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Enhancement of antifungal efficacy of *Bacillus subtilis* AS80 by cold plasma technology

Supachai Pothi [a], Kanta Sangwijit [b], Thamonwan Pankong [a], Somboon Anuntalabhochai [c] and Sugunya Suebsan [c*]

[a] *Department of Environmental Science, School of Energy and Environment, University of Phayao, Phayao, 56000, Thailand*

[b] *Plasma Bioengineering Unit, School of Science, University of Phayao, Phayao, 56000, Thailand*

[c] *Department of Biology, School of Science, University of Phayao, Phayao, 56000, Thailand*

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Abstract

Pathogenic fungi represented in avocado and other fruit orchards rely predominantly on the application of fungicides. However, the employment of synthetic fungicides is increasingly restricted due to the harmful effects of pesticides on human health and the environment. Therefore, this research aimed to enhance the antifungal efficacy of antagonistic bacteria and to elucidate the enzyme activity underlying their inhibition of fungal development. The antagonistic bacteria, *Bacillus subtilis* AS80, were induced to mutate by cold plasma technology, and an assessment of antifungal activity was performed. It was found that three mutants named MT144, MT163, and MT165 exhibited significant antifungal activity, with mycelial growth inhibition of 86.3%, 85.2%, and 82.8%, respectively, while the wild type showed an inhibition of 76.4% at 14 days after inoculation. In addition, *B. subtilis* AS80 and antagonistic mutants exhibited chitinase, cellulase, and amylase activities. Interestingly, the MT123 mutant, which was deficient in antifungal activity, showed a contrasting phenotype, with high amylase activity but no detectable chitinase or cellulase activities. This study suggests that chitinase and cellulase are key enzymatic factors in *B. subtilis* AS80 and its mutants, mediating their ability to inhibit the growth of pathogenic fungi.

*** Corresponding author** : sugunya.pi@up.ac.th

1. Introduction

Avocado (*Persea americana* Mill.) is a widely grown economic plant in Thailand, cultivated for both domestic and commercial use. The cultivation and production of avocados encounter annual growth. Thailand experienced a significant increase in avocado exports in 2021, with a growth rate of 125% compared to the previous year. Despite the fact that the domestic market is the largest consumer of avocados, Thailand's export market showed remarkable growth [1].

During the production period, fruit rot is one of the major diseases that leads to a reduction in fresh fruit yields and economic losses. Many reports indicate that *Fusarium solani* and *Lasiodiplodia theobromae* are responsible for causing fruit rot disease in avocados [2 – 4].

The *Fusarium* fungus species is responsible for soil-borne plant illnesses that have the ability to propagate through seeds and air and can also adhere to surviving plants. Consequently, *Fusarium* strains possess the capacity to infect a diverse array of host plants [5]. Traditionally,

fungicides have been used to manage the development of various diseases in avocado fruit. Nevertheless, concerns are growing about the potential hazards that fungicides pose to human health, environmental pollution, and the development of resistance to fungicides in diseases [6]. Thus, the use of microorganisms as an alternative to chemical control for disease management is highly prospective.

Microorganisms, including yeast, fungi, and bacteria, have been thoroughly researched for their ability to fight plant diseases. Antagonistic yeast has been established as a biocontrol agent against fruit rot induced by *L. theobromae* and anthracnose caused by *Colletotrichum gloeosporioides* in mango fruit after it has been harvested [7]. *Bacillus subtilis* strains obtained from cow dung have the potential to function as biocontrol agents against pathogenic fungi, specifically *Fusarium oxysporum* and *Botryodiplodia theobromae*, which are responsible for postharvest rots in yam (*Dioscorea rotundata*) tubers [8]. The avocado rhizobacteria, *Bacillus subtilis, B. amyloliquefaciens* species complex, exhibited antifungal properties against the pathogens that cause *Fusarium* dieback. In addition, these isolates and *B. mycoides* suppressed the growth of *Phytophthora cinnamomi*, leading to the development of avocado root rot [9]. Kim et al. [10] demonstrated that *Paenibacillus polymyxa* APEC128 inhibited the growth of anthracnose, a disease caused by *Colletotrichum gloeosporioides* and *C. acutatum*, in harvested apples. Moreover, *P. polymyxa* APEC136 and *B. subtilis* APEC170 have the capacity to reduce the symptoms of anthracnose disease as well as white rot disease caused by *Botryosphaeria dothidea*. The APEC136 exhibited high levels of amylase and protease, while APEC170 demonstrated high levels of chitinase, amylase, and protease, potentially contributing to the suppression of fungal mycelial development [11].

Cold plasma technology serves as a promising alternative approach for enzymatic enhancement. In recent years, cold plasma applications have gained significant traction in biological research, particularly for sterilization of both biotic and abiotic surfaces, as well as for enhancing enzymatic activity in various bacterial species. Moreover, cold plasma represents an excellent alternative technology due to its environmentally sustainable characteristics: it is clean, safe, and leaves no residual substances in the environment [12].

Recently, Suebsan et al. [13] demonstrated that the rhizobacterium *B. subtilis* AS80 isolated from avocado rhizosphere could control many genera of pathogenic fungi causing fruit rot and anthracnose disease in northern Thailand, including *Fusarium* sp., *Lasiodiplodia* sp., and *Colletotrichum* sp. The objectives of this work were to improve the antagonistic capabilities of this bacteria by plasma treatment and to elucidate the enzyme activities that inhibit the fungal growth.

2. Materials and Methods

Pathogenic fungi and antagonistic bacteria isolation

The pathogenic fungus *Fusarium* sp., which was isolated from the avocado fruit that exhibited fruit rot, was used in this study, along with the antagonistic bacterium *Bacillus subtilis* strain AS80, obtained from avocado rhizosphere soil as reported by Suebsan et al. [13].

Induction of mutation in the B. subtilis AS80 using cold plasma technique

A single colony of *B. subtilis* AS80 was cultivated in 3 mL of Luria-Bertani broth (LB) and incubated at 37 ^{o}C with agitation at 200 rpm overnight. The bacterial cells were collected through centrifugation at 6000 rpm for 5 minutes and were resuspended in 100 µL of sterile distilled water. Afterwards, the bacterial suspension underwent treatment using our developed atmospheric pressure plasma jet (APPJ) at the Plasma Bioengineering research unit, School of Science, University of Phayao. To initiate plasma treatment, bacterial suspension was dropped in holes of a sample holder for plasma bombardment. Argon and helium gas were used to generate plasma with an energy input of 120 W and gas flow rate of 2 SCCM (standard cubic centimeters per minute) at room temperature for all experiments. The bacterial samples underwent plasma treatment at time

intervals ranging from 30 seconds to 5 minutes. After plasma treatment, 1 mL of LB media was added to each bombarded sample and then incubated at 37 °C on a rotating shaker for 1 hour. Subsequently, each sample was diluted to a concentration of 10^{-2} and 10^{-3} and spread on a LB agar plate and left to incubate overnight at 37 °C.

In vitro assessment of B. subtilis AS80 mutants antagonistic activity towards Fusarium sp.

The antagonistic effect of bacterial growth on LB plates following plasma treatment was evaluated against pathogenic fungus on PDA by the dual culture method that was modified from Guevara- Avendaño et al. [9]. Pathogenic fungi were cultured on a PDA plate for seven days; subsequently, the tips of the fungal hyphae (0.5 cm in diameter) were excised with a cork borer and placed in the center of the new PDA plate. The bacterial isolates were cultured in 3 mL LB broth and incubated at 37 °C for 24 hours. The suspension of each antagonistic bacteria was adjusted to an OD600 value of 1.00 and 10 µL of the suspension was applied to sterilized filter paper positioned 25−mm from the fungal disc on the culture medium plate. Each plate was examined with *B. subtilis* AS80 (WT) and two distinct bacterial isolates. Additionally, sterile distilled water was used as a control on each experimental plate. The antagonistic assays were carried out in triplicate. Dual culture plates were incubated at 25 °C for a duration of 14 days. The mycelium's radial growth towards the bacterial and control treatments was measured at 7 and 14 days post-inoculation. The percentage of mycelial growth inhibition was calculated according to Idris et al. [14] as shown in equation (1);

$$\% \text{ Inhibition} = \frac{R-r}{R} x\ 100 \qquad (1)$$

When R equals the radial of fungal growth from the center of the plate towards the control treatment and r equals the radial of fungal growth towards the bacterial treatment. Subsequently, fungal hyphae were collected and stained with 0.5% methylene blue to examine for fungal morphology using a microscope at 40x and 100x magnification, and the isolate that expressed the highest score of % inhibition was selected for further study.

Evaluation of enzymatic activities of B. subtilis AS80 mutants

The antifungal mechanisms of the *B. subtilis* AS80 mutants were evaluated using several enzyme activity assays. Bacterial isolates were cultured in 3 mL LB broth and incubated at 37 °C for 24 hours. The bacterial cultures were centrifuged at 6000 rpm for 10 minutes after being adjusted to an OD600 value of 1.00. Chitinase, cellulase, and amylase activities were measured using the supernatant as a crude enzyme source. All enzyme activity were determined by measuring the release of reducing sugar groups using the dinitrosalicylic acid (DNS) method. Chitinase activity was performed following the protocol established by Roberts and Selitrennikoff [15]. The reaction mixture comprised 200 µL of 1% colloidal chitin (w v^{-1}) and 100 µL of 0.2 M phosphate buffer (pH6.5), incubated at 37 °C for 5 minutes. Subsequently, 100 µL of crude enzyme was added to the reaction and incubated at 37 °C for 30 minutes. Stop the reaction by boiling in boiling water for 10 minutes. For the measurement of amylase and cellulase activities, 200 µL of crude enzyme was added to 200 µL of 1% starch solution (w v−1) and 1 % carboxymethylcellulose (CMC) and incubated at 50 °C for 30 minutes [16]. Then the mixture was centrifuged at 13,000 rpm for 1 minute. The 300 µL of supernatant was transferred to a 1.5 mL centrifuged tube containing 300 µL of DNS solution then the reduced sugar was measured a wavelength of OD540.

The protein content in all tests was quantified using Bradford's method [17] described below: 1 mL of protein solution was mixed with 1X dye reagent and incubated at room temperature for 5 minutes. Then measure the absorbance of the samples at a wavelength of 595 nm and compare against a standard curve generated using protein solutions: Bovine Serum Albumin (BSA).

The specific enzyme activity was calculated as shown in equation (2) and (3);

$$\text{Enzyme Activity } (\text{U} \cdot \text{mL}^{-1}) = \frac{\text{Cons. of substrate} \times (\text{dilution factor x } 1000)}{\text{weight of glucose or NAG} \times \text{incubation time}} \quad (2)$$

$$\text{Specific enzyme activity } (\text{U} \cdot \text{mg}^{-1} \text{ protein}) = \frac{\text{Activity of enzyme } (\text{U} \cdot \text{mL}^{-1})}{\text{Total protein} (\text{mg} \cdot \text{mL}^{-1})} \quad (3)$$

Data analysis

The statistical analysis was analyzed by Duncan's multiple range test, and the values were considered significant at $p \leq 0.05$.

3. Results and Discussion

Mutation induction by cold plasma technique

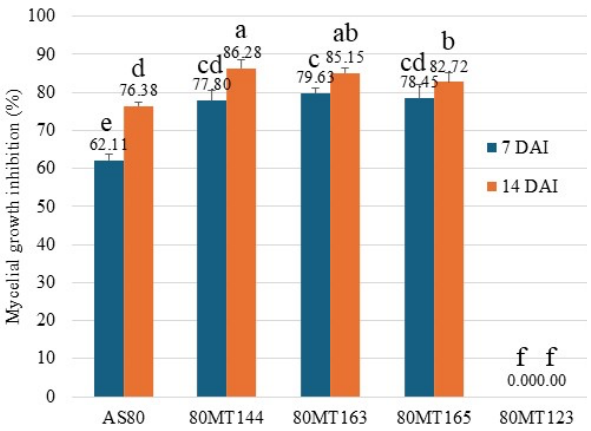
B. subtilis AS80 was subjected to plasma jet bombardment under diverse conditions followed by an investigation of the survival of each treated bacterium using a plate count method, which was compared to the bacterial control. The highest survival inactivation occurred after 5 minutes of treatment with helium plasma, resulting in a 1% survival rate. Five hundred eighty-one isolates of the bombarded bacteria were collected to assess their antagonistic activities for the screening of bacterial mutants.

In vitro assessment of B. subtilis AS80 mutants antagonistic activity towards Fusarium sp.

All the bombarded bacterial isolates were evaluated for their antagonistic activity against *Fusarium* sp. using the dual culture method. After the screening test, three mutants, MT144, MT163, and MT165, exhibited significant antifungal activity with inhibition of mycelial growth at 86.3%, 85.2%, and 82.8%, respectively, whereas the wild type showed 76.4% inhibition at 14 days after inoculation (DAI). Furthermore, one mutant (MT123) demonstrated a loss of antifungal activity as shown in Fig. 1. These results are consistent with previous reports which revealed that the cold plasma technique could induce mutation of *B. subtilis* [12] and *B. amyloliquefaciens* [18].

In addition, when observed under an optical microscope, the pathogen's hyphae in the confrontation zone with *B. subtilis* AS80 and 3 mutants (MT144, MT163 and MT165) caused abnormal morphology; the hyphae become coagulated, coiled, destructed, swelled, and the internodes were reduced whereas the fungal

A



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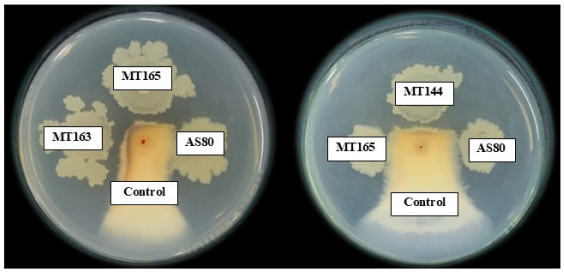

Fig. 1 Antifungal activity of *B. subtilis* AS80 (wild type) and mutants against *Fusarium* sp.. A. Percentage of mycelial growth inhibition at 7 and 14 days after inoculation (DAI). Values represent the average of three replicates ± standard error. Different letters indicate statistically significant differences ($P \leq 0.05$). B. Mycelial growth inhibition of *Fusarium* sp. by *B. subtilis* AS80 and mutants (MT144, MT163, MT165 and MT123).

hyphae that exposed to MT123 or were not exposed to the bacteria (control) did not show any signs of damage (Fig. 2). These results are consistent with previous reports which revealed that the antagonist bacteria against *Thielaviopsis paradoxa* (De Seynes) Hohn, a causal agent of butt rot disease by caused abnormalities in hyphae of; the hyphae became coagulated, coiled, destructed, malformed, and retarded after 7 days of incubation [19]. *Paenibacillus polymyxa*, *B. amyliquefaciens*, and *B. elezensis* have shown their potential ability to control *Fusarium oxysporum* which caused wilt disease in *Codonopsis pilosula***.** The observation under

an optical microscope showed that the pathogen's hyphae in the confrontation zone was enlarged and thickened, the internodes were reduced, and horizontal ridges even appeared [20].

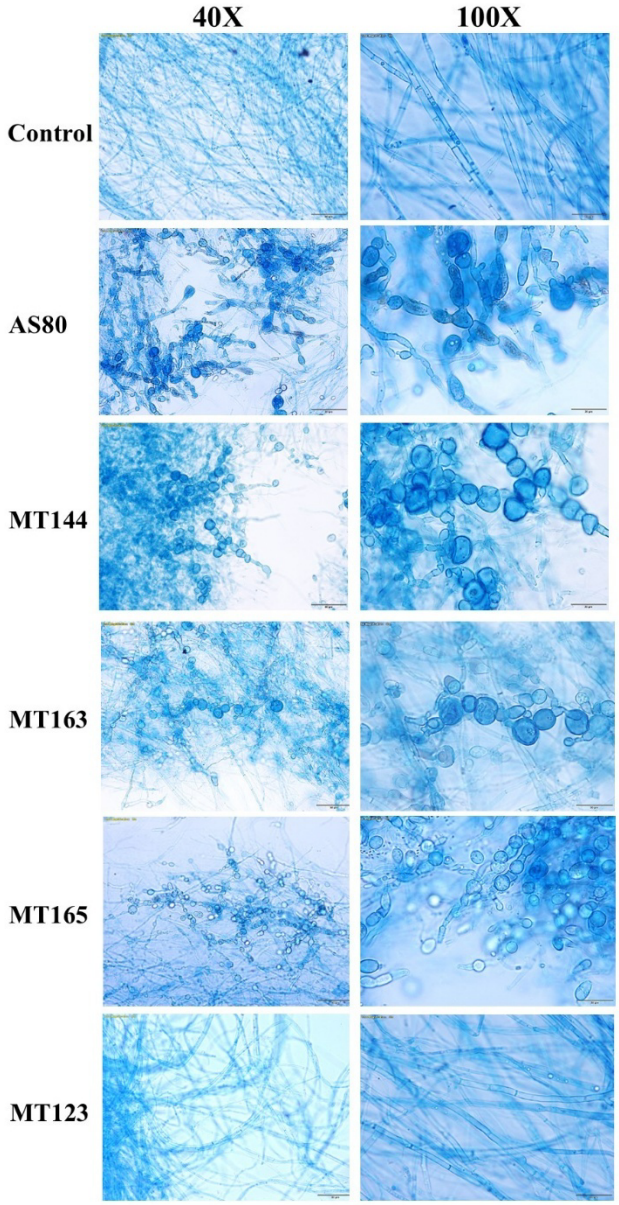

Fig. 2 Microscopic observation of fungal hyphae under 40× and 100× magnification of light microscope.

Enzymatic activities of B. subtilis AS80 mutants

The activities of chitinase, cellulase and amylase from *B. subtilis* AS80 (WT) and four mutants were assessed. The results indicated that the wild type and three mutants (MT143, MT163, and MT165), which demonstrated antagonistic activity, produced all enzymes activity (Fig. 3). Interestingly, these three mutants exhibited higher levels of chitinase activity. Recently, Li et al. [21] reported that *B. subtilis* LY-1, which significantly inhibits peanut root rot, was shown to produce cellulase, protease, amylase, chitinase, and β-1,3-glucanase activity. Hong et al. [22] investigated *B. velezensis* CE 100 inhibited the mycelial growth of *M. phaseolina* and *F. oxysporum* f. sp. *fragariae* by produced fungal cell wall-degrading enzymes, including chitinases and β-1,3-glucanases. In contrast the mutant MT123, which lost its antagonistic activity, showed no detectable chitinase or cellulase activity but exhibited a high level of amylase activity (Fig. 3). This study suggests that chitinase and cellulase are the key enzymes of *B. subtilis* AS80 and its antagonistic mutants, enabling them to inhibit the growth of pathogenic fungi.

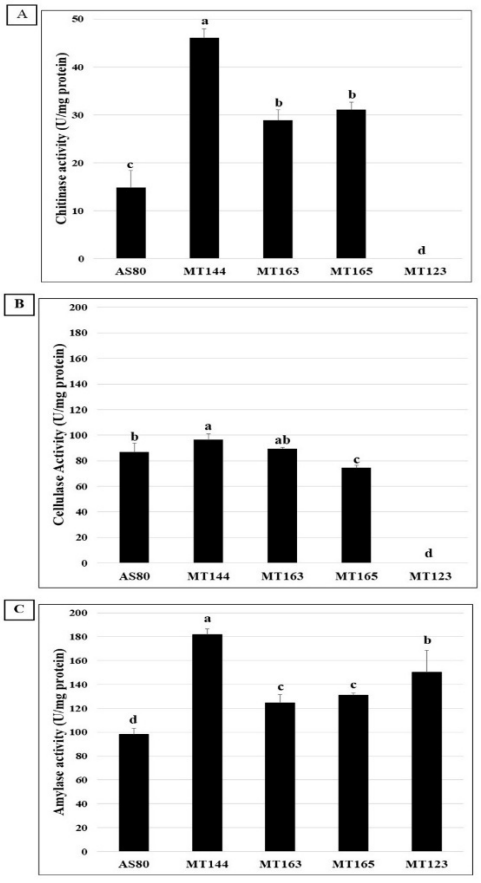

Fig. 3 Production of chitinase, cellulase, and amylase enzymes by *B. subtilis* AS80 (wild type) and mutants (MT144, MT163, MT165, and MT123). Different letters indicate statistically significant differences ($P \leq 0.05$).

4. Conclusion

Cold plasma represents an excellent alternative technology due to its environmentally sustainable characteristics: it is clean, safe, and does not produce residual pollutants in the environment. This study confirmed that atmospheric pressure plasma jet (APPJ) is an effective method for enhancing the antifungal activity of Bacillus subtilis AS80, making it a potentially valuable biocontrol agent. *B. subtilis* AS80 and its mutants MT144, MT163, and MT165 have been shown to significantly inhibit the growth of *Fusarium* sp. This inhibition is associated with hyphal destruction, distortion, or abnormality, and cell wall collapse resulting from chitinase and cellulase activities. These findings provide a theoretical foundation for the development of novel treatments for fungal infections in fruit orchards.

5. Acknowledgement

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