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Microbial Population and Nutrient Content of a Biofertilizer Containing *Azotobacter* sp. and *Pseudomonas fluorescens* with Different Carrier Materials After Storage

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Abstract. *Biofertilizers contain N-fixing and P-solubilizing bacteria. The microbial population is dynamic and influenced by nutrient availability and storage temperature. Maintaining microbial populations requires appropriate carrier media to maximize microbial viability. The aim of the research is to determine the appropriate carrier material for the biofertilizer after storage based on the nutrient content and microbial population. The experiment utilized a completely randomized design with seven treatments and four replications, resulting in 28 experimental units. The treatments were as follows: B0 = Compost, B1 = Compost + Bacteria (Azotobacter and Pseudomonas fluorescens), B2 = Compost + Bacteria (Azotobacter + P. fluorescens) + Molasses, B3 = Compost + bacteria (Azotobacter + P. fluorescens) + CMC, B4 = Compost + bacteria (Azotobacter + P. fluorescens) + Arginine, B5 = Compost + bacteria (Azotobacter + P. fluorescens) + Sugar + CMC, and B6 = Compost + bacteria (Azotobacter + P. fluorescens) + Molasses + Arginine. The study results showed that the highest bacterial colonies were observed seven days after storage in treatment B2, reaching 156.33 CPU. The highest bacterial population growth in the first month was recorded in treatment B5; however, in months 2, 3, 4, and 5, treatment B2 exhibited the highest bacterial colony population. The pH remained more stable in treatments B2, B4, and B6. The highest nutrient content, including pH, N, P, K, and C/N ratio, was recorded in treatment B2, respectively, with values of 6.67, 2.49%, 2.04%, 1.77%, and 20.01. Findings in this study suggested the potential biofertilizer can be applied in the field to reduce dependence on chemical fertilizers to support sustainable agriculture.*

Keywords: *azotobacter, biofertilizer; carrier materials; compost; pseudomonas fluorescens; molasses.*

Type of the Paper: Regular Article.

1. Introduction

Nitrogen and phosphorus are essential macronutrients for plant growth. Nitrogen is highly susceptible to decomposition, loss, leaching, and evaporation. The P element is mostly bound by Al and Fe, leaving only a small fraction available for plant uptake. This limited absorption increases production costs for farmers. Intensive use of chemical fertilizers depletes soil organic matter levels, damages soil structure, and causes environmental pollution [1].

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An effective solution is the use of biofertilizers containing rhizobacteria groups, which play a role in encouraging plant growth, maintaining soil health, and functioning well under biotic stress conditions [2]. Biofertilizers contain nitrogen-fixing microbes, phosphate solvents, potassium solvents, plant growth promoters, arbuscular mycorrhizal fungi, and increase the production of various enzymes, resulting in a better level of waste degradation [3-6]. The prospect of this beneficial biofertilizer can be used as a prerequisite in sustainable agriculture [7].

The use of biofertilizers saves production costs and improves plant growth performance [8,9]. The application of biofertilizers to rice plants using the System of Rice Intensification (SRI) method increases production by up to 10.3 tons/ha (24%) from conventional systems and can reduce the use of chemical fertilizers, especially P, by up to 50% [10].

Biofertilizer technology is constrained by the short shelf life of microbes in biofertilizers. The durability and efficiency of rhizobacteria microbial performance in biofertilizer formulations are highly determined by the carrier material and storage temperature [11,12]. Research on biofertilizer formulations has been widely conducted and evaluated, but to date very little data has been obtained on the shelf life of biofertilizers and is very inadequate. Research must be intensified to develop stable, functional, and reliable biofertilizer inoculants as a tool for sustainable agriculture [13].

Biofertilizer storage is feasible in a room so that it can be easily integrated into an agricultural distribution system that does not have a cooling room [14]. The novelty of this research is the microbes used in biofertilizer from the rhizosphere of indigenous rice plants. The formula for organic biofertilizer from rice harvest residues, there is no similar data from previous research

As biofertilizer effectiveness depends on microbial activity, it is essential to determine the duration of microbial viability in biofertilizers. Hence, the aim of the research was to determine the appropriate carrier material for biofertilizer after storage, based on nutrient content and microbial population.

2. Materials and Methods

2.1. Time dan place

The research was conducted at the Biology Laboratory of the Politeknik Pertanian Negeri Payakumbuh from March to August 2024. The materials used included organic residues from rice harvest (straw, husks, bran), cow dung, indigenous *Trichoderma* spp. [15], and indigenous bacteria (*Azotobacter, Pseudomonas fluorescens*) [16,17]. The equipment used included petri dishes, measuring cups, conductors, and aerators.

2.2. Experimental design

The study employed a Randomized Block Design with 7 treatments and 4 replications, resulting in 28 experimental units. The treatments were as follows: B0 = Compost, B1 = Compost

+ bacteria (*Azotobacter* + *P.fluorescens*) + Sugar, B2 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Molasses, B3 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + CMC, B4 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Arginine, B5 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Sugar + CMC, and B6 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Molasses + Arginine.

2.3. Work procedures

2.3.1. Microbial enrichment

Trichoderma harzianum, used as a decomposer, was propagated on a bran and husk medium (2:1). The husks were soaked overnight, drained, mixed with bran, and packed in 250 g plastic bags. The mixture was then sterilized in an autoclave at 1 atm for 1 hour, cooled, inoculated with *Trichoderma harzianum* culture, and incubated for 5 days. The spore density of *Trichoderma* sp. was 10^3 spores per gram.

Indigenous bacteria (*Azotobacter* and *P.fluorescens*) were re-cultured on NA medium supplemented with 0.01 ml FeCl3. The bacterial culture was then mass propagated in coconut water at a ratio of 1 ose of pure *Azotobacter and P.fluorescens* per liter of coconut water. The culture was incubated for 7 days using an aerator. A total of 100 ml (50 ml each mass propagation) was added.

The formula treatment included CMC (0.1 mg/30kg) and Arginine (0.1 mg/30kg) to prevent rhizocbacteri from becoming inactive during storage and to reactivate their metabolism upon application. Granulated sugar (10 gr/30 kg) and molasses (10 mL/30kg) served as microbial nutrition.

2.3.2. Compost making process

Compost was prepared using a formula consisting of organic materials from rice harvest residues (straw, husks, bran) and dried-cow dung that has been air-dried for one week in a ratio of 4: 6 (based on the dry weight). A total of 840 kg of compost was produced, with each treatment unit consisting of 30 kg. *Trichoderma harzianum* was used as a decomposer at a rate of 100 gr/30 kg.

At the beginning of compost preparation, a layered system of organic materials were layered with cow dung and *Trichoderma harzianum* as decomposers. Microbes and additives were applied according to the treatment, and the compost was then covered with black plastic and incubated. Temperature was monitored every three days using a thermometer for up to eight weeks. The compost was first turned on the seventh day and subsequently repeated once a week.

2.3.3. Biofertilizer Formulation Shelf Life Test

After a 56-day incubation period, 1 kg of compost from each treatment unit was sampled and packaged for storage tests based on the number of treatments. Observations were conducted

monthly for each treatment.

Observations included: (1) temperature during composting, (2) the number of bacterial colonies in each biofertilizer treatment after 7 days was counted in pure culture, (3) the number of bacterial colonies each month of storage in pure culture, (4) the pH value of the biofertilizer after storage, and (5) the nutrient value of the biofertilizer after storage.

2.3.4. Data analysis

Data were analyzed using analysis of variance (ANOVA). When significant differences were detected, Duncan's New Multiple Range Test (DNMRT) was performed at 5% significance level.

3. Results and Discussion

3.1. Temperature

The results of observations on temperature during the composting process can be seen in Fig. 1. The initial temperature of the pile of organic materials was 22°C, which was the same as the ambient temperature. The temperature of the pile was monitored every three days and reached a peak between 35°C and 47°C on the 15th day. After the peak, the temperature of the pile fluctuated between 23°C and 25°C. The temperature variation in each biofertilizer pile depended on microbial activity and the aeration provided during the turning process. Temperature plays a crucial role in regulating microbial activity during the decomposition of organic materials into compost. Proper aeration during compost turning enhances microbial growth, accelerating decomposition [18].

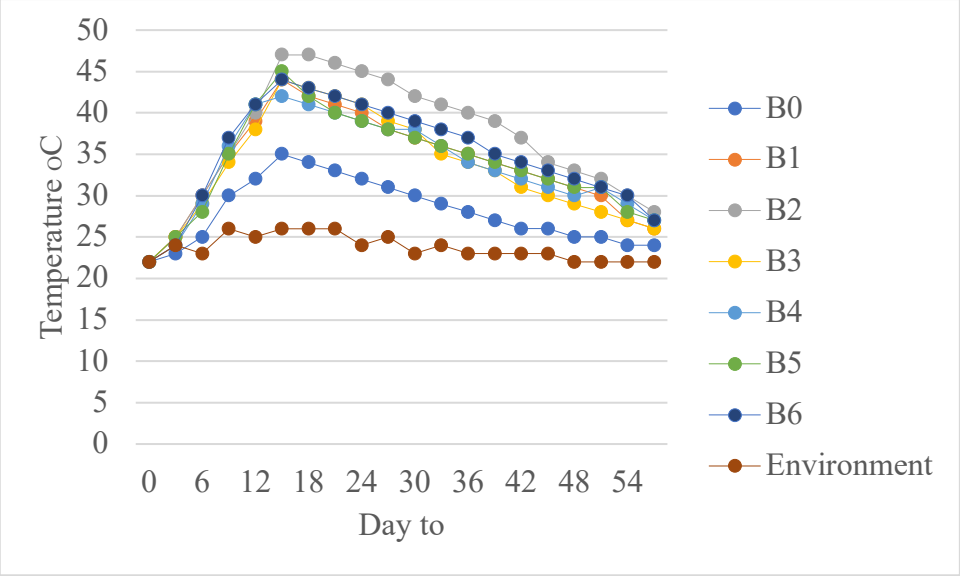

Fig. 1. There is an increase in temperature in the pile of composted organic materials.

On the 42nd day, the temperature of the biofertilizer pile decreased to between 30°C and 42°C. By the 57th day, the temperature stabilized, ranging from 24°C to 28°C. During the cooling phase, the biofertilizer temperature remained consistent within the 24°C to 28°C range. The

ambient temperature throughout the composting process fluctuated between 22°C and 26°C.

3.2. Microbial population after seven days of biofertilizer storage

The number of bacterial colonies in the formulated media containing indigenous *Azotobacter* and *Pseudomonas fluorescens* isolates after seven days, as determined through statistical analysis, is presented in Table 1.

Table 1. Number of bacterial colonies of *Azotobacter* and indigenous *P. flourescens* isolate formulations

Treatment	CPU Bacterial Colony Count
B0 = Compost	$10.33^{g} \times 10^{8}$
B1 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Sugar	$43.67^{de} \times 10^{8}$
B2 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Molasses	$156.33^{a} \times 10^{8}$
B3 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + CMC	$31.00^{f} \times 10^{8}$
B4 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Arginine,	$38.67^{ef} \times 10^{8}$
B5 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Sugar + CMC	$128.00^{c} \times 10^{8}$
B6 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Molasses + Arginine	$133.33^{bc} \times 10^{8}$

According to DNMRT, the numbers in the columns followed by the same capital letter are not significantly different at the 5% level of significance.

Table 1 shows that the highest number of indigenous *Azotobacter* and *P. fluorescens* bacterial colonies after seven days observation after storage was observed in treatment B2 (Compost + bacteria (*Azotobacter* + *P. fluorescens*) + Molasses). This shows that the addition of molasses promotes bacterial growth. Molasses, a byproduct of sugarcane processing, is applied in liquid form to biofertilizer, providing an accessible nutrient source and energy for bacteria. In addition, molasses contains essential vitamins and minerals absent in refined sugar, including manganese (13%), magnesium (12%), copper (11%), potassium (6%), selenium (6%), iron (5%), calcium (3%), and B vitamins (B3, B5, and B6).

Microorganism growth media consists of a mixture of nutrients essential for microbial growth. Microorganisms utilize these nutrients in their simplest forms to synthesize cellular components [19].

3.3. Microbial population in biofertilizer during storage

The results of biofertilizer storage tests on the bacterial colony population per unit after 5 months of storage are presented in Fig. 2. As shown in Fig. 2, the highest bacterial colony population with statistically significant ($p<0.05$) in the first month of storage was obtained in media B5 = Compost + bacteria (*Azotobacter* + *P. fluorescens*) + Sugar + CMC (158 CPU). A substantial bacterial colony population was also recorded in treatment B2 (Compost + Bacteria (*Azotobacter* + *P. fluorescens*) + Molasses) during the first month. Similarly, treatment B6 (Compost + Bacteria (*Azotobacter* + *P. fluorescens*) + Molasses + Arginine) exhibited high bacterial colony count in the first month. The addition of sugar and molasses as carrier materials appears to enhance nutrient availability, thereby accelerating bacterial population growth.

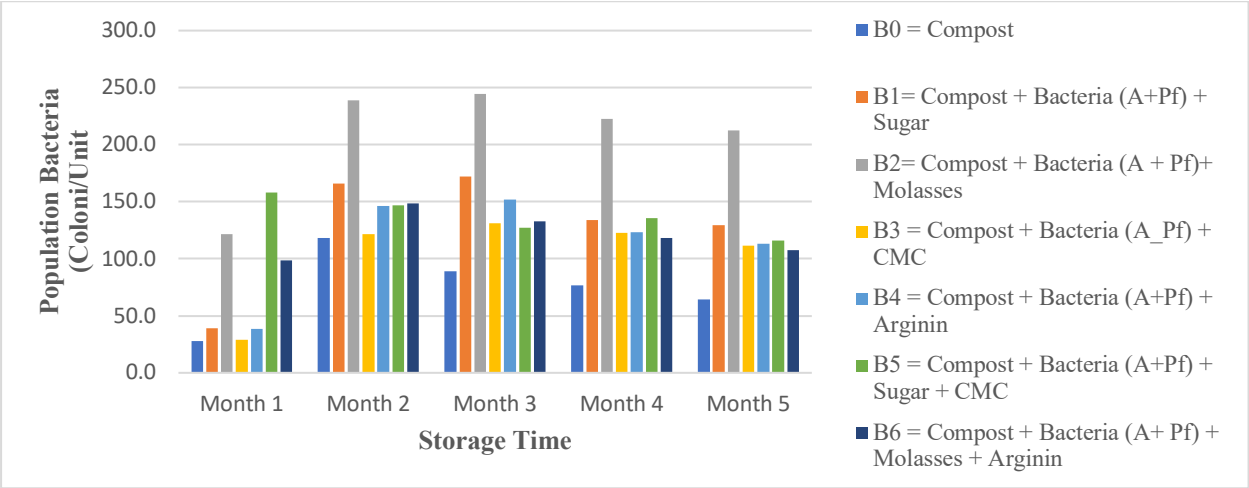

Fig. 2. Population growth of *Azotobacter* and *P.fluorescens* bacteria on various media

In the second month, the bacterial population increased in all treatments, with the highest bacterial count observed in treatment B2, consisting of compost, *Azotobacter, P. fluorescens*, and molasses. This trend continued in the third and fourth months, before a decline was noted in the fifth month. These results suggest that compost supplemented with molasses provides adequate nutrition to support bacterial growth.

In the third month, the highest populations of *Azotobacter* and *P. fluorescens* were observed across all storage treatments. The treatment B2, consisting of compost, *Azotobacter, P. fluorescens,* and molasses, exhibited the highest bacterial colony count.

Furthermore, during the 4th and 5th months of storage, the population of *Azotobacter* and *P. flourescens* bacteria began to decline across all treatments. However, the highest bacterial population of *Azotobacter* and *P. fluorescens* bacteria was still observed in the B2 treatment (Compost + Bacteria (*Azotobacter* + *P. fluorescens*) + Molasses). This suggest that biofertilizer supplemented with molasses and granulated sugar maintained a higher bacterial count compared to other treatments. This result indicate that the carrier materials molasses and granulated sugar serve as effective nutrient sources for microbial growth, enhancing their activity in the biofertilizer.

Microorganisms utilize nutrients for growth through biosynthetic processes, leading to the production of new cell material or biomass. This process results in an increase in the size of microbial cells over time. As microbial biomass and the number of microbial individuals grow, the overall population increases. The extent of this population growth largely depends on the composition and physical conditions of the growth environment, which must be conducive to supporting the microorganisms' biosynthesis of new biomass [20].

A major limitation of biofertilizers is their short shelf life, primarily due to challenges in long-term storage. However, the shelf life of biofertilizers can be extended through strategies such as the use of heat- and drought-tolerant bacterial strains, genetic engineering, and the development of liquid biofertilizer formulations [21].

3.4. pH

The results of the biofertilizer analysis after storage against pH are presented in Table 2. The results in Table 2 indicate that biofertilizers made solely from compost (B0) experienced a drastic decrease in pH after storage. This indicates that, in the absence of *Azotobacter* bacteria with *P. fluorescens*, the decomposition of organic matter occurs rapidly, leading to a pH decline. Treatments containing granulated sugar, CMC, and arginine also showed a decrease in pH. However, in the treatments with molasses, the pH of the biofertilizer remained stable before and after storage.

Table 2. Biofertilizer pH value after storage for 5 months

Treatment.	Biofertilizer stability during storage. Soil Acidity pH		
	Initial	Final	Status
B0 = Compost/control	6.24	5.63	Decreased drastically
B1 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Sugar	6.56	6.22	Decreased
B2 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Molasses	6.72	6.72	Stable
B3 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + CMC	6.53	6.35	Decreased
B4 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Arginine,	6.55	6.55	Stable
B5 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Sugar + CMC	6.53	6.24	Decreased
B6 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Molasses + Arginine	6.22	6.22	Stable

The pH change in the compost media as a carrier material for the inoculant is more drastic in the absence of *Azotobacter* and *P. fluorescens,* which serve as key regulators of the biofertilizer nutrient availability. This is due to the continued decomposition of organic matter during incubation or storage, leading to carbon release and increased concentration of H^+, as indicated by a significant pH decline (6.2 to 5.63). The high nutritional content of molasses such as Fe and Mn can produce H^+ so that it can stabilize the pH value.

The pH value of compost after storage is influenced by the availability of nutrients for microbes in the biofertilizer. Great energy availability for microbes results in more stable pH values [22]. During the initial composting process, the pH increases due to the activity of indigenous *Trichoderma* sp. Decomposer, which breaks down organic nitrogen into ammonia, creating alkaline conditions. By the end of the composting, the pH decreases toward a neutral range of 6.9-7.26, indicating reduced nitrogen decomposition [23]. The decrease in pH is associated with volatilization (ammonia) and microbial nitrification, which produces more CO2 and acid [24]. The ideal pH value for compost is generally 5.5-8 [25].

3.5. Biofertilizer nutrient content

Nutrient content with various substitute materials during storage for C-org, C/N, N, P and K is presented in Table 3.

Table 3. Characteristics of nutrient content analysis of C-org, C/N, N, P and K biofertilizer with *Azotobacter* and *P. fluorescens* bacteria after being stored for 5 months

Treatment	Characteristics of biofertilizer product components				
	C-org (%)	C/N	N-total (%)	P-total (%)	K-total (%)
B0 = Compost/control	49.15	21.56	1.88	1.13	1.45
B1 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Sugar	49.32	20.38	2.42	1.84	1.64
B2 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Molasses	49.84	20.01	2.49	2.24	2.12
B3 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + CMC	49.65	20.51	2.42	1.95	1.76
B4 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Arginine,	49.65	20.86	2.38	1.85	1.65
B5 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Sugar + CMC	49.65	20.51	2.42	1.85	1.75
B6 = Compost + bacteria (*Azotobacter* + *P.fluorescens*) + Molasses + Arginine	49.52	21.34	2.32	1.83	1.66

3.5.1. C-organik and C/N

The lowest C-organic nutrient content after storage was observed in B2 treatment. Prolonged storage led to a decline in carbon content, as carbon serves as an energy source for microbial reproduction. Microbes use energy derived from biochemical reactions to decompose organic matter. Carbohydrates in the biofertilizer break down into CO2 and H2O gases, a process that continues during the storage process, leading to reduction in carbon content. The C-organic content in biofertilizer plays a crucial role in improving soil properties.

The C/N ration in the B2 treatment was 20.01 after 5 months of storage. The B2 treatment is categorized as the best biofertilizer formulation, as the addition of molasses stimulates the activity of *Azotobacter* and *P. fluorescens*, ensuring nutrients availability remains high after storage. An optimal C/N ratio is crucial for maintaining a balanced and efficient nutrient profile in the compost mixture.

The optimum C/N ratio is important for maintaining an efficient nutrient balance in the compost mixture. As the composting process progresses, the C/N value varies due to carbon conversion into CO_2 during organic degradation. The ideal C/N ratio for composting ranges from 25 to 35 [26]. Microorganisms require approximately 30 parts C per unit N during the composting process [27]. Several researchers state that the favorable C/N ratio for the composting process falls between 20 and 50 [26].

3.5.2. Total - N

The total N value of biofertilizer after storage in all treatments with the addition of *Azotobacter* and *P. fluorescens* bacteria was > 2, with the highest value was recorded in treatment B2. Molasses additives helped sustain the viability of N-fixing microbes for a longer period. The total N value is closely related to carbon content and C/N ratio. The availability of total N nutrition results from the activity of N-fixing microbes (*Azotobacter*) in biological N2 Fixation (BNF), which converts atmospheric N2 into ammonia. Plants absorb nitrogen through a complex enzymatic process known as the nitrogenase process. The longer N-fixing remains viable, the N nutrient will always be available [28].

3.5.3. Total - P

The total P nutrient content value after storage was obtained in the B2 treatment. The P nutrient content of the compost is determined by the raw materials of the compost and the decomposing microorganisms. Compost serves as the key ingredient of biofertilizer plus *Azotobacter* and *P. fluorescens* bacteria, classified as phosphate-solubilizing microorganisms and other additional materials, particularly molesse. Phosphate-solubilizing microorganisms produce organic acids to dissolve unavailable P into available P [29]. The addition of phosphate-solubilizing microbes to the biofertilizer increases P nutrient utilization efficiency [30]. Besides supporting plant and soil health, these microbes also produce low molecular weight organic acids such as oxalic acid, propenadioic acid, acetic acid and arylic acid [31].

3.5.4. Total - K

The K-total value of biofertilizer after storage for all treatments with added Azotobacter and P. *fluorescens* bacteria ranged from 1.64% to 1.77%, with the highest K-total value observed in treatment B2. However, the K-total value remains relatively low. The incorporation of K-solubilizing bacteria into biofertilizers has the potential to increase the K-total value, thereby promoting plant growth and potentially replacing chemical potassium fertilizers. This approach can also contribute to reducing environmental pollution [32].

4. Conclusions

This study concluded that the B2 treatment, consisting of compost, Azotobacter, *Pseudomonas fluorescens*, and molasses, was the most effective biofertilizer formulation after five months of storage. B2 demonstrated the highest bacterial colony count (156.33 CFU/g) after seven days and consistently maintained the highest microbial population from the second to the fifth month. Additionally, B2 exhibited the most stable pH, reaching 6.67, and superior nutrient content with the highest total nitrogen (2.49%), total phosphorus (2.04%), total potassium (1.77%), and an optimal C/N ratio of 20.01. These results indicate that the B2 formulation supports excellent

microbial viability and nutrient stability, making it a promising biofertilizer for long-term use.

Abbreviations

CMC Carboxymethylcellulose

Data availability statement

Data will be shared upon request by the readers.

CRediT authorship contribution statement

Nelson Elita: Conceptualization, Methodology, Resources, Formal analysis, Investigation, Data curation. **Nor Aishah Hasan**: Writing – review & editing. **Rita Erlinda**: Writing – original draft, Validation. **Fri Maulina**: Data curation, Formal analysis, Conceptualization. **Yefriwati**: Conceptualization, Supervision, Project administration. **Rinda Yanti**: Resources, Formal analysis, Writing – review & editing. **Deliana Andam Sari**: Validation, Data curation. **Ayu Kurnia Ilahi**: Formal analysis, Methodology, Resources, and Formal analysis**.**

Declaration of Competing Interest

The authors declare no competing interest.

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