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Shelf Life of Biofilm Producing PGPR Liquid Inoculants in Different Enriched Media Composition

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Abstract. Selecting a carrier material compatible with microbes is crucial for ensuring the quality of biofertilizers. This research aimed to identify a carrier material that could sustain the population of inoculants from dryland (including *Delftia tsuruhatensis* strain D9, *Delftia* sp. strain MS2As2, and *Bacillus* sp.) for a specified period. After conducting the study, it was found that a formulation of 3% molasses, 3% glycerol, 1% potassium sorbate, and 1% Tween-20 enriched with 1% nutrient was the most effective carrier material in maintaining the number of live inoculant cells. This formulation was able to sustain the population of inoculants up to the twelfth week, with a total population of 21.60×10^8 CFU.mL^{-1} with pH value of media is 5.29, and fulfilled the main criteria for biofertilizers.

Keywords: viability of superior isolates rhizobacteria; liquid consortium biofertilizer; selection of carrier formulation

INTRODUCTION

The continuous application of chemical fertilizer in agricultural fields has a significant impact, causing soil degradation and environmental pollution. In recent years, biofertilizers have become a public concern as an effort to reduce the use of chemical fertilizers and are increasingly being applied (Tapia and Fuentes, 2015; Bhavya, 2017). Previous research with *Plant Growth Promoting Rhizobacteria* (PGPR) based on biofertilizers showed that the dose of 100% NPK fertilizer gave results that were not different from the 50% NPK dose with the addition of biofertilizers was tested on upland rice cultivation. Other studies have also demonstrated that inoculation results with selected PGPR isolates have a considerable positive impact on different rice growth parameters, including the percentage of sprouts, growth of shoots and chlorophyll content (Naher, 2018; Cavite et al., 2021; Bal et al., 2013).

Biofertilizers are not a direct source of nutrients for plants, but contain beneficial microbes that break down organic compounds in the soil into simple ion forms that plants can absorb (Bhavya, 2017). Plant Growth Promoting Rhizobacteria (PGPR) are a group of beneficial microorganisms that are often inoculated as biofertilizers. These bacteria have relevant properties that are essential for plant nutrients such as nitrogen (N) and phosphorus (P) and are known as N-fixing rhizobacteria and phosphate-solubilizing bacteria (Lobo et al., 2019). Additionally, PGPR has several functional properties, such as inhibiting soil pathogens by producing HCN, siderophore, and antibiotics, improving plant tolerance to drought, salinity, metal toxicity, and P dissolution, and producing phytohormone (Figueireo, et al., 2011; Basu et al., 2021; Vandana et al., 2020). Inoculation of PGPR isolates as biofertilizers requires a compatible carrier material from the laboratory to the field when applied to plants. The survivability and efficiency of rhizobacteria inoculants in biofertilizer formulations greatly depend on the choice of carrier materials and storage temperatures.

Designing effective biofertilizers with long storage shelf lives is the greatest bottleneck for biofertilizer technology (Aloo et al., 2022). The selection of a suitable carrier material is necessary to maintain the appropriate number of microbes. In addition to meeting the requirements of carrier materials in general, liquid carriers have advantages over solid carriers, such as not requiring additional binding agents, requiring fewer cells, and being easier to use (El-Ramady et al., 2018; Raimi et al., 2021).

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Glycerol and molasses are commonly used liquid carriers as carbon source for microbes during incubation (Allouzi et al., 2022). For example, the use of 2% glycerol in the actual liquid medium can increase the storage life of biofertilizers with the consortium of *Azotobacter chroococcum*, *Azospirillum* lipoferum, *Bacillus* megaterium, and *Bacillus* sporothermodurans, mostly compared to other carriers up to day 180 (Gopi et al., 2019). Molasses produced from sugarcane waste contain up to 55% sucrose (Olbrich, 2006).

The addition of molasses to the carrier material yeast extract mannitol agar (YEMA) with various concentrations can maintain a population of Rhizobium microbes up to 4.30×10^8 CFU.mL^{-1} for six months of incubation (Garcha et al., 2019). Knowing the combination of good carrier materials for the survival of microbes is the first step in using liquid biofertilizers. Therefore, this research was conducted to improve the shelf life of N-fixing and biofilm-forming bacteria (*Delftia tsuruhatensis* strain D9, *Delftia* sp. strain MS2As2, and *Bacillus* sp.) isolates, which were isolated from dry land using a suitable carrier material with the highest bacterial population.

METHODS

Experimental condition

A viability test was conducted to determine the survival ability of N-fixed PGPR isolates within a certain period using liquid carriers with different nutrient compositions and doses. The viability test was conducted at the Biotechnology Laboratory, Faculty of Agriculture, Padjadjaran University, from March to June 2022. The isolates were obtained from five different ecosystems, namely rainfed field, rice field, mix plantations, forests and savannas in West Nusa Tenggara Province, Indonesia, and were isolated using Okon's media. The coordinates for the isolation locations are S 80 48' 29.82"; E 1160 18' 52.81", and the elevation ranges from 34 to 1461 meters above sea level. There are consortium isolates consisting of: *Delftia tsuruhatensis* strain D9, *Delftia* sp. strain MS2As2 and *Bacillus* sp.

Formulation of liquid biofertilizer inoculants

The production of liquid biofertilizer is carried out at the Soil Biotechnology Laboratory, Padjadjaran University. Pure bacterial cultures are prepared by multiplying pure cultures from agar in a liquid Okon's medium. The culture was shaken then and harvested after 72 hours, when the cell count reaches 10^8 CFU.mL.$^{-1}$. All carrier materials are mixed according to treatment to obtain different formulations. All carrier materials were sterilized using an autoclave at 121℃ and 15 Psi for 15 minutes. After sterilization, each carrier was mixed with a pure culture of bacteria enriched with nutrients according to the treatment at a ratio of 9:1 (v/v). The nutrient provided were a mixture of Okons selective media and KNO_3 with a ratio 1:1 (v/v). Furthermore, the carrier material containing pure bacterial cultures was packaged in sterile high-density polyethylene (HDPE) bottles with a capacity of 100 mL. After that it is closed as not to allow contamination and stored at room temperature. The materials are packed in boxes and stored indoors at room temperature (25 ºC). There were a total of twelve liquid biofertilizer strain formulations used. There are : L_1 = 3% glycerol ; L_2 = 3% glycerol + 1% nutrient ; L_3= 3% glycerol + 1% nutrient + KNO_3 5% ; L_4= 3% molasses + 3% glycerol ; L_5 = 3% molasses + 3% glycerol + 1% nutrient ; L_6 = 3% molasses + 3% glycerol + 1% nutrient + 5% KNO_3 ; L_7 = 3% molasses + 3% glycerol + 1% Potassium sorbate ; L_8 = 3% molasses + 3% glycerol + 1% Potassium sorbate + 1% nutrient ; L_9 = 3% molasses + 3% glycerol + 1% Potassium sorbate + 1% nutrient + 5% KNO_3 ; L_{10} = 3% molasses + 3% glycerol + 1% Potassium sorbate + 1% tween 20 ; L_{11} = 3% molasses + 3% glycerol + 1% Potassium sorbate + 1% tween 20 + 1% nutrient ; L_{12} = 3% molasses

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+ 3% glycerol + 1% Potassium sorbate + 1% tween 20 + 1% nutrient + 5% KNO_3.

Values obtained were means of three replications. The viability of microorganisms was evaluated on a weekly basis up to 16 weeks after production with the Total Plate Counter (TPC) method, and the potential of hydrogen (pH) media was measured using a digital pH meter. Standard deviation and were statistically analyzed using Duncan's multiple range test ($p<0.05$) using a complete randomized design. Values are the mean of three replications ±SD; Means values followed by the same letter are not significantly different based on Duncan's multiple range test ($p<0.05$).

RESULTS AND DISCUSSION

Generally, bacteria have a division time of 72 hours after incubation. **Table 1**. shows that during the incubation, there was an increase in the number of bacterial cells from the initial incubation time (0 weeks after incubation) to 16 weeks after incubation.

The addition of nutrients significantly increased the number of microbes, as evidenced in all treatments. Formulation with 1% nutrient addition showed different and increased numbers of microbes than formulations without nutrient addition. The population of microbes continued to increase in weeks 8 to 12 in all carriers. The experimental data can be seen in **Table 1**. For instance, the carrier with glycerol 3% had a bacterial population of 11.93×10^8 $CFU.mL^{-1}$ at the sixth week after incubation, which decreased to 11.13×10^8 $CFU.mL^{-1}$. The bacterial population increased to 11.77×10^8 $CFU.mL^{-1}$ in week 12 with adding 1% nutrient, but decreased to 3.10×10^8 $CFU.mL^{-1}$ in week 16. The molasses and glycerol carriers were able to increase the population of microbes to a number of 22.53×10^8 $CFU.mL^{-1}$ until week 8, but in week 12 the population decreased by 28.22%. The decrease in the number of bacterial cells indicates that the cells have entered a phase toward death. In this phase, there are more dead cells than growing cells, which is suspected to be due to significantly reduced nutrients and unfavorable environmental conditions for bacterial cell survival resulting from the metabolites produced by the microorganisms themselves (White, 1995).

The population of microbes on a carrier material comprising 3% molasses, 3% glycerol, 1% potassium sorbate and 1%Tween-20 without added nutrients was found to be 18.23×10^8 $CFU.mL^{-1}$ at the twelfth week. This number was significantly less than the total population when 1% nutrient was added. The L_{11} treatment (3% molasses, 3% glycerol, 1% Potassium sorbate and 1% Tween-20 enriched 1% nutrient) was the most effective formulation, maintaining the highest number of microbes until the sixteenth week of incubation. This carrier material was also found to be stable, with the highest population number of 21.60×10^8 $CFU.mL^{-1}$ in the twelfth week, which was a 30.67% increase from the total microbial population in the eighth week. The high population count in this carrier material is suspected to be caused by the complete composition of the carrier as an energy and nutrient source for bacterial living. Every microorganism requires a carbon source for its growth by converting that carbon into cellular materials through the process of assimilation (Lim, 1998). Carbon sources that these bacteria can utilize include molasses. The population has been in line with the technical requirements for liquid biofertilizers determined by the Indonesian Ministry of Agriculture ($\geq 1 \times 10^7$ $CFU.mL^{-1}$ for consortium liquid biofertilizers) (Ministry of Agriculture Indonesia, 2019).

Several countries where the biofertilizers industry has been strongly developed in the last years have already enacted some regulations. China has defined the quality of biofertilizers with eight parameters and the number of live cells is considered the most important parameter to judge the quality of biofertilizers. Depending

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on the type of bacteria for liquid biofertilizers in this country, it requires a minimum number of live cells ranging from ≥ 0.5 × 10^9 CFU.mL^{-1} (Suh et al., 2006). India sets seven parameters for the quality of biofertilizers with the main requirement being the number of live cells for the bacterial group, which is above 1 × 10^8 CFU.mL^{-1} (Malusá & Vassilev, 2014).

Table 1. Log population of nitrogen-fixing bacteria isolates on different carrier compositions with shelf life ranging from 0 WAI – 16 WAI

Inoculant formulation	Log Population of Nitrogen Fixing Bacteria (× 10^8 CFU.mL^{-1})						
	Weeks After Incubation (WAI)						
	0 WAI	2 WAI	4 WAI	6 WAI	8 WAI	12 WAI	16 WAI
L_1	5.40 ± 0.36	4.40 ± 0.10 a	10.97 ± 0.45 bc	11.93 ± 0.71 ab	11.13 ± 1.00 ab	7.50 ± 5.11 a	3.00 ± 0.10
L_2	6.97 ± 0.15	8.13 ± 0.35 abc	8.13 ± 0.55 ab	9.53 ± 0.31 a	9.43 ± 0.70 a	11.77 ± 0.31 b	3.10 ± 0.10
L_3	7.73 ± 0.40	6.97 ± 0.50 abc	5.13 ± 0.21 a	9.33 ± 0.32 a	10.23 ± 0.45 ab	16.83 ± 0.31 c	2.20 ± 0.17 a
L_4	7.03 ± 0.25	7.70 ± 0.20 abc	10.30 ± 0.40 bc	25.50 ± 0.44 d	22.53 ± 0.21 d	16.17 ± 3.71 c	2.40 ± 0.36
L_5	8.87 ± 0.15	9.47 ± 0.49 c	10.47 ± 0.51 bc	19.20 ± 7.98 c	20.87 ± 2.34 d	16.60 ± 9.79 c	3.43 ± 0.15
L_6	7.37 ± 0.25	7.93 ± 0.93 abc	9.80 ± 0.66 bc	19.73 ± 10.29 c	13.50 ± 0.36 bc	16.43 ± 0.70 c	3.23 ± 0.12
L_7	8.40 ± 0.26	8.83 ± 0.06 bc	10.87 ± 0.31 bc	10.33 ± 0.47 a	10.40 ± 0.56 ab	18.83 ± 0.25 cd	3.97 ± 0.15
L_8	8.47 ± 0.35	5.60 ± 0.20 ab	12.43 ± 0.31 c	9.73 ± 0.21 a	9.20 ± 0.10 a	15.00 ± 0.70 bc	3.37 ± 0.12
L_9	6.20 ± 0.56	5.77 ± 0.55 abc	7.53 ± 0.38 ab	10.77 ± 0.47 a	9.00 ± 0.26 a	18.93 ± 0.15 cd	3.37 ± 0.32
L_{10}	8.13 ± 0.35	7.90 ± 0.44 abc	10.43 ± 0.31 bc	12.77 ± 0.31 ab	14.83 ± 0.15 c	18.23 ± 0.32 cd	3.83 ± 0.25
L_{11}	8.43 ± 0.25	6.47 ± 0.32 abc	9.67 ± 0.61 bc	15.37 ± 0.31 b	16.53 ± 0.75 c	21.60 ± 0.20 d	5.50 ± 0.20
L_{12}	8.80 ± 0.26	8.07 ± 0.31 abc	11.87 ± 0.57 c	12.30 ± 0.20 ab	13.60 ± 0.56 bc	12.50 ± 0.80 b	2.67 ± 0.25

Note: Values are the mean of three replications ±SD; Means values followed by the same letter are not significantly different based on Duncan's multiple range test ($p<0.05$).

In line with the research of (Goudar et al., 2017) showed that the shelf life of N-fixing bacterial isolates (*Azospirillum*) can last up to 180 days after incubation. The

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largest populations were found in culture broth media containing Tween-80, glycerol, xanthan gum, sorbic acid and potassium sorbate, reaching up to 58 × 10^8 CFU.mL^{-1}. This amount was higher than the carrier material without glycerol. Nitrogen-fixing bacteria inoculants can metabolize ethanol into a medium containing fructose or glycerol as a carbon source and contribute to their growth. In a minimal medium containing fructose or glycerol as a carbon source, ethanol supplementation increases the production of dehydrogenated alcohols and aldehyde dehydrogenases. Ethanol is used as a secondary carbon source when fructose and glycerol are the primary growth substrates (Singh et al., 2021).

Table 2. The pH value of the carrier during shelf life from 0 WAI – 16 WAI

Inoculant formulation	pH of Carrier						
	0 WAI	2 WAI	4 WAI	6 WAI	8 WAI	12 WAI	16 WAI
L_1	6.23	5.84	5.48	5.79	6.01	5.54	6.34
L_2	6.37	7.04	5.25	7.30	7.23	7.28	7.33
L_3	6.37	7.30	5.53	7.28	7.29	7.26	7.29
L_4	5.10	4.15	5.02	4.19	4.09	4.27	4.26
L_5	5.73	4.32	4.74	4.30	4.78	4.15	4.81
L_6	5.82	4.66	4.86	4.47	4.43	4.85	4.48
L_7	5.73	5.45	5.54	4.96	4.92	5.06	5.05
L_8	5.67	5.08	4.74	5.03	5.10	5.04	5.01
L_9	5.47	5.00	4.87	5.11	4.88	4.42	4.94
L_{10}	5.87	5.79	5.57	5.37	5.36	5.17	5.21
L_{11}	6.23	5.55	5.29	5.16	5.38	5.29	5.32
L_{12}	5.63	5.25	6.39	5.31	5.37	5.41	5.32

Evaluation of pH in liquid-based biofertilizers showed variations among different carrier materials. The pH value of nitrogen-fixing bacteria (NFB) carriers ranged from 4.19 to 7.33. Each carrier material has a different pH value. The biofertilizer inoculant on 3% glycerol media had an acidity degree of 5.48–6.34. The pH values of glycerol and nutrient carriers ranged from 5.25–7.33, indicating a slightly basic pH. However, glycerol and molasses carriers had a highly acidic pH from 5.10 to 4.26. The addition of nutrients to the glycerol and molasses carriers was not able to increase the pH, as evidenced by the pH value of the carrier until the 16th week remained 4.26 (very acidic). The carrier material consisting of glycerol, molasses and

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potassium sorbate having a pH of 4.92–5.73 also belongs to the acid category. The pH values of the glycerol, molasses, potassium sorbate, and 1% nutrient carriers remained relatively stable at the beginning of incubation, with a pH of 6.23 (close to neutral). However, from the second week until the sixth week, the pH value tended to stabilize at around 5, as shown in **Table 2**. Several factors can influence the pH of the culture medium, such as protein degradation, which can release amino acids, as well as the presence of inhibitors and the age of the culture. It is worth noting that the degree of acidity of each carrier material differed, with values ranging from 4.26 to 7.30. The pH of the biofertilizer carrier materials plays a crucial role in determining the viability and activity of the inoculants, as the pH and viscosity of the medium are physical and chemical properties that can impact the shelf life of rhizobacteria strains in the carrier media (Puwanto et al., 2019).

Bacteria are able to survive in media with a pH range of 4 to 8.5. If the pH in the medium is not optimal, it will disrupt metabolic activity, thus affecting the growth of the bacteria itself. The pH value of the environment helps control the progress of respiration and microbial growth, shaping the population composition of bacteria (Jin and Matthew, 2018).

CONCLUSION

The research findings indicate that the liquid fertilizer inoculants developed using carriers supplemented with nutrients successfully increased the microbial populations. For instance, the carrier material consisting of 3% molasses, 3% glycerol, 1% potassium sorbate, and 1% Tween-20 with 1% nutrient is the best carrier and was able to maintain the highest microbial population up to the twelfth week, with a total population of 21.60×10^8 $CFU.mL^{-1}$, and has a media pH of 5.29. The total microbial population meets the biofertilizer industry requirements and is in line with the technical requirements for consortium liquid biofertilizers determined by the Indonesian Ministry of Agriculture, which is $\geq 1 \times 10^7$ $CFU.mL^{-1}$.

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