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Enhancing Oil Palm Empty Fruit Bunch (EFB) Compost by Addition of Burnt Rice Husk as Carrier Material for Selected Nitrogen-Fixing Bacteria

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ABSTRACT

The current interest in minimizing the use of inorganic fertilizers and the increasing demand for the combined action of beneficial microorganisms and agricultural wastes is considered to have a positive impact on soil, crop productivity and sustainable agriculture. The objective of this study was to prepare a series of combinations of empty fruit compost (EFBC) and burnt rice husk (BRH) as carrier materials for selected nitrogen-fixing bacteria, evaluate their suitability, and determine the ability of the inoculated EFBC-BRH carrier materials. Different ratios of EFBC and BRH were used to prepare the carrier materials for the selected N_2-fixing bacteria, namely strains Sb35 and Sb42 of *Bacillus* sp. Both coarse and fine EFBCs were used for the preparations. The suitability of the combinations of EFBC-BRH as a bacterial carrier was tested by inoculating the different ratios of organic material combinations with the N_2-fixing bacteria and incubating them at room temperature for eight weeks, after which the effects on the bacterial population, pH, moisture content, and contaminants were determined. The results showed that the fine-textured EFBC-BRH carriers' formulation (T5 to T8) were better carriers compared to the formulation of coarse-textured EFBC-BRH carriers (T1 to T4). The inoculated carrier T6 (1 fine structured EFBC: 1 BRH) showed the best response with an increase in Sb35 and Sb42 populations of 7.34% and 7.47%, respectively. In conclusion, T6 as a carrier material for Sb35 and Sb42 is suitable to be developed as a biofertilizer.

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1. Introduction

The heavy reliance on nitrogenous fertilizers as a plant macronutrient in agriculture due to the loss of nitrates through leaching and denitrification has not only increased the cost of crop production, but more importantly has adversely affected the environment and human health. Alternative approaches to reduce this dependence are therefore essential. One such approach is the use of N_2-fixing bacteria to mitigate this situation. Biological nitrogen fixation not only reduces the use of synthetic nitrogen fertilizers, but also lowers the cost of crop production, preserves soil fertility, and protects the environment. To improve their survival and biological efficiency, and to protect N_2-fixing bacteria from biotic and abiotic stress, they require carrier materials. There are several types of agricultural by-product that have been recognized as the potential carrier materials for biofetilizers. For example, pea hulls, compost from plant waste, vermicompost, wheat bran, and cassava hulls (IRRI, 2002; Ogbodo, 2011; El-fattah *et al*., 2013). Compost from empty fruit bunches of oil palm and rice husk are widely used agriculture waste in Malaysia (MPOB 2019), and their potential as a carrier material for microorganisms has not yet been studied in detail.

This study was conducted with the following objectives: 1) preparation of EFBC-BRH combinations as carrier materials for nitrogen-fixing bacteria and 2) evaluation of the suitability of EFBC-BRH combinations as carrier materials for the selected nitrogen-fixing bacteria. The scope and limitation of this study focused on the use of oil palm EFB compost and burnt rice husk as a carrier for the N_2-fixing bacteria. The N_2-fixing bacteria used in this experiment were exclusively *Bacillus* sp. strains which are Sb36 and Sb42.

2. Materials and Methods

The aim of this study was to evaluate the ability of both *Bacillus* strains; Sb35 and Sb42 to grow and survive in selected carrier materials. The selected organic carrier materials consist of different formulation of EFBC with BRH. The survivability and abundance of bacteria in the carrier materials were thoroughly analyzed for week 2, week 4, week 6 and week 8.

2.1 Sources of Bacteria and Carrier Materials

Both *Bacillus* strains Sb35 and Sb42, previously isolated by Naher *et al*. (2013) from Department of Land Management, Faculty of Agriculture, UPM. The oil palm EFBC and BRH from Ladang 2, Taman Pertanian Universiti, UPM and Seberang Perak respectively were used in this study.

2.2 Preparation of EFBC-BRH Combinations as Carriers for Nitrogen Fixing Bacteria

An amount of 40 g of bacterial inoculant carriers was prepared using different ratios of empty fruit bunch compost (EFBC) to burnt rice husk (BRH) as shown in Table 1. The carriers were packaged in 10 cm x 10 cm autoclavable polyethylene bags and tightly sealed by sealer. The formulated carrier materials were 30 minutes sterilized at 121 ºC and 15 psi for three consecutive days (Okereke *et al*., 2007). Both texture coarse and fine EFBC were used for the preparations. Fine-textured EFBC was prepared by grinding the EFBC with an electric grinder and sieved with the sieve < with a sieve size of 2 mm.

Table 1. *The ratios of the carrier materials*

Ratio EFBC: BRH	Course textured EFBC (g)	Fine textured EFBC (g)	BRH (g)
1:0	40	-	-
1:1	20	-	20
1:3	10	-	30
3:1	30	-	10
1:0	-	40	-
1:1	-	20	20
1:3	-	10	30
3:1	-	30	10

2.3 Inoculation of EFB Compost- Burnt Rice Husk with Nitrogen-Fixing Bacteria

Strains Sb35 and Sb42 were transferred separately to 500 ml of sterilized Jensen broth and grown for 48 to 72 hours at 27°C and 300 rpm in a mechanical shaker. The cultures were then centrifuged at 4000 rpm for 40 minutes to separate the bacterial cells from the broth. After centrifugation, the supernatant was removed, and the bacterial cell pellet was used as inoculum for the carrier. The 5 ml bacterial cell pellets were then resuspended in 0.85% phosphate buffer saline (PBS). The Sb35 and Sb42 cultures were then inoculated into the carriers. The Sb35 and Sb 45 cultures were aseptically incjected into the sterilized bags of carrier material using a sterile plastic syringe with a needle tip (Okereke *et al*., 2007). The punctured hole on the autoclavable polyethylene bags was immediately sealed with sterilized tape. The sterilized inoculated carriers were thoroughly mixed to ensure even distribution of bacteria and incubated at 32 ^{0}C for five days in incubator (Pupathy and Othman, 2015). After five days of incubation period, the packages for both inoculated Sb35 and Sb42 were incubated at room temperature and sampled for every two weeks interval (week 2, week 4, week 6 and week 8) to determine the survival of the bacterial inoculants.

2.4 Sample and Experimental Design

Two-factor experiment of two (texture of EFB compost: coarse textured and fine textured) x four (formulated ratio with burnt rice husk: 1:0, 1:1, 1:3, and 3:1) x two (bacterial inoculants: Sb35 and Sb42) with four replication were conducted and arranged in a complete random design (CRD) as shown in Table 2.

Table 2. *Experimental samples of inoculated carrier materials*

Formulation Designation	Ratio EFC to BRH	Nitrogen Fixing Bacteria Inoculant	Replications
T1	1 course EFBC : 0 BRH	Sb35	4
		Sb42	4
T2	1 course EFBC : 1 BRH	Sb35	4
		Sb42	4
T3	1 course EFBC : 3 BRH	Sb35	4
		Sb42	4
T4	3 course EFBC : 1 BRH	Sb35	4
		Sb42	4
T5	1 fine EFBC : 0 BRH	Sb35	4
		Sb42	4
T6	1 fine EFBC : 1 BRH	Sb35	4
		Sb42	4
T7	1 fine EFBC : 3 BRH	Sb35	4
		Sb42	4
T8	1 fine EFBC : 1 BRH	Sb35	4
		Sb42	4
		Total samples of experiment	64

2.5 Evaluation of the Suitability of the EFBC-BRH Combinations as Carrier Materials for Sb35 and Sb42 Strains

The efficacy of the inoculated carrier materials in supporting the growing and survival of the Sb35 and Sb42 bacteria and their physicochemical properties were evaluated. The inoculated supports were subjected to fortnightly destructive sampling over an 8-week period and the biological (growth and survivability) and physicochemical properties were evaluated. The collected data were analyzed with analysis of variance (ANOVA) and Tukey's Test for honestly significant differences (HSD) to compare the mean separation at 5% significance.

2.5.1 Enumeration of Sb35 and Sb42 in Different Carrier Materials

The number of viable bacterial cells of Sb35 and Sb42 was determined on inoculated carrier at two-week intervals over an eight-week period using the spread-plate method (Jones, 1983). The serial dilutions of the inoculated carrier materials were perpared prepared by aseptically transferring 10 g of each inoculated carrier material into 95 ml of sterilized distilled water. The mixture was mixed thoroughly by using a mechanical shaker to produce a suspension of 10^{-1} dilution. The 0.1 ml of the 10^{-4} to 10^{-10} dilution was aseptically pipetted onto a petri dish containing nitrogen-free agar (NFA) and spread using a sterilized glass spreader. The petri dishes were inverted and incubated to 3 to 14 days at 32 ^{0}C in an incubator for colonies development. The petri dish containing 25 to 250 CFU per plate were selected (Scott, 2011) to be recorded. The contamination of the inoculated carriers was directly assessed for the presence of fungal contamination (Rozarina *et al*., 2013). The degree of contamination of inoculated carriers was also identified at a 10^{-5} dilution by the bacterial spreading plate method. The development of bacterial colonies on the NFA medium, which differed from those formed by Sb35 and Sb42, was observed for contaminants for up to 14 days.

2.5.2 Determination of Physicochemical Properties of Different Carrier Materials

The physicochemical properties of the carrier were examined for pH and moisture content in the first week (before inoculation with N_2-fixing bacteria) and at regular intervals of two, fourth, sixth, and eight weeks periodically after inoculation with bacteria. The pH of inoculated carriers was determined with a pH meter at 1:5 ratio (inoculated carriers to distilled water) (Coleman *et al*., 1967). The moisture content of inoculated carriers was determined using the gravimetric method (Black, 1965).

Include in the Method section information that provides definitions of all primary and secondary outcome measures and covariates, including measures collected but not included in this report. Describe the methods used to collect data (e.g., written questionnaires, interviews, observations) as well as methods used to enhance the quality of the measurements (e.g., the training and reliability of assessors or the use of multiple observations). Provide information on instruments used, including their psychometric and biometric properties and evidence of cultural validity.

3. Results

3.1 Total Population of Sb35 and Sb42 in the Different Carrier Materials Combinations

Table 3 shows the results of the total population of Sb35 and Sb 42 inoculants in different EFBC-BRH combinations of support materials after eight weeks of incubation. There is a significant difference ($p < 0.05$) between the different carriers with bacterial population (Sb35 and Sb42) after eight weeks of incubation from ANOVA analysis. The viability of bacterial strains, Sb35 and Sb42 gradually increased for EFBC-BRH carriers' combinations with storage time. In the T6 (1 fine textured EFBC: 1 BRH), the viability for both Sb35 and Sb42 was significantly highest after eight weeks of incubation which are at 7.32 and 7.34 log $_{10}$ CFU per gram respectively (Figure 1). The other treatments which consist of fine textured EFBC and BRH; T5, T7 and T8 were also able to increase the number of viable cells of inoculants after eight weeks of incubation from week 2 to week 8. Meanwhile for the treatment T1 with coarse textured EFBC, the viability of Sb35 and Sb42 was significantly decrease during the eight-week incubation. The population of Sb35 and Sb42 dropped to 0.40% and 0.33%, respectively, during the first four weeks of incubation. Thus, carrier materials made of finely textured EFBC support the growth of the vaccine better than those made of coarsely textured EFBC.

Table 3. *Population of Sb35 strain and Sb42 strain in different carriers for eight weeks period*

EFBC-BRH Carrier Materials Combinations	Total Bacterial Population in Inoculated carrier materials (Mean ± SD Log_{10} CFU/g)	
	Sb35	Sb42
T1	6.81 ± 0.05	6.83 ± 0.06
T2	6.91 ± 0.04	6.89 ± 0.04
T3	6.82 ± 0.04	6.83 ± 0.11
T4	6.91 ± 0.05	6.86 ± 0.06
T5	6.99 ± 0.06	7.08 ± 0.04
T6	7.32 ± 0.02	7.34 ± 0.03
T7	7.00 ± 0.05	6.98 ± 0.03
T8	7.07 ± 0.03	7.07 ± 0.03

Keys:

T1= 1 coarse EFBC: 0 BRH
T2= 1 coarse EFBC: 1 BRH
T3= 1 coarse EFBC: 3 BRH
T4= 3 coarse EFBC: 1 BRH
T5= 1 fine EFBC: 0 BRH
T6= 1 fine EFBC: 1 BRH
T7= 1 fine EFBC: 3 BRH
T8= 3 fine EFBC: 1 BRH

EFBC= EFB compost
BRH = Burnt rice husk

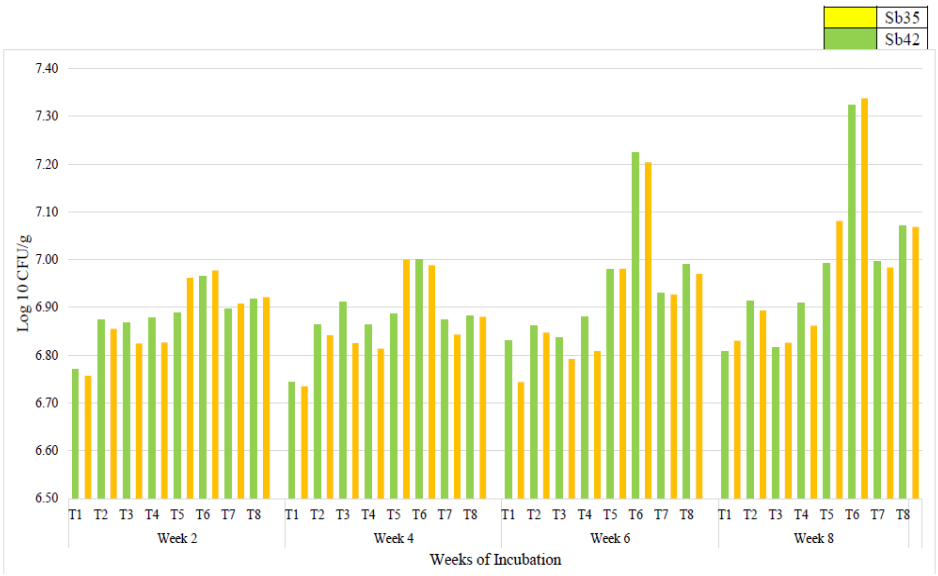

Figure 1. *Population of Sb35 strain and Sb42 strain recovered from the different inoculated carrier for eight weeks period*

3.2 Changes in pH of Different Inoculated Carrier Materials

Table 4 shows the results of the pH values of an inoculated carrier after eight weeks of incubation. There is a significant difference; $p < 0.05$ exhibited between the pH of different inoculated carriers during the eight weeks of incubation from one-way analysis ANOVA. The pH of Sb35 and Sb42 inoculated carriers showed the little

fluctuation and variation after incubation period (Figure 2). The fluctuations in pH in all the support materials could be due to the bacteria trying to adapt to the conditions of the support material in order to survive.

Table 4. *The pH value of different inoculated carriers after eight weeks period*

EFBC-BRH Carrier Materials Combinations	pH values (Mean ± SD) of Inoculated Carrier Materials	
	Sb35	Sb42
T1	7.05 ± 0.48	6.93 ± 0.35
T2	7.39 ± 0.64	7.65 ± 0.58
T3	7.52 ± 0.55	7.86 ± 0.50
T4	7.35 ± 0.53	7.27 ± 0.33
T5	6.78 ± 0.31	6.83 ± 0.13
T6	6.84 ± 0.19	6.93 ± 0.13
T7	7.69 ± 0.15	7.13 ± 0.24
T8	6.87 ± 0.29	6.66 ± 0.20

Keys:

T1= 1 coarse EFBC: 0 BRH
T2= 1 coarse EFBC: 1 BRH
T3= 1 coarse EFBC: 3 BRH
T4= 3 coarse EFBC: 1 BRH
T5= 1 fine EFBC: 0 BRH
T6= 1 fine EFBC: 1 BRH
T7= 1 fine EFBC: 3 BRH
T8= 3 fine EFBC: 1 BRH

EFBC= EFB compost
BRH = Burnt rice husk

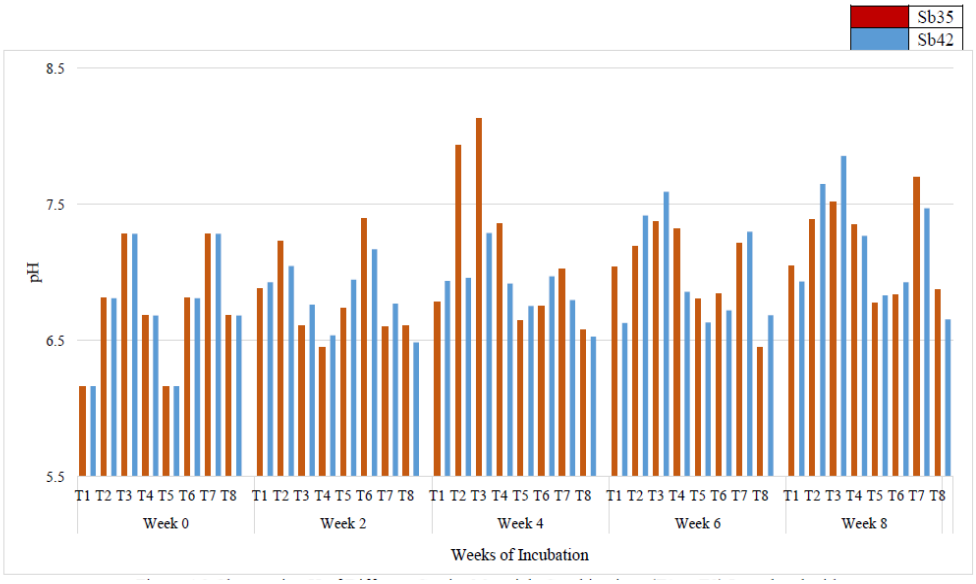

Figure 2. *The pH changes of different inoculated carriers for eight weeks period*

3.3 Changes in Moisture Content of Different Inoculated Carrier Materials

The moisture content of the inoculated substrates is shown in Table 5. There was a significant difference; $p<0.05$ between the moisture content of different inoculated carriers for eight weeks storage period (Figure 3). The moisture content in all inoculated carrier materials gradually decreased during the incubation period. The moisture contenct is one of the most important properties used to evaluate the carrier materials. According to Mishra, 2002, 35% to 40% recognized as the minimum range of carrier's moisture content for bacterial growth.

3.4 Contaminations of Different Inoclated Carrier Materials

The inoculated carrier contamination study was conducted up to an eight weeks period of incubation. All treatments of inoculated carriers; Sb35-inoculated carriers (T1 to T8) and Sb42-ionculated carriers (T1 to T8) had absence of contamination for eight weeks period. This demonstrates and proved that sterilization in an autoclave at 121 ºC and 151 psi for 30 minutes for three consecutive days effectively eliminated all microbes present in the carrier materials, both in vegetative and spore forms.

Table 5. *Moisture content in different inoculated carriers for eight weeks of period*

EFBC-BRH Carrier Materials Combinations	Moisture Content (Mean ± SD %) in Inoculated Carrier Materials	
	Sb35	Sb42
T1	37.13± 2.17	37.08 ± 2.03
T2	39.32 ± 1.98	38.10 ± 2.07
T3	43.42 ± 2.01	41.85 ± 1.95
T4	38.60 ± 2.05	38.85 ± 1.88
T5	33.98 ± 2.19	33.63 ± 2.43
T6	38.46 ± 2.33	35.74 ± 2.00
T7	39.19 ± 1.69	39.23 ± 1.76
T8	35.39 ± 2.25	35.29 ± 2.65

Keys:

T1= 1 coarse EFBC: 0 BRH
T2= 1 coarse EFBC: 1 BRH
T3= 1 coarse EFBC: 3 BRH
T4= 3 coarse EFBC: 1 BRH
T5= 1 fine EFBC: 0 BRH
T6= 1 fine EFBC: 1 BRH
T7= 1 fine EFBC: 3 BRH
T8= 3 fine EFBC: 1 BRH

EFBC= EFB compost
BRH = Burnt rice husk

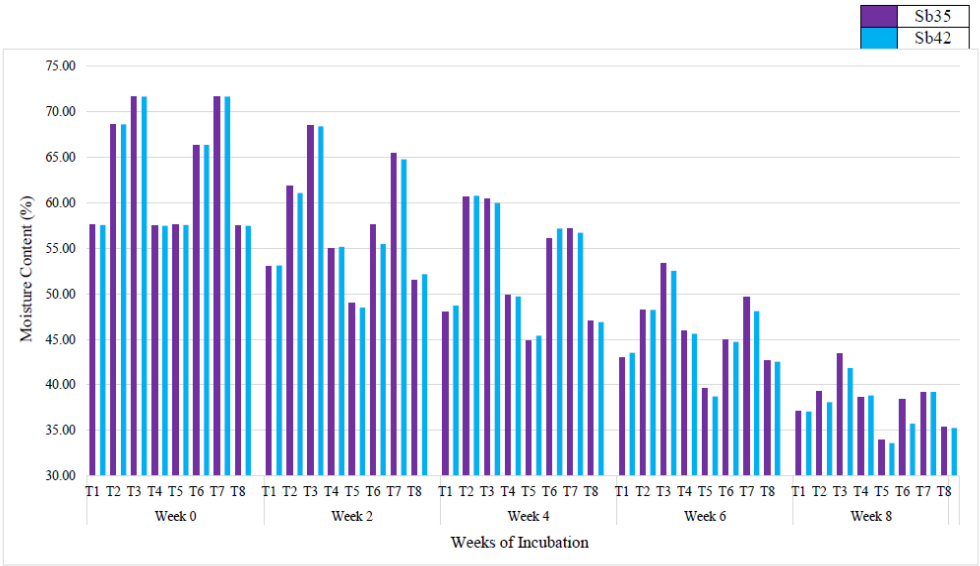

Figure 3. Changes in moisture content of different inoculated carriers (T1 to T8) for eight weeks period

4. Discussion

Regarding the coarse texture of the carrier materials, all treatments with fine textured carrier materials performed better than those with coarse textured carrier materials, especially at T6 (1 fine textured EFBC: 1 BRH). The fine-textured materials used for bacterial carriers have a friable consistency and a large surface area that allows thorough mixing of bacterial broth cultures so that they adhere to the surface of the carrier materials (Okerekeh and Okeh, 2007). This friable consistency may be less or absent in coarse textured EFBC compost material, making the attachment of bacterial cells to the surface of the support material less efficient. BRHs play the role of a nutrient-rich carrier material containing carbon, reducing sugars, nitrogen, and vitamins to stimulate microbial growth (Wang, 2012; Chen *et al*. 2016). The EFBC also play an important role in providing nutrients to the microorganisms. According to Chang *et al*., 2007, the EFBC materials contains the compound that are important for microbial growth such as hormones, vitamins, nutrients and growth regulators. The high carbon content in EFBC serve as a very good food sources for microbes (Schuchardt *et al*., 2008).

The fluctuations in pH in all the support materials could be due to the bacteria trying to adapt to the conditions of the support material in order to survive. It may also be due to the formation of the complex substances and compound by bacteria during their matabolisms in the carrier materials (Kaljeet *et al*., 2011). In general, the pH of varieties of carrier materials was stable and showed minor fluctuations. Previous study by Evans *et al*., 1980 indicated the N_2-fixing bacteria optimally survive between the ranges of pH 4.4 to 6.6. Nevertheless, in this research, the recorded range of pH for Sb 35-inoculated carriers and Sb42-carriers are 6.84 to 7.69 and 6.66 to 7.86 respectively. In this study, both bacteria tested were tolerant of slightly acidic to slightly alkaline pH values. The highest population of Sb35 and Sb42 was found at treatment T6 carriers with pH 6.83 ans pH

6.84 respectively.

The inoculated carriers with a dominant proportion of BRH had the highest moisture content after eight weeks of incubation, ranging from 39% to 43% (Figure 3). BRH materials can maintain moisture content because it has a higher water holding capacity (Hafeez *et al*., 1989). According to previous discovered by Kaljeet *et al*. (2011), to increase their suitability as a microbial carrier, the rice husks need to be added with other carrier materials which would enable them to increase, maintain and improve their water retaining capacity. In this study, BRH was shown to be as a suitable material to be supplemented to retain the moisture content of the inoculated carriers.

The carrier materials used were non-toxic as shown by the increase in bacterial population during the incubation period. The combination of EFBC-BRH carrier materials was also less susceptible to fungal attack. The Ogbo and Odo, 2011, stated the 19% of silica (SiO_2) in rice husk as antifungal which make the carrier materials less susceptibility to fungal and mould attack.

5. Conclusion

Agricultural wastes are commonly used as microbial carriers for soil fertility and crop productivity management. Compost is a suitable carrier material for microbial inoculants because it is environmentally safe, has high water retention capacity, is physicochemically uniform, is locally available at reasonable cost, and does not contain toxic substances. In summary, the evaluation of the suitability of carrier materials for N_2-fixing bacterial inoculants showed that fine-textured EFBC is the better carrier material compared to coarse-textured EFBC. The fine-textured EFBCs with their friable consistency provide a better adherent surface for the selected bacteria to grow. The addition of BRH is important to improve EFBC as a support material for selected N_2-fixing bacteria as it plays a role in improving water holding capacity. Based on these criteria, T6 treatment is the most suitable carrier material for both Sb35 and Sb42 strains. Further studies on T6-Sb35 and T6-Sb42 as a potential biofertilizer should be further investigated under field conditions to better evaluate their performance. Further studies could include optimizing the carrier materials by combining fine-structured EFBC and coarse-structured EFBC and using other genera and species of N_2-fixing bacteria. The suitability of T6-Sb35 and T6-Sb42 as soil amendments should be investigated.

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