Conference Paper

Biofilm Formation of *Pseudomonas geniculata* (Wright, 1895) Chester, 1901 on Three Fungals Species: Relationship with Incubation Time and Fungal Diameter Size

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Abstract

Pseudomonas geniculata has been isolated from uncontaminated vertisol in Kulon Progo district. The isolate is hydrocarbonoclastic bacterium capable of forming biofilm on the fungal hyphae. Sinergy of both microbe in the form fungal-bacteria biofilm produce high ability to degrading hydrocarbon and survive in its pollution environment. The purpose of this research was to evaluate ability of *Pseudomonas geniculata* (Wright, 1895) Chester, 1901 to form biofilm and its attachment on three fungals species such as *Penicillium* sp., *Penicillium funiculosum* and *Penicillium crustosum*. The diameter size of fungal hyphae was of 1.3 μm, 1.9 μm and 2.4 μm, respectively. *P. geniculata* required at least 48 h to form biofilms on *Penicillium* sp. hyphae when incubated in mineral Bushnell Haas Medium suplemented with 2% glucose at room temperature, with maximal biofilm formation being evident at 360 h. Biofilm attachment on *Penicillium* sp. hyphae was disrupted by the vortex power of 5 rpm for 20 s. Interaction of *P. geniculata* and *Penicillium* sp. that has a smallest diameter size of hypha were more successful on biofilm formation and attachment which could contribute to bacterial survival in environmental stresses.

Keywords: Biofilm; fungal hyphae; *Pseudomonas geniculata* (Wright, 1895) Chester; 1901; *Penicillium*.

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1. Introduction

In nature, free-living bacteria will interact with each other to form a microbial community, where they developed system of intercellular interaction and communication [1]. These microbial communities have the ability to persist as surface-attached communities surrounded by extracellular polymeric substances, called biofilm [2]. Biofilms communities have the adaptability and survival strategies were better against environmental stress due to the protection of the biofilm matrix [3, 4].

Bacterial biofilm attached on fungal hyphae surface called fungal-bacteria biofilms [5]. Physical interactions between bacterial and fungal within the biofilms are key

factors in their enhanced performance, compared to those attached to abiotic surfaces. Fungal-bacterial biofilms have been observed to have better growth and colonization abilities than their monocultures. For example, in an industrial flowing water system, interactions between fungi and bacteria showed significantly higher rates of colonization and growth over single cultures in a complex seven-species model community [6].

Biological performance of fungal bacteria biofilm is more effective than bacteria monocultures, fungal monocultures, and bacteria biofilm on the abiotic surface [2]. Utami [7] showed hydrocarbon degradation ability of each single isolates are 9.22% *P.geniculata*; 17.47% *P. aeruginosa*; 12.99% *Penicillium* sp. and the hydrocarbon degradation ability of co-culture are 18.68% *Penicillium* sp.-*P. geniculata* and 20.66% *Penicillium* sp.-*P. aeruginosa.* Mahardika [8] showed vegetable oil degradation ability of *P. aeruginosa* and *Eupenicillium javanicum* are 4.74% and 21.46%. The ability of *P. aeruginosa, Eupenicillium javanicum* co-culture is 94.04%.

Contact and adhesion between bacteria and fungi are likely to be important early events in the process of the formation of bacterial-fungal biofilms. Fungal-bacteria specificity may exist in this interaction [9]. Hogan and Kolter [10] reported about biofilm formation of *P. aeruginosa* on the surface of *Candida albicans* hyphae. When *P. aeruginosa* and *Candida albicans* are co-cultured in carbon-limited minimal medium, the bacteria readily attach to the fungal filament and forming a biofilm within 24 h to 72 h. While co-inoculated *P. aeruginosa* with yeast-form *C. albicans,* no attachment was observed.

This research was aimed to evaluate the ability of *P. geniculate* to form biofilm and its attachment on three fungal species. The results of this study may have an impact on the use of fungal-bacteria biofilm as agents of biodegradation in the environment.

2. Materials and Methods

2.1. Bacterial cultures

P. geniculata was the wild-type strain isolated from vertisol soil, strain was cultivated in Nutrient Agar Medium at 37°C. For co-culture, *P. geniculata* inoculum was preculture in Mineral Bushnell Hass broth suplemented with 2% glucose at 37°C, 120 rpm (1 rpm = 1/60 Hz), for 24 h.

2.2. Fungal cultures

Penicillium sp., *P. funiculosum* and *P. crustosum* were the wild-type strain. Strains were cultivated on potato dextrose agar at room temperature, For co-culture, each of fungal

inoculum was inoculated at 10^5 spores · mL^{-1} and cultivated in Mineral Bushnell Hass broth suplemented with 2% glucose at room temperature, 120 rpm, for 2 d until hyphae are formed.

2.3. Co-culture

Penicillium sp., P. funiculosum and P. crustosum were precultured for 2 d, then, added inoculant bacteria were 10^6 cells mL^{-1}. Co-culture incubated at room temperature for 15 d for microscopic observation.

2.4. Confirmation of the biofilm formation

Bacterial attachment and biofilm formation were observed at 48 h, 96 h, 144 h, 192 h, 240 h, and 360 h using microscopic. fungal hyphae were taken using a needle ose onto a microscope slide and staining with tryphan blue. Observations using the microscope optilab 1 000 × magnification. count a number of bacteria attached on the fungal surface with total plate count method.

2.5. Biofilm attachment strength on the fungal surface

A combination of fungal-bacteria co-culture can form biofilms were tested using a vortex at different speeds (1 rpm, 3 rpm and 5 rpm), each for 20 s. The results are then observed using a microscope optilab 1 000 × magnification. to observe biofilm attachment strength on the fungal surface, fungal hyphae were taken using a needle ose onto a microscope slide.

3. Result and Discussion

3.1. Confirmation of the biofilm formation

To determine the ability of the *P. geniculata* bacteria in attached and form a biofilm on the fungal surface, bacteria were grown with three different species of fungi such as *Penicillium* sp., *Penicillium funiculosum* and *Penicillium crustosum*. The diameter size of fungal hyphae were 1.3 μm, 1.9 μm and 2.4 μm, respectively. The bacteria with each of fungal was mixed in MBH medium. Due to growth rate differences, fungal was pregrown for two days for growing and propagation of fungal hyphae before *P. geniculata* was added to the medium. Observations of co-culture *P. geniculata-Penicillium* sp. was observed after 48 h incubation. Bacteria seen attached to the surface of some hyphae, while other hyphae were still free of bacteria. Many planktonic bacteria were

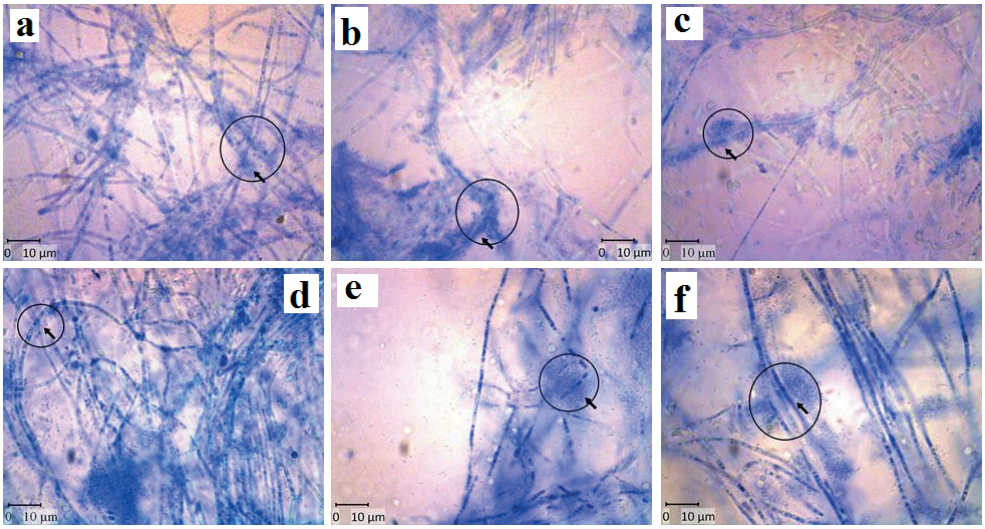

Figure 1: Results of co-culture growth between *Penicillium sp.* and *P. geniculata*. Photographs (a)–(f) were taken 48 h, 96 h, 144 h, 192 h, 240 h and 360 h after co-inoculations respectively. Magnification 1 000 ×. The arrow shows attachment of bacteria on the hyphae surface.

still moving around in the medium. Attachment and biofilm formation of *P. aeruginosa* on the surface hyphae of *Penicillium* sp remain visible up to 360 h of observation. Fungi may provide biotic support for the bacterial biofilm establishment [9]. Until the end of the observation (360 h incubation), not all hyphae are equally colonized by the bacterial cells and planktonic bacteria were still observed moving around the hyphae (Figure 1).

Interestingly, when *P. geniculata* was co-inoculated with *P. funiculosum* and *P. crustosum* that has bigger diameter size of hyphae, not show any attachment until the end of the observation (360 h incubation) (Figure 2 and Figure 3). During the first 48 h of incubation, planktonic cell was visible in co-culture medium. This planktonic cell is not visible after 96 h of incubation. Antagonism test results showed no antagonism reaction between *P. geniculata* bacteria and *P. funiculosum* and *P. crustosum* (data not shown), but their interaction does not support the attachment and biofilm formation. These data illustrate that *Penicillium* species have different surface characteristics or structures that will impact bacterial ability to physically interact. Moreover, hyphae may reflect a selection for bacteria capable of exploiting fungal metabolites or fungi have active mechanisms to resist bacterial colonization [11].

The amount of constituent bacterial biofilm on the hyphae surface of fungi continue to increase up to 144 hours of incubation, then decreased on subsequent observation (Figure 4). This indicates the maximum attachment and biofilm formation occurs after 144 h of incubation. Limitations of nutrients in the medium reduce the ability to grow both of these microorganisms.

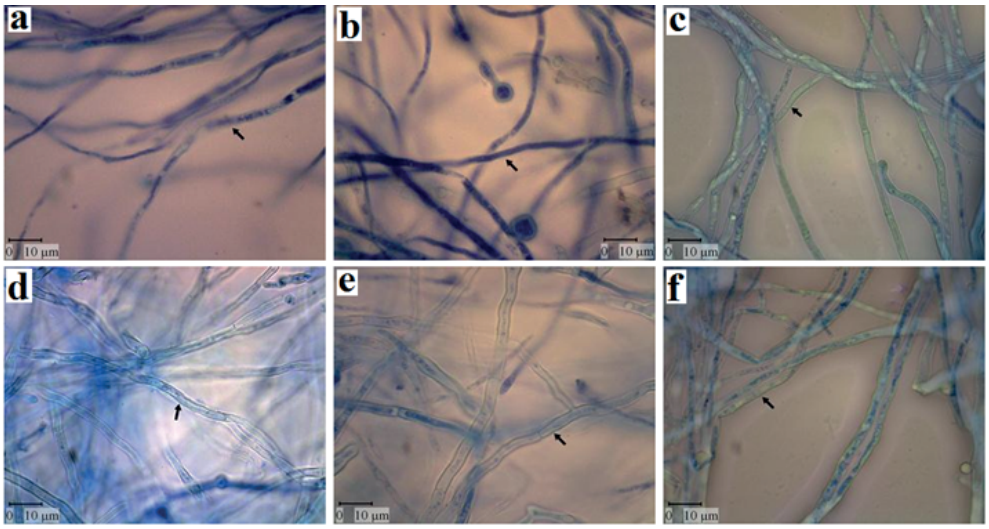

Figure 2: Results of co-culture growth between *P. funiculosum* and *P. geniculata*. Photographs (a)–(f) were taken 48 h, 96 h, 144 h, 192 h, 240 h and 360 h after co-inoculations respectively. Magnification 1 000 ×. The arrow shows the absence of bacteria attachment on the hyphae surface.

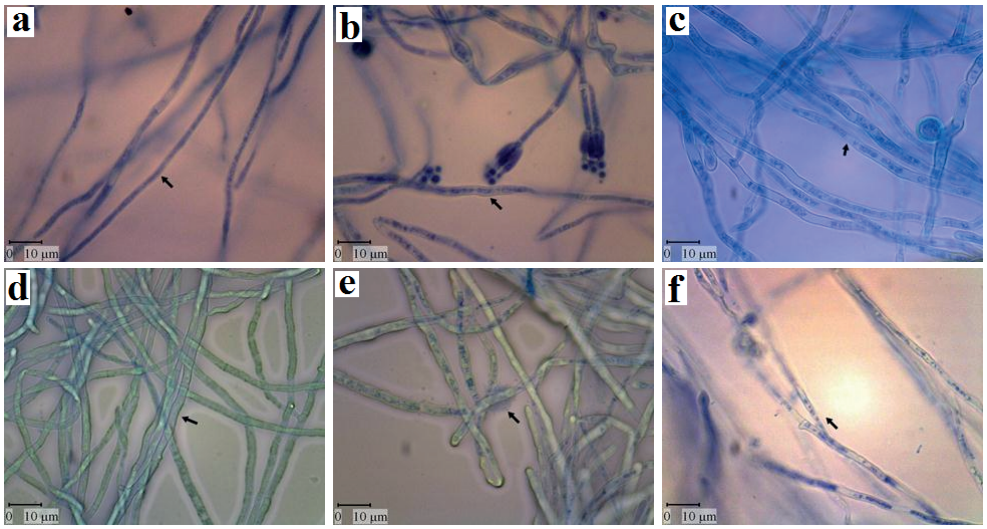

Figure 3: Results of co-culture growth between *P. crustosum* and *P. geniculata*. Photographs (a)–(f) were taken 48 h, 96 h, 144 h, 192 h, 240 h and 360 h after co-inoculations respectively. Magnification 1 000 ×. The arrow shows the absence of bacteria attachment on the hyphae surface.

3.2. Biofilm attachment strength on the fungal surface

Co-culture biofilm *P. aeruginosa-Penicillium* sp. were tested using a vortex to measure the biofilm attachment strength on the fungal surface. The vortex power of 5 rpm and 3 rpm causing biofilms disrupted from hyphae surface. Bacteria are seen being around hyphae of fungi. Biofilms are still seen attached to the surface of the hyphae of fungi when tested with vortex 1 rpm (Figure 5).

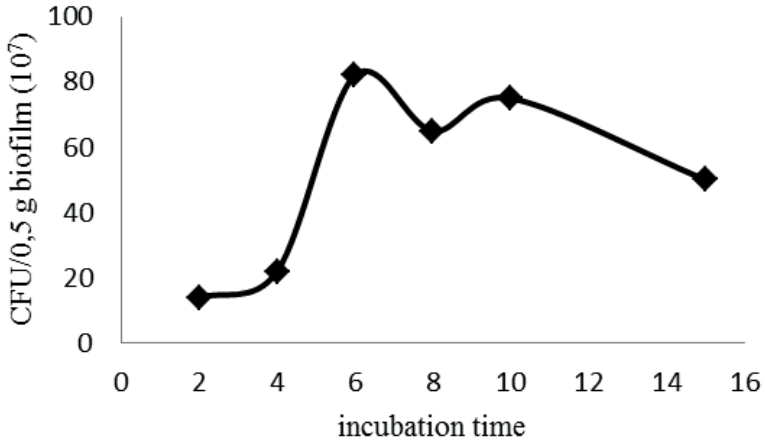

Figure 4: The amount of constituent bacterial biofilm on the hyphae surface. *Penicillium* sp. *P. geniculata* co-culture.

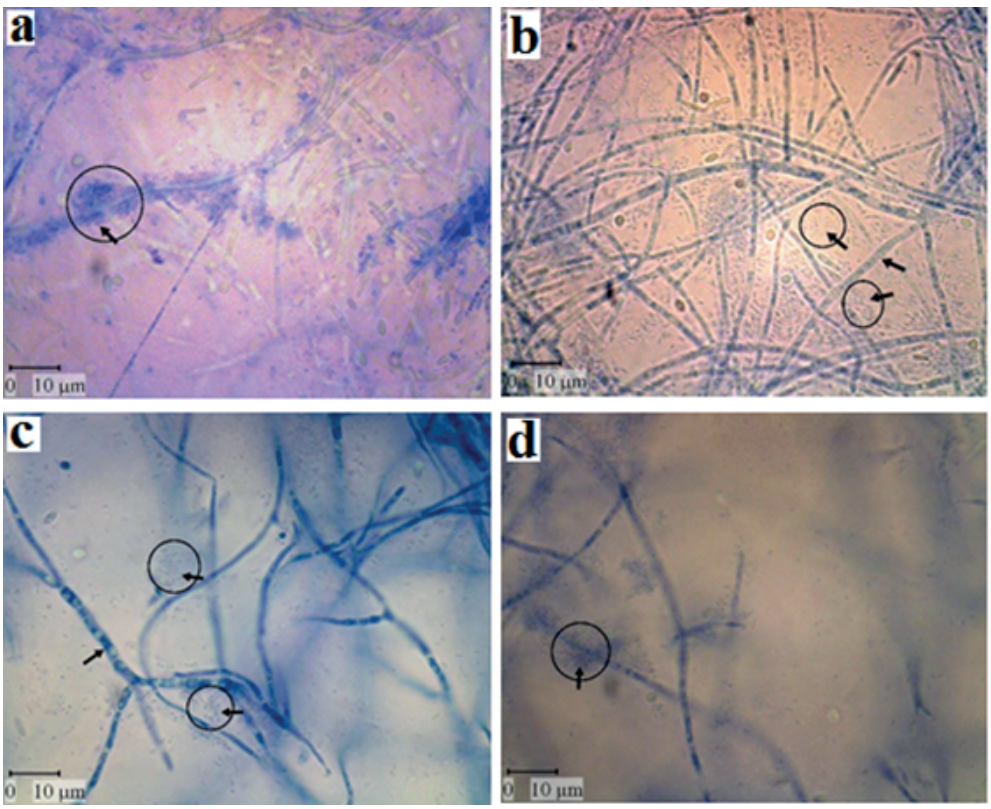

Figure 5: Result of biofilm attachment strength on the fungal surface, (a) *Penicillium* sp *P. geniculata* co-culture 144 h of incubation; (b)–(d) 5, 3, and 1 rpm of vortex power, respectively. Magnification 1 000 ×. The arrow shows the bacteria are attached or detached from the fungal hyphae.

4. Conclusion

In conclusion, fungal-bacterial biofilms can increase synergistic interaction between *P. geniculata* and *Penicillium* sp. Interaction of *P. geniculata* and *Penicillium* sp. that has a smallest diameter size of hypha were more successful on biofilm formation and

attachment. Selection of combination of specific bacterial-fungal pairs could greatly aid in the development of more effective species for survival in environmental stresses.

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