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SCREENING OF INDIGENOUS MYXOBACTERIA ISOLATED FROM SOIL AND DECAYING WOOD IN TOBA-SAMOSIR, NORTH SUMATERA AS ANTIMICROBIAL PRODUCER

[*PENAPISAN Myxobacteria PRIBUMI YANG DIISOLASI DARI SAMPEL TANAH DAN KAYU LAPUK DI TOBA-SAMOSIR, SUMATRA UTARA SEBAGAI PENGHASIL ANTIMIKROB*]

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ABSTRACT

Gram-negative myxobacteria is a member of Deltaproteobacteria with some unique characteristics, include moving by gliding motility, forming fruiting bodies when starving, and preying on other microorganisms. They are known to produce some of the potential and novel antimicrobial compounds. Therefore, in this study, we aim to isolate and screen indigenous myxobacteria as an attempt to bioprospect their potential as antimicrobial substances producer. Samples consist of soils, plant rhizospheres, and decaying woods collected from Toba -Samosir, North Sumatera were subjected to isolation procedure using baiting and filter membrane methods. A total of 23 isolates visually displaying myxobacteria features were selected and molecularly identified. Analysis on their 16S rRNA gene sequences resulted in grouping them in the genus *Corallococcus*, *Cystobacter*, and *Myxococcus*. Antimicrobial assay using the agar plug diffusion method revealed that they were able to inhibit the growth of *Candida albicans* among other microorganisms tested in this study. Some of the highest inhibition zones were produced by *Cystobacter velatus* isolates, hence indicating their potential as anti-Candida compound producer.

Key words: Antimicrobe**,** *Corallococcus*, *Cystobacter*, *Myxococcus, Candida,* Toba

ABSTRAK

Bakteri Gram-negatif, myxobacteria merupakan anggota Deltaproteobacteria yang memiliki beberapa karakter unik, diantaranya bergerak dengan motilitas meluncur, membentuk badan buah saat kelaparan, dan memangsa mikroorganisme lainnya. Bakteri tersebut diketahui menghasilkan sejumlah senyawa antimikrob yang potensial dan baru. Oleh karena itu, penelitian ini bertujuan mengisolasi dan menapis myxobacteria pribumi sebagai upaya bioprospeksi potensi myxobacteria tersebut sebagai penghasil zat antimikrob. Terhadap sampel yang terdiri dari tanah, rizosfer tanaman, dan kayu lapuk dilakukan prosedur isolasi menggunakan metode umpan dan membran filter. Total sebanyak 23 isolat yang secara visual memperlihatkan sifat myxobacteria diseleksi dan diidentifikasi secara molekuler. Analisis terhadap sekuen gen 16S rRNA mengelompokkan isolat-isolat tersebut ke dalam genus *Corallococcus*, *Cystobacter*, dan *Myxococcus*. Uji antimikrob menggunakan metode difusi *plug* agar-agar menunjukkan bahwa isolat tersebut mampu menghambat pertumbuhan *Candida albicans* diantara mikroorganisme lain yang diuji pada penelitian ini, sehingga mengindikasikan potensinya sebagai penghasil senyawa anti-Candida.

Kata kunci: Antimikrob**,** *Corallococcus*, *Cystobacter*, *Myxococcus, Candida,* Toba

INTRODUCTION

Antimicrobial resistance is increasing rapidly and has become a global concern. Novel antimicrobials are needed to replace ineffective antibiotics. Antibiotics can either be obtained from natural compound, semi-synthetic, or synthetic, and about two-third of these antibiotics are generated from microorganisms (Pancu *et al*., 2021). Drugs compound from microorganisms is regarded as the promising source to control antimicrobial-resistant pathogens. Myxobacteria can produce biologically active compounds important for human health and antibiotics. These bacteria belong to the order Myxococcales within the class Deltaproteobacteria group. Krug et al. (Krug *et al.,* 2008) reported that *Myxococcus xanthus* produced 37 metabolite profiles as novel compound candidates promising in the future. Krop et al. (Korp *et al*., 2016) also stated that there are 100 core structures and 500 derivatives of novel antibiotics produced by myxobacteria. In their study, they also revealed that genome sequences from 6 strains of myxobacteria which belong to *Myxococcus*, *Corallococcus*, *Sorangium* species, carried a specific sequence for drug compound biosynthesis. Through the molecular approach, about 24 gene clusters from the genome sequence of *Myxococcus xanthus* showed a sequence for secondary metabolite biosynthesis (Weber *et al*., 2015). Antifungal compound identified as haliangicin from

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myxobacterium was reported by Kundim et al in 2003 (Kundim *et al*., 2003).

Myxobacteria are Gram-negative bacteria and ubiquitous in nature, mainly in plant-enriched soil (Saadatpour and Mohammadipanah, 2020). They also live in the wood, decaying plants, dried trees, marine water, and the fruiting bodies of Basidiomycetes (Zagriadskaia *et al.,* 2013). They are distributed all over the world (Mohr, 2018). Myxobacteria are unique and distinguished from other Gram-negative bacteria by their gliding motility, advanced intercellular communication systems, as well as uncommon development biological process (Wrótniak-Drzewiecka *et al*., 2016). Naturally, they live on nutritional availability, fully consuming other bacteria or becoming saprophytes when the prey targets are lacking. In starvation conditions, they form fruiting bodies. This uniqueness is presumed to be involved in the synthesis of secondary metabolites (Pasternak *et al.,* 2013). Xiao et al. (Xiao *et al.,* 2011) reported that predatory behaviors of myxobacteria have a linkage with antibiotic synthesis. Some of the antibiotics produced by Myxobacteria including aurachins synthesized by *Stigmatella aurantiaca* strain a15, corallopyronins and myxopyronins, ripostatins and etnangien from *Sorangium cellulosum*, sorangicins from the genus *Sorangium*, Angiolam A from *Angiococcus disciformis,* Myxovalargins from *Myxococcus fulvus*, Carolacton and Myxovirescins, *M. virescens* (Schäberle *et al.,* 2014).

The biodiversity of myxobacteria is still poorly understood, particularly indigenous myxobacteria despite their potential antimicrobial compounds. The difficulty in the isolation and purification procedure might cause these bacteria to be underexplored. As much as 1 g of soil was estimated to have 10^6 or less and contained at least 5–10 myxobacterial species. (Vos and Velicer, 2008) successfully isolated *M. xanthus* from Sulawesi, Indonesia. Meliah and Lisdiyanti (Meliah and Lisdiyanti, 2018) also announced that the members of myxobacteria group, *M. fulvus, M. stipitatus,* and *Melittangium lichenicola* as the principal record in Indonesia. Furthermore, the DNA of Myxococcales group (*Sorangium* and *Haliangium*) was reported in the soil samples from Sumatera, Indonesia (Schneider *et al*., 2015). Those reports showed that the opportunity to explore myxobacteria diversity is promising. Therefore, we investigated the diversity of indigenous myxobacteria from soil and decaying wood as well as initial bioprospecting for their antimicrobial production.

MATERIALS AND METHODS

Samples

The samples, which consist of 9 decayed woods and 20 soils (including soil in rhizosphere) were collected from Taman Eden 100 and Arboretum Samosir in March 2018. Both places are ecotourism park that located in North Sumatra, Indonesia. The soil samples used to isolate myxobacteria were subjected to pre-treatment with the air-drying method overnight.

Microbial strains

Some microbial strains were used in this study. *Escherichia coli* InaCC B5 was used as bacterial prey to isolate myxobacteria. Gram-positive *Bacillus subtilis* InaCC B1, Gram-negative *Pseudomonas aeruginosa* InaCC B3, and yeast *Candida albicans* InaCC Y116 were used in a screening assay for antimicrobial activity.

Isolation and purification of myxobacteria

Isolation of myxobacteria conducted in two types of isolation media consists of Stan 21 Agar and Water Agar cross-streaked with living *E. coli* InaCC B5. Standard methods to isolate and purify myxobacteria, as described by (Reichenbach and Dworkin, 1992), were employed in this study. A small amount of soil and decaying wood samples were put onto a piece of Whatman No. 1 membrane on Stan 21 Agar medium and incubated at 30^0C for 2–8 weeks. Soil samples were also inoculated onto Water Agar medium at each tip of *E. coli* cross and then incubated at 30^0C for eight weeks.

Purification was performed by transferring formed fruiting bodies from both isolation media to VY/2 Agar medium supplemented by cycloheximide 25 µg/mL using a sterilized syringe needle under dissecting microscope. Most of the fruiting bodies can be observed clearly in 10 times magnification and more. The transferring process was repeated until a pure bacterial colony was obtained. All the purified isolates were preserved in glycerol 10% solution and store at -80 ^{0}C for further studies.

Identification of myxobacteria

Bacterial cells were transferred to 0.2 mL tube containing 20 µL of nuclease-free water and then boiled at 98^{o}C for 10 minutes to extract the genomic DNA. Cells debris was eliminated by spinning down the suspension. The genomic DNA in the upper phase was treated as a template to amplify the 16S rRNA gene using a universal bacterial primer set; 27F (5'- AGAGTTT-GATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (Lane, 1991). The polymerase chain reaction (PCR) program

includes pre denaturation at 94°C for 90 seconds, followed by 35 cycles of denaturing at 94°C, 30 seconds; annealing at 50°C, 30 seconds; elongation at 72°C, 90 seconds; and final extension at 72°C, 10 minutes. PCR products were separated on gel electrophoresis and visualized under ultraviolet light after staining them using ethidium bromide.

Good quality of PCR product confirmed by their visual was subjected to sequencing process. All the 16S rRNA gene sequences obtained were analyzed using BioEdit (Hall, 1999) and then aligned against other bacterial sequences in EzBioCloud server (https://www.ezbiocloud.net) (Yoon *et al.,* 2017). Phylogenetic analysis was performed using MEGA X program (Kumar *et al.,* 2018). Method and model applied to construct phylogenetic tree were neighbor-joining (Saitou and Nei, 1987) and Kimura 2-parameter (Kimura, 1980), respectively. Bootstrap value was generated based on 1000 replication. A member of *Firmicutes* phylum *Bacillus subtilis* NCIB 3610 (ABQL01000001) was used as an outgroup.

Screening for myxobacteria with antimicrobial activity

Antimicrobial assay was conducted using agar plug diffusion (Balouiri *et al*., 2016). Targeted microbes were inoculated on double-layer media. As many as 10^6 CFU/mL of bacterial cells were inoculated in semisolid Mueller Hinton Agar (MHA, Merck) and pour onto solid MHA treated as bottom layer. Meanwhile, 10^5 CFU/mL of yeast cells were inoculated in semisolid Sabouraud Dextrose Agar (SDA, Oxoid) and pour onto solid SDA also treated as bottom layer.

Myxobacterial isolates were inoculated on VY/2 Agar supplemented by cycloheximide 25 µg/mL for about three weeks. The fully grown isolates were cut using a sterilized straw. The agar plug containing myxobacterial cells was put on the bioassay medium prepared before. They were incubated at 4°C for an hour in order to let any chemical compounds present in agar plug diffuse better into the bioassay plate. The plates were transferred to incubator 30°C for further incubation that took 2 days in total. This procedure was performed in three replicates.

RESULTS

Bacterial colonies producing orange to brownish fruiting bodies were successfully collected from soil and decaying wood samples. They formed a flame-shaped colony with solitaire or clustered fruiting bodies. Some colonies produced vein-like structure (Figure 1D). As many as 23 isolates were selected for further analysis. The number of isolates recovered from open soil, plant rhizosphere, and decaying wood is 18, 3, and 2 isolates, respectively. Ninety percent of bacterial isolates from soil samples were isolated using baiting method with *E. coli* (Table 1).

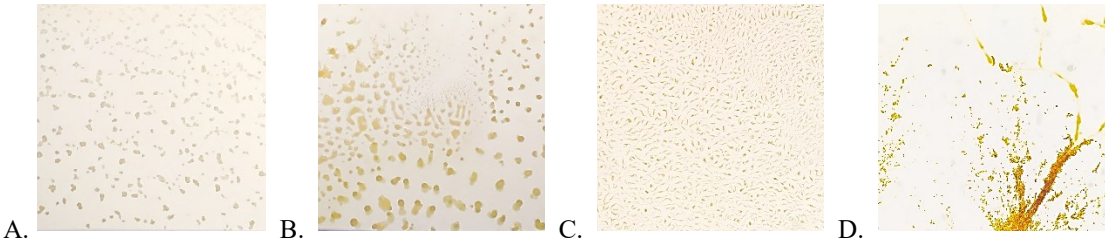


Figure 1. Morphological appearance of the fruiting bodies formed by bacterial isolates as observed using dissecting microscope; (A) DT06-TB6, (B) DT16-TS2, (C) TE29-TS1 and (D) TE09-TB4. The isolates were incubated at 30°C for three weeks. *(Penampilan morfologi badan buah yang dibentuk oleh isolat seperti yang teramati melalui mikroskop stereo; (A) DT06-TB6, (B) DT16-TS2, (C) TE29-TS1 dan (D) TE09-TB4. Isolat-isolat tersebut diinkubasi pada suhu 30°C selama tiga minggu*

Table 1. The number of isolates collected from soil and decaying wood in Toba-Samosir, North Sumatera. *(Jumlah isolat yang dikoleksi dari tanah dan kayu lapuk di Toba-Samosir, Sumatera Utara)*

Isolate *(Memisahkan)*	Sample origin *(Sampel Asal)*		Isolation methods *(Metode Isolasi)*
DT16-TS2	Arboretum, Samosir; 1483 masl	Soil	Membrane cellulose
DT05-KS1	Arboretum, Samosir; 1433 masl	Decaying wood	Membrane cellulose
DT16-KS1	Arboretum, Samosir; 1483 masl	Decaying wood	Membrane cellulose
DT06-TB2, DT06-TB3, DT06-TB4, DT06-TB5, DT06-TB6, DT06-TB7, DT06-TB8, DT06-TB10, DT06-TB11	Arboretum, Samosir; 1433 masl	Soil	Baiting with *E.coli*
TE29-TS1	Taman Eden 100; 1253 masl	Rhizosphere *Styrax* sp.; Kemenyan)	Membrane cellulose
TE08-TB1, TE08-TB2, TE08-TB3, TE08-TB4, TE08-TB6, TE08-TB8	Taman Eden 100; 1250 masl	Soil	Baiting with *E.coli*
TE09-TB2, TE09-TB4	Taman Eden 100; 1260	Rhizosphere *Schima wallichii*; Simartolu	Baiting with *E.coli*
TE19-TB1, TE19-TB6	Taman Eden 100; 1241 masl	Soil	Baiting with *E.coli*

Analysis on 16S rRNA gene sequences of these 23 isolates revealed that they belong to three genera of myxobacteria. They are *Myxococcus*, *Corallococcus* and *Cystobacter*, the member of suborder Cystobacterineae. Nine isolates were closely related to *M. stipitatus* based on alignment using online server and placement in a phylogenetic tree (Figure 2). Two isolates were closely related to the other *Myxococcus* member which is *M. fulvus*. The percentage similarity of these bacteria to the next relative is more than 99%. As many as eight isolates were closely related to *Cc. exiguus* and four others were closely related to *Cb. velatus*. The length of the sequences aligned in this study range from 1234 to 1400 bp.

Based on antimicrobial assay against bacterial strains, these myxobacteria were unable to inhibit the growth of tested *P. aeruginosa* and *B. subtilis*. However, some of the tested myxobacteria showed the ability to inhibit *C. albicans* growing on SDA plates. Clear zones appeared around the agar plugs containing myxobacterial cells after several days of incubation. Isolates encoded by TE09-TB2, TE09-TB4, TE19-TB1 and TE19-TB6 produced the largest clear zones (Figure 3). Those isolates belong to *Cystobacter* group based on their 16S rRNA gene analysis

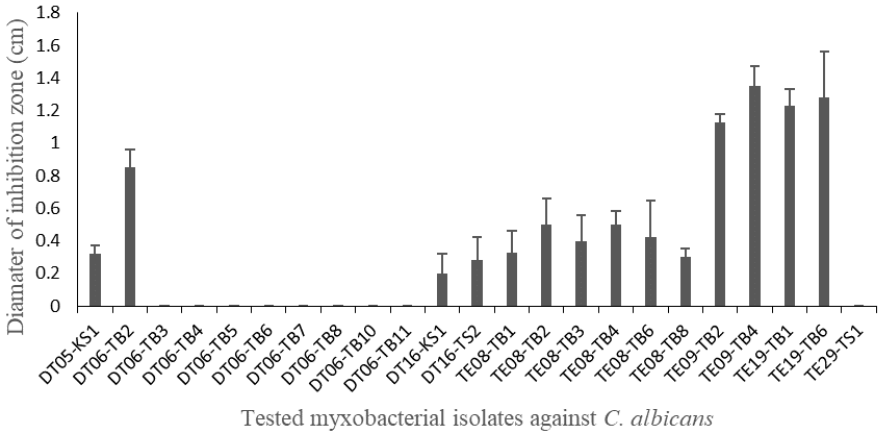


Figure 3. The ability of myxobacterial isolates in inhibiting tested yeast evaluated by the presence of clear zone. *(Kemampuan isolat-isolat myxobacteria dalam menghambat khamir uji yang dievaluasi berdasarkan keberadaan zona bening)*



Figure 2. Topology of the phylogenetic tree of isolated myxobacteria as constructed by neighbor-joining method and Kimura 2-parameter model. The bootstrap values were shown on each branch, and only those greater than 50% were displayed. *(Topologi pohon filogenetik myxobacteria terisolasi yang disusun berdasarkan metode neighbor-joining dan model Kimura 2-parameter. Nilai bootstrap ditunjukkan pada masing-masing cabang dan hanya nilai yang lebih tinggi dari 50% yang ditampilkan)*

DISCUSSION

The interest in searching for potential antibiotic-producing microorganisms exalts as the resistance cases arise. Myxobacteria have become one of the most sought-after microorganisms due to their enormous secondary metabolites. These secondary metabolites capable of inhibiting pathogens and tumor cells (Wang *et al.,* 2014). Myxobacteria can be found naturally in terrestrial samples, particularly in the soil, ranged from slightly acidic (Mohr *et al*., 2017), neutral (Rana *et al*., 2019) to alkaline (Zhang *et al*., 2013) soils. In this study, we collected 23 myxobacterial isolates from soil, plants rhizosphere and decayed wood in the highlands of Toba-Samosir, North Sumatera. They were mostly recovered from the water agar medium supplemented with *E. coli*. Only a small number of myxobacteria can be recovered from Stan 21 medium due to fungi on the membrane that overgrows targeted bacteria. Fungal spores and other fast growing microorganism cells from the samples can still be carried out by targeted bacteria during purification process. Therefore, the possibility of gaining a pure bacterial colony using series of direct purification decreased. Certain technique which includes freezing, heating, and multiple antibiotics can be used to purify myxobacteria. The additional of crystal violet in water agar medium used to purify myxobacteria was also reported capable of increasing the number of pure cultures obtained (Zhang *et al.,* 2003).

Morphologically, all the isolates which are obtained in this study displayed the characteristics of myxobacteria. They formed orange transparent film-like colonies that covered the medium as they grow or move across the medium. This movement on the solid surface is the typical gliding motility performed by myxobacteria (Mauriello *et al*., 2010). They also produced orange to brownish fruiting bodies after incubation for several days. The fruiting bodies of bacteriolytic myxobacteria may appear after 3–4 days, whereas the fruiting bodies of cellulolytic myxobacteria may appear after 7–21 days of the incubation period (Charousová *et al*., 2017). Some isolates showing brownish rough vein-like colonies, which were further identified as *Cystobacter velatus* based on their 16S rRNA gene analysis.

Using the same molecular analysis, the genus *Corallococcus* and *Myxococcus* were also identified among the purified isolates. Collectively, both genera make 83% of isolated myxobacteria. They were recovered from soil, plant rhizosphere, and decaying wood. Other studies also reported that the two genera are the most frequent myxobacteria isolated from the terrestrial samples using the standard isolation methods (Charousová *et al*., 2017; Gaspari *et al.,* 2005). However, *Myxococcus* can also be isolated from marine sample, as reported by (Brinkhoff *et al.,* 2012). This indicates their widespread distribution in nature.

All the three genera identified in this study were grouped in the suborder Cystobacterineae. The group members are mostly bacteriolytic myxobacteria with the ability to lyse other bacterial cells. Therefore, they are suggested to possess antimicrobial activity. Some of the antimicrobial compounds produced by these group of myxobacteria are corallopyronin, bithiazole, myxothioazol, and rhizopodin. Corallophyronin was reported to capable of blocking RNA polymerase of Gram-positive bacteria. This chemical compound is secreted by the member of genus *Corallococcu*s (Jansen *et al.*, 1985). Bithiazole and myxothioazol are secreted by *M. fulvus.* Bithiazole is known to inhibit C. albicans (Ahn *et al*. 2007), whereas myxothioazol is known to inhibit *B. subtilis* as well as *P. aeruginosa* (Schieferdecker *et al*., 2014). Rhizopodin is secreted by *M. stipitatus* and known to inhibit several filamentous fungi, including *Rhizopus orizae* (Sasse *et al*., 1993). The biosynthetic of secondary metabolites in myxobacteria mainly involves type I polyketide synthases (Li and Müller, 2009).

Based on the antimicrobial assay, 7 *Cc. exiguus*, 2 *M. fulvus*, 1 *M. stipitatus* and 4 *Cb. velatus* were able to inhibit the growth of *C. albicans.* All the four isolates of *Cb. velatus* showing higher inhibition zone on assay plate compared to the other tested myxobacteria. *Cc. exiguus, M. fulvus* and *M. stipitatus* were revealed to actively kill *C. albicans* during predation assay (Livingstone *et al*., 2017). Hence, it is expected that those bacteria possess biological activity against other microorganisms. In addition, we previously reported the ability of *Cc. exiguus* to inhibit the growth of *Fusarium* sp. which support their biological action against fungal group (Meliah *et al.*, 2020). However, we found so little information on *Cb. velatus* and their antimicrobial activity. Some members of the genus *Cystobacter* are known to produce antimicrobial compounds, such as cyrmenins (Sasse *et al.*, 2003) isolated from *Cb. armeniaca* and revealed to be an antifungal compound; cystodienoic acid (Raju *et al*., 2015) isolated from *Cb. ferrugineus*; althiomycin (Kunze *et al*., 1982) and myxalamide (Kundim *et al*., 2004) isolated from *Cb. fuscus*; and archazolid (Horstmann *et al*., 2011) isolated from *Cb. violaceus*. Therefore, this finding is important in providing initial data of *Cb. velatus* as potential antifungal compounds producer, particularly anti-Candida.

CONCLUSION

In this study, a total of 23 myxobacteria were successfully isolated from the samples using baiting and filter membrane methods. They are identified as *Corallococcus exiguus*, *Cystobacter velatus*, *Myxococcus fulvus*, and *Myxococcus stipitatus* based on 16S rRNA gene analysis. Fourteen myxobacterial isolates collected were able to inhibit *Candida albicans* with *Cystobacter* isolates showed the higher result. Therefore, this opens the possibility of exploiting their potential as anti-Candida compound producers.

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