

Optimization of culture medium and bioformulation of rhizobial actinomycetes to enhance soybean plant growth

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Abstract. Fatmawati U, Qurrata'aini MR, Rosyidi NW, Sari DP, Santosa S. 2023. Optimization of culture medium and bioformulation of rhizobial actinomycetes to enhance soybean plant growth. *Biodiversitas* 24: 2912-2918. Actinomycetes are a group of Gram-positive bacteria that are well-known for having plant growth-promoting activity. In an earlier study, *Streptomyces* sp. strain ASR 58 and *Streptomyces* sp. strain ASR 67, isolated from soybean rhizosphere, were found to be capable of producing Indole-Acetic Acid (IAA), dissolving phosphate, and stimulating soybean growth. Optimization of culture media and selection of carrier materials for formulation are necessary to maintain actinomycetes' cell viability and plant growth property stability. This study aimed to evaluate several culture media, determine the optimal carrier materials in actinomycetes formulation, and evaluate the effect of actinomycete bioformulation on the soybean plant growth *in planta*. The sugar potato broth medium was the best in increasing the cell biomass of ASR 67 and co-culture of ASR 58 and ASR 67 compared to other mediums (skim molasses and rice bran broth). The viability of ASR 58 cell (5.3×10^7 CFU/mL), ASR 67 (3.8×10^7 CFU/mL), and its combination of both (4.3×10^7 CFU/mL) was best maintained in talc powder carrier during 10 weeks of storage as revealed by the number of viable cells decreases. Applying ASR 58 and ASR 67 co-culture on talc powder formula enhanced soybean plant biomass four weeks after planting, including plant height, the number of leaves, and root dry weight.

Keywords: Actinobacteria, carrier, formulation, growth promoter

INTRODUCTION

In intensive farming systems, chemical fertilizers are used to obtain optimal yields. However, chemical fertilizers have low efficiency because they are prone to soil degradation, reduction in soil organic matter, volatilization, denitrification, leaching, and mineralization (Lin et al. 2019). Long-term chemical fertilizers can damage the ecology of soil fauna, reduce soil fertility, contaminate waters, and affect human health (Sundarasu and Neelanarayanan 2012). Plant Growth-Promoting Rhizobacteria (PGPR) can be used as an alternative fertilizer to reduce chemical fertilizers.

Several PGPR has been circulating in the market. Various consortia with several bacterial strains have been studied to obtain more advantageous characteristics than single-strain PGPR. Several studies have also revealed that multi-strain PGPR can increase nodulation and growth in several legume species (Flores-Duarte et al. 2022). It is because several types of bacteria can interact synergistically, providing nutrients, eliminating inhibitory products, and stimulating each other physical and biochemical activities to support plant growth (Vacheron et al. 2013; Grover et al. 2021).

Actinomycetes colonize the rhizosphere and interact with plants by producing growth-promoting compounds that can inhibit the growth of phytopathogens and promote plant growth (Boukhatem et al. 2022). Earlier studies reported that actinomycetes, especially the *Streptomyces* group, from soil and rhizosphere have activity in increasing plant

growth and inhibiting the spread of soil-borne phytopathogens, such as *Fusarium oxysporum* (Mariastuti et al. 2018; Sari et al. 2021) *Rhizoctonia solani* (Ebrahimi-Zarandi et al. 2021) and *Verticillium dahliae* (Xue et al. 2013). Furthermore, in several developed countries such as the United States, Canada, Finland, and New Zealand, biofertilizer agents and actinomycetes-based biocontrol of plant pathogenic bacteria and fungi have been used (Palaniyandi et al. 2013a). Apart from being a biocontrol of soil phytopathogens, the actinomycetes consortiums can increase plant growth in several commodities such as chickpea (Alekhya and Gopalakrishnan 2017), yam (Palaniyandi et al. 2013b), sorghum and rice (Gopalakrishnan et al. 2013) and wheat (Jog et al. 2012). However, using actinomycetes as biological fertilizer and biocontrol of plant pathogens is uncommon in Indonesia. Therefore, exploring the potential actinomycetes for effective and environmentally friendly biological agents is necessary.

Bioformulations are all biologically active substances derived from microorganism biomass or products containing microbes and their metabolite products that can increase plant growth, nutrient acquisition, and disease control in an eco-friendly manner (Aamir et al. 2020). Bioformulation is a mixture of active ingredients made with inert (inactive) substances in a formulated product. In a bioformulation, it is also possible to mix and combine several PGPR as a consortium (Ikhwan et al. 2021). Unfortunately, many bioformulation products are

ineffective due to inadequate or poor-quality formulations, including poor compatibility and stability of the carriers (Vassilev et al. 2020). In the previous study, *Streptomyces* sp. strain ASR 58 and *Streptomyces* sp. strain ASR 67 were isolated from soybean rhizosphere soil. These isolates could produce Indole Acetic Acid (IAA) and dissolve phosphate in vitro (Fatmawati et al. 2019). These isolates also produced higher IAA concentration and higher solubilizing phosphate when co-culture compared to a single culture.

According to this potential, the selection of culture medium and cultivation techniques are important for actinomycetes bioformulation in mass production in vitro. In addition, the selection of carrier material for actinomycetes is also necessary to maintain the viability of the actinomycetes cell during storage. In this study, optimization was done in alternative propagation media to grow actinomycetes inoculums. Moreover, this study intended to determine the viability of actinomycetes inoculums on various carrier mediums and the application of actinomycetes consortia and soybean *Rhizobium* bacteria. Soybean rhizosphere microbes are expected to have better adaptations and be optimal in increasing soybean plant growth. The results can be used for developing more effective and environmentally friendly growth-promoting agents.

MATERIALS AND METHODS

Rhizosphere actinomycete source and culture

Streptomyces sp. ASR 58 and *Streptomyces* sp. ASR 67 isolates were isolated from the soybean rhizosphere in Sukabumi, West Java, Indonesia (Fatmawati et al. 2019). The isolates were stored in 50% glycerol on semisolid international *Streptomyces* Project 2 (ISP2) media and stored at -20°C at the Laboratory of Biology Education, Faculty of Teacher Training and Education, Sebelas Maret University, Surakarta. Reculture of *Streptomyces* sp. was performed by growing them on 100 mL medium solid ISP2 (4 g L⁻¹ yeast extract, 10 g L⁻¹ malt extract, 4 g L⁻¹ dextrose, 20 g L⁻¹ agar, and 1,000 mL distilled water; pH 7.2) and incubated for seven days at ±25°C.

Optimization of alternative media for actinomycete cell propagation

The alternative media used for actinomycete cell propagation consists of skim molasses broth (8 g L⁻¹ of skim, 15 mL L⁻¹ of molasses, 1.5 g L⁻¹ of K₂HPO₄, and 1.5 g L⁻¹ of MgSO₄ in 1,000 mL of distilled water); and sugar-potato broth (200 g/L of potato, 20 g L⁻¹ of sugar, and 1,000 mL of distilled water); and 3) rice bran (10 g L⁻¹ of rice bran and 1,000 mL of distilled water) (Tridesianti et al. 2017). Two plugs of 7-day-old actinomycetes culture in the ISP2 agar media were inoculated into a 100 mL ISP2 pre-culture liquid medium. It was then incubated in a rotary shaker at 120 rpm for seven days at 25°C until a cell density of up to 10⁸ CFU/mL was obtained. Cell populations are determined using the Total Plate Count (TPC) method. As much as 0.5 mL of each strain (ASR 58,

ASR 67, and the combination of ASR 58 and ASR 67) were inoculated on the 50 mL of the alternative media and incubated for ten days at ± 25°C in a rotary shaker (120 rpm). The cell dry weight of each culture was measured in the liquid culture using sterile 150 mm filter paper (Whatman), and then the filtrate was dried in an oven at 60°C for 18 hours. The culture was periodically collected at 12, 24, 48, 96, 144, 192, and 240 hours. The dry actinomycetes cells were weighed and compared with the control filter paper. The best alternative medium resulting in the highest actinomycetes biomass was used for further actinomycete culture in the formulation stage (Sari et al. 2021).

Actinomycete cell growth estimation

The growth phase of actinomycetes was determined by the Total Plate Count (TPC) method, where two plugs of actinomycetes culture were inoculated on 100 mL of sugar-potato broth medium and incubated for seven days at ±25°C in a rotary shaker at 120 rpm. The number of cells was counted by standard serial dilution by plating the culture on the ISP2 agar medium (Sarma et al. 2011).

Preparation of inoculum and carrier media

Talc, clay, and sawdust (Simply Wood, Yogyakarta, Indonesia) were carriers for the actinomycetes formulation. A total of 3 kg of each carrier was mixed with Carboxymethyl Cellulose (CMC) (Sigma) at a ratio of 10:1 (w/w) and by adding CaCO₃ to adjust the pH to 7. Then the mixture was divided into three equal parts, 1 kg each, and sterilized by autoclaving for 60 minutes at 121°C. Each actinomycete culture (*Streptomyces* sp. ASR 58, *Streptomyces* sp. ASR 67, and a combination culture of these isolates) in the sugar potato broth medium was inoculated to 1 kg of the carrier medium. The inoculated carrier media were then air-dried for 12 hours in a Laminar Airflow Cabinet (LAF) and stored at room temperature for ten weeks (Novinscak and Filion 2020).

Evaluation of actinomycetes viability in various carrier materials

Actinomycetes viability was evaluated during the shelf life at two-week increments: 2, 4, 6, 8, and 10 weeks. First, one gram (1 g) of each formula was collected and added to 9 mL of sterile 0.85% NaCl solution. The suspension was then homogenized and serially diluted to 10⁻⁶. Next, 100 µL of each dilution was plated into a Petri dish containing ISP2 agar and incubated for five days at ± 25°C. The number of actinomycetes colonies was counted according to (Sari et al. 2021).

Evaluation of the actinomycetes formula in promoting soybean growth

The effectivity of the actinomycetes formula carried in three different materials was evaluated in a greenhouse with a Completely Randomized Design (CRD). Next, 1 g of each formula was inoculated into a pot containing 50 g of planting medium (soil : sand = 2 : 1). There were 11 treatments in this experiment, consisting of negative control (without inoculants) and positive control (planting medium with 0.2% dissolved inorganic NPK fertilizer (N :

P : K = 6 : 1 : 11); and 3) three types of carrier media (talc powder; bentonite clay and sawdust) x three types of inoculants (*Streptomyces* sp. strain ASR 58; *Streptomyces* sp. strain ASR 67, and the combination of those isolates). Based on the antagonistic assay, the two strains can coexist without showing antagonistic properties. Each treatment was repeated three times, and each replication used ten soybean seeds. Plants' growth was observed four Weeks After Planting (WAP) by measuring the stem height, the number of leaves, root dry weight, and shoot dry weight. The data were analyzed using one-way ANOVA. The difference between treatments was analyzed using the Tukey test at a 95% confidence level using SPSS 25.

RESULTS AND DISCUSSION

The growth of actinomycetes on various alternative culture media

The three types of actinomycetes cultures (*Streptomyces* sp. strain ASR 58; *Streptomyces* sp. strain ASR 67, and a combination of both) could grow in various alternative media, including skim molasses, sugar potato broth, and rice bran broth. The dry weight of actinomycete cells varied greatly in each medium. The highest dry weight of *Streptomyces* sp. ASR 58 isolate was achieved on the rice bran medium at 192 hours of incubation (day 8), namely 1.41 g/100 mL. The dry weight of cells decreased to 1.34 g/100 mL at the end of the incubation period (Figure 1). The highest dry weight of the ASR 67 isolate was seen in the potato broth medium at 96 hours of incubation (day 4) with a dry weight of 1.52 g/100 mL. After 240 hours of incubation (day 10), the ASR 67 biomass decreased to 1.23 g/100 mL (Figure 2). The highest cell dry weight of ASR 58 and ASR 67 combination culture was achieved in the potato broth medium at 96 hours of incubation (day 4), at 1.35 g/100 mL dry weight. After 144 hours (day 6), the mass decreased in all alternative media. That was thought to occur in a transitional or adjustment stage (lag phase), and the number of cells would increase again after eight days of incubation (Figure 3).

Number of actinomycete inoculant cells in the potato broth medium

The results showed that the actinomycetes culture (*Streptomyces* sp. strain ASR 67 and the combination of *Streptomyces* sp. ASR 67 and ASR 68) demonstrated maximal growth in the potato broth medium as indicated by the highest cell dry weight of these cultures. The cell density of each inoculant used for the formulation in the carrier materials is listed in Table 1. It can be observed that the number of cells for each inoculant reached $3.8\text{--}4.5 \times 10^8$ CFU/mL on the 7th day of incubation. Thus, it met the cell density standard needed in an inoculant of bioformulation, as suggested by the Regulation of the Indonesian Minister of Agriculture concerning Minimum Technical Requirements for Organic Fertilizers, Biological Fertilizers, and Soil Improvements.

Table 1. The cell density of inoculants per 100 g of formulation material

Culture	Carrier media (g)	Cell Counts (CFU/mL)
<i>Streptomyces</i> sp. strain ASR 58	100	4.5×10^8
<i>Streptomyces</i> sp. strain ASR 67	100	4.2×10^8
Co-culture of <i>Streptomyces</i> sp. strain ASR 58 and <i>Streptomyces</i> sp. Strain ASR 67	100	3.8×10^8

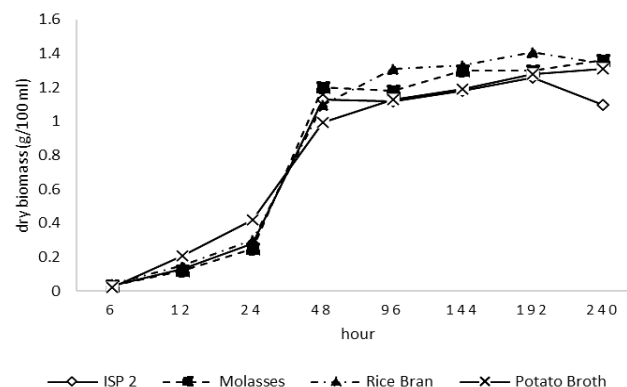


Figure 1. The dry weight of *Streptomyces* sp. ASR 58 isolate in various culture media for ten days (240 hours)

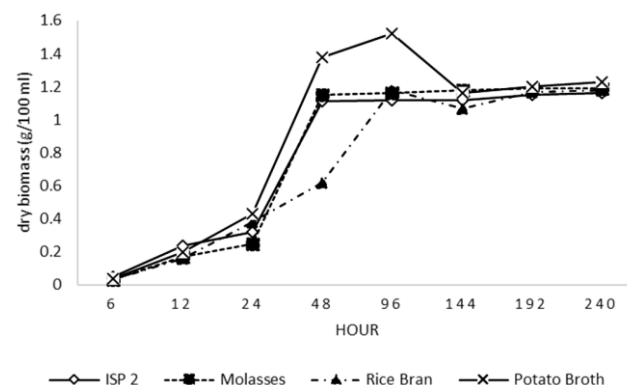


Figure 2. The dry weight of *Streptomyces* sp. ASR 67 isolate in various culture media for ten days (240 hours)

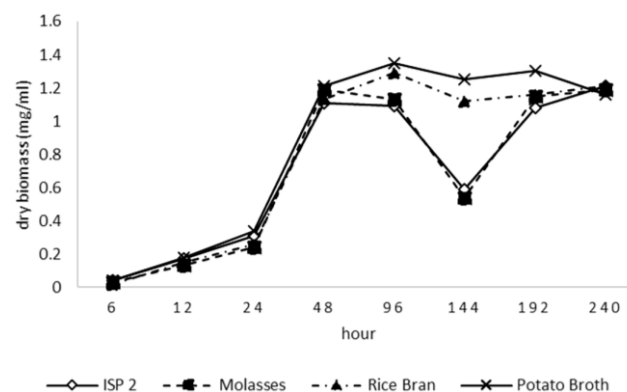


Figure 3. The dry weight of the ASR 58 and ASR 67 combination in various culture media for ten days (240 hours)

Actinomycete viability in various carrier materials during the shelf life

The number of viable actinomycete cells decreased during ten days of storage at $\pm 25^{\circ}\text{C}$ in all carrier material types. The highest number of viable cells was found after 2 weeks of storage in the $5.7\text{--}31.6 \times 10^7$ CFU/g range. However, the number of viable cells of *Streptomyces* sp. strain ASR 58 and *Streptomyces* sp. ASR 67 was highest in sawdust (31.6×10^7 CFU/g) and clay (1.9×10^7 CFU/g), respectively, after 10 weeks of storage. In the talc powder carrier, the ASR 58 and ASR 67 co-culture cell count was highest in week 2 at 8.9×10^7 CFU/g, which decreased after 10 weeks of storage to 4.3×10^7 CFU/g. Although there was a significant decrease in the number of cells during the storage period, the microbial populations were still above the standard established in the Regulation of the Minister of Agriculture on the number of inoculant cells in organic fertilizers, which is 10^6 CFU/g for actinomycetes consortium.

The effect of actinomycete formulation on soybean plant growth

The inoculation of the actinomycetes formula (which has been stored for two months at room temperature) on the soybean growing media showed a significant increase in soybean growth compared to the negative control (without inoculants). The results of one-way ANOVA showed an effect of various bioformulations on plant height, number of leaves, root dry weight, and shoot dry weight. The effect of the bioformulants on plant height is shown in Figure 4. ASR 58 and the combination of ASR 58 and ASR 67 in the talc powder carrier showed a significant increase in plant height compared to the control ($P > 0.05$). Both ASR 58 and ASR 67 co-culture and single ASR 58 in the talc powder medium also significantly increased the number of leaves of soybean plants after four weeks of planting ($P > 0.05$) (Figure 5). The ASR 58 and ASR 67 co-culture inoculants in talc powder medium also significantly increased root dry weight compared to the control ($P > 0.05$) (Figure 6). Shoot dry weight also increased with ASR 58 and ASR 67 combination treatment on talc powder medium by 10% from 5.10 g/plant to 5.6 g/plant, but this increase was not statistically significant ($P < 0.05$) (Figure 7).

Table 2. Actinomycete viability in various carrier mediums during 10 weeks of storage

Carrier Media	Isolates	Cell counts ($\times 10^7$ CFU/g)				
		Week #2	Week #4	Week #6	Week #8	Week #10
Talc	ASR 58	3.7a	4.3a	4.4a	4.7a	5.3a
	ASR 67	6.6a	5.1ab	4.8ab	4.2ab	3.8b
	ASR 58 + ASR 67	8.9a	6.4ab	5.2b	4.5b	4.3b
Clay	ASR 58	19.0a	7.7a	3.8bc	4.8bc	7.0c
	ASR 67	30.4a	15.1b	5.9c	1.4d	1.9cd
	ASR 58 + ASR 67	6.2a	5.7a	3.6b	2.0c	1.5c
Sawdust	ASR 58	31.6a	12.7b	6.9c	4.4c	2.9c
	ASR 67	12.6a	5.6b	5.3b	2.7c	2.4c
	ASR 58 + ASR 67	18.3a	4.2b	2.9bc	3.0bc	2.3c

Note: The same letter at the end of the number in the row from the same isolate indicates that the value is not significantly different in the Tukey test ($P \leq 0.05$)

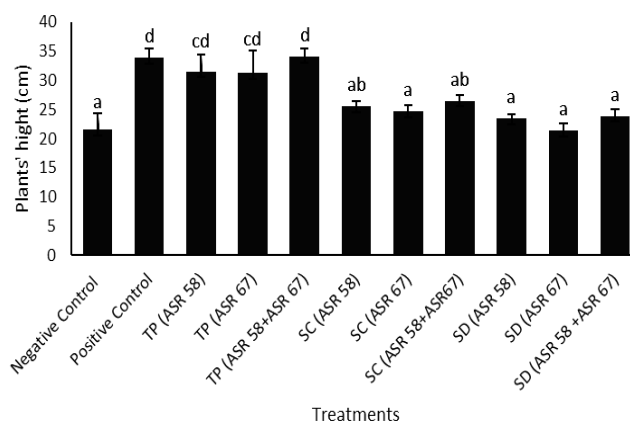


Figure 4. The effect of various actinomycetes formulas on soybean plant height after four weeks of planting. Bars with the same letter represent nonsignificant differences according to the Tukey test ($P \leq 0.05$). Note: TP (Talc Powder), SC (Sterile Clay), and SD (Sawdust)

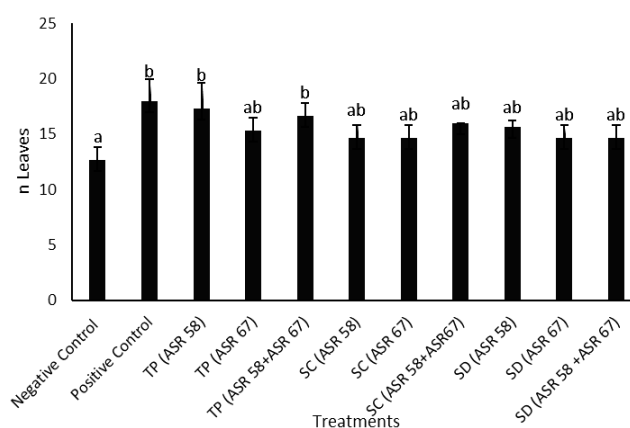


Figure 5. The effect of various actinomycetes formulas on the number of soybean plant leaves after four weeks of planting. Bars with the same letter represent nonsignificant differences according to the Tukey test ($P \leq 0.05$). TP (Talc Powder), SC (Sterile Clay), SD (sawdust)

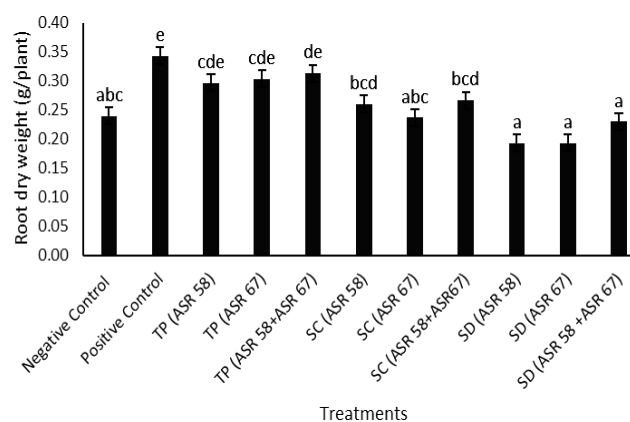


Figure 6. The effect of various actinomycetes formulas on the dry weight of soybean roots after four weeks of planting. Bars with the same letter represent nonsignificant differences according to the Tukey test ($P < 0.05$). TP (Talc Powder), SC (Sterile Clay), SD (sawdust)

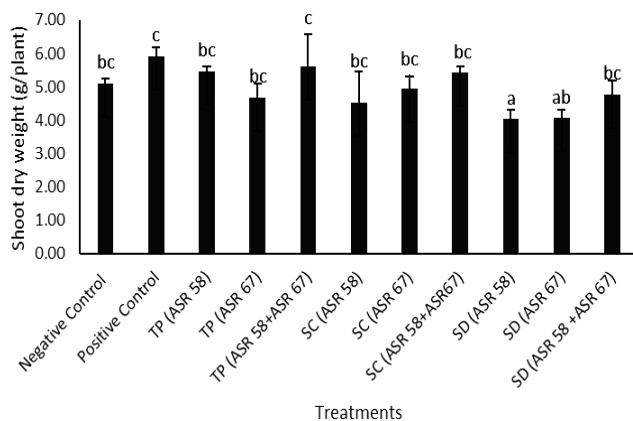


Figure 7. The effect of various actinomycete formulas on the dry weight of soybean shoot after four weeks of planting. Bars with the same letter represent nonsignificant differences according to the Tukey test ($P < 0.05$). TP (Talc Powder), SC (Sterile Clay), SD (sawdust)

Discussion

The actinomycetes group is commonly known to have a beneficial effect in enhancing plant growth (Franco-Correa and Chavarro-Anzola 2016). Three actinomycete cultures (ASR 58, ASR 67, and a combination of ASR 58 and ASR 67) could grow in an alternative medium (molasses, rice bran, and sugar-potato broth). However, the highest biomass of ASR 58 isolates, ASR 67, and a combination of these isolates were found in different alternative media. ASR 58 and ASR 67 biomass was highest in rice bran and sugar-potato broth, respectively. Both sugar-potato and rice bran media provides carbon, protein, and other nutrient supporting actinomycete growth. In addition, these materials are cheap and easy to obtain (Alrumman et al. 2014; Moon and Chang 2021). This study's results align with an earlier study reporting that sugar-potato broth was also effective in the culture of root nodule bacteria with the same effectiveness as Potato Dextrose Broth (PDB) (Martyniuk and Oroń 2011). This medium is relatively cheaper and contains complex nutrients like commercial PDB. Sugar-potato broth can also stimulate the production of antifungal, bio-pigment, and bioactive compounds in *Streptomyces* spp. (Schalchli et al. 2021). In addition, the results showed that sugar potato broth was the best alternative culture medium to enrich actinomycetes biomass. Inoculum preparation is needed to increase the actinomycetes cell number before inoculation to the carrier materials. Inoculum preparation is also important because the time required to colonize the substrate depends on the type and amount of biomass in the inoculum (Van Kuijk et al. 2015). Based on this result, it is suggested to use potato sugar broth medium in inoculum preparation in order to get the optimum number of inoculant cells.

Bioformulation of actinomycetes in various carrier materials could last at least two months of storage at room temperature. However, during storage, the cell viability of *Streptomyces* sp. ASR 58 in the sawdust materials decreased from 31.6×10^7 CFU/g in the early storage to 2.6×10^7 CFU/g. A decrease in the number of viable actinomycetes cells is a common issue in the

bioformulation of a biofertilizer agent. Another study reported that the viability of microbial cells is higher and more stable while storing is conducted at 4°C (Bazilah et al. 2011; Wong et al. 2019). Low temperatures and low humidity storage can minimize the metabolic rate of the bacteria (Aloo et al. 2022).

Actinomycete bioformulations were developed to be applied to agricultural practices. Bioformulations are developed by combining biologically active ingredients, such as live microbes inoculated in a carrier material. Some common carrier materials are peat (Novinscak and Fillion 2020), vermiculite, talc (Sarma et al. 2011), sawdust (Gopalakrishnan et al. 2016), and clay (Bashan et al. 2014). Good carrier materials can protect the microbial cells from contamination, provide a place for microbial attachment, support the growth of the target organism, and maintain the number of microbial populations during the storage period (Mącik et al. 2020). This study used organic materials (sawdust) and inorganic materials (bentonite clay and talc powder). These materials can maintain water capacity, and they are environmentally friendly, easy to apply, and cheap (Gopalakrishnan et al. 2016). In this study, talc powder was the best carrier material for maintaining *Streptomyces* sp.'s ASR 58 cell viability for 10 weeks of storage at room temperature. Talc powder contains a combination of minerals in the form of chloride, carbonate, and magnesium silicate (Nakkeeran et al. 2006), this material is commonly used in actinomycete bioformulation. The actinomycetes formulated in this carrier material effectively increased plant growth and reduced plant pathogen attack. Talc-based bioformulation of *Streptomyces cellulose* Aktino 48 was reported effective in reducing the attack of the *Sclerotium rolfsii* and increasing the biomass of peanut plants (Abo-Zaid et al. 2021). This study also showed that talc powder can maintain the cell viability of *Streptomyces* sp. ASR 67, *Streptomyces* sp. ASR 58, and the combination of both. This result is supported by (Zamoum et al. 2017), that the number of actinomycetes spore in talcum powder formulation remains relatively stable during the one-year storage at room temperature.

Furthermore, inoculating a combination of ASR 58 and ASR 67 formulated in talc powder significantly increased plant height, number of plant leaves, and root dry weight of soybean plant compared to negative controls, treatment without inoculant, and in the greenhouse assay. While the combination of ASR 58 and ASR 67 in talc powder formulation did not show a significant difference, it can be concluded that the supplementation of the combination of ASR 58 and ASR 67 can support the growth of soybean plants as well as the use of inorganic chemical fertilizers (NPK). Therefore, using potential microbial fertilizers as organic fertilizers can be used as substitutes for chemical fertilizers. In contrast, the excessive use of chemical fertilizers for a long period may cause rapid depletion in soil nutrient content (Sandrakirana and Arifin 2021). This research indicated that the combination of ASR 58 and ASR 67 can work synergistically as plant growth promoters, as shown in the previous research that these two isolates can produce IAA and dissolve the phosphate in the consortium culture (data not shown). Based on the results

of actinobacterial viability, it was observed that talc is the best medium for actinobacterial growth. Therefore, when growth is maximized, it is followed by its ability to produce plant growth-promoting bioactive compounds. Based on the result of this study, it proves that the bioformulation of a combination of ASR 58 and ASR 67 inoculum has the potential as a biofertilizer to enhance soybean plant growth.

In conclusion, sugar-potato broth was the best alternative medium for culturing ASR 67 and a combination of ASR 58 and ASR 67 inoculants which could produce the highest cell biomass after 96 hours of incubation. The ASR 67 also showed the highest cell number at the beginning of storage in clay medium, but the population decreased during 10 weeks of room temperature storage. The viability of cell biomass of ASR 58 (5.3×10^7 CFU/mL), ASR 67 (3.8×10^7 CFU/mL), and its combination (4.3×10^7 CFU/mL) was best maintained in talc powder carrier medium during 10 weeks of storage, showed by the low decreasing number of the cell. Supplementation of co-culture ASR 58 and ASR 67 formulated in talc powder on the planting medium significantly increased plant height, the number of leaves, and the dry weight of roots ($P > 0.05$) of soybean plants more significantly than the controls. Based on these results, it is obvious that bioformulation of *Streptomyces* sp. strain ASR 58 and strain ASR 67 in talc powder medium can maintain the number of viable cells and be a potential agent in promoting soybean growth.

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