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THE EFFECT OF MEDIA AND FERMENTATION CONDITION TOWARD ANTIBACTERIAL ACTIVITIES OF *BACILLUS* SP. DA11 ASSOCIATED WITH *SARCOPHYTON* SP.

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ABSTRACT: The emergence of antibiotic-multidrug resistant microorganisms is an essential issue in the health sector. Consequently, the exploration of new antimicrobial compounds from many promising resources is urgently required. Soft coral-associated bacteria are potential as antimicrobial compound producers. This study aims to get the best medium and fermentation conditions in the production of secondary metabolites having antibacterial activities and to identify antibacterial agents from a bacterium associated with soft coral. A soft coral sample was collected from Randayan Island, Indonesia, and was identified morphologically as *Sarcophyton* sp. One of the bacteria isolated from the *Sarcophyton* sp. showed antibacterial activities and identified as *Bacillus* sp. based on Bergey's Manual of Determinative Bacteriology. The best antimicrobial activities of *Bacillus* sp. DA11 were carried out on medium 1/10 Zobell supplemented with 1% of glucose (1/10ZG) which were able to against all of the test bacteria except on a test fungus, *C. albicans*. The extract was prepared on medium 1/10ZG with liquid state fermentation without shaking showed the best condition to produce secondary metabolites having antibacterial activities. The extract showed weak antibacterial activities based on minimum inhibitory concentrations (MICs) which were around 150-300 µg/disc for *A. hydrophila*; *Bacillus* sp., *C. freundii*, *Salmonella* sp., *P. aeruginosa*, *S. aureus*, *E. coli*, *V. cholerae*, *V. vara*, *B. subtilis*. The results of phytochemical analysis and thin layer chromatography (TLC)-bioautography showed that the secondary metabolite having antibacterial activities contained in the extract was a terpenoid.

INTRODUCTION: Infectious diseases are caused by human pathogenic microorganisms such as *Staphylococcus aureus*, *Streptococcus*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia*, *etc.* The diseases can be treated using antibiotics, but the issue of antibiotic-resistant microorganism crisis has driven scientists to explore novel antibiotics from various sources, for instance, from bacteria associated with soft coral.

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Soft corals have been studied and reported to be a rich source of potentially bioactive compounds such as antimicrobial activities, cytotoxicity, anti-inflammatory, and neuroprotective activities, but it is produced in low concentration [1]. Isolation of secondary metabolites in a large scale from a soft coral is impossible because the secondary metabolites are usually produced in very low quantity. One of the approaches to obtain a large secondary metabolite with homologous structures with a soft coral can be carried out by isolation of the bacteria associated with corals. Since most of the bioactive compounds produced by marine invertebrates are from bacteria associated with the marine invertebrate [2]. Therefore, isolation of bacteria from a soft coral is potential for drug discovery.

Most of the secondary metabolites are not produced in the laboratory. Some biosynthetic gene clusters involved in the secondary metabolite production are probably silent due to unsuitable media and fermentation conditions [3]. Therefore, this study aims to find the best media and condition for secondary metabolite production, having anti-bacterial activities and to identify the antibacterial agent from a bacterium associated with *Sarcophyton* sp., *Bacillus* sp. DA11.

MATERIALS AND METHODS:

Materials: A soft coral sample were collected from Randayan Island, Indonesia. It was collected on 3-4 m depth from sea level. pH, temperature, and salinity of seawater was 8.18, 30 °C and 36, respectively. The identification of the soft coral based on a morphological comparison between soft coral standard and the sample was conducted in the laboratory of Biology, Faculty of Mathematics and Natural Sciences, Universitas Tanjungpura, Indonesia. The sample was identified as *Sarcophyton* sp.

Identification of Isolate DA11: A bacterial isolate from *Sarcophyton* sp. DA11 was isolated using medium M13. The isolate was identified based on Bergey's Manual of Determinative Bacteriology [4]. The morphology of colony DA11 was observed on M13 medium, including shape, size, elevation, margin, and surface. DA11 was tested motility and Gram. The biochemical tests consist of fermentation tests (glucose, lactose, maltose, mannitol, and saccharose), catalase activity, decarboxylase activity (lysine, arginine, ornithine), protease activity, amylase activity, and citrate utilization test.

Preliminary Screening of Antimicrobial Activities*:* Preliminary screening of antimicrobial activities of *Bacillus* DA11 was carried out using a cross-streak method [5]. Each bacterium was inoculated to form a circle with diameter 4-6 mm using an inoculating loop on various solid media (M13, 1/10ZoBell (1/10Z), 1/10Z supplemented with glucose (1/10ZG), and 1/10Z supplemented with glycerol (1/10ZB)) and incubated at 37 °C. After 48 h, the plate was inoculated carefully with a test microorganism by a single streak close to *Bacillus* sp. DA11 and incubated at 37 °C for 24 h. Positive antimicrobial activities were indicated by the inhibition zone around *Bacillus* sp. DA11. The 1/10Z medium consists of peptone (0.5 g) and yeast extract (0.1 g) in 1L of seawater. M13 medium consists of peptone (0.25 g), yeast extract (0.25 g), glucose (0.25 g), seawater (25 mL) and aquadest (75 mL).

Extraction of *Bacillus* sp. DA11 Grown on the 1/10ZG Solid Medium: *Bacillus* sp. DA11 was inoculated on the 1/10ZG solid medium and incubated at 30 °C. After 5 days, *Bacillus* sp. DA11 on the solid medium was cut into small pieces and macerated with ethyl acetate for 48 hours and then centrifuged at 4.000 × g for 15 min to obtain a supernatant. The supernatant was dried using a rotary evaporator to get the crude extract.

Extraction of *Bacillus* sp. DA11 is Grown on Liquid Medium: *Bacillus* sp. DA11 is grown on liquid medium in 2 conditions, namely with and without shaking. A starter of *Bacillus* sp. DA11 was prepared with a colony of *Bacillus* sp. DA11 inoculated on the 1/10ZG liquid medium and incubated on a rotary shaker incubator at 140 rpm at room temperature for 14 h. A 500 µL of the starter was inoculated into 2 of 1/10ZG liquid media. One of the samples was fermented with shaking at 140 rpm, and another was without shaking at room temperature. After 7 days, each culture was extracted directly with ethyl acetate with a 1:1 ratio. Then the ethyl acetate extracts were centrifuged to remove the cells. The supernatants were evaporated using a rotary evaporator to obtain crude extracts. The crude extracts were de-fatted with n-hexane. The crude extracts fermented with and without shaking will then be mentioned as 1/10ZGS and 1/10ZGWS, respectively.

Antimicrobial Assay of *Bacillus* sp. DA11 Extract: The antimicrobial assay of *Bacillus* sp. DA11 extract was carried out using agar disc diffusion method [6, 7]. Test microorganisms were *Aeromonas hydrophila*, *Bacillus* sp., *C. freundii*, *K. pneumoniae*, *Salmonella* sp., *P. aeruginosa*, *S. aureus*, *E. coli*, *V. cholerae*, *V. vara*, *B. subtilis*, and *C. albicans*. A test bacterium was inoculated into the nutrient broth and incubated using a shaking incubator at 200 rpm, 37 °C for 14-16 h. The culture was spread on nutrient agar and a disc paper impregnated with the crude extract with

various concentrations (500 µg/µl, 450 µg/µl, 400 µg/µl, 350 µg/µl, 300 µg/µl 250 µg/µl, 200 µg/µl, 150 µg/µl, 100 µg/µl dan 50 µg/µl) was placed on it then incubated at 37 °C. After 16 h, the diameter of the inhibition zone around the disc was measured. Positive antimicrobial activities were indicated the bythe formation of the inhibition zone. The procedure of antimicrobial assay for a fungal test (*C. albicans*) followed the same as a protocol for the bacterial tests except for the media and incubation. The medium for this test was potato dextrose agar (PDA) and incubated at 30 °C for 48 h.

Phytochemical Analysis: The extract was applied on the thin layer chromatography (TLC) plate and eluted using ethyl acetate: methanol with a ratio of 2:1. The chromatogram was observed under UV light with a wavelength of 254 dan 365 nm. Finally, it was sprayed using cerium sulphate, ninhydrin dan Dragendorff reagents.

Direct Bioautographic Thin Layer Chromatographic (TLC) Assay: Direct bioautographic TLC assay was carried out based on Hamburger and Cordell method [8]. The extract was applied on a TLC plate (TLC silica gel 60G) and eluted using ethyl acetate: methanol (2:1) then dried to remove the solvent. The plate was placed on NA medium inoculated with a test microorganism (*K. pneumonia*) and incubated at 8 °C. After 2 h, the TLC plate was removed from the NA medium, and the NA medium was then incubated for 24 h at 37 °C. The clear zone was observed for zone inhibition around the separated molecule.

RESULTS AND DISCUSSION:

Identification of Isolate DA11: Most corals, marine invertebrates, contain various microorganisms such as fungi, cyanobacteria, viral, and bacteria in their tissues. For instance, a soft coral, *Alcyonium digitatum* contains *Firmicutes*, *Actinobacteria*, *Gamma proteobacteria*, and *Alphaproteo bacteria* [9]. Bacterial communities in most corals may be involved in nutrient cycling, degradation of xenobiotic, and defense against pathogens by antimicrobial compound production or coral health and survival [10, 11]. The bacterial communities can be abundant on the compartments of corals such as mucus layer, tissue, and skeletons and each compartment contains the different bacterial group [12]. High bacterial diversity in the mucus layer is caused by enormous nutrient sources which are needed for the growth of heterotrophic marine bacteria. It probably causes a significantly different bacteria diversity compared to the water and sediment environment.

The high diversity of bacteria among coral compartments induces competition among them or between bacteria and coral to produce secondary metabolites, for example, antimicrobial compounds [10]. The antimicrobial compounds can be used to protect the coral against the infection from coral pathogenic bacteria such as *Vibrio* sp. [13]. *Virgibacillus salaries* and *Virgibacillus marismortui* isolated from healthy acroporid corals and can inhibit the growth of the Black Band Disease (BBD) bacterial strain [14]. *Pseudo alteromonas* sp. isolated from the Mediterranean coral *Oculina patagonica* can inhibit an infection and bleaching bacterium, *Vibrio shiloi* [15]. Coral microenvironments probably induce most cryptic genes involved in the biosynthesis of secondary metabolites from bacteria, so it is a potential to obtain novel compounds with various structures and biological activities. Also, the transfer of genes between microorganisms and soft corals cause produce compounds with similar structures.

TABLE 1: BIOCHEMICAL CHARACTERISTICS OF ISOLATE DA11

Type of tests	Characteristics of the isolate
Morphology of the Colony	
Elevation	flat
Shape	circular
Size	2.5 µm
Colour	white
Margin	wavy
Shape	rod
Gram Staining	positive
Biochemical Tests	
Catalase activity	weak positive
Fermentation test	
a. Glucose	negative
b. Lactose	negative
c. Maltose	negative
d. Mannitol	negative
e. Saccharose	negative
Decarboxylase	
a. Lysine	negative
b. Arginine	negative
c. Ornithine	negative
Protease activity	positive
Amylase activity	positive
Citrate utilization test	negative

Therefore, most scientists focus on the exploration of secondary metabolites from the bacteria associated with marine invertebrates.

In this study, a bacterium of isolate DA11 was isolated successfully from a soft coral of *Sarcophyton* sp. Morphological characteristics of the isolate DA11 were having endospore, rod-shaped cell, Gram-positive, motile, catalase positive and aerobics which was predicted as Genus Bacillus based on Bergey's Manual of Determinative Bacteriology **Table 1** [4]. Its biochemical characteristics were positive catalase, amylase, and protease. Therefore, it is classified as *Bacillus* sp. DA11.

Marine Bacillus has been reported have various classes of secondary metabolite structures which were different from that of terrestrial Bacillus [16, 17]. Secondary metabolites of Bacillus also shows various biological activities, such as antiviral, antimicrobial, anticancer, *etc*. [17] Most bacteria associated with soft corals shows antimicrobial activities [9, 10]. *Bacillus* sp. associated with Brazillian coral species show 23.6% of anti-microbial activity [10]. Cereusit in the A produced by Bacillus cereus strain isolated from the soft coral Antillogorgia (syn. Pseudopterogorgia) elisabethae [18].

Preliminary Screening of Antimicrobial Activities: *Bacillus* sp. DA11 was preliminarily screened on antimicrobial activities in various media using cross-streak method [5]. The advantages of this method are easy, rapid, and cheap to screen bacteria [16, 19]. The best antimicrobial activities of *Bacillus* sp. DA11 was shown on the 1/10ZG medium **Table 2**. *Bacillus* sp. DA11 grown in the 1/10ZG medium was able to inhibit all of the test bacteria except a test fungus, *C. albicans*. *Bacillus* sp. DA11 cannot inhibit *C. albicans* in all of the solid media used in the tests.

TABLE 2: SCREENING OF ANTIMICROBIAL ACTIVITIES OF *BACILLUS* SP. DA11 IN VARIOUS SOLID MEDIA

Media	Test Microorganisms											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
M13	-	+	-	-	-	-	-	-	-	-	-	-
1/10Z	-	-	-	-	-	-	-	-	-	-	±	-
1/10ZG	+	+	+	+	+	+	+	+	+	+	+	-
1/10ZB	-	-	-	-	-	-	-	-	-	+	-	

Test Microorganisms: 1. *A. hydrophila*; 2. *Bacillus* sp.; 3. *C. freundii*; 4. *K. pneumoniae*; 5. *Salmonella* sp.; 6. *P. aeruginosa*; 7. *S.aureus*; 8. *E. coli*; 9. *V. cholerae*; 10. *V. vara* 11. *B. subtilis* 12. *C. albicans*. (+) antimicrobial activity. (-) no antimicrobial activity, (±) cloudy zone.

The screening of antimicrobial activities was carried out on various solid media (M13, 1/10Z, 1/10ZG, and 1/10ZB). All of the media consists of peptone and yeast extract, which are nitrogen sources but different carbon sources. The 1/10Z medium did not contain glucose as a carbon source. The 1/10ZG medium was the best medium for antibacterial activities of *Bacillus* sp. DA11. According to the composition and concentration of the media, the glucose contained in 1/10ZG medium probably played a pivotal role in the biosynthesis of secondary metabolites having the antibacterial activities. Low concentration of glucose contained in the M13 medium was likely not enough to induce the biosynthesis of secondary metabolites having antibacterial activities. *Moraxella* sp. RA15 also needed glucose to trigger the expression of biosynthetic gene clusters encoding its antimicrobial compounds [20]. Glycerol contained in the 1/10ZB medium was an unsuitable carbon source to produce the secondary metabolites with antibacterial activities. *Bacillus* sp. DA11grew in 1/10ZB medium only inhibited a bacterium, *V. vara*. Conversely, *Vibrio alginolyticus* MH66 and *Vibrio colalliilyticus* MB3 need glycerol to improve their growth and antimicrobial properties [21]. Therefore, the 1/10ZG medium was the best medium to produce the antibacterial compound of *Bacillus* sp. DA11.

Antimicrobial Activities of Extract *Bacillus* sp. DA11: *Bacillus* sp. DA11 exhibited the best antimicrobial activities on a 1/10ZG medium using the cross streak method **Table 2**. The further antimicrobial assay was carried out using *Bacillus* sp. DA11 extract using the disc diffusion method. The *Bacillus* sp. DA11 extract was prepared by fermentation on the 1/10ZG medium with 3 conditions which were fermented on 1/10ZG solid medium, 1/10ZG liquid medium with and without

shaking (1/10ZGS and 1/10ZGWS). In addition to the growth medium, the fermentation conditions also influence secondary metabolites production. Therefore, *Bacillus* sp. DA11 extract was prepared on 1/10ZG medium with various fermentation conditions (solid state fermentation, liquid state fermentation with and without shaking).

The *Bacillus* sp. DA11 extract fermentedona liquid medium without shaking was active against for all of the test bacteria while the other condition were not. The best antimicrobial activities from 3 extracts were obtained from *Bacillus* sp. DA11 extract fermented from the 1/10ZGWS liquid medium because it could inhibit all of the test bacteria **Table 3**. All of the extract of *Bacillus* sp. DA11 exhibited broad-spectrum antimicrobial because it could work against a wide range of bacteria either Gram-positive (*Bacillus* sp., *B. subtilis*, *S. aureus*) or Gram-negative (*P. aeruginosa*, *A. hydrophila*, *C. freundii*, *K. pneumoniae*, *Salmonella* sp., *E. coli*, *V. cholera*, *V. vara*) **Table 3**.

A. hydrophila, *V. vara*, and *V. Cholera* are fish bacterial pathogens [22]. *S. aureus*, *P. aeruginosa*, *K. pneumoniae*, *Salmonella* sp., and *E. coli* were categorized as human pathogenic bacteria.

TABLE 3: ANTIMICROBIAL ACTIVITIES OF EXTRACT *BACILLUS* SP. DA11 PRODUCED IN VARIOUS FERMENTATION CONDITIONS

Bacterial Tests	The diameter of Inhibition Zone (mm) from the extract			
	1/10ZG	1/10ZGS	1/10ZGWS	De-fatted 1/10ZG
A. hydrophila	7.4	10.8	14.9	5.34
Bacillus sp.	22.7	12.9	15.3	7.66
B. subtilis	14.9	10.0	13.6	10.96
C. freundii	7.6	7.0	11.9	5.50
E. coli	13.5	12.1	8.8	6.80
K. pneumoniae	13.3	10.5	20.5	13.70
P. aeruginosa	±	5.6	12.3	6.26
Salmonella sp.	±	±	9.3	6.34
S. aureus	9.4	16.2	13.5	3.19
V. cholerae	16.1	10.3	11.0	7.44
V. vara	±	9.8	12.6	5.64

Note: ± cloudy zone

TABLE 4: MICS OF THE DE-FATTED *BACILLUS* SP. DA11 EXTRACT

Test Bacteria	MICs (µg/disc)
A. hydrophila	150
Bacillus sp.	150
B. subtilis	150
C. freundii	300
E. coli	300
K. pneumoniae	50
P. aeruginosa	300
Salmonella sp.	200
S. aureus	500
V. cholerae	300
V. vara	350

Minimum inhibition concentrations (MICs) were calculated towards the extract. MIC value is defined as the lowest concentration of antimicrobial agent that inhibits the growth of test microorganisms [19]. MICs can be used to asses the effectiveness of an antimicrobial agent of an extract [7]. The disc diffusion method is one of the methods which can be used to determine MICs even though the dilution method is the most common method for most clinical microbiology laboratories [19]. MICs of the extract *Bacillus* sp. DA11 were carried out based on disc diffusion agar. The extract of *Bacillus* sp. DA11 showed weak antibacterial activities (MIC=150-500 µg/mL) against bacterial tests except for a bacterial test, *K. pneumonia* **Table 4**. The extract of *Bacillus* sp. DA11 showed strong antibacterial activity (MIC=50 µg/mL) against *K. pneumonia* **Table 4**.

Direct Bioautographic TLC and Phytochemical Analysis: TLC-chromatograms of the de-fatted 1/10 ZGWS extract produced good separation using ethyl acetate: methanol (2:1) as the eluent. There were 3 fluorescence spots observed under UV with λ254 nm. Phytochemical results exhibited that the top spot (R_f = 0.79) categorized as terpenoid, which was obtained from a positive reaction with cerium sulfate. The other spot showed negative reaction towards ninhydrin and Deggendorf reagents, which indicated that they did not contain peptide or alkaloid.

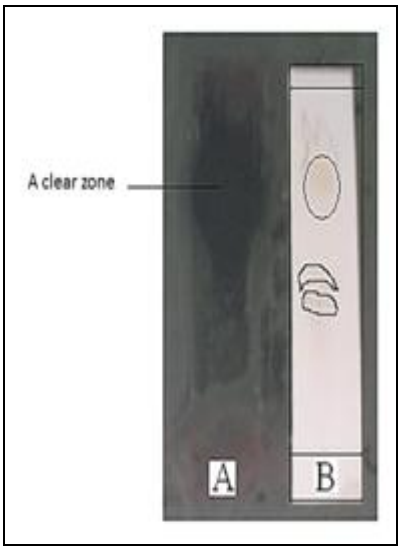

FIG. 1: TLC-BIOAUTOGRAM OF THE EXTRACT OF DE-FATTED 1/10ZGWS

Another the TLC-chromatogram was applied on a plate containing the test bacterium (*K. pneumoniae*). The result showed that only the top spot (R_f = 0.79) could inhibit the test bacterium. It can be concluded that the terpenoid likely contributes as an antibacterial agent. Some of the terpenoid compounds have been reported to function as antimicrobials [23]. Terpenoid is rarely isolated from bacteria, but the extensive result of bacterial genomic analysis identifies many genes of terpene synthases involved in terpenoid biosynthesis [24]. Bacterial terpene synthase generally shows a significant difference from fungal or plant terpene synthase, so it is a chance to obtain novel terpenoids.

Sarcophyton spp. of the Red Sea extract demonstrates strong antibacterial activities against *Bacillus* sp. and *Staphylococcus* sp. [25] A terpenoid derivate, (5S)-3-[(3E,5S)-5-hyroxyl-3-hepten-6-yn-l-yl]-5-methyl-2(5H)-furanone, is isolated from a soft coral of the Red Sea *Sarcophyton trocheliophorum* [26]. This compound can inhibit the growth of *S. aureus*, *Bacillus cereus*, *E. coli*, and *Salmonella typhi* and *P. aeruginosa.* This compound might be contained from our soft coral sample *Sarchophyton* sp. because both of them are in the same genus. The *Bacillus* sp. DA11 extract isolated from *Sarcophyton* sp could also inhibit the growth of *S. aureus*, *Bacillus* sp., *E. coli*, *Salmonella* sp., and *P. aeruginosa*. Interactions between *Sarcophyton* sp. and *Bacillus* sp. DA11 were probably induced *Bacillus* sp. DA11 to produce this terpenoid had homologous structures with the soft coral. Most of the secondary metabolites produced by marine invertebrates have a similar structure with microorganisms associated with marine invertebrates [2].

CONCLUSION: Bacteria associated with soft corals are promising sources to obtain the novel secondary metabolites. *Bacillus* sp. DA11 isolated from a soft coral, *Sarcophyton* sp. producing the secondary metabolite which could inhibit bacterial growth. However, secondary metabolite production was affected by the media and the condition of fermentation. The best medium and condition of fermentation for *Bacillus* sp. DA11 in the secondary metabolite production is having antibacterial activities was a liquid medium of 1/10 Zobell supplemented with 1% of glucose fermented without shaking. The secondary metabolite having antibacterial activities was identified as a terpenoid.

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