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Viability of *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30 in The Carrier Formula of Mocaf Solid Waste, Peat and Manure

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Abstract *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30 are bioremediation agent that can be used as functional bacteria in biofertilizers. The storage of these bacteria requires carrier. Not all carriers can support bacterial viability, so it was necessary to examine carrier formulas as basic ingredients for biofertilizers which the quality standards based on the Minister of Agriculture Regulation No. 70/2011. This research aimed to: (1) study the viability of *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30 in some carrier formulas; and (2) obtain the best carrier formula to support the viability of *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30. Research design was factorial using completely randomized design (CRD) as the based design, consisting of 2 factors: 1) Carrier Fornula (C): C1; C2; C3; C4; C5, 2) Bacteria (I): I1; I2; I3, so there were 15 treatment combinations each was repeated 4 times, thus there were 60 experimental units. The results showed that *Agrobacterium* sp. I26 has better viability than *Agrobacterium* sp. I30 during 90 days incubation period. The best bacterial viability with total bacterial as the indicator was C4 carrier formula: 74 x 10^{13} cfu.g^{-1} for *Agrobacterium* sp. I26 and C3 carrier formula: 155 x 10^{12} cfu.g^{-1}.

Keywords: Bacterial viability, *Agrobacterium* sp. I26, *Agrobacterium* sp. I30, carrier, biofertilizers

INTRODUCTION

Agrobacterium sp. I26 and *Agrobacterium* sp. I30 are bioremediation agents isolated from Cr-contaminated soil by study Rosariastuti *et al.* (2013). Treatment using *Agrobacterium* sp. I26 without chemical fertilization can reduce Cr uptake in rice by 92.38% compared with control (Utari, *et al.*, 2018). Combination of inorganic fertilizer with *Agrobacterium* sp. I26 has the highest weight of 1000 seeds: 31.95 g (14.96% higher than the control) (Rosariastuti *et al*., 2020).

In order to apply *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30 in large numbers with a wide location coverage, so need high quality carrier for supporting viability the bacterias during storage. Singh and Harikesh (2016) suggested that carriers can support and maintain the high viability of the bacteria contained in them. Not all carriers can support the high viability of bioremediating agents. It is important to choose a carrier, to maintain the viability of the bioremediating agent in a dormant state and suitable as a place to live before it is applied to the soil (Karnataka *et al.*, 2007).

Various kinds of organic materials have been widely used as carriers. One of the organic materials is peat, which has been widely used as a carrier material for a long time around the world (Rosariastuti *et al.*, 2017). Mocaf solid waste still contains high nutrients in the form of carbohydrates (63-68%) and water (20%) (Amalia, 2012). Provision of biofertilizers with the carrier material of manure and sand showed an increase in the P nutrient before planting from 40.8 ppm to 91.7 ppm after planting (Firdausi *et al*., 2016). Viability of *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30 in a carrier, as the functional bacteria in biofertilizer is interesting to be studied by examined several carrier formulas and referring to Regulation of the Minister of Agriculture Replubic of Indonesia No. 70/2011 about biofertilizers. This research aims to:

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(1) study the viability of *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30 in some carrier formulas; and (2) obtain the best carrier formula to support the viability of *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30 which is according to the Minister of Agriculture Regulation No. 70/2011.

METHODOLOGY

1. Place and Time Resesarch

The research was conducted from April to December 2019 at the Laboratory of Soil Biology and Biotechnology, Laboratory of Chemistry and Soil Fertility and Greenhouse, Faculty of Agriculture, Sebelas Maret University, Surakarta.

2. Materials and Tools

The materials needed were mocaf solid waste from PT. Sasindo Solo, peat from Rawa Pening Central Java, wheat flour Bogasari brand, manure from Jatikuwung Laboratory of UNS and *Agrobacterium* sp. I26 and *Agrobacterium*sp. I30 isolated by Rosariastuti *et al*. (2013). Additional and chemical materials used in laboratory analysis were Luria Bertani liquid media, alcohol, spirtus, physiological salts, PDA media (Potato Dextrose Agar), aquadest, lactose broth**.** The tools were used: 0.5 mm sieve, container boxes, loop needles, bunsen, Erlenmeyer, micropipette, autoclave, petri dish, shaker, hot plate and stirrer, ph meter, oven, vortex, AAS spectofotometry and etc.

3. Research Design

This research design was factorial using completely randomized design (CRD) as the based design, consisting of 2 factors: 1) Carrier Fornula (C): C1 (100% Solid Waste Mocaf); C2 (75% Peat + 25% Solid Waste Mocaf + 70 g Wheat Flour per kg Carrier); C3 (75% Peat + 25% Solid Waste Mocaf + 35 g Wheat Flour per kg Carrier + 35 g Clay per kg Carrier); C4 (50% Peat + 50% Solid Waste Mocaf + 70 g Wheat Flour per kg Carrier); C5 (75% Peat + 25% Manure + 70 g Wheat Flour per kg of Carrier, 2) Bacteria (I): I1 (without bacteria); I2 (*Agrobacterium* sp. I26); I3 (*Agrobacterium* sp. I30), so there were 15 treatment combinations each was repeated 4 times, thus there were 60 experimental units.

4. Procedure

a. Preparation of Agrobacterium sp. I26 *and Agrobacterium* sp. I30 *Inoculum*

Propagation was done using pure *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30 isolate, inoculated to Luria Bertani agar media. Inoculation of bacteria was carried out by taking one loop of pure culture, then each type of bacteria inoculated on 3 tubes new of Luria Bertani agar media. Isolates were then incubated for 3 days to grow and develop. Then, 3 tubes isolates transferred to 600 ml of liquid Luria Bertani media in Erlenmeyer. The bacterial inoculum was shaking using shaker at a speed of 55 rpm, until bacterial density reach of 10^{12} cell.ml^{-1}. Bacterial density measurement was done everyday using hemacytometer.

b. Carrier Preparation

Air dried peat, mocaf solid waste, and dry manure were mashed and sieved using a 0.5 mm sieve. Furthermore, they weighed according to the treatment dose. Each carrier formula was mixed until well blended, then packed into 0.5 PP plastic and sealed using rubber and sterilized using an autoclave with a temperature of up to 121^{o}C in ± 60 minutes.

c. Inoculation of Agrobacterium sp. I26 *and Agrobacterium* sp. I30 *in the carrier*

Ten (10) ml inoculum *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30 with a density of 10^{12} cfu.ml^{-1} were put into 1 (one) kg of the carrier, so that the bacterial density in the carrier was 10^{10}.g^{-1} carrier. Then the carrier was mixed until it was homogeneous. The outer plastic was labeled according to each treatment. Carriers were incubated in a container box for 3 months at room temperature 25^{0}C.

d. Laboratory Analysis

Observations were done once a month for three months with the main parameters of total bacteria (Total Plate Count Method), total fungal contamination (Total Plate Count Method), total *E. coli* contamination (Most Probable Number-Durham Method), water content (Gravimetric Method) and pH

(Electrometric Method). The supporting parameters analyzed were total of N (Kjeldahl Method), total of P (HCl Extraction Method), total of K (HCl Extraction Method), and C-organic (Walkley and Black Method).

5. Data Analysis

Observation data were statistically analyzed using ANOVA with a confidence level of 95%. If the treatment were significant, then analyzed using Tukey HSD with a 95% confidence level using Minitab software.

RESULTS AND DISCUSSION

1. Initial Characteristics of Carrier Formula

Table 2. Analysis Result of Initial Characteristic Carrier Formulas before being inoculated by bacteria

No	Observation Variable	C1	C2	C3	C4	C5
1	Bacteria Contaminants (cfu/g)	0	0	0	0	0
2	Fungal Contaminants (cfu/g)	0	0	0	0	0
3	E. *Coli* Contaminants (MPN/g)	0	0	0	0	0
4	Moisture Content (%)	12	14	14	13	12
5	pH	6,75	5,66	5,63	5,93	6,27
6	Total N (%)	2,41	0,31	5,35	3,01	4,01
7	Total P (%)	0,10	0,25	0,26	0,18	0,19
8	Total K (%)	0,07	0,044	0,049	0,13	0,03
9	Total C-Organic (%)	5,77	6,83	6,78	6,24	6,20

Analysis results (Table 2) showed that there was no bacteria, fungi and *E. coli* in each carrier base material (0 cfu.g^{-1}). The sterilization process has been done successfully deadly microorganisms in the carrier base material. Tittabutr *et al.*, (2012) said contamination by microorganisms was a major problem affecting the quality and shelf life of inoculants. Khavazi *et al*. (2007) said in their research, that the initial sterilization process had been carried out on carrier base materials (before the carrier was inoculated) successfully supported the growth of *Bradyrhizobium japonicum* strain CB1809 for 6 months storage period, with bacterial density $1x10^9$.g^{-1} carrier.

The parameters of water content and pH (Table 2) were in accordance with the Minister Of Agriculture Regulation No. 70/2011. High water content creates optimal humidity for bacterial growth and survival (Mohamed, 2010). The pH condition is one of the factors that influenced the viability of bacteria in the carrier media. The bacteria require an optimum pH in 5.0-8.0 (Ogbo, 2010).

The basic carrier material (Table 2) contains N, P, K, and C organic, this means that the carrier can support bacterial growth. Gelbrich (2009) said that nitrogen is an element needed by microbes in protein synthesis. The peat from Rawa Pening used has an excess of the organic matter content of more than 30%, this organic matter has a contribution to the longer life of bacteria (Tittabutr *et al.*, 2012). The high protein, starch and total sugar content in mocaf solid waste is a source of carbon and nitrogen for microbial growth (Rosariastuti *et al.*, 2017).

2. Growth Pattern of *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30

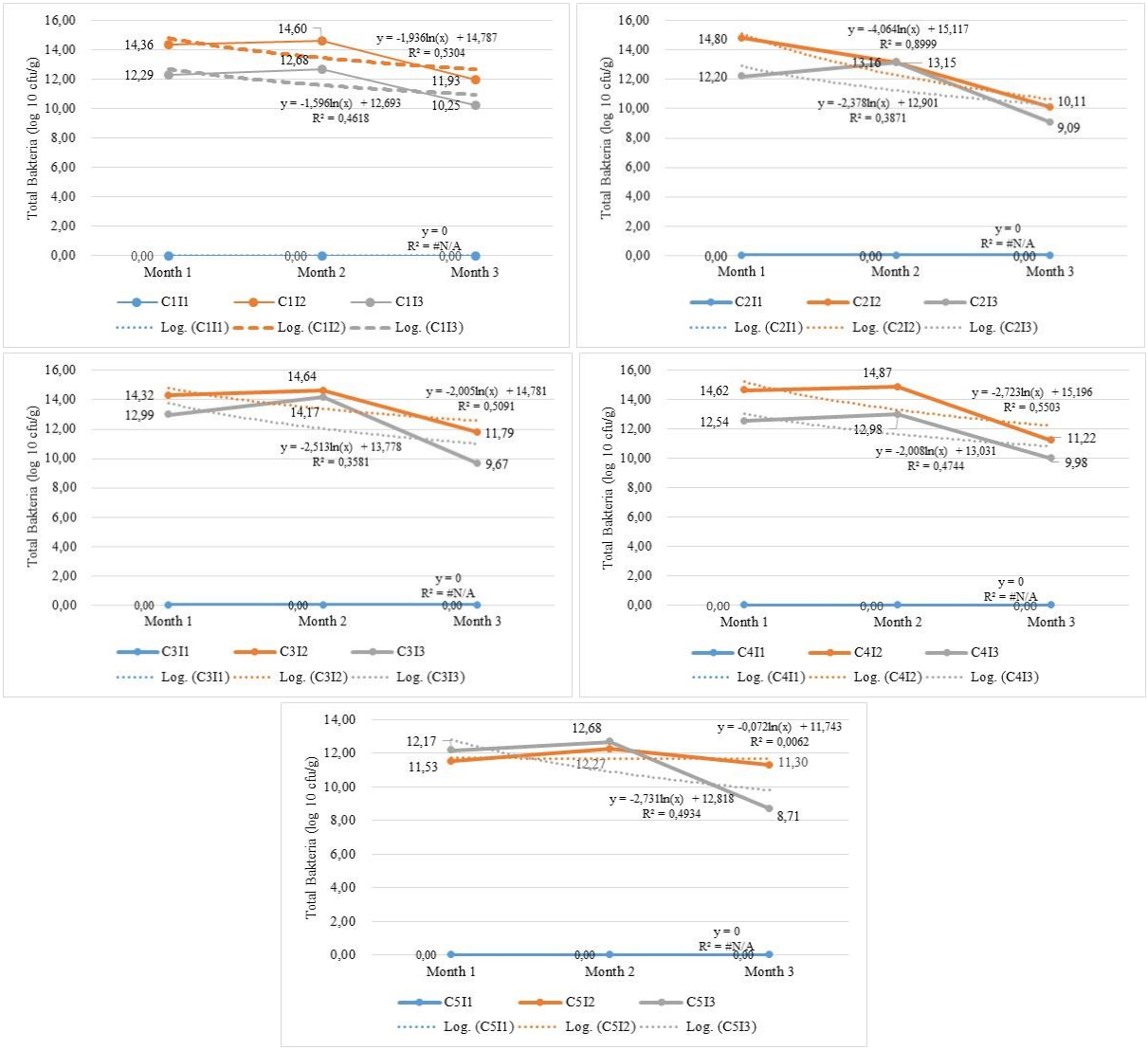

Figure 1. Pattern Growth of *Agrobacterium* sp I26 and *Agrobacterium* sp I30 in any Carrier Formulas

Bacterial growth with an indicator of total bacteria in this study was the main indicator of the viability of bacteria in the carrier. Based on Figure 1, the growth pattern of *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30 in all carrier formulas indicated slow growth in the first month incubation period reach to 10^{11} cfu.g^{-1}, because the bacteria were in the adaptation phase (lag). This slow growth occurred due to the differences in the characteristics of the media from the initial growth medium, liquid Luria Bertani media to a solid media. So that, bacteria took time to adapt the different environmental conditions (Bhattacharya *et al.*, 2018).

In the second month's incubation period (Figure 1) showed that *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30 were in log (exponential) phase. Duben-Engelkrik and Paul (2010) said that bacteria during the log phase growth very quickly so that the number of cells were multiply. This evidenced by the total bacteria of *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30 which have upgraded in almost all carrier formulas. Swinnen *et al.* (2004) said that the rapid enhancement of bacterial growth in the second month incubation period was due to the availability of nutrients in the carrier for bacterial growth. *Agrobacterium* sp. I26 has the highest viability in the C4 carrier formula (50% peat + 50% mocaf solid waste + 70 g wheat flour kg^{-1} carrier) with the average of total bacteri: 14.87 log10 cfu.g^{-1}. The high total bacteria in the carrier formula was influenced by the presence of a neutral pH factor and water content that supports the growth of *Agrobacterium* sp. I26. The highest Viability of *Agrobacterium* sp. I30 was found in the C3 carrier formula (75% peat + 25% mocaf solid waste + 35 g wheat flour kg^{-1} carrier + 35 g clay kg^{-1} carrier) with

the average of total bacteri: 14.17 log10 cfu.g^{-1}. *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30 can take advantage of carbon sources and energy sources in the carrier from peat and mocaf solid waste mocaf as base carrier materials (Rosariastuti *et al.,* 2017).

At the end of the third month incubation periode (Figure 1), the bacteria entered a decline phase, as evidenced by the decrease in total bacterial colonies. The decrease was due to growth limiting factors: limited nutrition and carrier water content (Liu, 2017). Besides that, a population selection process occurs due to competition for nutrients and other essential components such as oxygen and water (Doran, 2013).

3. Total Bacteria

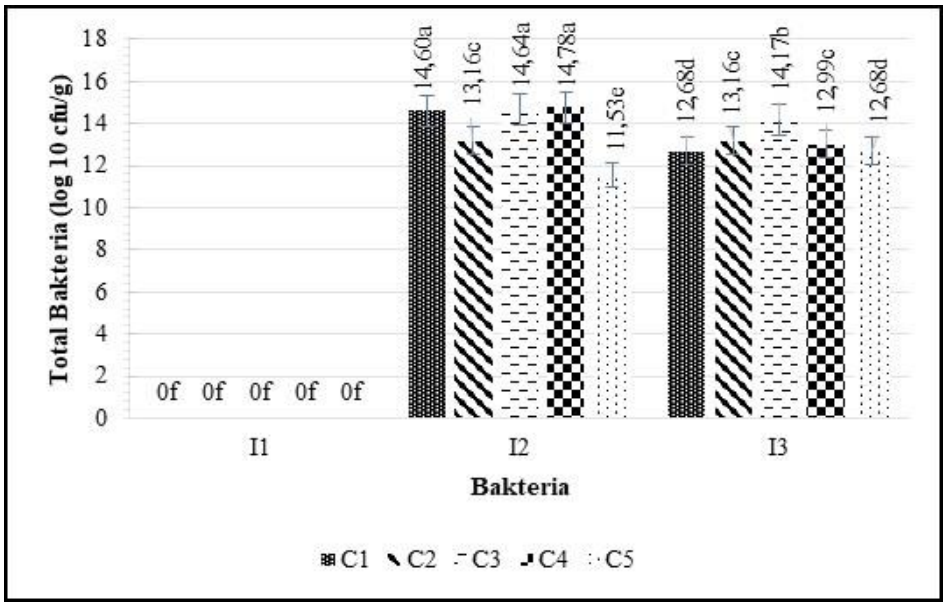

Figure 2. Treatmen Effect to Total Bacteria in The Second Month of Incubation Period

Statistical analysis were carried out for data of the end of second month incubation periode laboratory analysis with the observed parameters: total bacteria, total fungal contaminants, total *E. coli* contaminants, water content and pH. The ANOVA with a confidence level of 95% (Figure 2) showed that carrier treatment, bacterial treatment and the interaction between carrier and bacterial treatment had a very significant effect on total bacteria. *Agrobacterium* sp. I26 has a higher total bacteria than *Agrobacterium* sp. I30. This showed that the carrier formula treatment affects the total *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30. Inoculant can survive growth and development requires a carrier that can keep the highest number of total inoculants (Roldan *et al*., 2008).

The results of the Tukey HSD test with a confidence level of 95% (Figure 2) showed that C4I2 treatment (50% peat + 50% mocaf solid waste + 70 g wheat flour kg^{-1} carrier) had the highest total bacteria: 14.78 Log10 cfu.g^{-1} but not significantly different from C1I2 and C3I2. C3I3 treatment (75% peat + 25% solid waste mocaf + 35 g wheat flour kg^{-1} carrier + 35 g clay kg^{-1} carrier) was the treatment with the highest total bacteria inoculated by *Agrobacterium* sp. I30 and significantly different from other carrier treatments. Both peat and mocaf solid waste contain nutrients needed for bacterial growth. Amalia (2012) stated that solid waste from the mocaf industry still contains high nutrients in the form of carbohydrates around 63-68% and crude protein 2.80%. Rosariastuti *et al*. (2017) also argued that mocaf solid waste was high in protein, starch and total sugar content combined with peat can be used as a carrier and can increase the population of *Rhizobium* sp inoculum. Granner *et al*. (2003) said that carbohydrates contained in carriers were used by bacteria as the main source in energy-producing metabolism for bacteria and as build blocks of cells. Dickopp *et al.* (2018) said that the used of a carrier from peat can also provide an environmental support capacity for bacterial breed, because peat has a high water-holding capacity and creates moisture in the carrier.

4. Total Fungal Contaminants

Table 3. Total of Fungal Contaminants in Second Month of Incubation Period

Treatment	Fungal Contaminants (*cfu*/g)	Quality Standard (*cfu*/g)	Criteria
C1I1	0	$<10^5$	Suitable
C2I1	0	$<10^5$	Suitable
C3I1	0	$<10^5$	Suitable
C4I1	0	$<10^5$	Suitable
C5I1	0	$<10^5$	Suitable
C1I2	0	$<10^5$	Suitable
C2I2	0	$<10^5$	Suitable
C3I2	0	$<10^5$	Suitable
C4I2	0	$<10^5$	Suitable
C5I2	0	$<10^5$	Suitable
C1I3	0	$<10^5$	Suitable
C2I3	0	$<10^5$	Suitable
C3I3	0	$<10^5$	Suitable
C4I3	0	$<10^5$	Suitable
C5I3	0	$<10^5$	Suitable

Note: * Minister Of Agriculture RI regulation number 70/2011

The ANOVA with a confidence level of 95% (Table 3) showed that carrier treatment, bacterial treatment and the interaction between carrier and bacterial treatments had no significant effect on the total fungal contaminants. All treatments (Table 3) had a total fungal contaminant of 0 cfu.g^{-1}. It mean that the carrier formula was sterile from the presence of fungal contaminants and was accordance to Minister Of Agriculture RI regulation number 70/2011: $<10^5$ cfu.g^{-1}. Contamination in the carrier will be a major problem affecting the quality and storage capacity of the inoculant in the carrier (Tittabutr *et al*., 2012). As Balume *et al.* (2015) said that the decline in survival quality of *Rhizobia* inoculants in contaminated carriers was higher than in sterile carriers. Decreased survival of *Rhizobials* during storage due to antagonism by fungi. Khavazi *et al.* (2007) it is also said that *Rhizobia* bacteria have a high amount of inoculant while free from unwanted contamination.

5. Total *E. Coli.* Contaminants

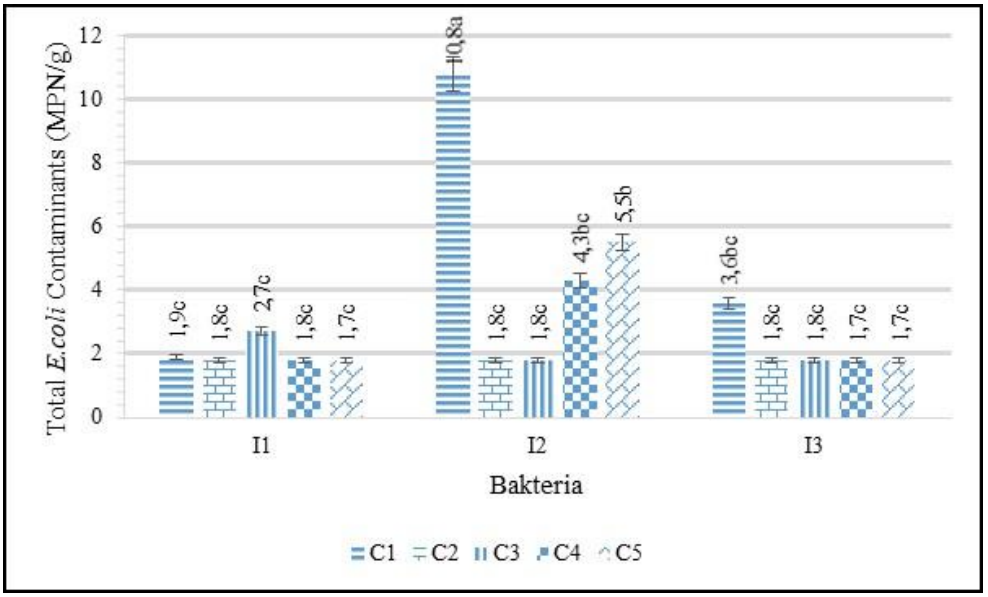

Figure 3. Treatment Effect to Total *E. Coli.* Contaminant in The Second Month of Incubation Period

The ANOVA with a confidence level of 95% (Figure 3) showed that carrier treatment, bacterial treatment and the interaction between carrier and bacterial treatment had a very significant effect on the total contaminants of *E. coli*. Total *E. coli* contaminants (Figure 3) are abundant in the carrier formula inoculated by *Agrobacterium* sp. I26 than *Agrobacterium* sp. I30. It means that *Agrobacterium* sp. I30 can suppress the growth of *E. coli.* higher than *Agrobacterium* sp. I26, althought the average of total *Agrobacterium* sp. I26 higher than *Agrobacterium* sp. I30. Putri *et al.* (2010) said that a good carrier material formula must be sterile from contaminants, so the microbes in the carrier can survive without competition from the contaminants themselves.

The results of the Tukey HSD test with a confidence level of 95% (Figure 3) showed that treatment C1I2 (100% solid waste mocaf) had the highest total *E. coli* of 10.8 MPN.g^{-1} and significantly different from other treatments. Khavazi *et al*. (2007) state that contamination was possible because of too often opened the plastic carrier when sampling. As a result, the carrier exposed to air from outside for too long which is possible that the air contains other unwanted microorganisms. Iswadi *et al*. (2014) said that the room air contains various bacteria that spread through the respiratory secretions of the room users and polluted dust.

Based on the Minister of Agriculture RI Regulation No. 70/2011, the total contamination of *E*. coli contained in biofertilizers was <10^3 MPN.g^{-1}, so even though it is contaminated, the carrier is still appropriate to be good carrier. Tittabutr *et al*. (2012) said that the ability to live of contaminants depends on cell physiology, available substrates and environmental factors such as temperature and humidity.

6. Water Content

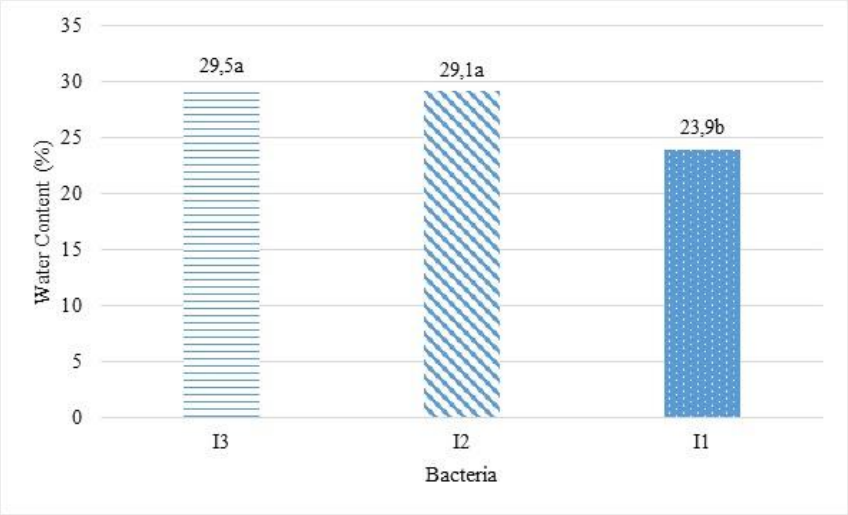

Figure 4. Bacteria Effect to Water Content in The Second Month of Incubation Period

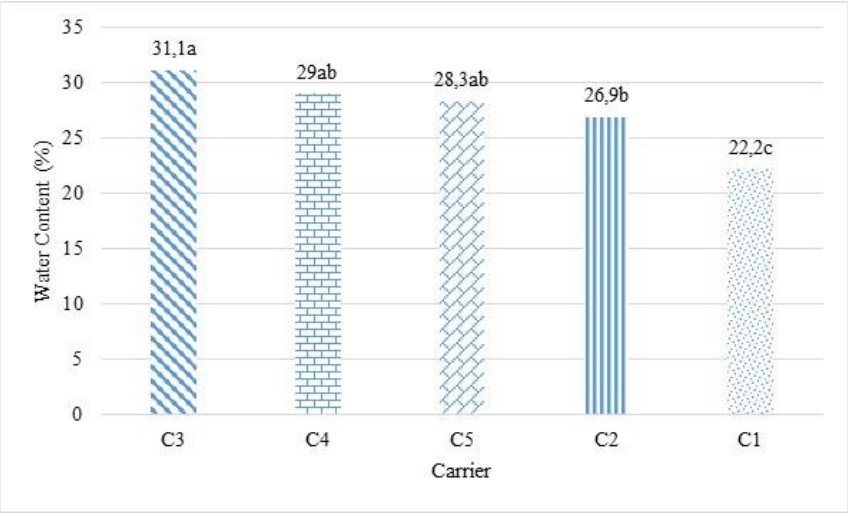

Figure 5. Carrier Effect to Water Content in The Second Month of Incubation Period

The ANOVA with a confidence level of 95% (Figure 4) showed that carrier treatment and bacterial treatment had a very significant effect on water content. The interaction between carrier treatment and bacteria had no significant effect on water content. Carrier inoculated by *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30 (Figure 4) had a higher water content compared to the control. Nwokocha *et al.* (2015) said that the criteria for a good carrier material formulation was has high water content.

The results of the Tukey HSD test with a confidence level of 95% (Figure 4) showed that bacteria has the higher water content than without bacteria. *Agrobacterium* sp. I26 and *Agrobacterium* sp. I30 have same water content. Bacteria carry out metabolic processes in their life, producing metabolite product. Bacterial metabolite also secretes out from the cell of bacteria that resulting a humid environment. Samudro *et al.* (2017) said that in the process of decomposing organic matter, microbes will decompose organic matter into carbon dioxide, water content and heat through the metabolic system.

The results of the Tukey HSD test with a confidence level of 95% (Figure 5) showed that C3 carrier formula has the highest water content (31,1%) but not significantly different with C4 and C5. The water content was according to the Minister Of Agriculture No.70 of 2011 that the water content is ≤ 35%. Sivasakthivelan and Saranraj (2013)stated that the carrier material from peat has a high water holding capacity, so that it was able to maintain moisture and create suitable environmental conditions for the inoculum in it. C1 carrier formula (100% Mocaf Solid Waste) has lowest water holding capacity and significantly different with other carrier formula.

7. pH of Carrier Formula

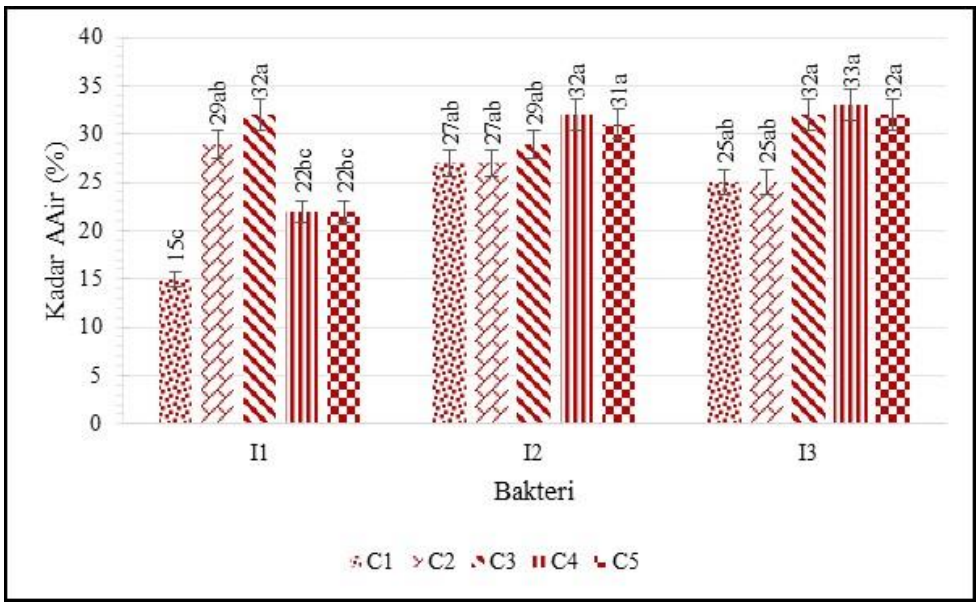

Figure 6. Treatment Effect to Carrier pH in The Second Month of Incubation Period

ANOVA with a confidence level of 95% (Figure 6) showed that carrier treatment, bacterial treatment, and the interaction between carrier and bacterial treatment had a very significant effect on the pH value. Carrier formula inoculated by *Agrobacterium* sp. I26 has a pH value according to Minister Of Agriculture RI Regulation 70/2011 than the carriers inoculated with *Agrobacterium* sp. I30. Abat (2006) said that the decrease and increase in pH indicates that there was a reaction activity process of bacterial growth. The pH condition was one of the factors that affect the viability of a bacterium in the carrier medium (Ogbo, 2010). The results of the Tukey HSD test with C5I3 treatment had the lowest pH value compared to other treatments: 4.7. This is because the C5 content is 75% peat. according to Naafs *et al*. (2017) the pH range on peat soil is 3-6. Carrier with lot of material of mocaf solid waste tend to have the higher pH than carrier with material of peat.

CONCLUSION

Agrobacterium sp. I26 has better viability than *Agrobacterium* sp. I30 during the 90 days incubation period. The best viability was in the end of second month incubation period. The highest viability of *Agrobacterium* sp. I26 was in the C4 carrier formula (50% Peat + 50% Mocaf Solid Waste + 70 g Wheat Flour kg^{-1} of Carrier): 74 x 10^{13} $cfu.g^{-1}$, while *Agrobacterium* sp. I30 was in the C3 carrier formula (75% Peat + 25% Solid Waste Mocaf + 70 g Wheat Flour kg^{-1} of Carrier): 155 x 10^{12} $cfu.g^{-1}$. The best carrier formula was C4 (50% Peat + 50% Mocaf Solid Waste + 70 g Wheat Flour kg^{-1} of Carrier).

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