Microbe population in biofertilizer with vermicompost as a carrier during the process and storage

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Abstract. The main problem with biofertilizer is a short storage life. Carrier materials of biofertilizer include determinants of quality and storage life. This study measured the ability of vermicompost to be used as a carrier of biofertilizers from various beneficial microbes. The study used a factorial randomized block design with 2 factors and 3 replications. The first factor was the worm and microbial application technique consisting of 4 treatments: no worm; worm applied one week after applying microbes; microbial and worm applications were applied simultaneously and microbes applied one week after applying worm. The second factor was the type of beneficial microbes consisting of 4 treatments: Azospirillum sp.; Azotobacter chroococcum; Trichoderma asperellum G strains and Talaromyces pinophilus. The results obtained that after 3 and 6 weeks incubation the highest population was found at T. pinophilus (59.5-210.5 x 10¹⁰ CFU mL⁻¹) and at A. chroococcum (83.5-190.5 x 10¹⁰ CFU mL⁻¹, respectively. After 1 year storage, the population of A. chroococcum in the carrier biovermi decreased to 50 - 90 x 10⁵ CFU mL⁻¹, T. asperellum 60 - 210 x 10⁶ CFU mL⁻¹ and Azospirillum 75 x 10⁷ - 32 x 10⁸ CFU mL⁻¹, while T. pinophilus 60 x 10⁶ - 45 x 10⁸ CFU mL⁻¹.

1. Introduction
Fertilizers are an important element in agriculture that must be given to plants to achieve production targets. Fertilizing using chemical fertilizers in Indonesia has long been carried out. The intensity of its use has increased since the green revolution and made it as one of the pillars of agricultural success. The improper use of fertilizers and other agricultural materials results in a decrease in the carrying capacity of the land. In addition, the manufacture of chemical fertilizers requires high energy, where the energy source comes from fossil fuel which will thin out if continuously mined. This decline in petroleum reserves will directly affect the production of chemical fertilizers. Furthermore, a decrease in fertilizer availability will reduce crop production. In addition to chemical fertilizers, there are also other sources of nutrients, namely organic fertilizers and biofertilizers. The problem with biofertilizer is the length of the manufacturing process and the relatively short storage life and quality that is still focused on microorganisms only.

Earthworms have the ability to vector microorganisms and their cast have high nutrient availability and microorganism populations. In the intestines of worms, various types of enzymes were found [1]. In earthworm cast, there are more microorganism populations than the surrounding soil [2], this makes the idea to utilize earthworms as carriers of biofertilizer. The results of [3] showed that epigeic earthworms can be used to make carriers, and of seven beneficial microbes that were tested only 5 of them were suitable for life in carriers using worms namely A. chroococcum, Azospirillum sp., T.
*pinophilus* and *T. asperellum* strain G. This research was conducted to determine the ability of carriers containing earthworms in maintaining microbial populations during storage.

2. Materials and Methods
This research was conducted at the Soil Biology and Biotechnology Laboratory of the Faculty of Agriculture, University of North Sumatra from February 2018 to March 2019.

2.1. Materials
The materials used were epigeic earthworm earthworm, *Azotobacter chroococcum* (180 x 10⁸ CFU mL⁻¹), *Azospirillum* sp (42 x 10⁹ CFU mL⁻¹), *Talaromyces pinophilus* (70 x 10⁸ CFU mL⁻¹), *Trichoderma asperellum* strain G (50 x 10⁸ CFU mL⁻¹), cow dung and paddy straw, Okon media, Pikovskaya media and Jensen media.

2.2. Methods
The study was conducted to evaluate the application techniques of various types of microbes that have the highest microbial population in the bio-vermic carrier. The study used a factorial randomized block design with 2 factors and 3 replications. The first factor was the worm and microbial application technique (A) consisting of 4 treatments i.e., A₀ = no worm; A₁ = worms applied one week after applying microbes; A₂ = Microbe and earthworm applied at same time, and A₃ = microbes applied one week after applying worm. Factor 2 was the type of microorganism (M) consists of 4 treatments, i.e., M₁ = *Azospirillum* sp; M₂ = *Azotobacter chroococcum*; M₃ = *Trichoderma asperellum* strain G and M₄ = *Talaromyces. pinophilus*. Data were analyzed using Analysis of Variance (ANOVA) for each parameter measured and tested further on treatments that significantly affected by Duncan's Multiple Distance Test (DMRT) at the level of 5%.

2.3. Implementation
The media used as a carrier material was a mixture of cow dung and a paddy straw with a ratio of 1:7 (350 g of cow dung: 50 g of mashed paddy straw) sterilized by wet sterilization method. The results of the initial analysis of pH, nutrient content from organic matter mixed with cow dung and straw were as follows pH 7.23; organic C 40.31%; N total 0.89%; P₂O₅ total 0.83%; K₂O 1.25%; C/N ratio of 45.29 with water content of 66.43%. A total of 20 mL of 10 microbial liquid inoculums was put into earth-mixed medium of cow manure + sterile rice straw according to the treatment.

2.4. Observation Parameters
Parameters observed were:1. Scanning Electron Microscope biovermi of media with and without earthworm: Analysis was carried out using Scanning Electron Microscope EVO MA-10 ZEISS. 2. Microbial population during manufacturing process 3 and 6 weeks after microbial application. 3. Microbial population dynamics: Microbial population dynamics are known from the difference between the first observation population and the second observation population (Δ population). 4. Microbial population during storage power test: The storage power test is stored in one (1), three (3) and twelve (12) months.

3. Results

3.1. Characterization of Scanning Electron Microscope (SEM) EDS
The treatment that became the SEM sample of EDS was treatment with / without earthworms which were inoculated with *Azospirillum* sp and in the treatment with / without earthworms which were inoculated by *T.asperellum.*
Figure 1. Micrograph (left) and analysis of EDS (right) on treatment without worms with *Azospirillum* sp.

Figure 2. Micrograph (left) and analysis of EDS (right) on the treatment of worms with *Azospirillum* sp.

Figure 3. Micrograph (left) and analysis of EDS (right) on treatment without worms with *T. asperellum*
Figure 4. Micrograph (left) and analysis of EDS (right) on treatment of worms with *T. asperellum*

Micrograph treatment without earthworms with *Azospirillum* sp. at 1299 x magnification, the compost structure was formed from 40 μm of fine assembled granules (Figure 1). Worm treatment with *Azospirillum* sp. 1000x magnification has a 50 μm compost structure (Figure 2). The treatment without earthworms with *T. asperellum* at 1000x magnification has a structure of 50 μm (Figure 3). Micrographs of earthworm treatment with *T. asperellum* at 1000x magnification having a size of 50 μm seemed more homogeneous than treatment without worm application (Figure 4).

3.2. Microbial Population during incubation period

Worm application techniques, types of microbes and their interactions significantly influence microbial populations at 3 WAA and 6 WAA (Figure 5 a,b).

![Micrograph and EDS analysis](image)

**Figure 5.** Worm application techniques, types of microbes and their interactions significantly influence microbial populations at (a) 3 weeks incubation and, (b) 6 weeks incubation (WAA)

Note: A₀ = no worm; A₁ = worms applied one week after applying microbes ; A₂ = Microbe and earthworm applied at same time, and A₃ = microbes applied one week after applying worm ; M₁ = *Azospirillum* sp; M₂ = *Azotobacter. chroococcum*; M₃ = *Trichoderma asperellum* strain G ; M₄ = *Talaromyces. pinophilus*

Three weeks after microbial application (WAA), the population of all types of microbes in all application techniques increased. The results showed the growth of each type of microbe was different
for each application method. *T. cepacia, T. asperellum* and *Azo spirillum* fungi were growth well if applied a week after applying worms. However this was not similar happen for *Azotobacter*. The microbial population at 6 MSA also showed different growth responses for different application techniques. The population of *Azospirillum* and *T. cepacia* were high on application techniques 1 week after the worm was given. At 6 weeks after application, *Azotobacter* growth was best in application techniques after earthworms (Figure 5 a, b).

### 3.3. Dynamic of microbial population

The microbial population dynamics during the process of making biofertilizers using the carrier bio-vermi (Figure 6). Fluctuations occur in microbial populations from observations of 3 WAA and 6 WAA (Weeks After Application). Mostly, the microbial population increase in the treatment worm application followed by microbial application. The highest increase was found at *A. chroococcum*. Whereas the most extreme microbial population reduction was found in the treatment of worm application followed by microbial application with *T. asperellum*, and the population dropped in almost all application techniques except for microbial application along with earthworms.

![Figure 6. Microbial population dynamics during the manufacturing process (3 and 6 WAA)](image)

### 3.4. Microbial population after storing 1, 3 and 12 months

Worm application techniques and types of microbes in carrier production have a significant effect but their interactions have no significant effect on microbial populations in a one-month shelf life test (Table 1). The highest microbial population was found in carriers with microbial application after 1 week of worm application $129.6 \times 10^{10}$ CFU mL$^{-1}$ which was significantly different from other application techniques.
Table 1. Microbial populations of several types of microbes on earthworm application techniques after being stored for 1 month

| Treatments                              | Azospirillum | Azotobacter chroococcum | Trichoderma asperellum | Talaromyces pinophilus | Mean      |
|-----------------------------------------|--------------|--------------------------|------------------------|------------------------|-----------|
| Without earthworm                       | 32.5         | 97.5                     | 28.5                   | 46.0                   | 51.1d     |
| Microbe applied before earthworm        | 102.5        | 173.0                    | 81.5                   | 78.0                   | 108.8b    |
| Microbe and earthworm applied at same time | 75.5       | 142.5                    | 47.0                   | 65.5                   | 82.6c     |
| Microbe applied after earthworm         | 131.5        | 195.0                    | 112.5                  | 79.5                   | 129.6a    |
| Mean                                    | 85.5b        | 152.0a                   | 67.4c                  | 67.3c                  |           |

Note: Numbers followed by the same notation in the same column show no difference according to Duncan's Multiple Distance Test at the level of 5%.

The lowest microbial population was found in carriers without worm application $51.1 \times 10^{10}$ CFU mL$^{-1}$. The highest microbial population was found in microbes $A. chroococcum 152 \times 10^{10}$ CFU mL$^{-1}$ which was significantly different from other types of microbial populations. While the lowest population was found in $T. asperellum 67.4 \times 10^{10}$ CFU mL$^{-1}$ which was not significantly different from $T. pinophilus$ microbes $67.3 \times 10^{10}$ CFU mL$^{-1}$.

All microbial populations declined after 3 months of storage. The results showed that the carrier-making technique (application of earthworms and types of microbes) significantly affected the population. The population of $T. pinophilus$ was highest compared to other microbes, the population of $Azospirillum$ was higher than $Azotobacter$. Microbial application 1 week before the worm resulted in the best population for $T. pinophilus$ (Figure 7). The population of each microbe after 1 year of storage showed a drastic decrease especially in $Azotobacter$ and $T.asperellum$. The population of $Azospirillum$ and $T. pinophilus$ was still $10^8$, meaning that it was still suitable as a biofertilizer, but not for $Azotobacter$ and $T.asperellum$ whose population were $<10^8$ CFU mL$^{-1}$ (Table 2).
Figure 7. Microbial populations of several types of microbes on earthworm application techniques after being stored for 3 months. Note: A₀ = no worm; A₁ = worms applied one week after applying microbes; A₂ = Microbe and earthworm applied at same time, and A₃ = microbes applied one week after applying worm; M₁ = Azospirillum sp; M₂ = Azotobacter chroococcum; M₃ = Trichoderma asperellum strain G; M₄ = Talaromyces pinophilus.

Table 2. Microbial populations of several types of microbes on earthworm application techniques after being stored for 12 months

| Treatment                                      | Azospirillum | Azotobacter chroococcum | Trichoderma asperellum | Talaromyces pinophilus |
|------------------------------------------------|--------------|--------------------------|-------------------------|------------------------|
| Without earthworm                              | 75x10⁷ CFU mL⁻¹ | 50x10⁵ CFU mL⁻¹            | 60 x 10⁶ CFU mL⁻¹          | 60 x 10⁶ CFU mL⁻¹        |
| Microbe applied before earthworm               | 17x10⁸ CFU mL⁻¹ | 60 x 10⁵ CFU mL⁻¹            | 50 x 10⁶ CFU mL⁻¹          | 110 x10⁷ CFU mL⁻¹        |
| Microbe and earthworm applied at same time     | 32x10⁸ CFU mL⁻¹ | 80 x 10⁵ CFU mL⁻¹            | 210 x 10⁶ CFU mL⁻¹          | 45 x 10⁸ CFU mL⁻¹        |
| Microbe applied after earthworm                | 12 x10⁸ CFU mL⁻¹ | 90 x 10⁵ CFU mL⁻¹            | 90 x 10⁶ CFU mL⁻¹          | 31x10⁸ CFU mL⁻¹          |

4. Discussion
Observations using an electron microscope show that the particle and pore size found in the carrier uses smaller and uniform earthworms. Earthworm casts are more stable than soil macroaggregates [4]. They can also use the parameters for a long time, depending on temperature and moisture conditions, changing their nutrient contents and microbial properties in a process known as cast aging [5][6]. So that the microbial population (bacteria and fungi) in carriers with earthworms is higher than without earthworms. Population fluctuations from microbes from the time of manufacture and storage are natural. During the manufacture of 6 weeks after application, the population of *A. chroococcum* is the highest population compared to other microbes. With increasing age from the carrier the population of *A. chroococcum* decreased to the lowest (10⁵ CFU mL⁻¹) from other microbes. While the population of *Azospirillum* and *T. pinophilus* at 1 year storage age still reached 10⁸ CFU mL⁻¹, which according to the biological fertilizer
category is still feasible to use. A change in microbial composition in casting depending on the age of the casting [7]. Fresh cast microbiomes were composed of Bacteroidetes and Proteobacteria (62% of the total sequences) and in a lesser extent, of Acidobacteria, Chloroflexic Actinobacteria, Planctomycetes and Verrucomicrobia (36% of the total sequences). Copiotrophic bacteria (Alphaproteobacteria but not Bacteroidetes) were significantly (P <0.0001) decreased in abundance with cast aging, while oligotrophic bacteria (Actinobacteria, Acidobacteria and Deltaproteobacteria) were increased (P <0.03) in their proportion. The cause of this change in bacterial composition corresponds to decreased patterns in labile C and N pools in cast aging. T.asperellum fungus can be said to be unsuitably stored in this carrier by the presence of earthworms or without earthworms. At 3 months of storage the population was only 10^8 CFU mL^-1, and 1 year population storage decreased to 10^6 CFU mL^-1. The relative abundance of firm and bacteria that were increased by earthworms and zeolite addition in the late stage was related to increased temperature, decreased NH_4^+ contents [8]. Temperature showed a negative relationship with NH_4^+ exhibited positive associations with Georgenia, Devosia, Lithuania and Mycobacterium. These results are indicated by earthworm casts and zeolite addition benefited from the keystone species and enhanced the metabolic capacity of bacterial community.

5. Conclusion
The results obtained that after 3 and 6 weeks incubation the highest population was found at T.pinophilus (59.5-210.5x10^10 CFU mL^-1) and at A. chroococcum (83.5-190.5x10^10 CFU mL^-1), respectively. After 1 year storage, the population of A.chroococcum in the carrier biovermi decreased to 50-90x10^5 CFU mL^-1, T. asperellum 60 - 210 x10^6 CFU mL^-1 and Azospirillum 75x10^7 - 32x10^8 CFU mL^-1, while T.pinophilus 60 x10^6 - 45x10^8 CFU mL^-1.

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