

Shelf-life of *Bacillus subtilis* B298 inclusion in biopesticide microencapsule formula and its efficacy in suppressing anthracnose disease on chili

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Abstract. *Bacillus subtilis* B298 is an antagonistic bacterium isolated from the rhizosphere of potatoes. It is capable in controlling diseases both caused by fungi and bacteria. Inclusion of *B. subtilis* B298 in biopesticide microencapsule formula (BMF) is an effort to extend its shelf life, which maintain its stability and relatively more resistant to environmental change conditions. This study aimed to determine the shelf life of *B. subtilis* B298 in BMF, and its effectiveness in BMF to suppress chili anthracnose disease. Application of *B. subtilis* B298 was conducted by chili seed coating for 24 hr, after seed sowing the biopesticide was applied again by watering the roots of plants, i.e. 10, 20 and 30 d after planting. The experiment was arranged in a completely randomized block design with 4 treatments (control, *B. subtilis* B298 BMF, fungicide and combination of *B. subtilis* B298 BMF and fungicide), each treatment was repeated 6 times. Variables observed were; *B. subtilis* B298 population in the BMF, the disease intensity and infection rate by *Colletotrichum* sp. The study showed that *B. subtilis* B298 in BMF was capable to survive for 5 wk with a population of 14.4×10^7 cfu/g and suppressed chili anthracnose by 53.71%

1. Introduction

Bacillus is a genus of bacteria that is abundant in the rhizosphere, has a role as inducer of plant growth and agents in controlling plant pathogens. *Bacillus subtilis* is a bacterium that can be found in soil, plants, water and air. According to [1] this bacterial strain showed several mechanisms involved in its role as a plant growth promoter (PGPR, plant growth promoting rhizobacteria), which is shown by producing metabolite activity as an IAA producer, a phosphate solvent, a siderophore producer. Furthermore, it was known that *Bacillus* is a biocontrol agent by producing antibiotics, lipopeptides and hydrolytic enzymes. The activity of these bacteria has an effect on pathogens and plant growth. *B. subtilis* isolated from potato are capable to produce siderophores, phosphate solvents and IAA-producer and are capable to control bacterial wilt disease in chili [2, 3]. Naturally, *Bacillus* sp. colonize the roots, can be stable in contact with plants and settle as a rhizosphere microbe that can stimulate plant growth; therefore it can be functioned as a biofertilizer [4, 5].

In addition, as biocontrol agents and plant growth boosters; it also can increase plant resistance or induce resistance (Induced Systemic Resistance). Five isolates of *B. subtilis* (B46, B 209, B211, B298,



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and B315) derived from the potato rhizosphere were capable to control the bacterial wilt disease of potatoes [6]. It has been reported that the bacterial is safe for plants and can even increase plant growth and resistance because it produces siderophores, IAA, as a phosphate solvent and produces antimicrobial compounds such as hydrolitic enzyme chitinase, protease, and amylase [7, 8, 9]. *B. subtilis* B298 is capable to control bacterial wilt disease and chili anthracnose with suppressive effectiveness of 74.66% and 53.94% with a liquid formula [2]. However, in liquid form there are some limitations; such as its stability, shelf-life, flexibility application and transportation. Microencapsulation formula is an innovation in biopesticide formulation with the objective of a more stable active ingredient of *B. subtilis*, longer viability an effectiveness. In the microencapsulation formula, *B. subtilis* will have higher viability than in liquid formula. The formulation of *B. subtilis* B298 microencapsulant biopesticide was made with the aim to make it more stable, more viable, have better durability and effectiveness in controlling pathogens, more practical in transportation and application. It has been reported that inclusion of *B. subtilis* B298 in microencapsulation biopesticide formula induced plant systemic disease resistance on chili as total phenol of the treated plant increased [10]. Furthermore, the microencapsulation formula is more efficient and environmentally friendly. Microencapsulation formula showed higher viability compared to liquid formula [11].

Anthrachnose disease is a major disease in chilli that is still difficult to control because the pathogen is airborne and has several species. In Indonesia, losses from chili due to anthracnose disease can reach 50-100% [12]. The disease development is quite fast because of its spread through the air, as average temperature and humidity in Indonesia is suitable for the development of anthracnose disease. Control of anthracnose disease carried out so far is by synthetic fungicides which, if applied continuously and unwise, can have a negative impact on the environment. The use of biological agents *B. subtilis* B298 in the microencapsulant formula (*B. subtilis* B298 in BMF) is expected to suppress the development of this disease.

The objectives of the study were to: (1) determine the shelf life of *B. subtilis* B298 in BMF, (2) determine the effectiveness of *B. subtilis* B298 in BMF in suppressing anthracnose chili disease.

2. Methods

2.1. Shelf-life test of *B. subtilis* B298 in BMF

Microencapsulant material called encapsulant consists of maltodextrin and arabic-gum with a ratio of 1:1, 2:3 and 3:2, then water was added to form paste. Each microencapsulant formula weighing 50 g plus 30 mL water then sterilized at 121°C and pressure 15 psi for 25 min. *B. subtilis* B298 was grown on liquid Yeast-peptone medium, shaken at room temperature for 2x24 hr. Suspension of *B. subtilis* B298 0.1 v/w (density 10^8 cfu/mL) was added to the microencapsulant material, then dried using a benchtop K type freeze-drier at -73 °C, for 14 hr. The results of this drying are then blended until smooth and stored as a microencapsulant formula (*B. subtilis* B298 in BMF).

Variables observed: viability of *B. subtilis* population observed every week for 5 wk. *B. subtilis* viability evaluation in the microencapsulant formula was conducted by dissolving 1g of the formula in 9 mL of sterile water and a series of dilution with 0.85% physiological NaCl. Until dilutions of 6,7 and 8, 10 µL were grown on the YPGA medium by drop plate method [13]. Viability testing also included confirmation of Gram's properties with 3% KOH and the ability to produce amylase and chitinase enzymes, using starch hydrolysis medium and medium containing colloidal chitin [14].

2.2. Application of *B. subtilis* B298 in BMF to control anthracnose disease on chili

Application on red chili plants to control anthracnose disease, with treatments arranged by completely randomized block design consisting of 4 treatments with 6 replications, as follows:

K : control (without biopesticide formula)

B : application of *B. subtilis* B298 in BMF

F : application of fungicides with active ingredients Carbendazim

BF: application of a combination of *B. subtilis* B298 in BMF and fungicide

This experiment was carried out at 2 locations, i.e. Tambaksari Kidul (110 m asl) and Gandatapa (400 m asl), in order to examine the effectiveness of *B. subtilis* B298 in BMF to control anthracnose which growth both in low and high lands. The *B. subtilis* B298 in BMF used was the best formula from the previous experiment, that is formulation in maltodextrin: arabic gum (3:2). The application of this biopesticide is to cover the seeds, and when the plants are 10, 20 and 30 d after transplanting, they are poured on the soil around the plants with a concentration of 2 g formula/L water. The variables observed were incubation period (IP), the intensity of anthracnose disease. For anthracnose disease using the formula according to [15] as follows:

$$IP = \frac{\sum n \times v}{Z \times N} \times 100\%$$

with damage score values based on symptoms in the fruit (anthracnose) and leaves (leaf spot) as follows: **0**: no damage, **1**: damage area on fruit and or leaves > 0-<10%, **2**: damage area >10-<20%, **3**: damage area >20-<40%, **4**: damage area >40-<60%, and **5**: damage area > 60% (modification of [16]).

Infection rate was calculated using the van der Plank formula [17] with the type of disease development of "compound interest disease" $r = 2.3 / t_2 - t_1 [\log 1 / (1 - X_2) - \log 1 / (1 - X_1)]$ unit / d. Effectiveness of disease suppression is calculated based on comparison of control disease intensity and treatment disease intensity according to [18], as follows:

$$\text{Effectivity} = \frac{(\text{IP control} - \text{IP treatment})}{\text{IP control}} \times 100\%$$

3. Results and discussion

3.1. Shelf life test of *B. subtilis* B298 in BMF

Results of the viability test of *B. subtilis* B298 in BMF were seen from the colonies on the YPGA medium isolated from the formula every week by calculating the population in total plate counting (TPC). The population of *B. subtilis* B298 in BMF can be seen in Fig1.

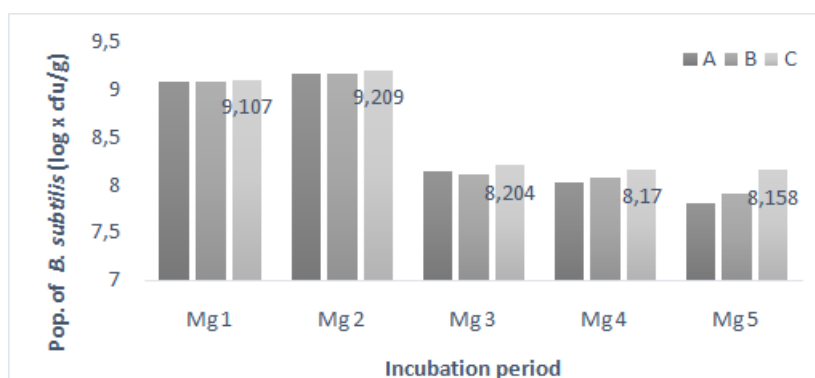


Figure 1. *B. subtilis* B298 in BMF population in 5 wk incubation time; A) M (maltodekstrin) : GA (Arabic GOM)= 1:1; B) M:GA= 2:3; C) M:GA= 3:2.

The best population is treatment no.3 (M: GA = 3: 2) with a population of 14.4×10^7 cfu/g formula. *B. subtilis* B298 can still survive the shelf life at BMF for up to 5 wk.

3.2. Evaluation of *B. subtilis* B298 in BMF application on chili anthracnose disease

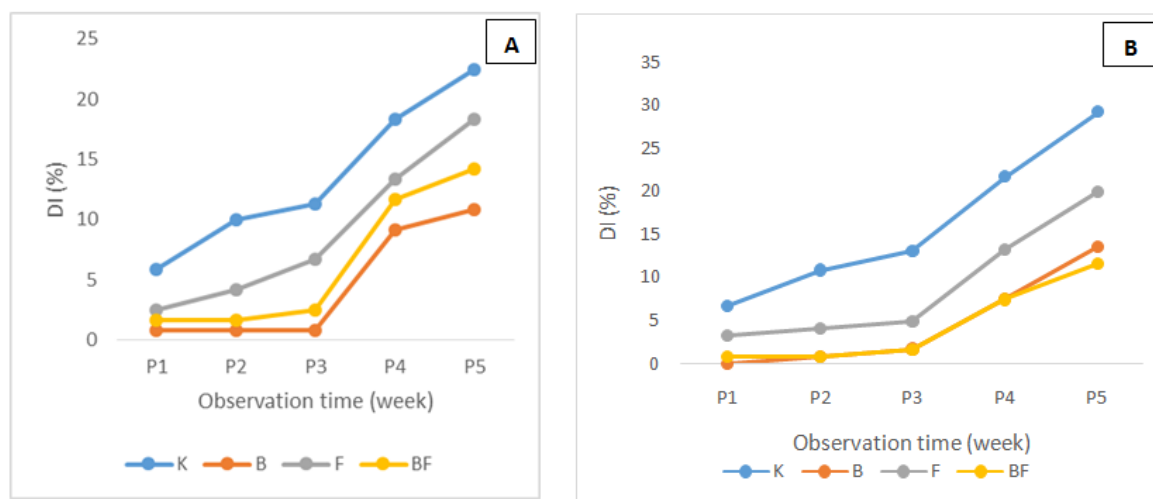
The main disease of chili is anthracnose by the fungus *Colletotrichum* sp. The intensity of the disease at 2 altitudes is shown in Table 1.

Table 1. Incubation period (Ip), disease intensity (DI) and effectiveness of disease control in Tambaksari Kidul Village (110 m asl) and Gandatapa (400 m asl).

Treatment ^a	‘Tambaksari Kidul’ village			‘Gandatapa’ village		
	Ip, day after planting	DI (%)	Effectiveness (%)	Ip, day after planting	DI (%)	Effectiveness (%)
K	55	22.5 a	-	58	29.17 a	-
B	62	10.83 b	51.87	65	13.50 b	53.71
F	62	18.33 a	18.53	66	20 ab	31.44
BF	64	14.17 b	37.02	66	11.67 b	59.99

^a K: control; B: *B. subtilis* B298 in BMF; F: fungicide with active ingredient of carbendazyme; BF combination of *B. subtilis* B298 in BMF and fungicide. Values followed by the same letters in the same column show no significant difference in the 5% level LSD.

The effectiveness of anthracnose disease control in the combination treatments of *B. subtilis* B298 in BMF and fungicide formula at 2 altitudes was not significantly different from the treatment of *B. subtilis* B298 in BMF alone. In Gandatapa the effectiveness of the control reached 53.71%. The development of anthracnose disease in the Tambaksari Kidul and Gandatapa villages for 5 observations up to 83 d afterwards was shown in Fig 2. The pattern of disease development at both altitudes was relatively the same, with the highest control and the average speed of disease development is 0.024 units/d.

**Figure 2.** Anthracnose disease development at 2 locations, (A) Tambaksari Kidul (110 m asl); (B) Gandatapa (400 m asl).

The yields differences in these 2 locations was due to weather differences, especially the temperature and humidity. In Gandatapa with a higher elevation, plants were greener and survive longer, therefore the harvest period was longer. According to previous report [19] anthracnose disease developed well under optimal conditions, namely temperature 27 °C and humidity 80%.

In order to protect an active ingredient, the coating through microencapsulation process was done in which the liquid active ingredient processed into very small particle sizes in the range 1-5000 µm. By microencapsulated, the active ingredient could be distributed evenly and extends to the location of the target of application. Inclusion an active ingredient in microencapsulation resulted that it is

protected from the environment, separated from incompatible components, protected and maintained in the formulation, finally it is released slowly (slow release) for long period of time [20].

Microencapsulant biopesticide formula (MBF) is an innovative biopesticide formulation. *B. subtilis* B298 in BMF is more durable, more practical in storage and transportation, as [11] has been stated that the microencapsulant formula is more stable, and *B. subtilis* as an active ingredient has a longer and more effective viability compared to the liquid formula.

4. Conclusion

This study revealed that *B. subtilis* B298 in BMF has a shelf life of up to 5 wk after the formulation with a population of 14.4×10^7 cfu/g formula. BMF showed the effectiveness of controlling anthracnose chili by 53.71%. Further studies needed including an evaluation of the presence and viability of *B. subtilis* B298 after the crop is harvested, to support sustainable agriculture.

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