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# Biocontrol Activity, Mode of Action, and Colonization of *Aureobasidium pullulans* Dmg 30 DEP on Controlling Early Blight Disease on Tomato Plant

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**ABSTRACT**

**We investigated the biocontrol activity of *A. pullulans* Dmg 30 DEP against *Alternaria solani* causal agent of early blight. Biocontrol activity was tested by the *in vivo* and *ad planta*. Biocontrol activity were tested by investigating the antibiosis capabilities with dual culture method, paper dish assay, two-compartment petri dish assay, and trapping and identification of volatile organic compounds (VOCs) with GC-MS. Lysis activity was examined by observing the clear zone formed by growing yeast on chitin agar and skim milk agar. The ability of hyperparasitism was assessed by the agar block method, and observed by light microscopy and scanning electron microscopy (SEM). The results showed that *A. pullulans* Dmg 30 DEP plays a role in the suppression of early blight disease at $10^6$ cells/ml and $10^7$ cells/ml yeast cell density. The mechanism involved in biocontrol activity is the production of VOCs, the production of chitinase and protease enzymes, the production of siderophore and hyperparasitism. The result shows that *A. pullulans* Dmg 30 DEP was colonizing the tomato leaves following the areole.**

## 1. Introduction

*Alternaria solani* is a fungal plant pathogen and the causal agent of early blight disease. Early blight is one of the important diseases of tomato in several countries where reductions in yield ranging from 27 to 75% (Sahu *et al.* 2014). To control early blight disease, farmers still depend heavily on synthetic fungicides usage. However, the fungal pathogen is reported to have resistant to some fungicides (Ishii 2006; Egüen *et al.* 2016). Furthermore, fungicide residue has negative side effects on the environment and human health (Nicolopoulou-Stamati *et al.* 2016). Therefore, it has generated interest in the development of alternative more environmentally friendly methods to control early blight disease. Biological control has emerged as one of the most promising alternatives to fungicides.

Among some known biocontrol agents, yeast is promising for its effectiveness and having the appeal to be studied. As biocontrol agent, yeast has some advantages, such as rapid growth, non-production of allergenic mycotoxins, and tolerant to extreme conditions (temperature, humidity, and UV radiation) (Sui *et al.* 2015). Specifically, yeasts have phenotypic adaptations for colonizing leaves, fruits and vegetable surfaces that give them an advantage as biocontrol agents (Filonow 1998). *A. pullulans* is one of the yeast types that has been widely used as an antagonistic yeast and has shown its effectiveness against some plant pathogens such as *Botrytis cinerea* on Strawberry (Adikaram *et al.* 2002), *Phytophthora infestans* on tomato (Di Francesco *et al.* 2017) *Penicillium expansum* and *Botrytis cinerea* on Apple (Vero *et al.* 2009). In Indonesia, the use of *A. pullulans* has been evaluated in controlling anthracnose disease in post-harvested of chili pepper. *A. pulluans* DMG 30 DEP isolated from chili pepper leaf in Bogor was reported to have the ability to decrease the anthracnose disease severity until 78% (Hartati *et al.* 2015). However that yeast has not been evaluated for controlling disease in the field experiment, especially for controlling early blight disease on tomato.

Another aspect that also determine the efficacy of biological control is the success of colonization

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of the antagonist agents used. The tropics have distinctive climatic conditions, such as temperature, humidity, and the intensity of relatively high rainfall. This condition will affect the development and colonization of microorganisms in the filoplan area. The success of yeast colonization in the host shows that the yeast has the ability to adjust, take nutrients and settle on the host. In the practical aspect, this will be related to the success of the application and recommendations for the interval of application of antagonistic agents.

Knowledge of the mode of action of biocontrol agents is important for its successful application. The mechanisms of biocontrol agents comprise antibiosis, lysis, competition, induced resistance and hyperparasitism. The antibiosis mechanism of yeast generally occurs through the production of volatile organic compounds (VOCs), not through the production of soluble metabolites (Di Francesco *et al.* 2014; Hartati *et al.* 2015). However, the mode of action of *A. pullulans* in the field has not been reported. Therefore the objective of this study was to investigate the biocontrol activity of *A. pullulans* against *Alternaria solani* (causal agent of early blight) and revealed its putative mode of actions.

## 2. Materials and Methods

### 2.1. Isolation and Identification of *A. solani* Originated from the Tomato-infected Site

*Alternaria solani* isolation was carried out using a single spore isolation method (Noman *et al.* 2018). The samples were collected from tomato plantations in Bogor (6° 42' 5" S, 106° 52' 28" E), Indonesia. The symptomatic leaves were cut to 0.5 x 0.5 cm (±1 g) and put into a test tube containing 10 ml sterile distilled water. As much 10 µl of spore suspension was dripped on potato dextrose agar (PDA) (Difco, Livonia, MI, USA) and incubated at room temperature for 24 hours. The hyphae formed from single spore germination was cut off and transferred into new PDA media. The pure isolate was stored for further treatment. The morphological characterization was done by observing the color and shape of *A. solani* colony on PDA media and observing the characteristic of hyphae and conidia under the light microscope (Olympus BX51). Further, genotypic identification was carried out by PCR amplification and sequencing of the ITS region for strengthening the morphological identification. The DNA extraction was carried out using ilustra phytopure™ DNA extraction kit according to the manufacturer's indications. The rDNA sequence of ITS region used universal primers; ITS 1(5'- TCC GTA GGT GAA CCT GCG G- 3') and ITS 4 (5'- TCC TCC GCT TAT TGA TAT GC- 3') primers (Chakraborty *et al.* 2010). The resultant sequence was edited using Bio Edit version 7.2.0 and was subjected to BLAST search analysis at NCBI.

### 2.2. Preparation and Re-identification of *A. pullulans* Dmg 30 DEP

The yeast *A. pullulans* Dmg 30 DEP from previous research (Hartati *et al.* 2015) was grown on PDA media and identified morphologically with a light microscope (Olympus BX51). Molecular identification was determined by sequence analysis. D1/D2 region of nuclear large-subunit ribosomal DNA was amplified and sequenced using primers NL1 (GCATATCAATAAGCGGAGGAAAAG) and NL4 (GGTCCGTGTTCAAGACGG) (Fell *et al.* 2000). Sequencing of D1/D2 of LSU rDNA was determined with Big Dye terminator v3.1. cycle sequencing ready reaction kit (Applied Biosystems) following the manufacturer's instructions. The LSU gene sequences determined in this study were manually aligned with published sequences of reference strain available from the EMBL/GenBank/DDBJ databases (Altschul *et al.* 1990). The phylogenetic tree was made with MEGA 7 software using the neighbor-joining method with Tamura 3-parameter distances. Numbers at branch nodes are bootstrap values, indicating support based on 1,000 replications (Kumar *et al.* 2016). Sequences with a similarity greater than 95% were considered to belong to the same species.

### 2.3. Biocontrol Activity of *A. pullulans* Dmg 30 DEP against *A. solani*

Biocontrol activity test divided into two parts (*in vivo* and *ad planta*) according to Di Francesco *et al.* (2017) with slight modification. The experiment was conducted with five treatments, comprise K0: control, K1: yeast cell density $10^4$/ml, K2: yeast cell density $10^5$/ml, K3: yeast cell density $10^6$/ml, K4: yeast cell density $10^7$/ml. The *in vivo* test consisted of preparation of healthy tomato leaves, sterilization of tomato leaf surface with NaOCl 2% and alcohol 70%, yeast treatment, pathogen inoculation at 2 hours after yeast treatment ($10^5$ hyphae fragments/ml), incubation for 5 days, and extensive symptom observation with the ImageJ application. The ad planta test was carried out in the following stages below; tomato plants (Tymoti) seeded in small pots filled with a 2:1 of peat moss, soil, transplanted after 14 days, and grown in the same substrate (1 plant per pot) until 6 weeks, under daylight conditions in an experimental greenhouse station (Research Center for Biology, Cibinong Science Center, Indonesian

Institute of Sciences). Plants were sprayed on surfaces of the leaves (25 ml per plant) by *A. pullulans* Dmg 30 DEP suspension $10^4$-$10^7$ cell/ml using a hand sprayer. The control consisted of inoculated plants treated with potato dextrose broth (PDB) (Himedia M403-500G) only. Plants were inoculated with the pathogen suspension ($10^5$ hyphae fragments/ml). Maintenance was conducted intensively such as watering, weeding, etc. The parameters observed were the number of infected leaves and the disease severity.

## 2.4. Antibiosis Activity

### 2.4.1. Dual Culture Test

This test referred to the method used by Spadaro (2003). Yeast was streaked on PDA media perpendicularly to the center of the petri dish (Ø 9 cm). A pure culture of *A. solani* was taken with a cork borer (Ø 0.6 cm) and placed next to yeast scratches (two per petri dish), then incubated for 7 days at room temperature in dark conditions. Observations were made by measuring the inhibition zone formed.

### 2.4.2. Antimicrobial Metabolite Production

*A. pullulans* Dmg 30 DEP was grown on the PDB media and incubated for 10 days. The yeast was grown in the PDB media for 10 days. Thereafter, the yeast suspension was centrifuged at 14,000 rpm to separate the supernatant from yeast cells. The supernatant was taken and filtered with a 0.22 µm filter (McMaster 2008). Coarse metabolites from filtration were dripped as much as 10 µl in a paper dish placed around *A. solani* colonies as many as four per petri dish. Observations were made by measuring the inhibition zone formed.

### 2.4.3.Volatile Organic Compounds (VOCs) Production

The ability of VOCs production by yeast was carried out using the two-compartment petri dish assay. The 5-day *A. pullulans* were inoculated at the right of the petri dish and *A. solani* (Ø 0.6 cm) which was 10 days old inoculated at the left of the petri dish. Observations were made at the end of the incubation period, by comparing the diameter of *A. solani* colony with control. Furthermore, trapping and VOCs identification are carried out. Yeast was grown on PDB media in the reactor. The first hole in the reactor was connected to the aerator to give air pressure, and the second hole was connected to the hexane solvent. The aerator was switched on for 3-4 hours per day and the incubation is carried out for 10 days. The composition of VOCs contained in hexane was analyzed using GC-MS devices (Buzzini *et al.* 2005; McMaster 2008).

## 2.5. Siderophore Production

The siderophore production test was conducted on CAS media (Louden *et al.* 2011). Blue Dye solutions preparation, dissolve 0.06 g of CAS in 50 ml of dd$H_2O$ (solution 1). Dissolve 0.0027 g of $FeCl_3$-6 $H_2O$ in 10 ml of 10 mM HCl (solution 2). Dissolve 0.073 g of HDTMA in 40 ml of dd$H_2O$ (solution 3). Mix Solution 1 with 9 ml of Solution 2. Then mix with Solution 3. Autoclave and store in a bottle. CAS agar preparation, add 100 ml of MM9 salt Solutions(15 g $KH_2PO_4$, 25 g NaCl, and 50 g NH4Cl in 500 ml of dd$H_2O$) to 750 ml of dd$H_2O$. Dissolve 32.24 g piperazine-N, N'-bis(2 ethanesulfonic acid) PIPES. Add 15 g Bacto agar, autoclave and cool to 50°C. Add 30 ml of sterile Casamino acid solution and 10 ml of sterile 20% glucose solution to MM9/PIPES mixture. Slowly add 100 ml of Blue Dye solution along the glass wall with enough agitation to mix thoroughly. Aseptically pour plates. Siderophore production test on CAS media, *A. pullulans* Dmg 30 DEP was scratched on the CAS media. Incubation is carried out at room temperature for 5 days. Siderophore production was indicated by the formation of orange color around yeast colonies.

## 2.6. Lysis Activity

The chitinolytic activity test was conducted on chitin agar media 0.5% (Agrawal and Kotasthane 2012). Twenty-gram chitin from shrimp skeleton was dissolved in 100 ml HCl. The solution was left for 24 hours at low temperature, and then it was strained with glass wool. The filtrate was added with 100 ml cold distilled water and 125 ml 10 N NaOH, to pH 7. The filtrate was then centrifuged at 14,000 rpm for 20 min, and suspended again in cold distilled water and re-centrifuged three times. The pellet of the colloidal chitin obtained was placed at 4°C. The chitin agar media was made by mixing colloidal chitin (5 g) and agar (20 g) in 1,000 ml mineral salt and sterilized using an autoclave. The mineral salts composition were 0.7 g $K_2HPO_4$; 0.5 g $KH_2PO_4$; 0.5 g $MgSO_4 \cdot 7H_2$ crystalline; 0.001 g $FeSO_4$; and 0.001 g $ZnSO_4$. The yeast was scratched on chitin agar media perpendicularly. Incubation was carried out at room temperature for 3-5 days. Observations were carried out every day during the incubation period of the clear zone formed on the edge of the yeast colony.

The proteolytic activity test was conducted on skim milk agar (SMA) (Kazanas 1968). PDB (30 g) + bacto agar (15 g ) + distilled (water 900 ml) were mixed and sterilized using autoclave. After the media temperature drops to 40-50°C, added with milk (10 g of skim milk in 100 ml of distilled water) sterilized at 100°C using an autoclave. *A. pullulans* Dmg 30 DEP was scratched on the skim milk agar media.

Incubation is carried out at room temperature for 3-5 days. Proteolytic activity was indicated by the formation of clear zones around yeast colonies.

### 2.7. Microscopy Examination on the Interaction between *A. pullulans* Dmg 30 DEP Cells and *A. solani* Hyphae

This test was carried out using the agar block method (Andrews *et al.* 1993; Allen *et al.* 2004). The yeast was inoculated on agar blocks that had been overgrown with *A. solani* hyphae, then dripped with sterile water (10 µl) and covered with a cover glass. Incubation was carried out for 4-5 days then observed under a light microscope (Olympus BX51). The interaction of yeast and *A. solani* in the leaves was observed by TM3030 tabletop scanning electron microscope (Hitachi High Technologies-JPN). The yeast with $10^7$/ml cell density was sprayed on leaves placed on petri dishes and *A. solani* isolate with $10^5$/ml hypha fragment density was dripped on the leaf surface (10 µl), then incubated for two days. Leaves are cut in areas between symptomatic and healthy ones and then dehydrated in stages with alcohol. The leaves are then coated with palladium-gold by using MC1000 ion sputter coater (Hitachi High Technologies-JPN) ad observed using TM3030 tabletop scanning electron microscope.

### 2.8. Colonization Observation on Tomato Leaves

Tomato leaves were taken from plants and sterilized on the surface. Tomato leaves were treated with yeast with cell density of $10^7$/ml, then incubated in moist conditions. After 48 hours, the leaves are taken and cut to size 2-3 $mm^2$. Furthermore, the samples were dehydrated with ethanol serially, then coated with palladium gold. Samples were observed using TM3030 tabletop scanning electron microscope (Chan and Tian 2005).

### 2.9. Observation of Yeast Development in Tomato Leaves

Tomato plants were grown in a greenhouse until they were six weeks old. *A. Pullulans* Dmg 30 DEP was inoculated by spraying on tomato leaves with cell density $10^7$/ml. Re-isolation was carried out by taking a composite of 1 g of tomato leaves from each treatment and then put into erlenmeyer which was 30 ml of water. Erlenmeyer was shaken using a rotary shaker at a speed of 140 rpm for 2 hours. The rinse water is diluted in stages by taking 100 µl and put it in 900 µl sterile water. Then each series of dilutions was dropped as much as 10 µl on PDA media containing antibiotics and incubated for two days. Re-isolation was carried out at 2-day intervals for 6 days (Elead *et al.* 1994).

## 3. Results

### 3.1. Isolates and Morphology

*A. solani* isolate was obtained from tomato leaves and stems that showed a symptom of early blight disease. Colonies growing on PDA medium were generally cottony to woolly while the color was gray on the top of brown to black on the reverse. Conidia of *A. solani* formed in sporulation medium at 18°C were ellipsoid or clavate, brown in color, and 6-9 horizontal septae (Figure 1B). The isolate was identified using ITS regions (ITS 1 and ITS 4 praimer) of rDNA and the search for sequence similarity in BLAST for nucleotide, it showed 99.30% similarity with Alternaria solani F10 (Accession no. KT.721914.1) (Figure 2).

The yeast antagonist colony formed on PDA medium was white to brown, convex, glossy and uneven/rough surface. Yeast cells were oval, hyaline, and buds form in some of the cells. Yeast cells were 5.3-10.7 x 2.1-3.6 µm in size. The yeast isolate was re-identified using D1/D2 regions of rDNA and the search for sequence similarity was done using BLAST nucleotide. The isolate of *Aureobasidium pullulans* Dmg 30 DEP gave 98% similarity with *Aureobasidium pullulans* EF595769.1. The phylogenetic analysis was based on the data from NCBI database, and outgroup sequence confirming that the isolates were grouped with the families and the closest BLAST identity. The phylogenetic analysis is presented in Figure 3.

### 3.2. Biocontrol Activity of *A. pullulans* Dmg 30 DEP against *A. solani*

The results of the biocontrol activity test showed that the cell density of *A. pullulans* Dmg 30 DEP had affected the number of infected leaves and the disease severity. The yeast cell density $10^4$/ml and $10^5$/ml was not significant to the control treatment. The treatment of yeast cell density $10^6$/ml and $10^7$/ml showed significant differences to control, both in the number of infected leaves and in the severity of the disease. Yeast cells density $10^7$/ml was the best treatment for suppressing the development of early blight disease (Table 1).

### 3.3. Mode of Action of *A. pullulans* Dmg 30 DEP

The results of the dual culture test showed that there was an inhibition zone between the yeast and *A. solani* colonies. There was no effect of metabolites produced by *A. pullulans* Dmg 30 DEP on the growth of *A. solani*. In the two-compartment petri dish assay,

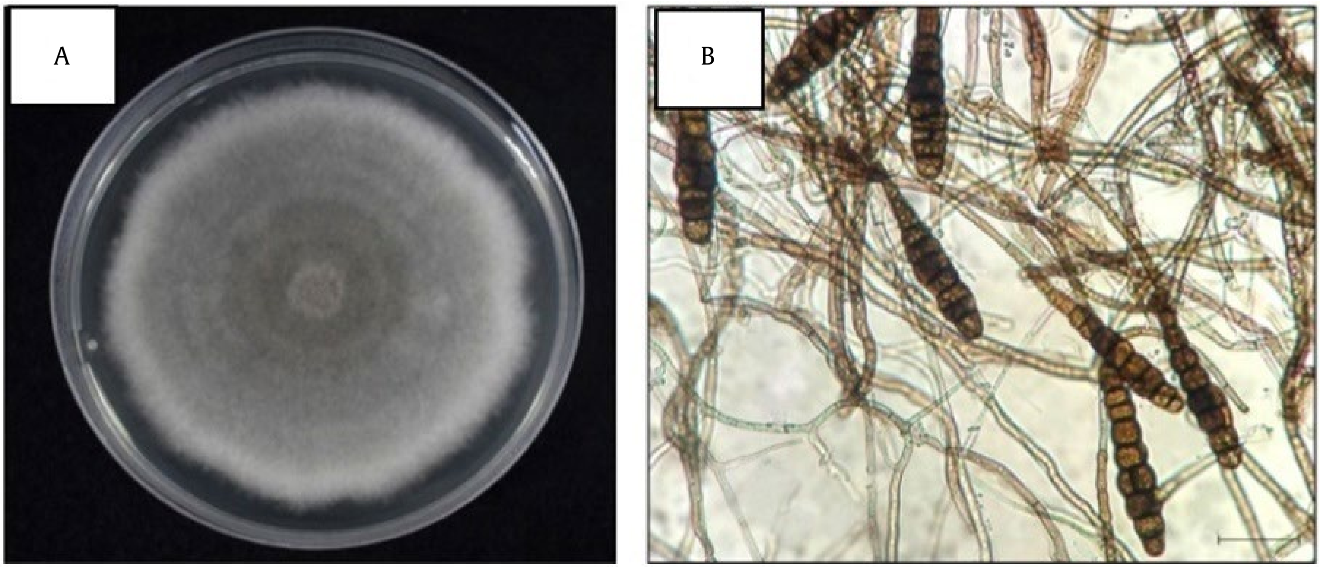


Figure 1. The morphology of *A. solani* B02 isolated from tomato leaves (A) the mycelium of *A. solani* in PDA medium (B) the ellipsoid (clavate), brown conidia with horizontal septate

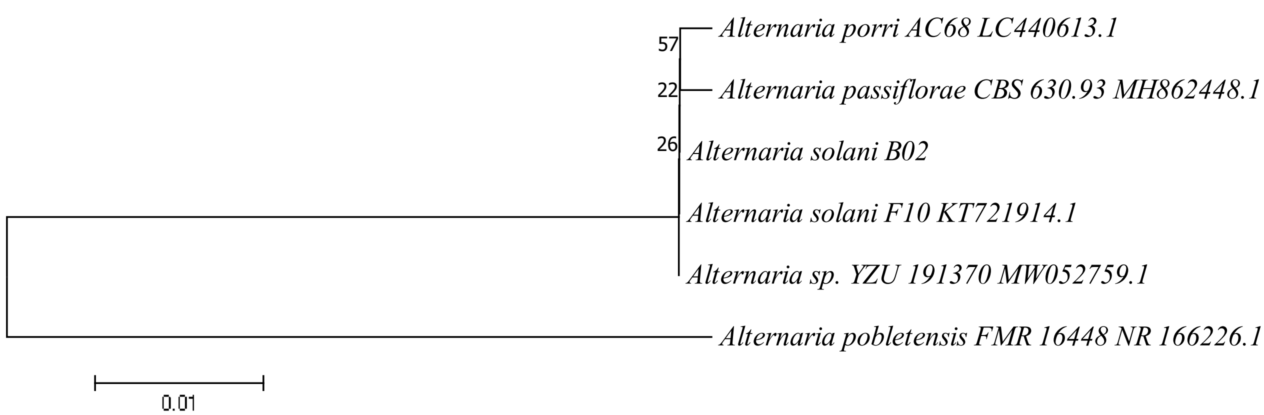


Figure 2. Neighbor-joining tree of isolate *A. solani* B02 according to ITS1/ITS4 region. The tree was generated using Tamura 3 parameter model with 1000x bootstrap replicates

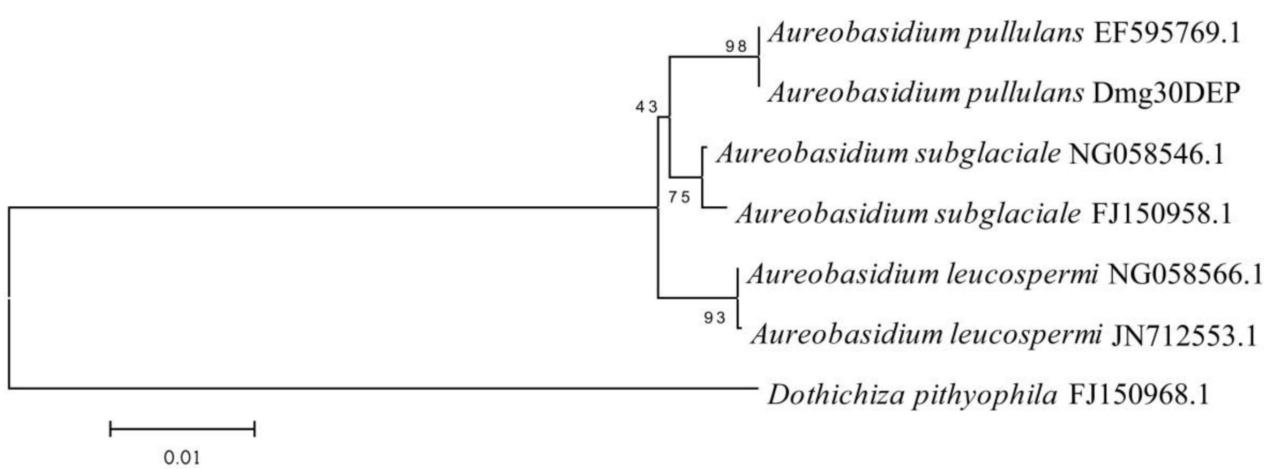


Figure 3. Neighbor-joining tree of isolate *A. pullulans* Dmg 30 DEP according to D1/D2 region. The tree was generated using Tamura 3 parameter model with 1000x bootstrap replicates

*A. solani* colony was completely inhibited by VOCs produced by yeast antagonist (Table 2). Identification of *A. pullulans* Dmg 30 DEP VOCs was investigated using GC-MS technique and results were shown on Table 3.

Orange pigments formed around yeast colonies that were grown on chrome azurol sulfonate (CAS) media means that *A. pullulans* Dmg 30 DEP was able to produce siderophore. The proteolytic and chitinolytic plate assay demonstrated that *A. pullulans* Dmg 30 DEP was able to produce protease and chitinase that characterized by the presence of clear zones at both of colony edges.

It has been known that volatile compounds produced by *A. pullulans* Dmg 30 DEP playing antimicrobial roles. The inhibition of *A. solani* in the two-compartment petri dish test was most likely due to the activity of those compounds. The compounds belong to the group of fatty alcohol (n Tetracosanol-1), long-chain hydrocarbon (Eicosane, Octadecane, 1-Nonadecene, Heneicosane, and Docosane), ester halogenated compound (Octadecyl Trifluoroacetate) and phenol. The three highest areas were shown by n tetracosanol-1 (37.52%), 1-Nonadecene (17.84%) and Eicosane (9.71%).

The results of the agar block assay showed that yeast *A. pullulans* had hyperparasitism activity. The contact between yeast cells and *A. solani* hyphae (Figure 4) occurred 24 hours after treatment and the hyphal damage began after 96 hours of incubation. The interaction between *A. pullulans* and *A. solani* were also observed in symptomatic tomato leaves, the results showed that hyphae of *A. solani* became undeveloped and space competition occurred. Apparently, most of the space of the leaves had been colonized by yeast and only a small portion was occupied by hyphae of *A. solani*.

The result shows that *A. pullulans* Dmg 30 DEP is able to colonize the surface of tomato leaves (Figure 5A). The pattern of yeast development in leaves tends to spread following the areole of tomato leaves (Figure 5B). Periodic observations of yeast populations indicate that the population of *A. pullulans* Dmg 30 DEP drops to the end of observation. Application of cell density $10^7$ ml$^{-1}$ and $10^4$ m$^{l-1}$ on the surface of tomato plants obtained the number of yeast cells attached to the leaves at 0 days estimated at 8.2 x $10^5$ cells cm$^{2\ -1}$ leaf area and 2.4 x $10^3$ cells cm$^{2\ -1}$ leaf area. The population tends to decrease until the end of the observation (six days), that is 2.1 x $10^4$ cm$^{2\ -1}$ cells for treatment $10^7$ and for treatment $10^4$, there are no yeast cells have been found (Table 4).

Table 2. Mode of actions of *A. pullulans* Dmg 30 DEP involved on suppressing *A. solani* infection on tomato

| Mode of action | | Activity |
|---|---|---|
| Antibiosis | Dual culture | + |
| | Antibiotic metaboltite | - |
| | VOC production | + |
| Siderophore | | + |
| Lysis | Chitinase | + |
| | Protease | + |
| Hyperparasitism | | + |

*) The symbol +/-

Table 1. Effect of yeast cell density to early disease development on leaf and tomato plant

| Yeast cell density/ml | *in vivo* | *ad planta* | |
|---|---|---|---|
| | Chlorosis area (%)*) | Number of infected leaves *) | Diseases severity (%) *) |
| Control | 48.06±9.06$^a$ | 10.4±1.38$^a$ | 48.33±6.32$^a$ |
| *A. pullulans* ($10^4$) | 44.15±6.69$^a$ | 9.7±1.68$^{ab}$ | 49.17±4.56$^a$ |
| *A. pullulans* ($10^5$) | 47.69±6.94$^a$ | 9.1±1.56$^{ab}$ | 31.67±4.75$^{ab}$ |
| *A. pullulans* ($10^6$) | 16.68±3.84$^b$ | 6.8±0.97$^b$ | 27.5±2.28$^b$ |
| *A. pullulans* ($10^7$) | 16.25±4.37$^b$ | 1.6±0.22$^c$ | 9.17±1.68$^c$ |

*) The value is the average with ± standard deviation, and the value followed by the same letter in the same column shows no significant difference

Table 3. Volatile organic compounds (VOCs) of *A. Pullulans* Dmg 30 DEP identified by GC-MS

| Peak number | Name | Retention time | Area (%) | Chemical structure |
|---|---|---|---|---|
| 1 | Eicosane | 15,592 | 9.71 | $C_{20}H_{42}$ |
| 2 | Octadecane | 19,290 | 7.31 | $C_{18}H_{38}$ |
| 3 | 1-Nonadecene | 20,378 | 17.84 | $C_{19}H_{38}$ |
| 4 | Octadecyl Trifluoroacetate | 24,548 | 7.64 | $C_{20}H_{37}F_3O2$ |
| 5 | Heneicosane | 25,511 | 8.33 | $C_{21}H_{44}$ |
| 6 | Docosane | 27,399 | 6.10 | $C_{22}H_{46}$ |
| 7 | n Tetracosanol-1 | 28,387 | 37.52 | $C_{24}H_{50}O$ |
| 8 | Phenol | 29,618 | 5.55 | $C_{14}H_{22}O$ |

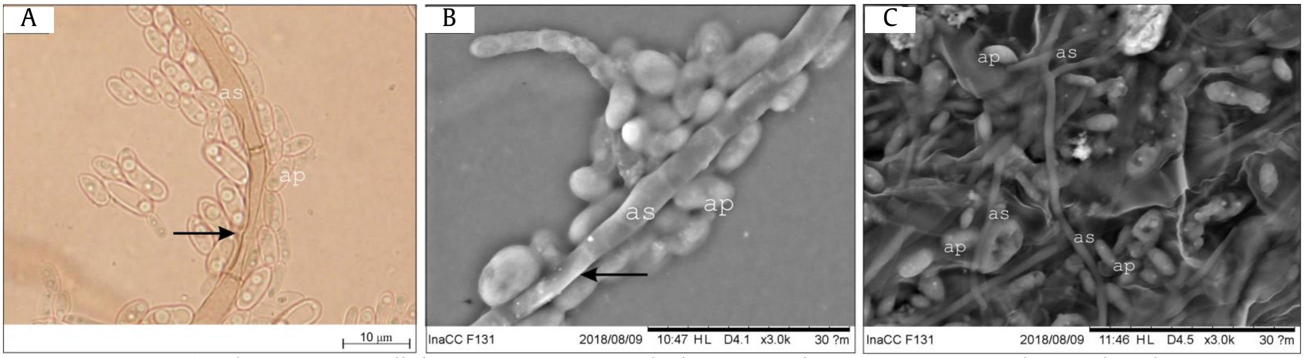


Figure 4. Interaction between *A. pullulans* Dmg 30 DEP and *Alternaria solani* on water agar observed under a compound microscope (A) observed under scanning electron microscope on 2000x magnification (B). Interaction between *A. pullulans* and *Alternaria solani* on symptomatic leave, visualization using SEM, on 3000x magnification (C), (ap: cell of *A. pullulans*, as: hyphae of *A. solani*)

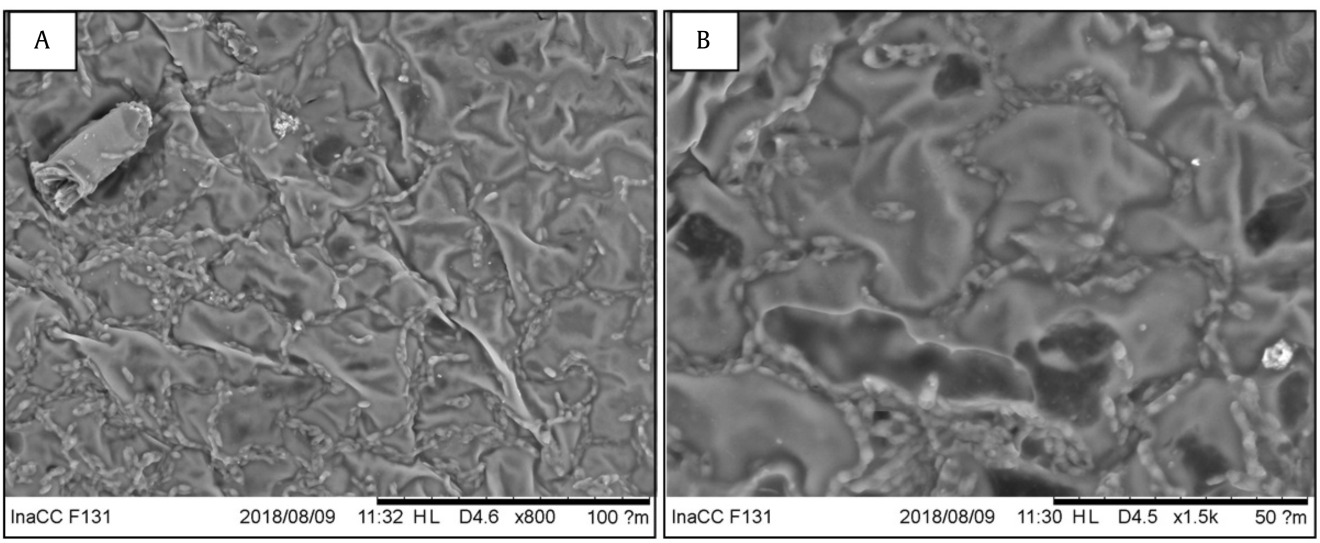


Figure 5. (A) Colonization of *A. pullulans* Dmg 30 DEP on tomato leaf surface, on 800x magnification, (B) on 1500x magnification

Table 4. Population dynamic of *A. pullulans* Dmg 30 DEP on tomato leaves for 6 days

| Days | Yeast cell density (cell $cm^{2\ -1}$ leaf area) | |
|---|---|---|
| | $10^7$ | $10^4$ |
| 0 | $8.2 \times 10^5$ | $2.4 \times 10^3$ |
| 2 | $1.8 \times 10^5$ | $1.2 \times 10^3$ |
| 4 | $2.7 \times 10^4$ | 0 |
| 6 | $2.1 \times 10^4$ | 0 |

## 4. Discussion

The effectiveness of yeast biocontrol activity is influenced by many factors, one of them is the yeast cell density on its application. Schisler *et al.* (1997) reported that the ability of bacterial antagonists against dry rot of potato was affected by cell density. The biocontrol activity of antagonists on controlling postharvest disease increased with the increasing concentrations of antagonists. *Candida saitoana* was effective at a concentration of $10^7$ CFU/ml for controlling *P. expansum* on apples (McLaughlin 1990). *A. pullulans* at $10^7$ and $10^8$ cells/ml were able to control *Penicillium digitatum* on grapefruit, *B. cinerea, R. stolonifer* and *Aspergillus niger* on grapes, and *B. cinerea* and *R. stolonifer* on tomatoes (Schena *et al.* 2003). The high density of yeast cells provides opportunities to yeast to occupy leaf surfaces more quickly so that they can compete with pathogens for nutrient and space (Filonow 1998; Di Francesco *et al.* 2017). Similarly, it should have the ability to survive even under conditions that are unfavorable to the pathogen (Hirano and Upper 2000).

Antagonism between biocontrol agents and pathogens can occur through direct contact or the presence of compounds produced by antagonistic agents in the form of antimicrobial secondary metabolites. The occurrence of antagonism on dual culture test is most likely due to direct contact. A previous study stated that most antagonistic yeasts do not produce non-volatile antimicrobial metabolites to *Colletotrichum acutatum* (Hartati *et al.* 2015), but some yeasts can produce VOCs that play a role in inhibiting the growth of pathogens (Buzzini *et al.* 2005).

The results of this study indicated that *A. pullulans* Dmg 30 DEP can produce VOCs. N tetracosanol-1 and 1-nonadecene are the two highest volatile compounds produced by *A. pullulans* Dmg 30 DEP in axenic culture condition (Table 3). With an alcoholic functional group, N tetracosanol-1 is categorized as a fatty alcohol. Alcoholic compounds can reduce ergosterol levels in the membrane and increase intracellular acidity, which may affect the stability of the membrane. It also can interfere with cell wall integrity (CWI) and the regulation of cell wall synthesis signals. In addition, alcohol compounds can be inhibitors in the process of hyphal morphogenesis, inhibiting the development of hyphae (Ansari *et al.* 2013).

The cell walls of Ascomycota group are generally composed of chitin (39%), glucan (29-60%), protein (7-13%) and lipids (6-8) (Webster and Weber 2007; Ruiz-Herrera 2012). In this study, *A. pullulans* Dmg 30 DEP able to produce protease enzymes (Table 2). The protease may not play a role when it works alone (Banani *et al.* 2014), but it can enhance the glucanolytic ability. Scott dan Schekman (1980) reported that the activity of glucanase enzyme increases when working with proteases. These results are related to the role of proteins in the cell wall, which is a glucan-binding structure (Ruiz-Herrera 2012). Chitin may be referred to as the main component of fungal cell walls. The production of chitinase enzyme by *A. pullulans* allows lysis of the *A. solani* cell wall. Several studies have previously expressed yeast antagonist *A. pullulans* (Di Francesco *et al.* 2015), *Pichia membranefaciens*, and *C. albidus* (Chan and Tian 2005) may produce the enzyme chitinase and has strong biological control ability.

This research also indicated that yeast *A. pullulans* Dmg 30 DEP can produce siderophore (Table 2). Siderophores are extracellular, low molecular weight compounds (<1000 Dalton), which selectively bind iron ($Fe^{3+}$). Siderophores are generally produced by anaerobic and aerobic facultative microorganisms. Siderophore-producing microbes are usually used as biocontrol agents and plant growth-promoting rhizobacteria (PGPR). Siderophore compounds are reported to also induce plant resistance, making them more resistant to pathogen infection. In addition, the main mechanism of the siderophore-producing microbes is the competition of $Fe^{3+}$ element uptake with pathogens (Maindad *et al.* 2014). Several microbes that were exposed could produce siderophore such as *Trichoderma viride, Trichoderma harzianum* and *Candida famata, Bacillus subtilis, B. megatericus, Pseudomonas aeroginosa* (Ghosh *et al.* 2015) and *Acinetobacter calcoaceticus* (Maindad *et al.* 2014).

The ability of hyperparasitism has been reported by several other types of antagonistic yeast, such as *P. membranfaciens, C. albidus* (Chan and Tian 2005) and *A. pullulans* (Hartati *et al.* 2015). The attachment of yeasts to the pathogen structure is closely linked to their biocontrol activity. The damage to hyphal cells outwardly related to another mode of action. The mechanism of hyperparasitism allows yeast cells to make more contact with the pathogen structure so that the performance of another mode of action will increase.

According to (Allen *et al.* 2004), the ability of hyperparasitism of yeast antagonists was affected by the initial number of given cells, yeast species, the presence of nutrients, and the length of the incubation period. The attachment of *A. pullulans* cell to its substrate is closely related to the role of extracellular polysaccharide (pullulans) (Andrews *et al.* 1993). Pouliot *et al.* (2005) also found that the adhesiveness of *A. pullulans* was affected by uronic acid and pullulans. Communication among yeast cells to form colonies and parasite hyphae cells is assisted by various signals, such as pheromone signals, quorum sensing signals, and ammonium signals (Honigberg 2011).

Enzymatic activity, particularly enzymes related to the cell membrane and wall lysis, has been reported to have a role in hyperparasitism interaction between fungi. During mycoparasitism of *Ganoderma boninense* by fast-growing filamentous mold *Trichoderma harzianum*, chitinase gene in *T. harzianum* was expressed (Naher *et al.* 2018). Furthermore, in agar dual culture during antagonistic interaction with *Lentinula edodes*, the production of extracellular enzyme N-acetyl-β-glucosaminidase and laminarinase of *Trichoderma* spp., were induced (Hatvani *et al.* 2002). Similarly, our result showed that *A. pullulans* was able to produce chitinase as well as protease that might be played role in mycoparasitism interaction with *A. solani*.

The success of biological control will be largely determined by the successful colonization of the antagonistic microorganisms used in the host. Colonization can be interpreted as settling and developing a microorganism in a host. To be able to settle on the surface of a leaf, a microorganism must be able to use nutrients and overcome fluctuating and extreme micro-climatic conditions.

Decreased yeast population is suspected because the adaptability of yeast to the environment has not yet been formed and the reduced source of nutrients available on the leaf surface. According to Di Francesco *et al.* (2017) report, *A. pullulans* L1 and L8 populations tend to decline at the beginning of the application and the population can last up to 10 days on tomato leaves. Inácio *et al.* (2002) reported a decrease in yeast population in leaves is largely determined by temperature, humidity and nutrient availability. Population density of *Sporobolomyces roseus* and *Cryptococcus laurenti* was reported to increase at a temperature of 12-24°C when given an adequate supply of nutrients and in vapor pressure deficit (VPD) conditions of 0,00 kPa. The population will decrease when no additional nutrients are given and VPD is at 0.61 kPa (Dik *et al.* 1992).

In conclusion, this study has demonstrated that *A. pullulans* Dmg 30 DEP plays a role in the suppression of earl blight disease at $10^6$ cells/ml and $10^7$ cells/ml yeast cell density. The mechanisms involved in biocontrol activity were the production of volatile organic compounds (VOCs), the production of chitinase and protease enzymes, the production of siderophore and hyperparasitism.

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