^7 CFU/g as a minimal population in Indonesia Regulation on Biofertilizer.

Table 3. Properties of compost-based multi-strain biofertilizer at different zeolite concentration inoculated with 10% and 15% liquid inoculant of *Bacillus* and *Azotobacter*

Formulation in 200 mesh compost	Acidity	EC* (ds/m)	Contaminant (MPN/g)	
			E. coli	Salmonella
1% zeolite, 10%	7.8	4.7	< 10^3	< 10^3
1% Zeolite, 15%	7.8	5.5	< 10^3	< 10^3
5% Zeolite, 10%	8.1	5.1	< 10^3	< 10^3
5% Zeolite, 15%	8.1	5.8	< 10^3	< 10^3

*Electrical Conductivity, All values were calculated from three replication.

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CONCLUSION

Different particle size and water content of compost as carrier material has been used successfully for formulation of multi-strain of *Bacillus-Azotobacter* biofertilizer. Particle size and water content of compost as carrier material only influence the population of *Bacillus* spore that will be germinated to high water content. The population of *Bacillus* spore and *Azotobacter* vegetative cell in carrier-based formulation was increased by mixing the compost with 1% or 5% zeolite powder. After 28-day storage, the spore population of 200 mesh compost with 10% water content enriched with 100 mesh zeolite powder was $>10^{10}$ CFU/g; while the *Azotobacter* population was 10^8 CFU/g.

Using compost cow manure enriched with zeolite for solid biofertilizer production is prospective since compost and zeolite powder are available and the price are affordable. The importance of biofertilizer development in tropics is to maintain soil health and hence ensure sustainable agriculture. Moreover, biofertilizer may reduce the chemical fertilizer doses and minimize the soil damage due to excess chemical fertilizer**.** Indonesia is a tropical country where agriculture is often carried out in low fertility soil, mixed fertilization using biofertilizer and chemical fertilizer is suggested to maintain the optimum availability of nutrient for root uptake.

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