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Screening of anti-*Candida albicans* metabolites produced by marine sponge-associated bacteria

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ABSTRACT

This study selected bacteria with high anti-*Candida albicans* (CA) activity among ten bacteria isolated from marine sponges. Bacteria were cultivated using the basal medium to produce the extract. Minimum Inhibitory Concentration (MIC) microdilution broth was used as an anti-CA assay followed by TLC-direct bioautography to characterize their active compound with spray reagents. The bacteria determination was done by molecular approaches using Repetitive-Element Sequences-based-PCR (rep-PCR) and amplification of 16S rDNA partial gene sequences, continued with BLAST analysis. The four out of ten tested bacteria had high anti-CA compounds and were potentially to be produced on a larger scale using the basal medium, which was BYT5C4, BYT5C5, BYT1A, and BYT7, with MIC of 1 mg/mL against 7.5×10^6 CFU/mL CA. TLC-bioautography test results showed that all metabolites from each isolate had different Rf and types of metabolites. Rep-PCR test showed that four bacteria had a low similarity index, indicating that they were different species. Based on molecular identification results, the BYT5C4, BYT5C5, BYT1A, and BYT7 isolates are strictly related to *Brevibacterium casei*, *Exiguobacterium profundum*, *Micrococcus lylae*, and *Bacillus firmus*, respectively. The active metabolites identified in this study can be isolated to determine the active molecules and their inhibitory routes to fungal growth. It is worth noting that additional research might be conducted to compare the activity of each antifungal metabolite to the synergistic activity of numerous antifungal metabolites detected in plant extracts.

1. Introduction

The oceans cover more than 70% of the earth's surface, and an estimated 99% of all marine bacteria have not yet been cultured. Therefore, many researchers tried hard to culture prospective marine bacteria because they found so many bioactive metabolites from marine bacteria. Since 2008, more than 1000 new chemical compounds have been discovered from marine organisms each year (Gerwick *et al.*, 2012; Solntsev *et al.*, 2019). Based on these reasons, great interest arises among scientists and biotechnology companies in the research and development of new medicines from the sea, especially from marine invertebrates in coral reef ecosystems (Dewi, *et al.*, 2008).

One of the most studied marine organisms, because of its diverse chemical contents and generally has a value in pharmacology, is a sponge. Sponges (Porifera) are a group of invertebrates, the oldest metazoans that have extraordinary abilities as living fossils (Hooper, 2002; Thakur and Müller, 2004). The high-pressure environment remains kept them alive. The mechanism that supports the life of sponges is their ability to symbiosis with the microbes in the sea. Sixty percent of the sponge's body mass is microbes. Sponges provided habitat for microbes in their body, and the microbes produced the variety of antimicrobial secondary metabolites and various enzymes that made sponge spared of competition, predation, or infection (Lee *et al.*, 2001; Thakur and Müller, 2004; Gandhimathi *et al.*, 2008; Dharmaraj and Sumantha, 2009). Intensive pressure during evolution makes the sponge take possession of strong chemical defense against competitors, which always threatens with overgrowth,

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toxicity, infection, or predation. Some evidence shows that many bioactive compounds found in sponges are synthesized by themselves or as symbiosis products with microorganisms with sponges (Faulkner *et al.*, 2000; Thakur and Müller, 2004).

Bacteria and fungi can develop a defense system toward some chemical compounds, make them resistant to many antibacterial or antifungal drugs (Center for Disease Control and Prevention, 2018). The broad-spectrum antifungal agents are essential in medicine nowadays. The fungal infection may be infecting the mouth, throat, skin, vagina, fingers, nails, trachea, lung, or gastrointestinal tract. Severe fungal infection can damage the immune system, causes invasive candidiasis includes blood-derived infections (candidemia), and deep-seated tissue infection. Invasive candidiasis can affect individuals with various, and, recently, 27–55% mortality has been reported due to invasive candidiasis (Nami *et al.*, 2019). Over the past two decades, the cases of fungal infection in immunosuppressive patients highly increase up to 400%. The elderly, and people with cancer or HIV are at a higher risk. It is caused by the formation of CA resistance toward some antifungal agents (Titmarsh, 2018; Alimuddin *et al.*, 2010; Lemriss *et al.*, 2003; Alexander and Perfect, 1997).

Murti *et al.* (2010) obtained sea sponges from Barang-Lompo Island waters by SCUBA Dived. The sponge was cut into 1×1 cm pieces and then soaked in the sodium perchlorate solution to remove bacteria outside the sponge. The sponge pieces are then rinsed clean cut into pieces, and grown in a SNA medium. Each type of bacteria that grows next was isolated and tested for antibacterial activity. Ten bacterial isolates found had high antibacterial activity as preliminary screening. This study intended to know their anti-CA activity through bacterial culture using the basal medium to determine their production potency on a larger scale. The extracts from each bacterial culture with potential anti-CA were then further characterized for the determination of its group metabolites. Bacteria produced extracts with potential anti-CA were further identified molecularly to determine its phylogenetic relationship.

2. Materials and Methods

2.1 Bacterial culture conditions and active metabolites extraction

The groups of bacteria were cultivated to obtain metabolites extracts. Each bacterial isolate (1.5×10^{12} CFU/mL) was grown in 250 mL of Marine Broth medium (40%) supplemented with starch 1% (as carbon source) and were incubated, with shaking, at room temperature for two weeks. After incubation of the inoculated broths, derived metabolites were extracted three times with ethyl acetate 1:1 (v/v). In the next step, the ethyl acetate solvent was evaporated using a rotary evaporator at 60 °C temperature at 115 rpm speed. Finally, the extracts were placed into a 1.5 mL microtube and weighed to determine the dry weight.

2.2 MIC Determination

Candida albicans (isolate number LIPIMC D382) used in this study was purchased from the Indonesian Research Institute (Bogor, Indonesia). Determination of minimum inhibitory concentration (MIC) was conducted using the microdilution broth method as described by Valgas *et al.* (2007) with minor modification. This analysis was performed to ascertain the lowest concentration of metabolites derived from bacterial isolates that are able to prevent the growth of *Candida albicans*. Initially, Mueller-Hinton (MH) broth was prepared with the addition of 1:10 DMSO (5%). The medium was used to make "two-fold" dilution series starting from 400 µg as the highest level up to 3.125 µg as the lowest and put them into the same column in the microplate contained 96 wells. The concentration of *Candida albicans* (CA) in each well was 7.5×10^6 CFU/mL. Amphotericin B (AMB) was used as a positive control, which was also made into two-fold dilution series, from 8 µg /well (32 µL) as the highest concentration. The wells contained antibiotic dilutions were filled with CA 7.5×10^2 CFU/mL. Subsequently, the microplate was placed in a shaking incubator for 24 hours. The growth of CA was observed after the addition of 30 µL MTT (5 mg/mL) into the microplate wells and incubated for approximately one hour. Column wells (vertical) that showed no color change into purple were interpreted as the borders of the MIC levels of each extract.

2.3 Thin layer chromatography-bioautography

TLC-bioautography was conducted according to the method described by Jacob and Walker (2005) with some modifications in extract levels spotted. TLC-bioautography was selected due to its rapid and efficient detection reaction between metabolites and *Candida albicans* (CA). Briefly, one microliter of extract solution (in methanol) was equivalent to 400 mg of each extract. The silica TLC plates (Silica gel 60 F 254, Merck) were developed using a solvent $CHCl_3$: MeOH in a ratio (96:4) with the addition of formic acid 25 mL per 5 mL of an eluent. Direct bioautography was performed by dipping the TLC plate in a medium containing CA inoculum at 1.5×10^4 CFU in 20 mL of MH broth medium for 15 seconds. The petri dishes were incubated in a chamber at 37 °C for 18-24 hours. The plates were dipped in a solution of 0.25% MTT for 15 seconds. The TLC plates were then put back into the sterile petri dishes containing wet cotton to keep the humidity and further incubated in a chamber at 37 °C for 24 hours. An active spot showing inhibition zones was visualized under UV light λ_{254} and λ_{366} nm and the *Rf* was calculated. The elution results obtained from the plates which were not dipped into the CA inoculum were used to determine the type of metabolites using several commercial spray reagents, including Dragendorff reagent, $FeCl_3$, anisaldehyde-sulfuric acid, ninhydrin, cerium sulfate, and 2.4-dinitrophenylhydrazine. The spot colors were observed and interpreted.

2.4 Molecular identification of bacterial isolates

Each microbial strain or isolate has a repetitive sequence located in a different area in its genome. The rep-PCR technique will produce several amplicons that differ in size and proportion of the distance between the locations of adjacent repetitive elements. The distribution of the amplicons will form a unique DNA genome fingerprint pattern for each microbial strain (Ishii and Sadowsky, 2009). This Rep-PCR test is intended to select bacterial similarity. If at least two bacteria are found with very high similarity using Rep-PCR then to reduce research costs, only one of them will be identified by 16S rRNA. However, if the similarity is low, all of the four bacteria will be tested for 16S rRNA amplification of 16S rRNA Partial Gene sequences

The genomic DNA of bacterial isolates was extracted using the Wizard DNA Extraction Kit (Promega) and was carried out based on the protocol from the manufacturer. Repetitive-Element Sequences-based-PCR (Rep-PCR) method was used to determine the genetic differences between isolates of bacteria. The bacterial genomic DNA was used as a template in a Rep-PCR amplification using BOXA1R primer (5′-CTACGGCAAGGCGACGCTGACG-3′) as described by Genersch and Otten (2003). Analysis of rep-PCR was performed in a microtube containing Go Taq Green Master mix (Promega). Microtube with 0.2 mL capacity was filled with 10 µL Go Taq Green PCR Mix, 6 µL of nuclease-free water, 2 µL of genomic DNA template, and 2 µL primer (20 pmol) in a total volume of 20 µL. The following PCR condition was predenaturation (95 °C, 15 min), 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 72 °C for 2.5 min, and final extension 72 °C for 10 min. The PCR product was visualized on 10% polyacrylamide gel electrophoresis (PAGE). The data obtained from PAGE were analyzed by using Unweight Pair Group with Mathematical Average (UPGMA).

The primer set for PCR amplification of 16S rRNA Partial Gene sequences were 27F (5'-AGAGTTTGATCCTGGCTC-3') and 1385R (5'-CGGTGTGTACAAGGCCC-3') (Hentschel *et al.*, 2001). PCR microtube containing PCR kit Go Taq Green Master Mix (Promega) 12.5 mL, Primer 27F 16S rRNA Gene 2.5 mL (0.5 mM) and the primer 16S rRNA Gene 1385R 2.5 mL (0.5 mM), 1-3 mL DNA template genome and Nuclease Free Water to a total volume 25 µL. The following PCR condition was performed by predenaturation (95 °C, 2 min), 30 cycles of 95 °C for 1 min, 57 °C for 1 min and 72 °C for 1.5 min, and final extension 72 °C for 5 min. The PCR products were showing the correct size of the amplicon were sent to the sequencing provider institution, 1st BASE (Malaysia). Sequencing data then compared with existing sequences deposited in the GenBank nucleotide database using BLAST (http://www.ncbi.nlm.gov/Blast). The phylogenetic tree was constructed using the MEGA 4.0 program.

3. Results

3.1 Anti-*Candida albicans* activity test by broth microdillution

As stated earlier, new compounds with antibacterial and antifungal activity were highly explored from nature by pharmacists due to the resistance of many commercial drugs. Based on the results of the broth microdilution test (shown in Table 1) in this study, all the extracts had anti-CA activities with different MIC ranging from 1 mg/mL to 4 mg/mL against CA levels of 7.5×10^6 CFU/mL. Previously, similar results were reported by Murti *et al.* (2010), in which the extracts had anti-CA activities with MIC values ranging from 0.11 to 11.60 mg/mL for CA (ATCC 10231) levels of 10^5 CFU/mL (Table 2). The antibiotic agent was not included in the experimental design of previous research.

Table 1. Minimum Inhibitory Concentration of each extract by broth microdilution test

No	Extract/Antibiotic	Minimum Inhibitory Concentration (mg/mL)	CA Concentration (CFU/mL)
1	BYT8C1	2	7.5×10^6
2	BYT8C2	2	7.5×10^6
3	BYT8C3	2	7.5×10^6
4	BYT5C3	>4	7.5×10^6
5	BYT5C4	1	7.5×10^6
6	BYT5C5	1	7.5×10^6
7	BYT7	1	7.5×10^6
8	BYT1A	1	7.5×10^6
9	BYB16	2	7.5×10^6
10	102TP	4	7.5×10^6
11	Amphotericin B	>0,08	7.5×10^2

Table 2. Minimum Inhibitory Concentration of each extract by broth microdilution test based on Murti *et al.* (2010)

No	Extract/Antibiotic	Minimum Inhibitory Concentration (mg/mL)	CA Concentration (CFU/mL)
1	BYT8C1	7,13	10^5
2	BYT8C2	11,6	10^5
3	BYT8C3	2,5	10^5
4	BYT5C3	0,11	10^5
5	BYT5C4	3	10^5
6	BYT5C5	6,4	10^5
7	BYT7	5	10^5
8	BYT1A	5,05	10^5
9	BYB16	2,38	10^5
10	102TP	3,6	10^5

Our study used Amphotericin B as a comparison to the commercial antifungal drug since the previous report conducted by Mohamadi (2014) showed that Amphotericin B effectively function as antifungal for 248 oral candidiasis and diaper dermatitis samples tested compared to nystatin, itraconazole, fluconazole, ketoconazole, clotrimazole, voriconazole, and posaconazole which showed various resistance. If we compared Table 1 (this research) and Table 2 (previous research), almost all MIC values of the extract from our study were lower than in the previous study, except the BYT5C3 extract, which showed a higher MIC value. However, it is still possible for BYT5C3 to produce active secondary metabolites as other potential drug candidates such as anti-parasitic, anti-viral, and antibacterial. Thus, further exploration researches are worth to be done.

The antifungal growth control of CA used in this study was Amphotericin B, which represented inhibition of CA activity at levels higher than 0.08 mg/mL against CA at level 7.5×10^{2} CFU/mL. The CA level used for the extract was 7.5×10^{6} CFU/mL, 10,000 times CA concentration higher than the antifungal drug standard representing the potential extract as novel anti-CA compound compared with Amphotericin B antifungal agents.

Based on the method recommended by CLSI, the dilution method was used to determine the sensitivity of the fungi against CA at levels of 1.5×10^{3} CFU/mL (CLSI, 2002). *Candida albicans* ATCC 90028 is considered for being sensitive if the MIC in a range of 0.5 to 2 mg/mL, while *Candida albicans* ATCC 24433 is considered for being sensitive at MIC range of 0.25 to 1 mg/mL (Pfaller *et al.*, 1995).

The extracts derived from ten bacterial isolates were then tested based on the highest inhibitory for CA growth ability, and four were selected (BYT5C4, BYT5C5, BYT1A, and BYT7) for the next analysis. They were further tested for direct bioautography analysis combined with thin layer chromatography followed by the characterization of its metabolites using spray reagents.

3.2 TLC-direct bioautography and metabolites characterization using spray reagents

The Bioautography test allowed the detection of active components in the crude extract. Meanwhile, the bioautography technique was used to determine the inhibition zone against the sample tested test that could visually be observed for finding the active compounds from an extract (Chomnawang *et al.*, 2009). As shown in Figure 1, four extracts exhibited spots of CA inhibition. Extracts BYT5C4 had two spots on *Rf* 0.30 and *Rf* 0.85, extract BYT5C5 had two spots on *Rf* 0.28 and *Rf* 0.83, extract BYT1A had four spots at *Rf*0.28, *Rf*0.63, *Rf*0,70 and *Rf*0.82 and extract BYT7 had three spots at *Rf*0.28, *Rf*0.60 and *Rf*0.78.

Both bioautography spots on BYT5C4 appeared as a dark spot under UV λ_{254} and emitted blue light under UV λ_{366} (Figure 1). Suppression of UV light at λ_{254} showed that the metabolites were able to absorb UV light at λ_{254} resulted in a dark spot with a bright green background (Waksmundzka-Hajnos *et al.*, 2008). Spot at *Rf* 0.3, showed red in anisaldehyde-sulfuric acid reagent, indicated that the active metabolites on the spot were terpenoids, while spot at *Rf* 0.85 showed red against anisaldehid-sulfuric acid, brown toward cerium sulfate reagent, orange toward Dragendorff and yellow toward 2,4 DNPH indicated that the active metabolites had terpenoids, aldehydes/ketones as well as alkaloids groups.

Bioautography analysis revealed that BYT5C5 extract produced two spots. Both bioautography spots were able to extinguish λ_{254} UV light and emit blue light under UV light λ_{366} (Figure 2). Spot at R*f* 0.28 showed a positive reaction using anisaldehyde-sulfuric acid reagent, Dragendorff, 2,4 DNPH, and ninhydrin. This result showed that the metabolites had a terpenoids, alkaloids as well as aldehyde/ketones groups. Meanwhile, the spot of R*f* 0.83 showed a positive reaction against anisaldehyde-sulfuric acid reagent, cerium sulfate and 2.4 DNPH represented terpenoid and aldehyde/ketones group metabolites.

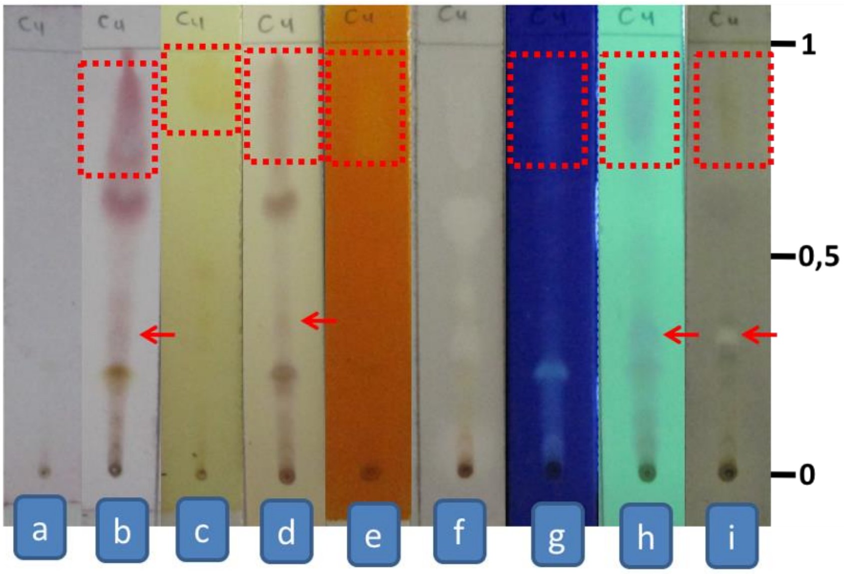


Figure 1. Visualization of bioautography results; UV irradiation and spraying TLC plates of BYT5C4 extract: a. Ninhydrin; b. Anisaldehid-Sulfuric Acid; c. 2.4 DNPH; d. Cerium Sulfate; e. Dragendorff; f. $FeCl_3$; g. λ254 UV; h. UV λ366; and i. Bioautography. Spots were indicated by red arrows and boxes.

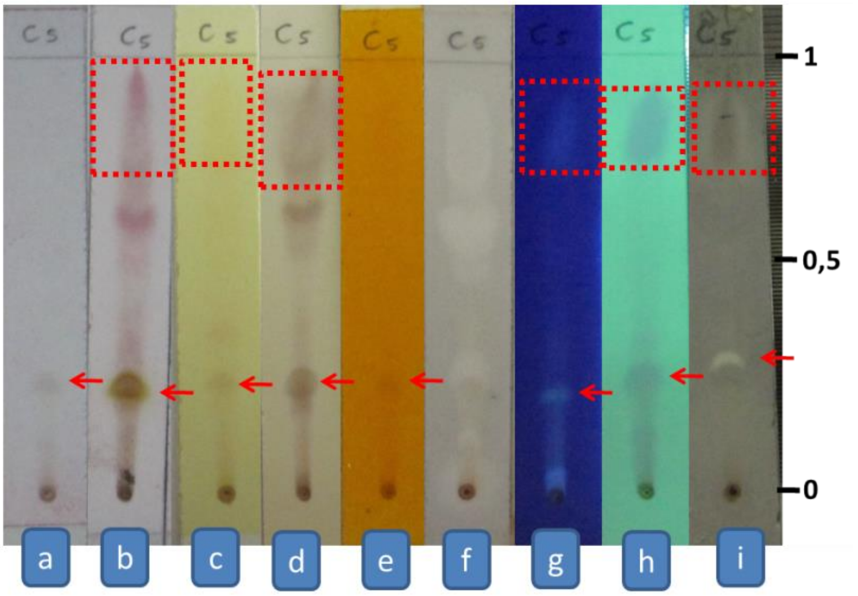


Figure 2. Visualization of bioautography results; UV irradiation and spraying TLC plates of BYT5C5 extract: a. Ninhydrin; b. Anisaldehid-Sulfuric Acid; c. 2.4 DNPH; d. Cerium Sulfate; e. Dragendorff; f. $FeCl_3$; g. λ254 UV; h. UV λ366; and i. Bioautography. Spots were indicated by red arrows and boxes.

Figure 3 showed that the metabolites extracted from BYT1A produced 4 spots of CA inhibition on *Rf* 0.28; 0.63; 0.70 and 0.83. However, not all spots showed extinguishing or emitting under UV light. *Rf* 0.28 and 0.63 were able to extinguish under λ_{254} UV light and emit the blue fluorescence under UV light λ_{366}, whereas the others were not able to leave a spot. *Rf* 0.28 and *Rf* 0.63 showed red spots against anisaldehyde-sulfuric acid reagent and brown spots on cerium sulfate, represented positive terpenoids content, whereas *Rf* 0.70 only had red spot against anisaldehyde-sulfuric acid represented terpenoids group. Spot *Rf* 0.82 showed a positive reaction to some reagents, anisaldehyde-sulfuric acid, cerium sulfate, and Dragendorff represented the existence of terpenoids and alkaloids.

BYT7 extracts showed CA inhibition on R*f* 0.28; 0.6 and 0.78 spots (Figure 4). Two of three spots showed the extinction of UV λ_{254} and emission under UV λ_{366}, i.e., Rf 0.28 and 0.78. The spot of R*f* 0.28 showed yellow on 2.4 DNPH sprayer indicating positive aldehyde/ketones groups. Spot R*f* 0.6 and 0.78 were suspected as terpenoid metabolites based on reaction against anisaldehyde-

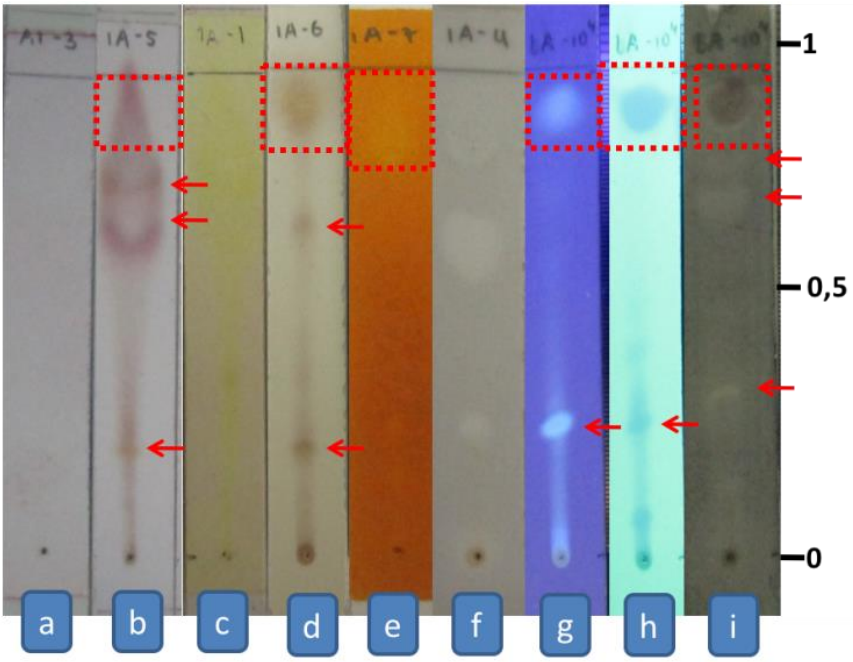


Figure 3. Visualization of bioautography results; UV irradiation and spraying TLC plates of BYT1A extract: a. Ninhydrin; b. Anisaldehid-Sulfuric Acid; c. 2.4 DNPH; d. Cerium Sulfate; e. Dragendorff; f. $FeCl_3$; g. λ254 UV; h. UV λ366; and i. Bioautography. Spots were indicated by red arrows and boxes.

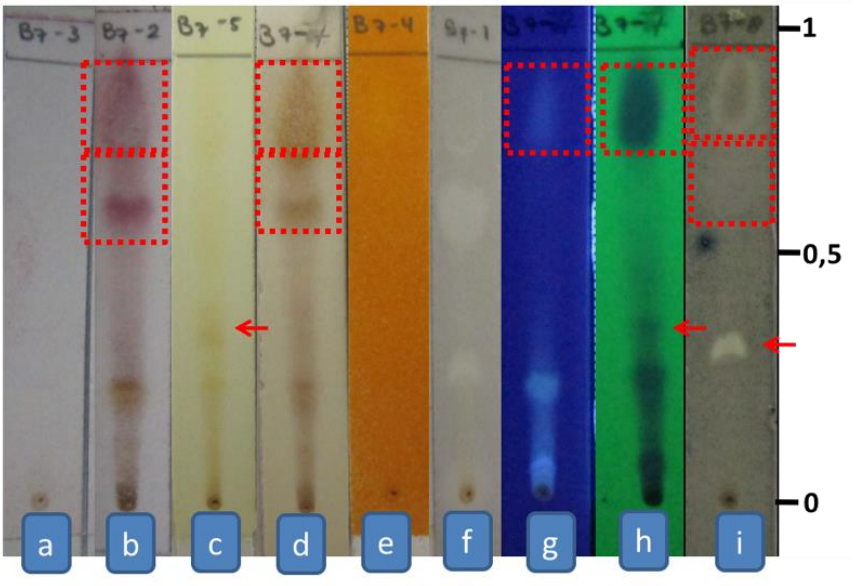


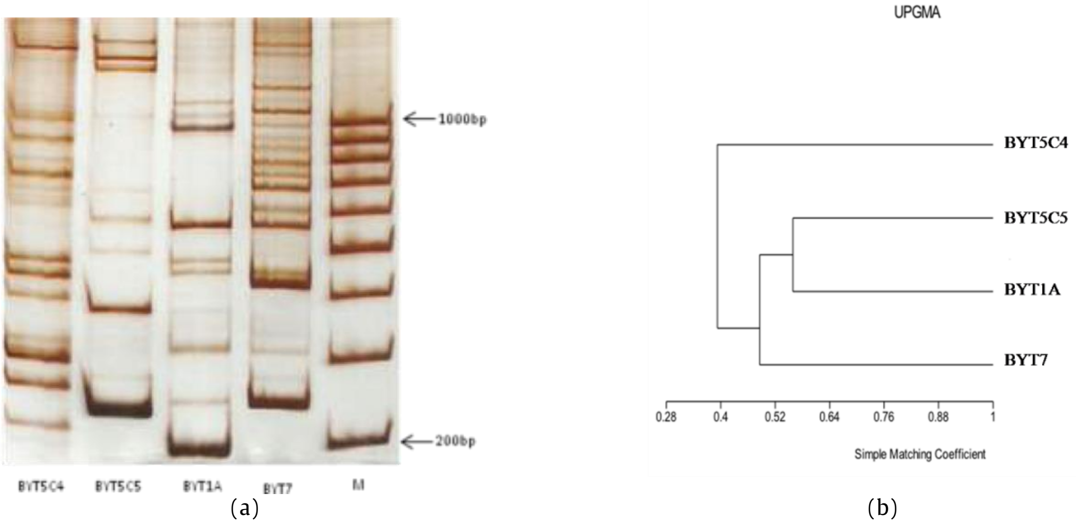
Figure 4. Visualization of bioautography results; UV irradiation and spraying TLC plates of BYT5C7 extract: a. Ninhydrin; b. Anisaldehid-Sulfuric Acid; c. 2.4 DNPH; d. Cerium Sulfate; e. Dragendorff; f. $FeCl_3$; g. λ 254 UV; h. UV λ 366; and i. Bioautography. Spots were indicated by red arrows and boxes.

sulfuric acid, which showed red and brown to cerium sulfate. The compilation result of metabolites characterization was illustrated in Table 3.

3.3 Molecular identification of selected bacterial isolates

In the first molecular assay, we used rep-PCR for DNA fingerprinting among selected bacteria. The repetitive element sequence-based PCR (rep-PCR) method can differentiate microbes by using primers complementary to interspersed repetitive consensus sequences that enable amplification of diverse-sized DNA fragments consisting of sequences between the repetitive elements. Multiple amplicons of different sizes can be fractioned by electrophoresis, and the resulting DNA fingerprint patterns, specific for individual bacterial clones, can be compared (Hiett & Seal, 2009). The rep-PCR result on polyacrylamide gel illustrated in Figure 5a.

Analysis of bacterial differences was performed based on UPGMA analysis (Unweighted Pair-Group Method Arithmetic Average) obtained from mapping DNA bands on PAGE (Poly-Acrylamide Gel Electrophoresis) from the results of DNA amplification using the rep-PCR method illustrated in Figure 5b. The four bacterial isolates were clustered into three groups. BYT5C5 and BYT1A were in the first group with a similarity index <64%. The second group was BYT7, with a similarity index of <54%, and the last group was BYT5C4 with a similarity index of 40%. All three groups revealed a low similarity index and indicated that all of the four bacteria were evolutionary originated from different species. To confirm in more detailed approach, thus the molecular approach was conducted for identification of each bacterial isolate using the 16S rDNA, and followed by BLAST analysis to determine the bacterial DNA homology with other bacterial DNA deposited in GenBank.



(a) (b)

Figure 5. Visualization of DNA bands amplified using rep-PCR with similarity index result. (a) the *results of rep*-PCR electrophoresis using PAGE, (b) dendrogram diversity of the four selected isolates bacteria based on UPGMA

Table 3. Compilation result of metabolites characterization using reagent spray

Metabolites extract	*Rf*	UV light λ254	UV light λ366	Group compound
BYT5C4	0.30	estinguish	emit	Terpenoids
	0.85	estinguish	emit	Terpenoids, Aldehides/Ketones & Alkaloids
BYT5C5	0.28	estinguish	emit	Terpenoids, Aldehides/Ketones & Alkaloids
	0.83	estinguish	emit	Terpenoids, Aldehides/Ketones
BYT1A	0.28	estinguish	emit	Terpenoids
	0.63	estinguish	emit	Terpenoids
	0.70	-	-	Terpenoids
	0.82	-	-	Terpenoids & Alkaloids
BYT7	0.28	estinguish	-	Aldehides/Ketones
	0.60	-	-	Terpenoids
	0.78	estinguish	emit	Terpenoids

The taxonomic identification of organisms is vital. As stated by Martin *et al.* (2006), taxonomic information on microbes producing secondary metabolites will be useful to know the biological character of the organism and able to examine the enzymes owned by the organism to produce a particular molecule. Taxonomic information can also provide information on the pathway of synthesis of secondary metabolites and genes that may play a role in it, thus opening up opportunities in the field of genetic engineering.

The test for using partial gene of 16S rDNA was performed based on the opinion of Gillis *et al.* (2005) in Bergey's Manual of Systematic Bacteria and Madigan *et al.* (2011) in Brock Biology of Microorganism, which stated that if the DNA sequence of the 16S rDNA gene in a bacteria had more than 97% similarity to a genus found in GenBank, it could be assumed that the bacteria is a strain of the genus members.

Based on the phylogenetic tree analysis, the BYT5C4 isolates bacteria is most closely related to *Brevibacterium casei*, *Brevibacterium celere,* and *Brevibacterium oceani* with similarity percentage sequences of 99%, 98%, and 98%, respectively (Figure 6). This bacterium was first isolated from livestock milk products by Collins *et al.* (1983). According to Kumar *et al.* (2011), *B.casei* is the most commonly reported opportunistic species as a clinical specimen. However, there is no report for *B.casei* for anti-CA activity until to date.

BLAST analysis of BYT5C5 bacteria showed that this isolate has closest to *Exiguobaterium profundum*, *Exiguobaterium aestuarii* and *Exiguobaterium marinum* by percentage similarity of 99%, 99%, and 98%, respectively

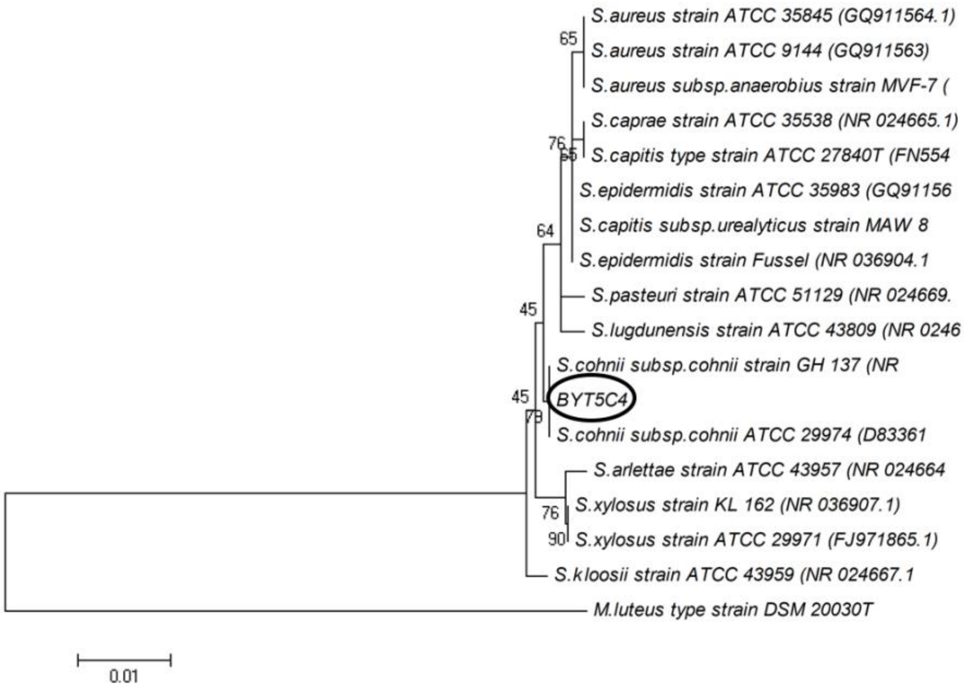


Figure 6. Brevibacterium phylogenetic dendrogram based on 16S rRNA partial gene. Branching pattern was obtained by neighbor-joining. *Staphylococcus aureus* subsp. *aureus* ATCC 12600 was used as outgroup. Numbers at branch nodes indicate bootstrap values based on 1000 resamplings. The scale bar represented 0.01 substitutions per nucleotide position (*K*nuc)

(Figure 7). Many Exiguobacterium reported originated from many water areas, especially the sea. The closest species to BYT5C5, namely *Exiguobacterium profundum*, was isolated from marine hydrothermal, facultative anaerobe, Gram-positive, colonies with a round shape diameter 1-2 mm orange, and able to grow in aerobic or anaerobic conditions (Crapart *et al.*, 2007). Other closest species are *Exiguobacterium marinum* and *Exiguobacterium estuarii*, which were isolated from the tidal regions of the Korean Yellow Sea. They have identical characteristics, short bacilliform at the beginning of the growth phase or coccus in the stationary phase, Gram variable, and anaerobic. Another characteristic of this bacteria is the rounded colony if grown over MA for two days, yellow to the deep orange aerobic atmosphere, and white in an anaerobic atmosphere (Kim *et al.*, 2005). Various Exiguobacterium isolates from different niches have been explored for biotechnological and industrial purposes, including enzyme production, bioremediation, and degradation of toxic substances released into the environment (Kasana & Pandey, 2017). However, research on anti-CA activity of the genus Exiguobacterium is not yet reported.

Phylogenetic tree analysis showed that BYT1A bacterial isolate is closely related to the species *Micrococcus lylae* with a sequences similarity percentage of 99% (Figure 8). Three species of Micrococcus members of bacteria producing secondary metabolites that are successfully isolated from sponge Callyspongia spp (Qian *et al.*, 2006). Some Micrococcus species also have antifungal activity against *Fusarium oxysporum*, *Pythium* group G, *Gelasinospora cerealis*, *Penicillium viridicatum*, *Trichoderma viride*, and *Zygorhynchus vuilleminii* (Kerr, 1999). Anti-CA activity studies have not been reported from the bacteria *Micrococcus lylae*.

BYT7 phylogenetic tree construction (Figure 9) is referred to the research conducted by Hestiyani, (2012), who tested the antimalarial activity of the BYT7 fermentation extract. As shown in Figure 9, the BYT7 bacteria has a close relationship with *Bacillus firmus, Bacillus infantis* dan *Bacillus thioparans* with sequences similarity percentages of 99%, 98%, and 97%, respectively. *Bacillus firmus* species were isolated by Loni and Bajekal (2011) from the Lake Lonar in India with characteristics of circular colony morphology, the elevation of raised colonies, opaque and creamy with positive motile, Gram-positive, possess catalase, oxidase, and chitinase enzymes. This enzyme is used in agriculture, food, and waste treatment. Kerr (1999) stated that the genus Bacillus produces various metabolites with antifungal activity, including rhizocticin A, a peptide that actively inhibits Candida spp., Aspergillus spp., and some phytopathogenic fungi. Bioactive compounds with antibacterial and antifungal activities from genus Bacillus are reported to be produced in the form of peptide molecules.

4. Discussions

The extracts derived from ten bacterial isolates tested can be considered to be a new potential candidate of the anti-CA agent, since the value of the MIC was smaller than the anti-CA commercial agent. The CA level used in this study was 5000 times higher than the CA level recommended by the CLSI (2002).

Based on the bioautography test, each of the four extracts had more than one spot of CA inhibition, which

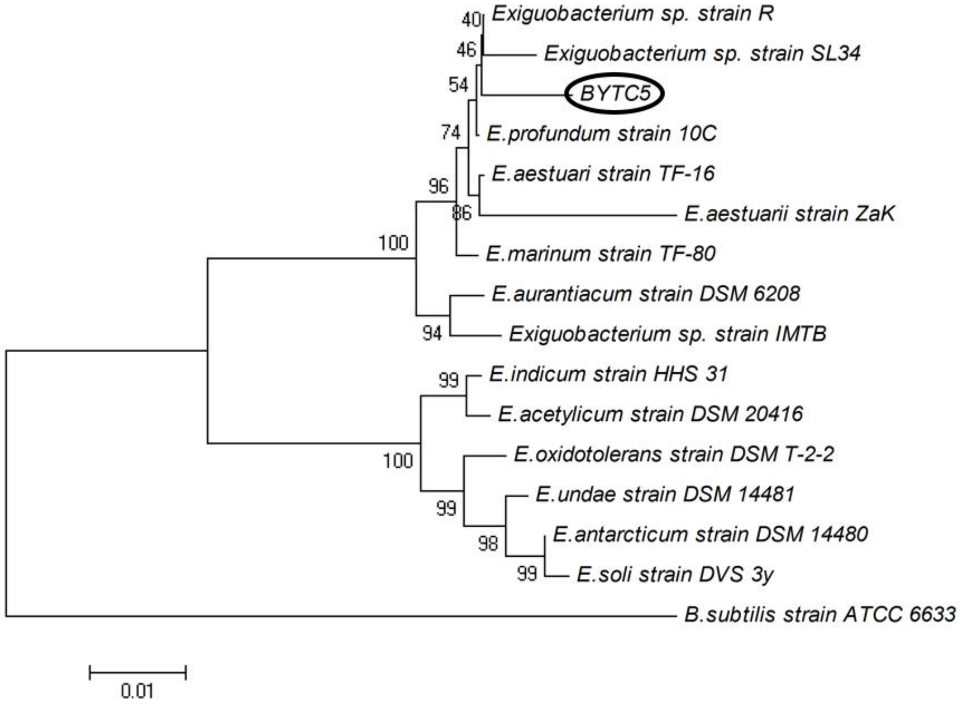


Figure 7. Exiguobacerium phylogenetic dendrogram based on 16S rRNA partial gene. Branching pattern was obtained by neighbor-joining. *Bacillus subtilis* ATCC 6633 was used as outgroup. Numbers at branch nodes indicate bootstrap values based on 1000 resamplings. The scale bar represented 0.01 substitutions per nucleotide position (*K*nuc)

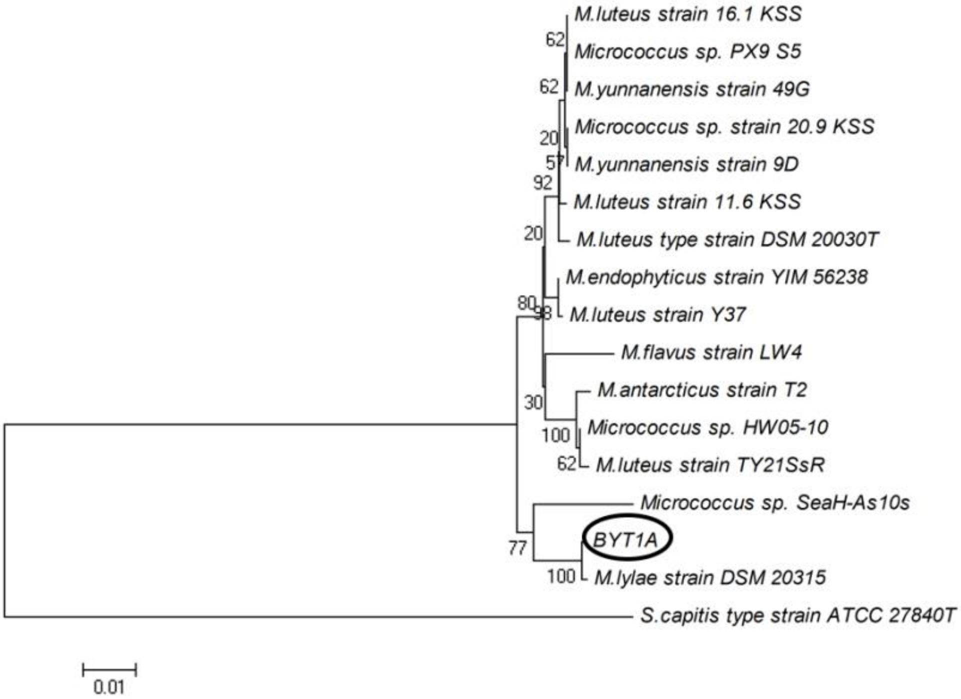


Figure 8. Micrococcus phylogenetic dendrogram based on 16S rRNA partial gene. Branching pattern was obtained by neighbor-joining. *Staphylococcus capitis* type strain ATCC 27840^T was used as outgroup. Numbers at branch nodes indicate bootstrap values based on 1000 resamplings. The scale bar represented 0.01 substitutions per nucleotide position (*K*nuc)

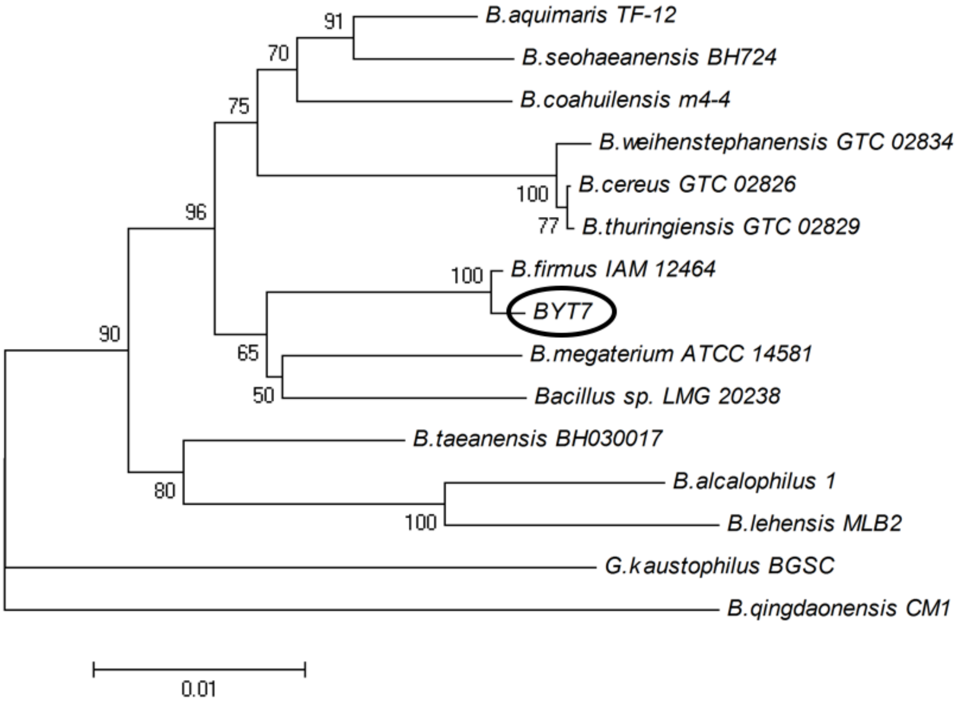


Figure 9. Bacillus phylogenetic dendrogram based on 16S rRNA partial gene. Branching pattern was obtained by neighbor-joining. *Geobacillus kaustophilus* BGSC was used as outgroup numbers at branch

means more than one metabolite has existed in every extract. Waksmundzka-Hajnos *et al.* (2008) classified secondary metabolites based on chemical structure, composition, and biosynthesis pathways into three main groups, i.e., terpenoids, phenolics, and nitrogen-containing metabolites. Active metabolites could contain multiple classes of metabolites at once because it came from the same main synthesis pathway.

Based on the results obtained from this study, it showed that each extract contains more than one metabolite with antifungal activity. Therefore, it is worth to notify that there is an opportunity for further research for testing the activity of each antifungal metabolite compared with the synergy activity of some antifungal metabolites-the synergy phenomenon of several antimicrobial compounds found in plant extracts. Inactive compounds in extracts can optimize the reaction rate and bioavailability of the active composition. On the other hand, the inactive compound can also be a medium for non-target microbial growth. Some active compositions can have a synergistic effect, which will be lost when separating constituents (Tadesse, 2010; Toroglu, 2011) as occurs with antimalarial extracts (Rasoanaivo *et al.*, 2011). The existence of a synergy effect of several compounds has been long applied in traditional Chinese medicine (Ulrich-Merzenich *et al.*, 2010).

From this study, we know that four out of ten bacteria isolated from sponges have the potential to produce new antifungal agents. Since there is no specific research on anti-CA activity or isolation of anti-CA metabolites from the four closest bacteria with our selected bacterial isolates, namely *Brevibacterium casei*, *Exiguobacterium profundum*, *Micrococcus lylae,* and *Bacillus firmus*. Therefore, the four bacteria that were grown on the basal medium were still able to produce high anti-CA metabolites showed the potential of bacteria for production on a larger scale and also for further research purposes, for example, anti-microbial, anti-viral, anti-parasitic, antioxidant, and anti-cancer.

In the recent study, antifungal treatments are the antibody-based mechanism by interrupting various supporting mechanisms which called immunotherapy strategy has cured various infectious diseases in example invasive fungal infection and also cancer treatment. Nami *et al.* (2019) report many inhibitory pathways against fungal growth. For example, immunotherapy using antibodies, immunotherapy using cytokine therapy-recombinant human IFN-γ, and immunotherapy using cell therapy. Antifungal compounds often damage healthy cells, leading to some side effects, such as fever, digestive, and liver disorders, which may less likely occur during immunotherapy. Immunotherapeutic side effects vary based on the type of immunotherapy. The side effects of immunotherapeutic have various diverse from minor symptoms of inflammation (e.g., fever) to critical conditions similar to autoimmune disorders. However, further research is needed to expand immunotherapeutic approaches for curing various infectious diseases. Related to this research, active metabolites in this study can be isolated to know the active compounds for further study to know their inhibitory pathways to fungal growth.

5. Conclusion

The screening of ten bacteria resulted in four selected bacteria, which produced higher anti-CA metabolites, BYT5C4, BYT5C5, BYT1A, and BYT7, with minimum inhibitory concentration (MIC) of 1 mg/mL against 7.5×10^6 CFU/mL. According to molecular identification, BYT5C4, BYT5C5, BYT1A, and BYT7 are closely related to *Brevibacterium casei, Exiguobacterium profundum, Micrococcus lylae, and Bacillus firmus*, respectively.

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Conflict of interest

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