Bacillus and azotobacter counts in solid biofertilizer with different concentration of zeolite and liquid inoculant

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Abstract. Bacillus and Azotobacter sp are Plant Growth Promoting Rhizobacteria widely used as biofertilizer. The success of commercial carrier-based biofertilizer is depend on formulation which dictate the shelf live and cell viability as well. A laboratory experiment was conducted to evaluate the population of Bacillus and Azotobacter in compost-based solid biofertilizer with several zeolite and liquid inoculant concentration. The experiment was conducted in completely randomized design which test the combination treatment of three concentration of zeolite (0.1, 1 and 5%) and four concentration of initial liquid mixed inoculant (1, 5, 10 and 15%). All formulations were incubated at room temperature prior to count the Bacillus and Azotobacter population at day 7, 14, 21 and 28. The results verified that, the population of both bacteria in compost-based inoculant were higher than initial population. The zeolite and liquid inoculant concentration influenced Bacillus count at 7 days and Azotobacter count at 7 and 14 days. Nonetheless the higher population of both bacterial species was in the compost-based biofertilizer contained 1% zeolite and 10% of liquid inoculant as well as 5% zeolite and 5% liquid inoculant. Both formulations maintain the Bacillus and Azotobacter population up to 12 and 10 log of cfu/g respectively.

1. Introduction
Due to conversion of agricultural land to mainly infrastructure, factory and building, food production requires an intensive input in limited area to maintain sustainable agriculture. Proper fertilization with balance composition of chemical, organic and biofertilizer is a key to the success of increasing production. Nowadays the used of Plant Growth Promoting Rhizobacteria (PGPR) as biofertilizer is in progress. The rhizobacteria enable to colonize the rhizosphere, and supply primary nutrient through mainly Nitrogen (N) fixation, phosphorus solubilizing and siderophore production; and plant growth factor to the plants [1]. The phosphate-solubilizing Bacillus sp. and nitrogen (N)-fixing Azotobacter sp are widely used in biofertilizer formulation. Both bacteria produced phytohormones [2], [3].

Biofertilizer formulation is an important step before the microbes being introduced into the soil. Generally either single or multi-strain biofertilizer are developed in liquid or carrier-based formulation. The selection of media should assure the growth of target microbes and physiologically active microbes under certain conditions, and support plant responses [4]. The formulation of multi-strain biofertilizer is an important step to increase the effectiveness of the inoculant. Different function of PGPR in single formulation assure the multiple role of PGPR to increase plant growth and yield. The Bacillus has the ability to liberate phosphate from unavailable P inorganic by excreting organic acids.

The B. megaterium cultured with fish bones (as the source of unavailable P) released 483 ± 5 mg/L of available P [5]. The genus of Azotobacter is well known nonsymbiotic N-fixing bacteria; activity of six Azotobacter isolates were 6-15 mmol C2H4/mg protein/d [2].

Survival of the microbes in the formulation will dictate the effectiveness of microbes after being inoculated to the soil [6]. The main active ingredients of biofertilizers are living cells that are more
susceptible to environmental changes than chemical fertilizers. In solid inoculants, microbes are intolerant of UV rays and temperatures of more than 30 °C [7]. It was also explained that the density of the microbes in solid inoculants was only 10^{8} cfu/mL at the time of production which decreased to 10^{6} cfu/mL at the fourth month during storage. So that the selection of carrier material for solid inoculant is important.

Carrier-based biofertilizers generally being formulated in crop residue such as compost, manure compost, peat, and also inert materials of lime, zeolite and vermiculite. Locally available carriers are recommended to ensure the availability. The research about single strain formulation of Bacillus and Azotobacter has been done elsewhere. The cocharcoal and talc-based and charcoal-based formulation of Bacillus has been developed [8]. Biogas sludge mixed with rock phosphate were used as carrier material for B. endophyticus and B. sphaericus [9]. In sterilized carrier materials, the population of Azotobacter during 3-month storage increased up to 15.3-15.7 log10 cfu/g in clay, peat moss, rice grain, wheat bran and wheat bran + vermiculite carrier material [10]. Mixed inoculant of Azotobacter, Azospirillum, Bacillus and Pseudomonas in fine wood charcoal powder contained at least 2×10^{6} CFU/g [6].

Formulation of Bacillus-Azotobacter mixed biofertilizer has not been developed. In preparing to delopped microbe-coated urea, the previous study we found that composted cow manure with particle size of 200 mesh and around 20% of humidity supported the growth of Bacillus and Azotobacter [11]. Inorganic materials such as zeolite, talc or calcium carbonate are required to reduce the moisture of solid inoculants. In dry condition, Bacillus and Azotobacter formed the dormant endospores and cysts respectively. The low humidity will maintain the dormancy of Bacillus and Azotobacter cells so that the viability of bacteria being maintained when applied to the soil. Zeolites are natural hydrated aluminosilicates utilized in agriculture due to the ability in retaining water and minimizing nutrient losses [12]. The objective of this experiment was to evaluate the population of Bacillus and Azotobacter in compost-based solid inoculant with several zeolite and liquid inoculant concentration during 28-day storage.

2. Material and method

The research has been done in Soil Biology Laboratory, Faculty of Agriculture, Universitas Padjadjaran located in Jatinangor Campus, Sumedang, Indonesia. The experiment was carried out at December 2019 to February 2020. The Bacillus subtilis, B. megaterium, A. chroococcum and A. vinelandii were the collection of Soil Biology Laboratory. All bacteria were isolated from different food crops rhizosphere.

Liquid inoculant was prepared by using molasses-based media [13]. The basal liquid media consisted of 1% of molasses and 0.1% ammonium chloride. The media then was sterilized at 121°C for 20 minutes and stored overnight at room temperature (23-24°C). A total of 1% mother culture of each species were inoculated to separated 250 mL molasses-based liquid media, and placed on the gyraction shaker at room temperature for 3 days. The equal volume of each Bacillus and Azotobacter species were mixed thoroughly and then stored for another 7 days. The population of Bacillus and Azotobacter in mixed liquid inoculant at day 7 were 5×10^{11} cfu/mL and 7×10^{8} cfu/mL respectively.

2.1. Experimental design

The laboratory experiment was arranged in completely randomized design which test 12 combinations of three level of zeolite concentrations (0.1%, 1% and 5%; w/w) and four level of inoculant concentration (1%, 5%, 10% and 15%; v/w). All treatments were repeated three times. The different formula was stored for 28 days at room temperature without direct sunlight. At day 7, 14, 21 dan 28 the population of Bacillus and Azotobacter were counted by serial dilution plate method. The media for Bacillus was Tryptone Soy Agar (15 g Pancreatic digest of casein, 5 g Enzymatic digest of soya bean, 5 g Sodium chloride, 15 g agar, pH 7), while for Azotobacter was N-free Ashby mannitol (20 g Mannitol, 0.2 g Dipotassium phosphate, 0.2 g Magnesium sulphate, 0.2 g Sodium chloride, 0.1 g Potassium sulphate, 5 g Calcium carbonate, 15 g Agar, pH 7). All data were subjected to Analysis of variance (p<0.05) and Tukey honestly significant difference test (p<0.05).

2.2. Experimental setup

The carrier material for solid biofertilizer was 50 g of 200-mesh compost powder with 22.3% moisture content mixed with 100-mesh zeolite. The concentration of zeolite was depend on the treatment. The mixture was then put into the aluminum bag of 14 cm in width and 23 cm in height, then sealed with
electric sealer and sterilized for 20 minutes at 121°C. All carrier materials were stored overnight at room temperature without direct exposure to sunlight prior to inoculate with the bacterial consortium liquid inoculant. The inoculation of bacterial consortium was carried out by using a sterile 10 mL syringe with the volume according to the treatment. The carrier and liquid inoculants in aluminum bags were mixed evenly by hand shaking. All solid biofertilizers formulations were incubated at room temperature without direct sunlight for 28 days.

3. Results
The Bacillus and Azotobacter as well were proliferate in manure-based solid inoculant irrespective of zeolite and bacterial liquid inoculant. The viability of both PGPR were clearly showed in the plate agar (Fig. 1). There was the increased in both bacterial counts from days of incubation to 28 days after storage (Table 1 and Table 2).

![Figure 1. The colony morphology of Bacillus on tryptone soy plate agar and Azotobacter on free-N Ashby plate agar.](image)

Three hours after inoculation, the number of Bacillus and Azotobacter counted by serial dilution plate method was between 6-7 log cfu/g and 3-4 log cfu/g (Table 1 and 2). The population was increased at 7 to 21 days after inoculation but both bacterial number remained constant at day 28 compared to day 21 day. Based on Tukey test, the compositions of solid inoculant affected the population of Bacillus population significantly at 7 days after storage; in general carrier material with 1% zeolite contained lower Bacillus population (Table 1). Between 14 – 28 days, the population of Bacillus was similar in all compositions. At 28 days, regardless of zeolite and liquid inoculant concentration, Bacillus population in solid inoculant was around 12 log cfu/g. Solid inoculant with 1% zeolite mixed with 10% liquid inoculant showed the highest Bacillus counts although was not significantly different with other composition.

Composition of solid inoculant affect Azotobacter number at day 7 and 14 (Table 2). At day 7, addition of 5% zeolite leads to increase Azotobacter population but the increase in Azotobacter count at 14 days was depend on certain combination of zeolite and liquid inoculants. Similar to Bacillus population, the composition of zeolite and liquid inoculant in solid biofertilizer didn’t affect Azotobacter population at day 21 and 28. Irrespective of statistical analysis, the highest count of Azotobacter at day 28 was showed by solid biofertilizer with 5% initial liquid inoculant enriched with 5% zeolite; followed by 1% zeolite and 10% liquid inoculant.
Table 1. Effect of zeolite and liquid inoculant concentration on Bacillus population up to 28 days after inoculation.

| Zeolite and liquid inoculant concentration in carrier material | Bacillus population at 7-28 days after inoculation (log of cfu/mL) |
|---------------------------------------------------------------|---------------------------------------------------------------|
|                                                               | Three hours after inoculation | 7      | 14    | 21    | 28    |
| A: 0.1% Zeolite + 1% LI                                       | 6.96 a                        | 8.44 b | 9.23 a | 11.80 a | 11.65 a |
| B: 0.1% Zeolite + 5% LI                                       | 6.98 a                        | 9.08 a | 9.35 a | 11.75 a | 11.96 a |
| C: 0.1% Zeolite + 10% LI                                      | 8.03 a                        | 9.21 a | 9.36 a | 11.55 a | 11.46 a |
| D: 0.1% Zeolite + 15% LI                                      | 8.8 a                         | 8.28 b | 9.33 a | 11.91 a | 12.05 a |
| E: 1% Zeolite + 1% LI                                         | 6.02 a                        | 8.93 b | 9.48 a | 11.84 a | 11.82 a |
| F: 1% Zeolite + 5% LI                                         | 8.04 a                        | 8.97 b | 9.27 a | 11.76 a | 12.02 a |
| G: 1% Zeolite + 10% LI                                        | 7.85 a                        | 8.85 b | 9.23 a | 11.89 a | 12.11 a |
| H: 1% Zeolite + 15% LI                                        | 9.98 a                        | 9.08 a | 9.53 a | 11.89 a | 12.03 a |
| I: 5% Zeolite + 1% LI                                         | 6.01 a                        | 9.09 a | 9.19 a | 11.94 a | 12.06 a |
| J: 5% Zeolite + 5% LI                                         | 8.07 a                        | 9.11 a | 9.50 a | 11.85 a | 12.09 a |
| K: 5% Zeolite + 10% LI                                        | 7.64 a                        | 9.08 a | 9.27 a | 11.93 a | 12.05 a |
| L: 5% Zeolite + 15% LI                                        | 8.15 a                        | 9.15 a | 9.26 a | 11.75 a | 12.00 a |

*Liquid inoculant Numbers in a column followed by the same number were not significantly different based on Tukey different test at p<0.05

4. Discussion

Biofertilizers are microbial inoculants which usually contain living or inactive cells from beneficial microbial strains. The experiment demonstrated the increase in both Bacillus and Azotobacter population in all solid inoculant composition. Both bacteria are heterotrophic that use organic matter as energy, carbon and even N sources to maintain the metabolisms. The increase of bacterial number is a prove that they proliferate in the compost during storage. Before the trial, the compost powder contained 1.8% N, 1.2% P2O5 and 3.71% K2O with acidity of 6.21 and water content of 22.3%. The compost was grounded to 200-mesh particle size. The lower particle size has higher surface area and leads to higher bacterial-organic matter interaction. In early stages of incubation, the heterotroph decomposed dissolved organic matter of compost powder in order to obtain C and energy and leads to change of microbial composition [14]. The interaction between organic matter particle and microbes might be bidirectional; soil particle controls the survival and biological activity by controlling the physicochemical properties, microbes modified the organic matter particle mainly by weathering and biodegradation of particle which release available nutrients [15].

The humidity of organic matter was 22.3% and will be decreased when zeolite was added since the later adsorb the humidity. The solid formulation with zeolite ensure the cell protection by zeolite surface adsorption. The micro pores volume and surface area of zeolite powder were reported around 0.25 cm³/g and 754 m²/g [16] higher than clayed soil surface area of 208 m²/g [15]. The bacterial cells adsorbed on zeolite surface and protect them from harsh environment. Adhesion of Gram-negative bacteria to zeolite was less than Gram-positive bacteria because of different structure of cell wall [17].

The results showed that the population of Bacillus and Azotobacter at 21-day storage was not differ with that at the end of storage. The proliferation of both bacteria was being ceased due to limited nutrient and space; then the vegetative cell of both bacteria produces dormant cell. Bacillus species produce endospores [18] while Azotobacter formed cysts [19]. At day 28, Spore germination in plat agar was triggered by nutrient availability initiated by spore rehydration and protective spore break up [18] while cysts germination might be induced by exogenous carbon source in N-free media [20].
Table 2. Effect of zeolite and liquid inoculant concentration on Azotobacter up to 28 days after inoculation.

| Zeolite and liquid inoculant concentration in carrier material | Azotobacter population at 7-28 days after inoculation (log cfu/mL) | Three hours after inoculation |
|---------------------------------------------------------------|---------------------------------------------------------------|-------------------------------|
|                                                               |                                                               | 7    | 14   | 21   | 28   |
| A: 0.1% Zeolite+1% LI                                        |                                                               | 3.73 a | 5.52 b | 6.75 a | 9.89 a | 9.30 a |
| B: 0.1% Zeolite+5% LI                                        |                                                               | 3.94 a | 5.62 b | 6.59 b | 10.18 a | 9.35 a |
| C: 0.1% Zeolite+10% LI                                       |                                                               | 4.15 b | 5.82 a | 7.58 b | 10.01 a | 10.26 a |
| D: 0.1% Zeolite+15% LI                                       |                                                               | 4.47 b | 5.53 b | 7.44 a | 9.97 a | 10.39 a |
| E: 1% Zeolite+1% LI                                          |                                                               | 3.71 a | 5.59 b | 7.45 b | 9.94 a | 10.01 a |
| F: 1% Zeolite+5% LI                                          |                                                               | 3.48 b | 5.57 b | 7.52 b | 10.01 a | 10.29 a |
| G: 1% Zeolite+10% LI                                         |                                                               | 4.13 b | 5.53 b | 8.13 a | 9.89 a | 10.42 a |
| H: 1% Zeolite+15% LI                                         |                                                               | 4.64 a | 5.61 b | 7.99 a | 9.96 a | 10.40 a |
| I: 5% Zeolite+1% LI                                          |                                                               | 4.72 a | 5.73 a | 7.72 a | 10.11 a | 10.41 a |
| J: 5% Zeolite+5% LI                                          |                                                               | 4.18 b | 5.68 a | 7.63 b | 9.88 a | 10.48 a |
| K: 5% Zeolite+10% LI                                         |                                                               | 4.10 a | 5.69 a | 7.67 b | 10.09 a | 9.37 a |
| L: 5% Zeolite+15% LI                                         |                                                               | 4.65 a | 5.68 a | 8.36 a | 10.18 a | 10.34 a |

*Liquid inoculant
Numbers in a column followed by the same number were not significantly different based on Tukey different test at p<0.05

The composition of solid inoculant didn’t affect Bacillus and Azotobacter population at the end of experiment. The ranges of liquid inoculant concentration in this experiment were short (1, 1.5, 5 and 10%) so that the number of Azotobacter cell of different inoculant treatment in the carrier was quite similar. The concentration zeolite also didn’t influence the cell number. Nonetheless the results showed that all carrier-based formulation of Bacillus-Azotobacter formulation supported the cell number above the threshold suggested by Agricultural Minister Regulation no 261 year 2019. According to that regulation, the population of bacteria in solid biofertilizer is ≥ 10^7 cfu/g (7 log10 of cfu/g).

To ensure the cell dynamic of this solid inoculants, storing the inoculant for at least 6 months at the same room condition should be done. After six months the bacterial population in general will be decreased but the population of Azotobacter of single strain inoculant has been shown to increase after 3-month storage [10], in the contrary, the decline in the bacterial population of carried based Rhizobium and Phosphate solubilizing bacteria inoculant was significant after 3-month storage [21].

5. Conclusion
This experiment verified that at day 28 statistically the population of both bacteria was similar. At 28 days, the population of both Azotobacter and Bacillus in compost-based inoculant enriched with zeolite were higher than the population at 3 days after inoculation. In general, the zeolite and initial liquid inoculant concentration increased Bacillus count at 7 days and Azotobacter count at 7 and 14 days although statistically were non significant. The relatively higher population of both bacterial species was in the two composition of solid biofertilizer: 1) 1% zeolite and 10% of liquid inoculant and 2) 5% zeolite and 5% liquid inoculant. Both formulations keep the Bacillus and Azotobacter population up to 12 and 10 log of cfu/g respectively, higher than bacterial population of Indonesia standard for solid biofertilizer.
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