Bio efficacy of frog skin bacteria as biological control agents against chili anthracnose disease

Bioefikasi bakteri dari kulit katak sebagai agensia biokontrol terhadap penyakit antraknosa pada cabai

Lela Susilawati[1,*], P. Afrizka Sari[1], Maulana Septiani[1], E.S. Purnomo[1,2]

1) Department of Biology, Faculty of Science and Technology, UIN Sunan Kalijaga, Yogyakarta
   Jl. Marsda Adisutjipto No.1, Yogyakarta-Indonesia, 55281
2) Lab. Microbiology, Integrated Laboratory, UIN Sunan Kalijaga, Yogyakarta
   Jl. Marsda Adisutjipto No.1, Yogyakarta-Indonesia, 55281
*Email: lela.susilawati@uin-suka.ac.id

ABSTRACT

Amphibian skin e.g., frog carry bacterial symbionts on their skin that protect the frog from invasion of pathogen infection. This study aimed to evaluate antifungal activity of five bacterial skin frog (namely KSMD3; KSMD9; KSMD10; KSMV12; KSMV15) of Indonesian origin, *Fejervarya limnocharis,* against phytopathogen (*Colletotrichum capsici*) TCKr2. Primary screening for their antifungal activity was performed using dual culture method on nutrient agar contained 2% (w/v) of dextrose. The alteration of hyphal morphology on media and the detached chili fruit bioassay were observed. Isolate of KSMD3 was selected based on its significant performance in inhibiting the growth of the chili anthracnose pathogen, *C. capsici*. In addition, the KSMD3 showed low severity of disease incidence on detached chili fruit. Based on the analysis of 16S rDNA, the isolate of KSMD3 was identified as member of genera of *Pseudomonas*.

Kata kunci: antifungal activity, Colletotrichum sp. *bioassay, Indonesian frog*, *Pseudomonas* sp.

INTISARI

Kulit amfibi seperti katak membawa simbion bakteri pada kulitnya yang melindungi katak dari serangan infeksi patogen. Penelitian ini bertujuan untuk mengevaluasi aktivitas antifungi dari lima bakteri kulit katak yaitu KSMD3; KSMD9; KSMD10; KSMV12; KSMV15 yang disiolasi dari spesies katak asal Indonesia, *Fejervarya limnocharis* terhadap fitopatogen (*Colletotrichum capsici*) TCKr2. Skrining utama aktivitas antifungi dilakukan menggunakan metode *dual culture* pada media agar yang mengandung 2% (b/v) dekstrosa. Adanya perubahan morfologi hifa dan bioassay buah cabai diamati. Isolat KSMD3 terpilih berdasarkan aktivits antifungi dalam menghambat pertumbuhan *C. capsici*. Selain itu, KSMD3 menunjukkan tingkat keparahan penyakit yang rendah pada buah cabai. Berdasarkan analisis 16S rDNA, isolat KSMD3 diidentifikasi sebagai anggota genus *Pseudomonas*.

Kata kunci: aktivitas antifungi, *Colletotrichum* sp., bioassay, katak Indonesia, *Pseudomonas* sp.

INTRODUCTION

Amphibian skin is frequently exposed to the environment including physical factors, microorganisms and predators (Xu & Lai, 2015). The amphibian skin is relatively thin and permeable (Varga et al., 2019), hence, their skin is vulnerable to pollutants (McCoy & Peralta, 2018) and pathogen (Madison

et al., 2017). Therefore, the essential physiological processes occurred in their skin play an important role for maintaining their health (Varga et al., 2019).

Amphibian skin can secret some remarkable substances such as antioxidants (C. Liu et al., 2010), and antimicrobial peptides (AMPs) (Conlon, 2011). Those substances not only has the essential role for gas exchange but also host defense against some invasive organisms (Jared et al., 2018). Mostly, those discovered peptides from frog skin demonstrated high antimicrobial and antifungal activities (Abbassi et al., 2008) that effectively against Gram-positive and Gram-negative bacteria. In addition to several studies indicated that these peptides from frog skin considerably have attracted to be developed as a promising therapeutic alternative in the future. For example, frog's peptide brevinine-1, brevinine-2, esculantin-2, and temporin from *Hylarana erythraea* (the green frog) (Al-Ghaferi et al., 2010) showed effectively inhibit the growth of *Escherichia coli*, *Staphylococcus aureus*, *Candida albicans*, and *Acetinobacter baumannii*. Recently D'Auria et al. (2022) reported that Temporin G (TG), originally isolated from *Rana temporaria* (Europian red frog), demonstrated inhibitory activity against fungi *C. albicans* and *Cryptococcus neoformans*. Moreover, TG showed high activity that inhibited the virulence factor of *C. albicans*.

These antimicrobial compounds are secreted by microbial symbiont on amphibian skin (Mangoni et al., 2001). These communities of microbiota occupies the mucous layer forming a microbiological barrier for skin protection (Smith et al., 2018; Varga et al., 2019). High diversity of microbes can grow and reside on amphibian skin surface because the skin provides a suitable habitats for them by producing a glycoprotein-containing mucus (Austin, 2000).

Previous studies have been reported of the recovery of frog-skin bacterial symbiont from several area and species. Belden et al. (2015) successfully isolated diverse species of bacteria from the skin of frogs, *Craugastor fitzingeri* (Fitzinger's robber frog), *Dendropsophus ebraccatus* (pantless treefrog), and *Agalychnis callidryas* (red-eyed leaf frog) in Panama. From Japan, Susilawati et al., (2021) obtained a total 102 bacterial isolates from three native species of wild frog *Hyla japonica*, *Pelophylax porosus porosus* and *Buergeria buergeri*. Yet, to our knowledge, no study has been reported in Indonesian frog. Susilawati & Sari (2014) obtained 20 culturable bacteria isolates from local species of frog *Fejervarya limnocharis* with no further study on the identification and their potentiality against invasive pathogen.

In fact, Indonesia is one of the megadiverse countries in the world comprises about 17,000 islands with high diverse of endemic species of floras and faunas (Iskandar & Erdelen, 2006). This provides an opportunity to preserve species of frog in Indonesia by applying its microbial community for controlling other microbial pathogen such as bacteria, fungi and virus in agriculture, wildlife, and human. Here, we investigate the bacterial community on endemic Indonesian frog and evaluating their antifungal activities against phytopathogen *Colletotrichum*, causing agent of chili anthracnose disease.

Chili is an essential horticultural commodity in tropical and subtropical countries, including Indonesia. Indonesia together with other Asian countries such as Thailand, China, Turkey, and India are known as the largest chili producers in the world (Saxena et al., 2016). However, there are obstacles in chili production, especially anthracnose disease caused by *Colletotrichum* sp. (De Silva et al., 2017). This fungus causes the black spots on the surface of the chili fruit, thus reducing its marketability (Than et al., 2008). Chili Anthracnose disease causes significant yield losses globally; for example, in Thailand, yield

losses of up to 80% (Montri et al., 2009), 10% in Korea (Kim et al., 2008), about 10-80% and 2-35% in the rainy season and dry season respectively in Indonesia (Than et al., 2008). To control this disease, we investigate the antifungal activity of bacteria isolated from frog skin against *Colletotrichum*. This study is the first report using frog skin bacteria as a potential biological control agent.

MATERIALS AND METHODS
Microorganisms and culture conditions

Five bacterial isolates from Indonesian's frog skin species *Fejervarya limnocharis* namely KSMD3; KSMD9; KSMD10; KSMV12; KSMV15 were used in this study. Pure bacterial cultures stored at –80ºC in 10% skim milk were revived on NA agar plates and incubated at 28ºC for 48 h. *Colletotrichum capsici* TCKr2, the chili anthracnose fungus was used. The fungi was cultured on PDA (potato dextrose agar) medium at 28°C in the dark (Kubota and Abiko, 2000). Ten-day old PDA plate cultures of *C. capsici* TCKr2 was flooded with 10 ml sterile distilled water and carefully scraped with a paint brush to harvest conidia. The conidial suspension was filtered through two layers of sterilized cheesecloth, and the density was adjusted to 10^5 conidia/ml using a haemocytometer under microscope (Olympus, Tokyo, Japan).

Primary screening of antagonistic bacteria by dual culture method

All five bacterial isolates were initially screened for their antifungal activity against the chili anthracnose pathogen *C. capsici* TCKr2 by the dual-culture method (Damasceno et al., 2019) with a slight modification. Briefly, a 10-day old mycelial plug of *C. capsici* (ø3 mm) obtained from the PDA culture was placed on the centre of plate contained a fresh NA added with 2% of dextrose (ø90-mm Petri dish) and incubated in the dark at 29ºC for 24 h. A loop full of bacteria was streaked 3.0 cm away from the fungal pathogen on plate, incubated in the dark at 29ºC for 3 days. Percent inhibition of mycelial growth (GI) was calculated as: GI (%) = $[(R\text{-}r)/R] \times 100$; where, R represents the colony radial size (mm) of the fungus distal to the bacteria (as a control) and r represents the colony radial size (mm) proximal to the bacteria.

The alteration of fungal morphology was examined by taking a treated aerial mycelium of fungi with a sterilized needle and put on the clean slide glass. Then the fungus was stained with 0.01% (w/v) trypan blue in lactophenol dye. Fungal morphology was observed using a light microscope.

Chili anthracnose disease resistance assay

The fresh chili pepper (*Capsicum frutescens* L.) fruit washed with distillate sterile water and allowed to air dry. The surface of dried chili were sterilized with 70% of ethanol and wiped them with a sterile tissue paper (Rajapakse & Ranasinghe, 2002). All sterilized chili pepper, were wounded in two site (4.0mm in diameter) and dropped 3µl of bacterial suspension (10^{10} cfu/ml). After 2h, the 3µl (10^5 conidia/ml) fungal pathogen was inoculated to each wound. The fungicide mancozeb was used as a control. Then, all treated chili were kept in a transparent box for 7 days with wet filter paper inside to maintain the humidity. After incubation period, the disease incidence was measured using the formula described by Cardoso et al. (2004) $I = \Sigma x/N$ where x represents the number of diseased plants divided by the total number of evaluated plants.

Molecular identification of bacteria using analysis 16S rDNA gene

Genomic DNA of selected bacteria was extracted using the *Wizard Genomic DNA Purification Kit* following the manufacturer's instructions. Bacterial isolates were cultured in nutrient broth at 37°C for 24h. The bacterial cells were harvested by centrifugation (536 ×g) at 4°C for 15 min. An approximately 1.5-kb fragment of the bacterial 16S rRNA gene was amplified using the universal primer set 27F (5'-AGAGTTTAGTCCTGGCTCAG-3') and 1529R (5'-CAIAAAGGAGGTGATCC-3). The total reaction volume per vial was 10.0 µl using kit MegaMix Blue (Microzone Ltd). Reactions were held at 94°C for 5 min to denature the DNA, with amplification proceeding for 30 cycles at 94°C for 1 min, 55°C for 1.5 min, and 72°C for 2 min, and a final extension at 72°C for 10 min. The amplicon was visualized in a 2% (w/v) agarose gel and subjected to DNA sequencing (ABI PRISM310 Genetic Analyzer, Thermo Fisher Science, Waltham, MA, USA). Primers used for sequencing were the same as those used for amplification of the 16S region (27F and 1492R).

Sequence analysis

The sequence of each isolate was analyzed for its similarity with the sequences in the NCBI GenBank database (http://www.ncbi.nlm.nih.gov) and aligned by Clustal W (Thompson et al., 1994). A phylogenetic tree was constructed with Neighbor-Joining analysis (NJ) using MEGA 11 (Kumar et al., 2018).

RESULTS AND DISCUSSION
Primary screening of bacterial frog-skin isolates for antagonistic activity against *C. capsici*

Two of the five bacteria, KSMD3 and KSMD10, showed high inhibitory activity against C. *capsici* (Fig. 1.A.). The percentage of mycelial growth inhibition achieved by KSMD3 was 50.0% followed by KSDM10 with 45.0% (Fig. 1.B).

Frog-skin bacteria has been widely investigated for protecting the amphibian fungal pathogen, *Batrachochytridium dendrobatidis* and *B. salamandrivorans* by producing antifungal compounds (Woodhams et al., 2018). In this study, we evaluated the efficacy of frog-skin bacteria in controlling the phytopathogen. Two selected isolates, KSMD3 and KSMD10 showed high capability inhibited the growth of *C. capsici*. It is indicated that these two isolates releases antifungal compound. However, the study on the mechanisms involved in this inhibition is required.

Colletotrichum has been considered as one of the most important fungal pathogen genera among other ten genera which significantly reduce the productivity (Dean et al., 2012). Application of synthetic fungicide is still popular to control the disease caused by *Colletotrichum*, however the excessive application can promote the fungal resistance to fungicide (Jamalizadeh et al., 2011). Thus, the application of biological control agent for instance bacteria can help to tackle the attacking of fungi.
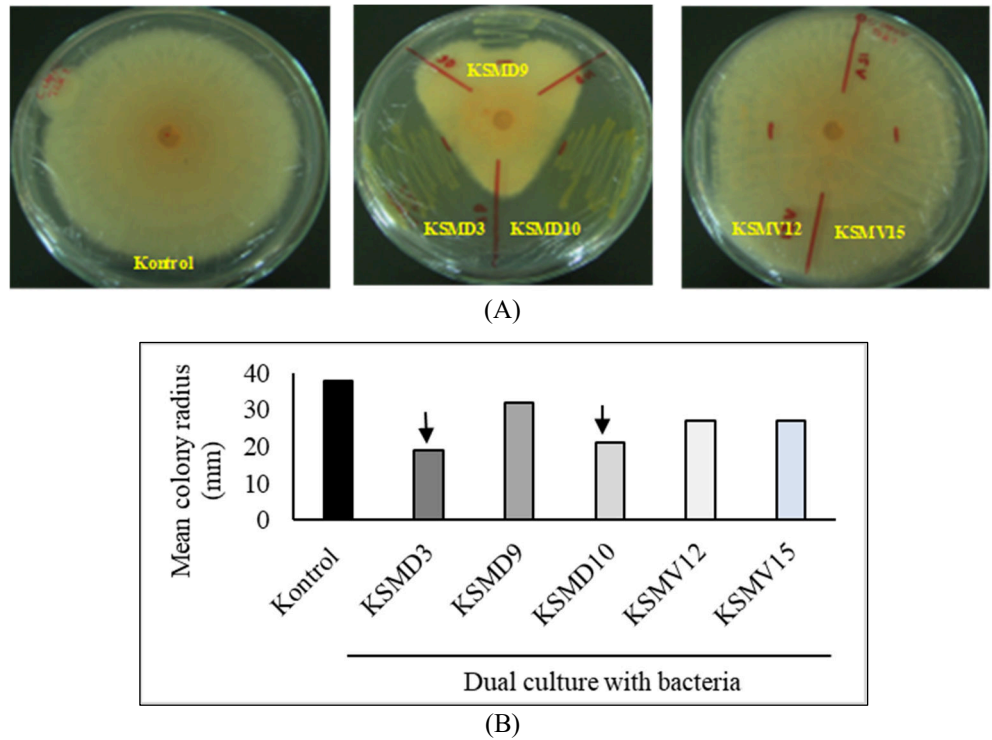
(A)

(B)

Figure 1. Five tested frog-skin bacterial isolates, KSMD3, KSMD10, KSMD9, KSMV12 and KSMV15 streaked on PDA medium inhibited the mycelial growth of *C. capsici* TCkr2 in dual cultures incubated at 29°C. 3-days post inoculation of inhibited fungal pathogen viewed from above of the PDA medium (A). The radius of colony on the side nearest the bacteria was compared with the control (B). KSMD3 and KSMD10 showed the strongest inhibitory toward *C. capsici*.

In vitro interactions between the frog-skin bacteria and *C. capsici*

Treated mycelial fungal with frog-skin bacteria was observed with a light microscope. *C. capsici* treated with one of the isolates (KSMD3 and KSMD10) resulted in swelling and lysis of hyphae (Fig. 2).
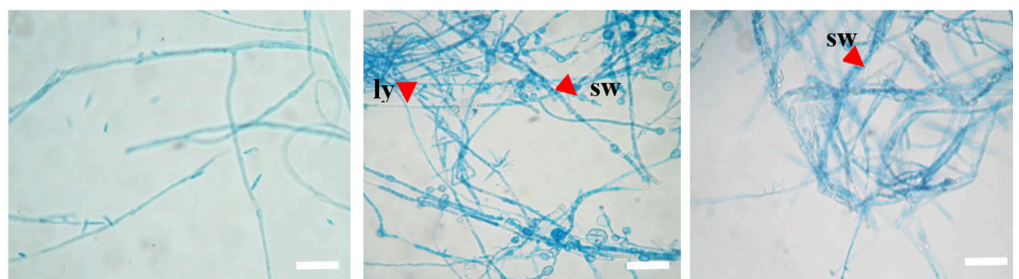

Figure 2. Treated Colletotrichum capsici TCkr2 with selected bacterial isolates KSDM3 (middle) and KSMD10 (left) after 3-days incubation at 29°C. No abnormal hyphae were observed in the control (right) without bacteria; whereas in the presence of bacteria, some abnormal mycelia formed, including swollen hyphae (sw) and lyzed (ly). Scale bars 20 µm.

The suppressive effect of two selected bacteria KSMD3 and KSMD10 presented the alteration of fungal hyphae after co-culture with *C. capsici*. Hyphal morphology was observed with a light microscope resulted in swelling hyphae (sw) and lysis of hypae (ly) (Fig. 2). These two bacteria indicated the induction of morphological changes. A previous study by Kwon et al., (2022) showed that

Bacillus tequilensis GYUN-300 induced the changes of mycelial *Colletotrichum acutatum*, where the conidia has swollen. Another study by Zepeda-Giraud et al., (2020) demonstrated the damage of *C. gloesporoides* mycelia after treated with biocontrol agent yeast *Wickerhamomyces anomalus*. Likewise, Susilawati et al., (2021) reported that three bacterial isolates frog skin causes severe changes in mycelia of *C. orbiculare* A-19. The mode of action involved in this mycelial alteration included the production of hydrolytic enzymes (Zepeda-Giraud et al., 2020), nutrient competition, hyperparasitism and antibiosis (Köhl et al., 2019).

Chili anthracnose disease resistance assay

　　Two selected bacteria, KSDM3 and KSMD10 were tested for their resistance activity toward *C. capsici* using fresh chili pepper. KSMD3 showed the lowest disease incidence percentage with 30% compared to control using SDW and the commercial fungicide with 76% and 40% respectively (Fig. 3.B). The mycelial of *C. capsici* growing well covered whole the fruit of chili pepper after 7 days-inoculation in control treatment, while chili fruit treated with KSMD3 the mycelial fungi did not show high severity disease (Fig. 3.A). The treatment of chili with KSMD3 proved satisfactory results, with the percentage of DI (30%) lower than the control (76%) and KSMD10 by 38%. The high inhibition demonstrated by KSMD3 and KSM10 could be due to the production of antifungal substances, however the mode of action of the inhibitory activity of both isolates is still unknown. Therefore, the further study is required.
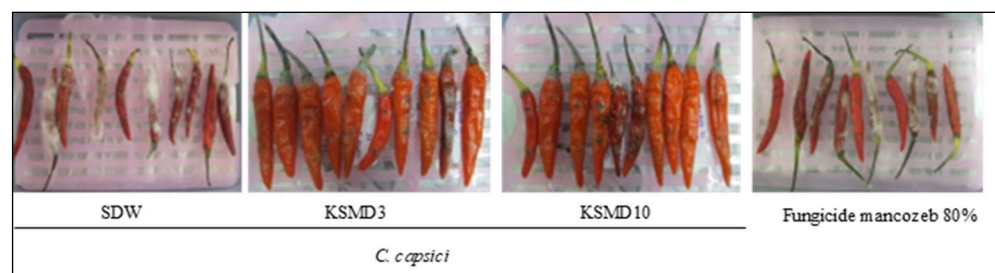
(A)
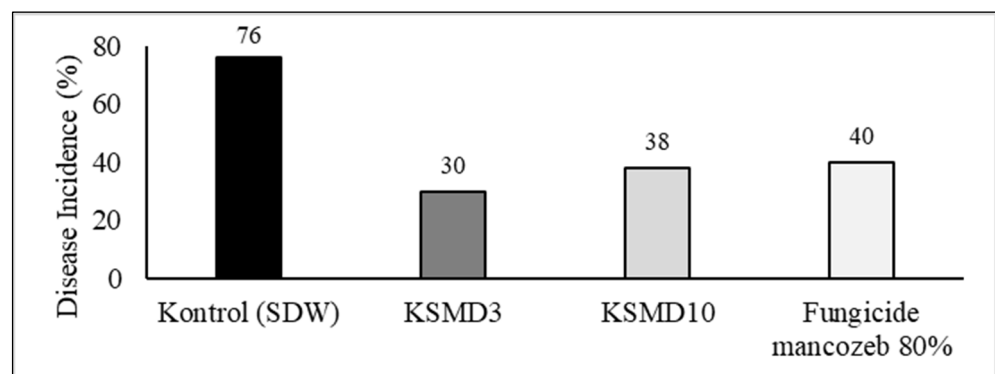
(B)

Figure 3. Two selected frog-skin bacteria KSMD3 and KSMD10 were dropped on wounded healthy chili fruit after 24h inoculated with C. capsici. controlled chili fruit with SDW showed high severity disease with chili fully covered with mycelium of C. capsici after 7-days inoculation compared to KSMD3 treatment (A). The lowest percentage of disease incidence showed by treatment with KSMD3 followed by KSMD10, while application of fungicide mancozeb 80% presented slight disease incidence compared to KSMD3 (B).

Molecular identification of best bacteria using analysis 16S rDNA gene

We sequenced the 16S rRNA of best bacteria KSMD3. The sequence was 99.28% identical to those of Pseudomonas aeruginosa (for example NR114471.1; Fig. 4). We identified KSMD3 as a *Pseudomonas* sp. *Pseudomonas* are widely known as effective biological control (Höfte, 2022). According to Lahlahi et al. (2022) genus of *Bacillus* and *Pseudomonas* are mostly important genera which high capability in secondary metabolites production. *Pseudomonas* has been reported able to inhibit several most important plant diseases such as *P. chloroaphis* zm-1 isolated from rhizosphere showed high inhibition of fungal pathogen causing peanut stem rot, *Sclerotium rolfsii* by producing 1-hydroxy phenazine (Liu et al., 2022), *P. aeruginosa* Ld-08 effectively suppressed the growth of three tested phytopathogens, *Fusarium oxysporum*, *Botrytis cinerea*, *Botryosphaeria dothidea*, and *Fusarium fujikuroi* due to its ability of several bioactive substances production such as quinolones; 3,9-Dimethoxypteroc arpan; cascaroside B; dehydroabietylamine; epiandrosterone; nocodazole; oxolinic acid; pyochelin; rhodotulic acid; 9,12-octadecadienoic acid; di-peptides; tri-peptides; pinolenic acid methyl ester; ursodiol; and venlafaxine (Khan et al., 2022). From these studies, *Pseudomonas* isolated from common niche of environment for instance soil, rhizosphere, or water (Lahlahi et al., 2022). In this study, we isolated *Pseudomonas* from amphibian skin. To date, this study is the first report of bacteria isolated from frog skin showing the antagonistic effect on *Colletotrichum* sp.
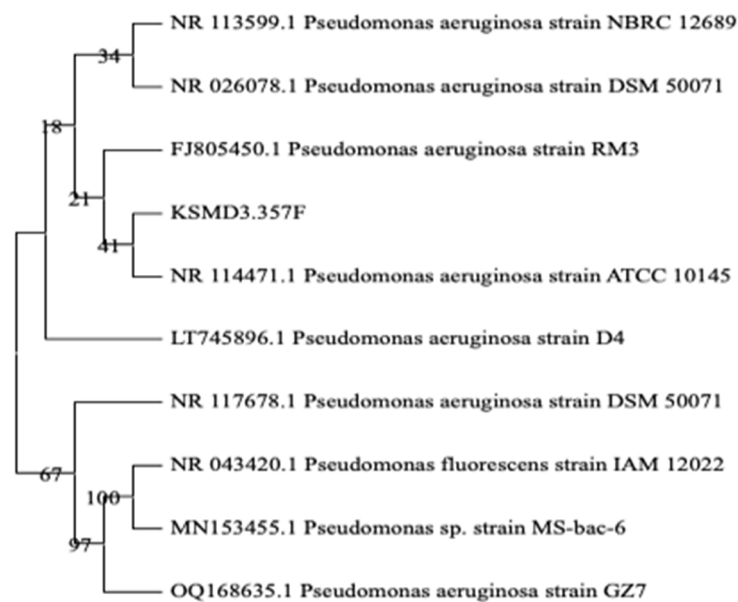

Figure 4. Neighbor joining (NJ) phylogenetic trees constructed based on 16S rRNA gene sequence indicate relationships between KSMD3 and Pseudomonas spp. Values at the nodes represent the percentage NJ bootstrap values from 1000 replicates; values ≥ 50% are shown. Bars indicate phylogenetic distances of 10%.

CONCLUSION

In conclusion, *Pseudomonas* sp. KSMD3 isolated from the skin of the Indonesian native frog species, *F. limnocharis*, is a candidate for biocontrol agents of plant disease caused by *Colletotrichum capsici*. The mode of action responsible for the control of disease seemed to be antibiosis, however further analysis to study this mode of action is necessary to be done.

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