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Research Article

Evaluation of the antifungal activity of marine actinomycetes isolates against the phytopathogenic fungi *Colletotrichum siamense* KA: A preliminary study for new antifungal compound discovery

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Abstract

Marine actinomycetes are being explored to discover potential actinomycetes that produce antifungal compounds. In a previous study, marine actinomycetes isolates from the mangrove ecosystem were found to inhibit growth of the phytopathogenic fungi *Colletotrichum siamense* KA. In this study, the three of these isolates with the highest antagonistic activity—SM11, SM14, and SM15—were evaluated for their antifungal activity using antibiosis assay. The fermentation was performed in SCB:PDB medium (1:1) for 6, 9, and 12 days. The results showed that SM14 was the strongest potential isolate; it inhibited the growth of *C. siamense* KA on average up to 64.90% for 12 days on PDA filtrate medium. Molecular identification showed SM14 was closely related to *Streptomyces sanyensis*, but had differences in morphological and biochemical characteristics compared to SM11 or SM15. This indicated that the three isolates were different strains and may challenge further research on identifying and analyzing their antifungal compounds.

Keywords

antibiosis assay, antifungal activity, marine actinomycetes, *Streptomyces sanyensis*

Introduction

Actinomycetes produce two-thirds of the antibiotics used today (Prudence et al. 2020). Following the successful exploration of actinomycetes in terrestrial habitats, marine actinomycetes have been used to produce various bioactive compounds with anticancer, antitumor, antibacterial, and antifungal properties (Solanki et al. 2008; Subramani and Aalsbersberg 2012). It is therefore not surprising that marine actinomycetes are a fascinating source of various

novel bioactive compounds in recent decades (Sharma et al. 2019). One of the actinomycetes habitat that remains little explored is the mangrove ecosystems, despite the large variety of potential actinomycetes that may produce antimicrobial compounds previously found in such areas (Azman et al. 2015; Sengupta et al. 2015; Rosmanie and Varghese 2016). The potential actinomycetes from the mangrove ecosystem might produce a new antifungal compound that can be used for controlling the phytopathogenic fungi.

Evangelista-Martinez et al. (2020) recently reported that *Streptomyces* sp. CACIS-1.5CA as one of the actinomycetes genus can produce bioactive compounds that inhibit the growth of *Colletotrichum* spp. and other several fungal phytopathogens. *Colletotrichum* spp. are endophytic and hemibiotrophic phytopathogenic fungi that infect various crops, causing fruit decay and serious losses to the agricultural sector (Connor et al. 2014; da Silva et al. 2020). *Colletotrichum siamense* has been reported to infect avocado crops in southeastern Brazil, the infection, called anthracnose, causes round brown lesions that become dark brown to black (Soares et al. 2021). Therefore, the infection of *Colletotrichum* in agricultural fields should be controlled.

The preliminary study to discover new antifungal compounds had been done by screening the marine actinomycetes isolates that have antifungal activity. Our previous study for discovering potential actinomycetes reported that 80% of the marine actinomycetes isolated from the mangrove ecosystem in Pramuka Island, Jakarta, Indonesia demonstrated antagonistic activity against the phytopathogenic fungi, *Colletotrichum siamense* KA (Fadhilah et al. 2021). The aim of the present study was to evaluate the antifungal activity of the three marine actinomycetes isolates (SM11, SM14, and SM15) with the highest antagonistic activity using antibiosis assay. The three isolates were fermented for 6, 9, and 12 days to determine the optimal percentage for inhibiting *C. siamense* KA. Moreover, the isolates will be characterized based on biochemical and morphological characters and also identified based on the 16S rRNA gene sequences for supporting our further study on seeking antifungal compounds from actinomycetes.

Material and methods

Microorganisms

The marine actinomycetes isolates SM11, SM14, and SM15, along with the fungal phytopathogen *C. siamense* KA, were provided by the Laboratory of Microbiology, Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia. *Colletotrichum siamense* KA was used to test the isolates' anti-fungal properties. The marine actinomycetes isolates were maintained in oatmeal agar at 27°C and *C. siamense* KA was maintained in Potato Dextrose Agar (PDA) at 27°C.

Fermentation of the antifungal compounds

The antifungal compounds from the isolates were fermented in modified Potato Dextrose Broth (PDB) medium containing Starch Casein Broth (SCB) with PDB medium (1:1) (Shrivastava et al. 2017). Ten percent (v/v) spore suspensions from a 7-day-old culture of isolates on an oatmeal agar slant were used as the inoculum. The spore suspension was inoculated in 100 mL of the SCB:PDB contained in a 500 mL Erlenmeyer flask and incubated for 6, 9, and 12 days at 27°C. After fermentation, the medium was centrifuged at 3,500 rpm for 20 minutes to obtain a cell-free filtrate. This filtrate, which was assumed to contain the antifungal compound, was used as the solvent to dissolve PDA powder. The PDA medium with the filtrate, called PDA filtrate medium, was used for the antibiosis agar assay.

Antibiosis agar assay

The antibiosis agar assay was conducted using the paper disc method on PDA filtrate medium in 2 batches; the procedure was replicated in triplicate. The spore suspension of *C. siamense* KA (10 µl) was inoculated on a paper disc, which was placed in the center of the plate containing the PDA filtrate medium. Meanwhile, for the control, an inoculated paper disc was placed in a normal PDA medium and incubated for 5 days at 27°C. After incubation, the diameter growth of *C. siamense* KA was measured using calipers. The growth inhibition of *C. siamense* KA caused by antifungal compounds in the PDA filtrate medium was described as the percentage of inhibition. The percentages were calculated according to Song et al.'s (2020) formula, where R1 was the diameter of the treated colony and R2 was the diameter of the control colony.

$$Percentage\ of\ inhibition = \frac{(R2 - R1)}{R2} \times 100\%$$

Characterization of the marine actinomycetes isolates

The marine actinomycetes isolates were characterized based on morphological and biochemical characteristics, including their extracellular enzyme activity and carbon utilization. The characteristics obtained completed the profiles of the three isolates.

Morphological observation

The isolates were grown on International *Streptomyces* Project (ISP) medium 2, 3, and 4 for 6 days at 30°C to observe their macroscopic morphology (Shirling and Gottlieb 1966). The colors of the soluble pigment and mycelium were determined using a Faber Castell Polychromos color chart. The microscopic morphology of the isolates was also observed using the slide culture method in ISP medium 2.

The microscopic structures of the isolates, such as aerial and substrate mycelia, were observed under a microscope Leica ICC50 HD (Leica Microsystems, Switzerland).

Biochemical characterization

The biochemical characteristics of the isolates tested were catalase, oxidase, H_2S production, phosphate solubilization, extracellular enzyme activity, and carbon utilization. The phosphate solubilization activity of isolate was determined using Pikovskaya's agar medium (Dastager and Damare 2013). The extracellular enzymes of isolates were qualitatively characterized in various media. The isolates were inoculated in a medium and incubated for 5–7 days at 30°C. The protease activity was observed in skim milk agar medium according to Fernandez et al. (2018). Following the gelatinase activity assay in a medium of modified nutrient gelatin (Elavarashi et al. 2017). The amylase activity was evaluated in starch agar medium (Kafilzadeh and Dehdari 2015).

The lipase activity of isolates was evaluated on Tween 20 agar supplemented with 0.1% (v/v) Tween 20 after being autoclaved (Gopinath et al. 2005). The isolates were also streaked on CMC agar to observe the cellulase activity according to Prasad et al. (2013). Chitinase activity was evaluated in colloidal chitin medium (Saima and Roohi 2013). Carbon utilization was determined using ISP medium 9 supplemented with 1% various carbon sources (Shirling and Gottlieb 1966).

Molecular identification

The molecular identification of the marine actinomycetes isolates was performed based on the 16S rRNA gene sequences. The isolates were identified by Macrogen (Seoul, South Korea). The 16S rRNA gene was amplified by a total reaction of 30µl using EF-Taq (SolGent, Korea) with the universal primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The PCR condition consisted of activating Taq polymerase at 95°C for 2 minutes, followed by 35 cycles of amplification at 95°C for 1 minute denaturation, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute; final extension was performed for 10 minutes at 72°C.

The DNA was sequenced using primers 785F (5'-GGATTAGATACCCTGGTA-3') and 907R (5'-CCGTCAATTCMTTTRAGTTT-3') and a PRISM BigDye Terminator v3.1 Cycle sequencing kit. The samples were analyzed using an ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA).

The DNA sequences were trimmed and assembled into a contig using ChromasPro. The contig sequence was compared to the available database at GeneBank in NCBI to identify the most closely related strain according to sequence similarity. The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987) in MEGA X with a bootstrap value based on 1,000 replications, and distance was determined based on the Kimura two-parameter model (Kimura 1980).

Results

Antibiosis agar assay

The three marine actinomycetes isolates demonstrated antifungal activity, as shown by the percentage of inhibition of *C. siamense* KA (see Table 1). The average percentages of inhibition for 6 to 12 days were 49.98–34.62% (SM11), 58.85–64.90% (SM14), and 48.92–52.02% (SM15). Fig. 1

Table 1. Results of the antibiosis assay of the marine actinomycetes isolates.

Isolate	Batch	Growth inhibition (%) Incubation time of fermentation (days)		
		6	9	12
SM11	1	66.68 ± 1.04	45.22 ± 1.02	41.82 ± 1.31
	2	33.28 ± 1.42	31.90 ± 1.43	27.41 ± 1.29
	average	49.98 ± 18.32	38.56 ± 7.38	34.62 ± 7.98
SM14	1	61.43 ± 1.65	67.68 ± 1.89	68.94 ± 1.46
	2	56.27 ± 1.54	56.63 ± 1.77	60.85 ± 3.43
	average	58.85 ± 3.17	62.15 ± 6.27	64.90 ± 5.01
SM15	1	47.72 ± 0.60	42.38 ± 1.15	48.79 ± 0.11
	2	50.12 ± 1.16	54.57 ± 0.32	55.25 ± 0.94
	average	48.92 ± 1.55	48.47 ± 6.72	52.02 ± 3.59

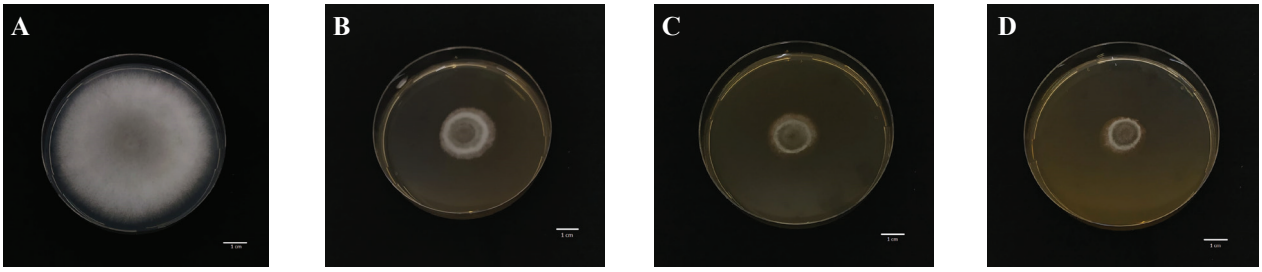

Figure 1. Results of the antibiosis assay of SM14 isolate in PDA filtrate medium. **A.** control, **B.** 6 days, **C**. 9 days, **D**. 12 days.

above shows the antibiosis assay results for SM14 isolate against *C. siamense* KA.

The results of the antibiosis agar assay, illustrated in Fig. 2, revealed that the inclination of percentage inhibition of batches 1 and 2 was consistent in all isolates. The percentage inhibition of SM14 and SM15 tended to increase, while SM11 tended to decrease along with incubation time. The percentage inhibition of both SM11 and SM14 was higher in batch 1, whereas that of SM15 was higher in batch 2.

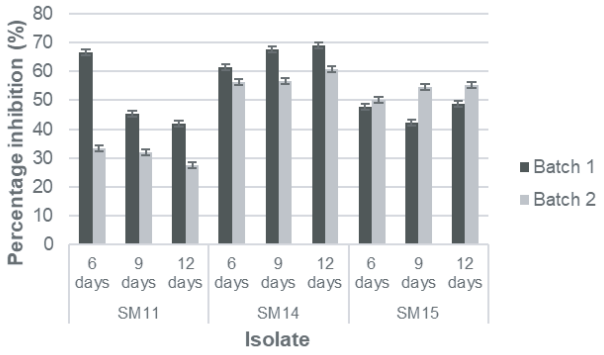

Figure 2. Graph of the antibiosis assay of the three marine actinomycetes isolates.

Characterization of marine actinomycetes isolates

The macroscopic characters of three marine actinomycetes isolates in ISP media are presented in Table 2. All of the isolates were circular in shape with an entire to undulate margin, and flat to raise elevation with a chalky texture on the surface of the colonies in various media. The soluble pigment was only observed in SM11 in ISP medium 2. Microscopic observation showed that all of the isolates produced branching substrate mycelia and or aerial mycelia. The spore chains of all isolates were rectiflexibilis which formed in aerial mycelia. The results of the biochemical characterization showed that all isolates demonstrated catalase, oxidase, and various extracellular enzyme activity, i.e. protease, amylase, lipase, and chitinase. However, only SM15 demonstrated gelatinase activity. Moreover, the isolates can utilize all the carbon sources i.e. glucose, galactose, fructose, sucrose, arabinose, xylose, mannitol, inositol, rhamnose, and raffinose.

Table 2. Macroscopic characters observation of marine actinomycetes isolates.

Characters	SM11	SM14	SM15
ISP medium 2			
Aerial mycelia	Cold grey I	Cold grey I	White
Substrate mycelia	White	White	White
Soluble pigment	Terracotta	None	None
Exudate drops	Present	Absent	Absent
ISP medium 3			
Aerial mycelia	Cold grey II	Cold grey I	Warm grey II
Substrate mycelia	White	White	White
Soluble pigment	None	None	None
Exudate drops	Present	Present	Absent
ISP medium 4			
Aerial mycelia	Warm grey II	Warm grey I	Cold grey I
Substrate mycelia	White	White	White
Soluble pigment	None	None	None
Exudate drops	Absent	Present	Absent

Molecular identification

The molecular identification based on the 16S rRNA gene sequences revealed that the three marine actinomycetes were closely related to *Streptomyces sanyensis*. The sequences of the isolates have 99.66% similarities with *S. sanyensis* 219820^T. The phylogenetic analyses of all isolates are presented in Fig. 3.

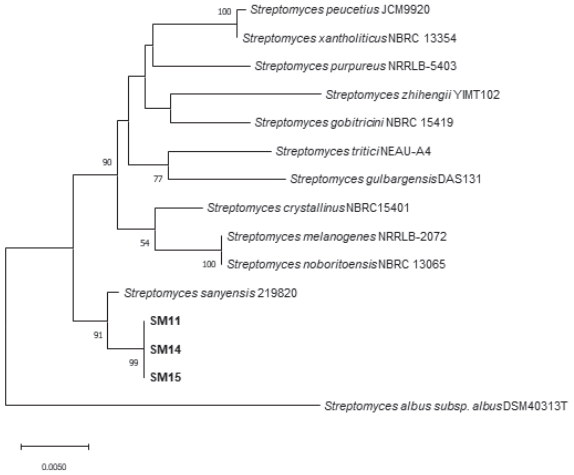

Figure 3. Phylogenetic analysis of marine actinomycetes isolates. The neighbor-joining tree of the three marine actinomycetes isolates (SM11, SM14, and SM15) was based on the 16S rRNA gene sequences. *Streptomyces albus subsp. albus* DSM 40313^T is represented as an outgroup. The bootstrap values, based on 1,000 replications, are shown at the nodes; only values above 50% are given. The scale bar indicates 0.0050 substitutions per nucleotide position.

Discussion

In this study, the result of antibiosis assays showed that all marine actinomycetes isolates could inhibit the growth of phytopathogenic fungi *C. siamense* KA (Table 1). Belonging to actinomycetes, the genus *Streptomyces* can control plant pathogens through various mechanisms, such as the production of antimicrobial and other metabolite compounds (Olanrewaju and Babalola 2019). The percentage inhibition of all isolates in antibiosis assay was lower than obtained in our previous study of antagonist assay using plug technique, for example, SM14 at 12 days was only 64.90% in antibiosis assay, whereas in antagonist assay was 82.34% (Fadhilah et al. 2021). This phenomenon may be due to the inhibition in antibiosis assays is represented

solely by the antifungal compound activity itself. Meanwhile, concerning the antagonism assay, it was assumed that the inhibition was caused not only by antifungal compounds but also by direct interspecific competition with the involved species for food and space (Fadhilah et al. 2021). As a biocontrol agent, *Streptomyces* has been reported to have various antagonistic mechanisms, including competition for nutrients and space and antibiosis, as well as parasitism (Law et al. 2017).

The fermentation periods for the antifungal compounds produced from the isolates lasted 6, 9, and 12 days based on our previous study. It was assumed that the marine actinomycetes isolates had been in a stationary growth phase (Fadhilah et al. 2021). Bioactive compounds such as antibiotics are synthesized during stationary growth phases when the nutrients in the medium are depleted (Fedorenko et al. 2015). The fermentation for producing bioactive compounds is affected by microorganisms, substrates, and processes (Pham et al. 2019). In our study, the substrate and process of fermentation relatively could be controlled, except for the microorganism, related to the genetic stability of the microorganism. The genetic instability probably occurred in SM11 which showed a significant difference in percentage inhibition between batch 1 and 2. The genus *Streptomyces* is, for the most part, genetically unstable, with more than 0.1% of colony-forming spores. This genetic instability affects different phenotypic characteristics, such as the morphological structure differentiation and production of secondary metabolites, including antibiotic compounds (Volff and Altenbuchner 1998; Peng and Liang 2020). The genetic instability of *Streptomyces* will affect the production of spores resulting in a "bald colony". The bald colonies of *Streptomyces* will form aerial hyphae but do not sporulate efficiently (Chandra and Chater 2014). In our antibiosis assay, probably the condition of the spore inoculum in batch 2 decreased compared to batch 1 due to the formation of bald colonies, which in turn will affect its production of antifungal compounds. The decrease in percentage inhibition in batch 2 may also have been caused by a decrease of antifungal production as a result of genetic instability.

Among the 3 isolates, the percentage inhibition values of SM14 and SM15 increased in parallel with the incubation time of fermentation, indicating an accumulation of antifungal compounds in the medium during 12 days of fermentation. However, SM11 exhibited a different curve, achieving optimal percentage inhibition at 6 days of fermentation. This is probably due to the degradation of the antifungal compounds. The actinomycetes produce bioactive compounds during a stationary phase. However, the efficiency of the antimicrobial compounds they produce could decrease after a long incubation period, due to the degradation (Sengupta et al. 2015). The antimicrobial compound as a secondary metabolite might be degraded due to being re-utilized or converted into other compounds by the bacteria (Horak et al. 2019).

The molecular identification of the marine actinomycetes isolates was done using 16S rRNA gene sequences because it conserves sequences and is commonly used to identify *Streptomyces* (Bhattacharjee 2012). The results showed that all of the isolates were closely related to *S. sanyensis* 219820^T, with 99.66% identical sequences (Figure 3). According to Woo et al. (2008), molecular identification using 16S rRNA is accurate if the species of identified bacteria has >99.5% similarities. Furthermore, the percentage similarity of *Streptomyces* for the identification of species is ≥99%, whereas a percentage similarity between 95–99% means that only its genus can be assigned (Sing et al. 2009). The novel *S. sanyensis* 219820^T was isolated by Sui et al. (2011) from mangrove sediment in Sanya, Hainan, China; meanwhile, we obtained our isolates from mangrove leaf litter samples from Pramuka Island, Jakarta, Indonesia (Fadhilah et al. 2018). In terms of bioactive compounds, *S. sanyensis* 219820^T has been reported to produce compounds that are active against the human colon tumor cell line HCT-116 (Hong et al. 2009).

Supporting the description of Sui et al. (2011), the macroscopic and microscopic characteristics of the three isolates were for the most part similar to the species *S. sanyensis* 219820^T. *Streptomyces sanyensis* 219820^T had grey aerial spores on ISP medium 2, 3, and 4, and formed branching substrate mycelia and aerial mycelia with rectiflexibilis spore chains. The spore chain structure indicates that the spore was straight to flexible (Barka et al. 2016). *Streptomyces sanyensis* 219820^T was positive for gelatin liquefaction, as well as the SM15 isolate. Nevertheless, *S. sanyensis* 219820^T did not produce diffusible pigment, unlike SM11 isolate, which produced terracotta pigment. The pigment production of actinomycetes is influenced by many factors, such as the pH and aeration of the medium, particularly the carbon and nitrogen sources it contained (Kheiralla 2016). Other different characteristics between the three isolates and *S. sanyensis* 219820^T were H_2S production and carbon utilization (specifically, arabinose, fructose, galactose, inositol, mannitol, sucrose, raffinose, rhamnose, and xylose). The differences of those characters might be due to alteration of genotype that reflects variation in strain level (Rosum et al. 2020). These give us the challenge to explore the three actinomycetes isolates in terms of antifungal compound discovery even though they belong to the same species.

In conclusion, all three isolates demonstrated antifungal activity against fungal phytopathogen *C. siamense* KA. The strongest potential isolate was the SM14 isolate, which inhibited the growth of *C. siamense* KA up to 64.90%. Twelve days of incubation time produced the optimal percentage inhibition. The three marine actinomycetes isolates were *S. sanyensis* as determined by molecular identification. The differences in morphological and biochemical characteristics indicated that all isolates were a different strain of *S. sanyensis*. This challenges further research on identifying and analyzing the antifungal compounds produced by the three marine actinomycetes isolates.

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Conflicts of interest

The authors have no financial conflicts of interest to declare.

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