

Antimicrobial Activity of *Pseudomonas fluorescens* and *Bacillus subtilis* on Different Dilution Concentrations Against Various Citrus Post-Harvest Pathogens

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ABSTRACT

Pseudomonas fluorescens and *Bacillus subtilis* are common antagonistic bacteria to various pathogens. These bacteria can produce various antimicrobial substances such as chitinase, siderophore, and antibiotic. This research aims to obtain the optimum dilution concentrations of *Pseudomonas fluorescens* and *Bacillus subtilis* that can effectively inhibit the growth of *Penicillium digitatum*, *P. paradoxum*, and *Aspergillus* sp. This research was carried out in a complete randomized design with five different dilution concentrations of *Pseudomonas fluorescens* and *Bacillus subtilis* ranging from 10^{-1} to 10^{-5} . The antimicrobial activity tests against fungal pathogens were carried out in triplicate using a dual culture method. *P. fluorescens* and *B. subtilis* displayed strong antimicrobial activities against all target pathogens. The highest inhibition activity of *P. fluorescens* and *B. subtilis* was recorded against *Aspergillus* sp. with 45.7 % and 35.92 % and cell density of 168×10^6 CFU/mL and 14×10^6 CFU/mL, respectively. The highest antimicrobial activity was recorded at the 10^{-1} and 10^{-4} for *P. fluorescens* and *B. subtilis*, respectively. The antimicrobial activity of *P. fluorescens* and *B. subtilis* against *P. digitatum* and *P. paradoxum* are ranging from 17.59 % to 27.73 %.

Keywords: Antimicrobial, *Bacillus subtilis*, dilution, post-harvest, *Pseudomonas fluorescens*

1. INTRODUCTION

Citrus is one of the most economically important crops in the world. Citrus is known for its high nutritional values that are found beneficial to improve immunity and source of vitamins and minerals. Citrus can also be made into various food products, namely marmalade and jam. Due to its benefits and versatilities, the worldwide demand for high-quality citrus fruit is very high. To meet this target, every phase in citrus productions needs to be maintained in stringent condition to ensure its quality and quantity, starting from its land managements to its post-harvest handling.

One of the most essential phases in determining the quality of a citrus fruits is its post-harvest phase. During this phase, the harvested citrus fruits are handled, processed, stored and transported in a

delicate way to prolong its commercial period and reduce the risk of postharvest loss due to contaminations by various microorganisms such as fungi and bacteria [1]. In the developing country like India and Indonesia citrus loss due to postharvest mishandling and contamination are very high, reaching the range of 25-30% annually [2]. These are mostly caused by the contaminations of various foodborne postharvest fungal pathogens such as *Penicillium italicum*, *P. digitatum* and *Colletotrichum gloeosporioides*. These isolates are known to be cause of blue mold, green mold and anthracnose diseases that are commonly found during citrus postharvest period.

Controlling fungal contaminations during postharvest period is difficult and have caused concerns among public, farmers and scientists [3]. Often times, fungal contaminations in postharvest

period is controlled through fungicide treatments [4,5]. However, this method is no longer considered effective because not only it can be dangerous if consumed, it will also contaminate the environment if used in long run [6-8]. To replace fungicide, several feasible and environmentally friendly alternatives have been proposed to control or avoid the contaminations of these pathogens [9].

The use of microorganisms as biopreservative agent for food products have been gaining its popularity lately. Biopreservative is considered safe because it doesn't leave chemical residue to the environment and normally will not cause harm to human if consumed. Biopreservative microorganisms work by directly inhibiting the growth target foodborne pathogens through the means of toxic secondary metabolites production and competition for space and nutrient uptake [10]. *Pseudomonas fluorescens* and *Bacillus subtilis* are one of the most common microorganisms used to control foodborne postharvest pathogens. *P. fluorescens* reported to have antifungal activity against various fungal foodborne pathogen such as *Aspergillus niger*, *A. flavus*, *Penicillium italicum*, *P. digitatum* and *Fusarium* sp. [11,12]. On the other hand, *B. subtilis* also exhibited antifungal activity against various foodborne pathogen such as *P. digitatum*. [13] reported that *B. subtilis* can inhibit the growth *P. digitatum* with 82.4 % inhibition zone when tested in in vivo dual culture tests by inhibiting the mycelial growth *P. digitatum*. However, despite showing promising antifungal activities against various fungal foodborne pathogens, the potency of *P. fluorescens* and *B. subtilis* as antifungal for citrus postharvest pathogen have not yet reported. Therefore, this research aim to study and evaluate the antifungal activity of *P. digitatum* and *B. subtilis* against citrus postharvest pathogens namely *P. digitatum*, *P. paradoxum* and *Aspergillus* sp.

2. MATERIALS AND METHODS

2.1 Research Design and Culture Preparation

This research is carried out in complete randomized design with three replications. Bacterial culture *P. fluorescens* and *B. subtilis* are challenged against three citrus postharvest pathogens namely *P. digitatum*, *P. paradoxum* and *Aspergillus* sp., *P. fluorescens* and *B. subtilis* were cultivated in King's B medium at room temperature for 48 hours. Both bacterial cultures were diluted to 10⁻⁵ serial dilutions. The cell density of each bacterial culture was

measured using hemocytometer. Target fungal pathogens were grown in *Potato Dextrose Agar* (PDA) medium for further use.

2.2 Antagonistic Tests of *P. fluorescens* and *B. subtilis* against Various Citrus Postharvest Fungal Pathogens

In vitro antagonistic tests of *P. fluorescens* and *B. subtilis* against various citrus postharvest pathogens were carried out using dual culture method proposed by [14] with modifications. Sterile filter papers (Ø: 0.5 mm) were dipped into each bacterial culture for 15 minutes. Filter papers then placed on PDA plate alongside the target fungal pathogens with 3 cm distances. The plate was incubated at room temperature for 7 days. Fungal colony diameter was measured and used to determined the inhibition activity (%) for each bacterial isolate. The inhibition activity (%) was determined according to:

$$I = \frac{R1 - R2}{R1} \times 100 \% \tag{1}$$

- I: Inhibition activity (%)
- R1: colony diameter of untreated pathogen (control)
- R2: colony diameter of treated pathogen

2.3 Data Analysis

Statistical significance in this experiment was determined using Analysis of Variance (ANOVA) at 5 % significance level followed by Tukey HSD test in SPSS.

3. RESULT AND DISCUSSION

Table 1. Cell density of both isolates in different dilution level

Dilution level	Bacterial isolates	
	<i>P. fluorescens</i>	<i>B. subtilis</i>
10 ⁻¹	169,8 x 10 ⁶	140,05 x 10 ⁶
10 ⁻²	53,6 x 10 ⁶	77,8 x 10 ⁶
10 ⁻³	51 x 10 ⁶	24 x 10 ⁶
10 ⁻⁴	46,2 x 10 ⁶	14 x 10 ⁶
10 ⁻⁵	17,25 x 10 ⁶	7,5 x 10 ⁶

The increase in serial dilution level can significantly reduce the cell density within the bacterial culture (Table 1), and these cultures were later evaluated for its antifungal activity against target pathogens. All bacterial culture in this experiment displayed good antagonistic activity against all target pathogens. These antagonistic can

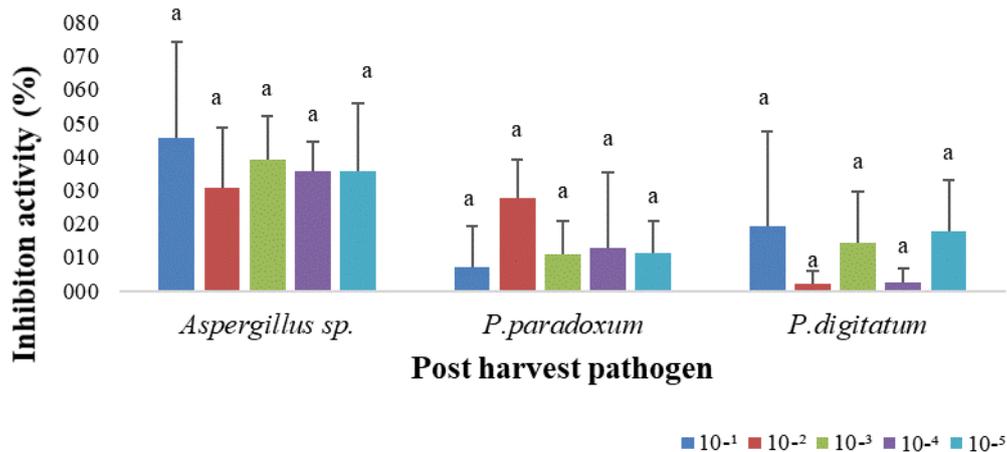


Figure 1. Inhibition activity (%) of *P. fluorescens* in different dilution level against three citrus postharvest pathogens

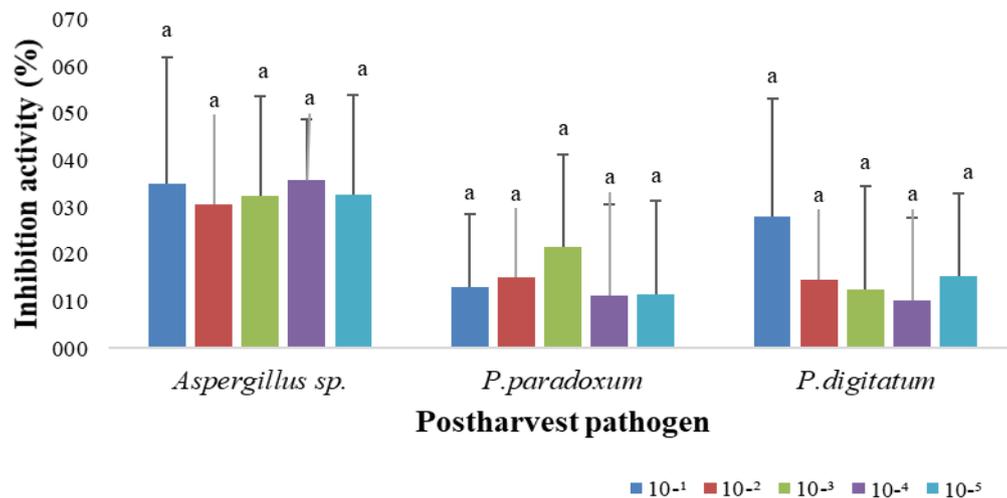


Figure 2. Inhibition activity (%) of *B. subtilis* in different dilution level against three citrus postharvest pathogens

be seen by the impaired growth and significant colony diameter reduction of the target pathogens when treated with *P. fluorescens* and *B. subtilis*.

The ability of *P. fluorescens* to inhibit various kinds of fungal pathogens also have been reported before. [15] reported that *P. fluorescens* isolated from wheat rhizosphere could inhibit the growth of its host pathogens notably *Fusarium sp.* and *Aspergillus sp.* with high efficacy and inhibition zone up to 20 mm in diameter. In other study, [16] reported that *P. fluorescens* cell culture suspended in growth medium or sterile distilled water could significantly inhibit the growth of the citrus postharvest pathogen *P. digitatum* based on in vitro and in vivo treatments. In

their research *P. digitatum* treated with *P. fluorescens* experienced significant loss in its mycelial growth, spore germinations and germ tube elongations.

Similar to its counterpart, *B. subtilis* also demonstrated strong inhibition activity against all target pathogens. The highest inhibition activity of *B. subtilis* was recorded against *Aspergillus sp.* at 10⁻⁴ dilution level with 35.92 % of inhibition. *B. subtilis* displayed slightly weaker inhibition activities against *P. paradoxum* and *P. digitatum* with 21.60 % and 28.02 % inhibition respectively (Figure 2).

Antifungal activity of *B. subtilis* have been reported in many studies. In a study conducted by [17] revealed that *B. subtilis* 355 have strong

inhibition activities against various postharvest and wood surface contaminant fungi such as *Aspergillus niger* and *Penicillium* sp. In their research, *B. subtilis* displayed its strongest antifungal activity when it was incubated for 6-11 days before challenged with target pathogens. [18] also reported that cell culture and cell free supernatant of *B. subtilis* demonstrated strong inhibition activity against *Aspergillus* sp. *Aspergillus* sp. treated in their experienced adverse effect on its mycelial growth and spore germinations significantly. In other study conducted by [19], *B. subtilis* also revealed to have inhibition activity against *P. digitatum* with >80 % inhibition zone, even when treated at 1:32 dilution (v/v).

Antibiosis is one of the most common mechanism involved in the fungal inhibition by bacteria through primary or secondary metabolite or competition for nutrients and space. In *Bacillus* spp. the mechanisms in controlling various postharvest pathogen is still largely unknown although some research also revealed that fungal inhibition by this species also involve of the induction of the systemic resistances [20]. To compete with its antagonists *P. fluorescens* forms a biofilm and produce inhibitory metabolites that specifically target spore germination and mycelial growth [21]. These inhibitory metabolites consist of volatile compounds, lytic enzymes, siderophore and other toxic chemicals [22].

Zamani et al [23] and Nunes et al [24] observed that there was a positive relationship between the population density of an antagonist and its biological efficacy. Contrary to the findings of previous research, several findings here suggest that the highest inhibition activity of each biocontrol and its target pathogens sometimes could be observed in a higher dilution level. This finding can be related to the number of viable cells. In a higher concentration culture, more bacterial cells are in multiplication and competition for nutrients and space at the same time hence resulting in more dead bacterial cells. These bacterial cells, when challenged with target fungal pathogens, may no longer producing antifungal metabolites as it is no longer alive.

4. CONCLUSION

Both *P. flourescens* and *B. subtilis* have the potency as the biological control agent of three citrus postharvest pathogens namely *Aspergillus* sp., *P. paradoxum* and *P. digitatum*. The inhibition activity of these isolates is affected by its dilution level. The highest dilution level of 10^{-1} for *Aspergillus* with *P. flourescens* and 10^{-4} for *B. subtilis*. These results suggest that both *P. flourescens* and *B. subtilis* is an

effective biocontrol for citrus fungal post-harvest pathogen.

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