RESEARCH ARTICLE

Antibacterial activity in secondary metabolite extracts of heterotrophic bacteria against *Vibrio alginolyticus, Aeromonas hydrophila,* and *Pseudomonas aeruginosa* [version 1; peer review: 2 approved]

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Abstract

Background: Disease causing bacteria such as *Vibrio alginolyticus, Aeromonas hydrophila,* and *Pseudomonas aeruginosa* present a problem for fish farming. Treatment to remove them are generally carried out using antibiotics which have side effects on fish, the environment and humans. However, the use of antibacterial compounds derived from heterotrophic bacteria serve as a good alternative for antibiotics. Therefore, this study aimed to explore antibacterial activity in the secondary metabolite extracts of heterotrophic bacteria against *Vibrio alginolyticus, Aeromonas hydrophila,* and *Pseudomonas aeruginosa*.
Methods: Heterotrophic bacteria namely *Bacillus sp.* JS04 MT102913.1, *Bacillus toyonensis* JS08 MT102920.1, *Bacillus cereus* JS10 MT102922.1, *Bacillus* sp. JS11 MT102923.1, *Pseudoalteromonas* sp. JS19 MT102924.1, *Bacillus cereus* JS22 MT102926.1, and *Bacillus* sp. strain JS25 MT102927.1 were used in this study. The sequences of these bacteria have been deposited and are available from NCBI GenBank. Each heterotrophic bacterium was cultured on 6L nutrient broth for 8 days, and extracts produced using ethyl acetate to obtain their secondary metabolites. These extracts were tested for their phytochemical contents using FT-IR and also tested for their inhibitory property in pathogenic bacteria by agar diffusion method.
Results: Phytochemical test results showed that the seven heterotrophic bacterial isolates produced terpenoid compounds. Based on the inhibitory test, the secondary metabolite extracts from *Bacillus* sp strain JS04 had the highest inhibitory effect on the growth

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of pathogenic bacteria namely, *V. alginolyticus* (17.5 mm), *A. hydrophila* (16.8 mm), and *P. aeruginosa* (17.3 mm).
Conclusion: It was concluded that the secondary metabolite extracts of heterotrophic bacteria inhibit the growth of *V. alginolyticus, A. hydrophila,* and *P. aeruginosa*.

Keywords
antibacterial, fish pathogens, heterotrophic bacteria, secondary metabolites

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Introduction

Bacteria diseases in fish stocks constitute a major problem for fish farming, since they cause significant economic losses[1–3]. Common pathogenic bacteria that affect fish include *Vibrio alginolyticus, Aeromonas hydrophila* and *Pseudomonas aeruginosa*[4–9]. *V. alginolyticus* is a gram-negative bacteria which is an opportunistic pathogen in marine animals[10–13]. Bacterial diseases cause different fish infections such as, exophthalmia, ulcers, septicemia, and corneal damage[14–16]. *Aeromonas hydrophila* is found to be the main cause of the septicemia epidemic in freshwater fish[17,18]. Its outbreak causes tissue damage of the spleen, gills, and the fish's stomach[19]. *A. hydrophila* is found to frequently infect various fish species namely, catfish (*Ictalurus punctatus)*[20], carp (*Cyprinus carpio)* and catfish (*Pangasius hypophthalmus)*[21], tilapia (*Oreochromis niloticus)*[22], salmon (*Oncorhynchus masou masou)*[23], snapper (*Lates calcarifer)*[24], striped snakehead (*Channa striata)*[25], cod (*Gadus macrocephalus),* nd tank goby (*Glossogobius guris)*[26]. Meanwhile, *P. aeruginosa* is found to infect freshwater and marine fish[27,28], with infection being characterized by the expression of red spots due to bleeding, skin darkens, loose scales, protruding eyes, fin erosion[29], behavioural changes due to disruption of locomotor activity[30], and abnormal swimming[31].

Bacteria disease treatment is generally carried out using antibiotics, however, these can have adverse effects on the fish and their environment[32–37]. The accumulation of antibiotics in the fish increase the risk of bacterial resistance[38,39]. *Escherichia coli* bacteria isolated from the digestive organs of catfish showed high resistance levels towards tetracycline, ampicillin, and chloramphenicol[40]. Therefore, it is necessary to explore natural compounds with antibacterial activity[41]. Sea water is a potential source of heterotrophic bacteria that produce antimicrobial compound[42], and have probiotic activity[43]

Sea bacteria such as, *Bacillus* sp. *B. cereus, B. toyonensis,* and *Pseudoalteromonas* sp., are known to inhibit the growth of pathogenic bacteria namely, *V. alginolyticus, A. hydrophila,* and *Pseudomonas* sp[43]. They also produce antimicrobial compounds such as, *Pseudoalteromonas*[44]. *Pseudoalteromonas piscicida* produces antimicrobial substances that inhibit the growth of different pathogenic bacteria namely, *Vibrio vulniosis*[45], *Bacillus* sp[46], *B. pumilus*[47], and *B. subtilis*[48,49]. *Bacillus amyloliquefaciens* shows antibacterial activity towards pathogenic bacteria such as, *Aeromonas hydrophila, Vibrio harveyi, V. vulnificus,* and *V. parahaemolyticus*[50]. Meanwhile, *Bacillus subtilis* shows antibacterial activity towards the pathogens *Vibrio parahaemolyticus*, *V. vulnificus*, and *Aeromonas hydrophila*[51].

Heterotrophic bacteria extracted from Riau sea waters were examined and found to able to inhibit the activity of pathogenic bacteria *Aeromon* as *salmonicida*, *Edwarsiela tarda* and *Edwarsiela ictalur*ias previously reported by Setiaji *et al.*[52]. However, the antibacterial activity of these heterotrophic bacteria extracted from Riau sea waters on the pathogenic bacterial namely, *Vibrio alginolyticus, Aeromonas hydrophila,* and *Pseudomonas aeruginos* have never been examined for its potential against pathogenic bacteria. Therefore, this study aims to explore antibacterial activity in secondary metabolite extracts of heterotrophic bacteria isolated from Riau sea water, against pathogenic bacteria namely, *V. alginolyticus, A. hydrophila,* and *P. aeruginosa.*

Methods

Bacterial culture

The heterotrophic bacteria isolates were collected from sea waters in Sungai Pakning Bengkalis Regency Riau Province Indonesia (North latitude 01°21'36,8" and East longitude 102°09'34,1"). 1 liter of the sea water was collected at 50 cm depth by using a water sampler (Tiolan Lab, type: WSV-BIT22), then was transferred into a sample bottle and was put into a cool-box filled with ice at 15°C, before being transported by car for 1 hour to the laboratory. The heterotrophic bacteria was cultured using nutrient Agar (NA; Merck-1.05450.0500). The heterotrophic bacteria cultured were used for an antagonist test against pathogen bacteria. The antagonist test procedure is as follows, 1 ml of pathogenic inoculants (OD_{600nm} = 0.08–0.1) (OD measured with Thermo scientific, Genesys 10S UV-Vis) was added to 15 ml liquid nutrient Agar media at 50°C, then homogenized, and poured into a petri dish to solidify. Furthermore, Oxytetracycline antibiotic disc paper (Oxoid, CT0041B, OT30 mcg) was used as the positive control, while 30 µl aquades (Kimiapedia id-1720602804) was dripped to a disc paper (Macherey-nagel, MN827 ATD) as the negative control. 30 µl heterotrophic bacterial isolate taken from bacteria culture in nutrient Broth (NB; Merck-1.05443.0500) was dripped to a disc paper and incubated at 30°C for 24 hours. The inhibitory power of heterotrophic bacterial isolate was measured from the diameter of clear zone formed around the disc. From the antagonist test, eight isolates with the best inhibition were collected, and the heterotrophic bacteria was identified using 16S rDNA technique[43]. The sequenced products were run through BLAST (NCBI Basic Local Alignment Search Tool) and registered to GenBank.

The pathogenic bacteria were obtained from the collection at the Marine Microbiology Laboratory of the Faculty of Fisheries and Marine Science, University of Riau, Indonesia. The heterotrophic and pathogenic bacteria were cultured on the nutrient Agar (NA; Merck-1.05450.0500). The cultured medium was sterilized in an autoclave at a pressure of 15 psi and 121°C for 15 minutes. After 1 hour at room temperature, the medium was inoculated by the heterotrophic bacteria and the pathogenic bacteria. Then the bacteria were incubated in an incubator (Memmert, Model 30–1060) at 30° C for 24 hours.

Isolates test

Previous studies showed that eight heterotrophic bacterial isolates possessed the potential to produce pathogens. Seven of these species were used in this study namely, *Bacillus sp.* JS04 MT102913.1, *Bacillus toyonensis* JS08 MT102920.1, *Bacillus cereus* JS10 MT102922.1, *Bacillus* sp. JS11 MT102923.1, *Pseudoalteromonas* sp. JS19 MT102924.1, *Bacillus cereus* JS22 MT102926.1 and *Bacillus* sp. strain JS25 MT102927.1 have been deposited in GenBank.

Then, each bacterium was cultured in a 6 L nutrient Broth (NB; Merck-1.05443.0500) diluted with sea water of salinity 29 ppt and aerated for 8 days. After this, the bacteria were mixed with ethyl acetate (P.a) at ratio 1:1 and shaken vigorously to homogenize. Subsequent filtering was performed until a clear filtrate was obtained using funnel and filter paper (Whatman 41, no. 1441–125), and evaporated with a rotary evaporator (Cole Parmer, N-1300) at 50°C and a speed of 50 rpm. This allowed thick secondary metabolite extracts to be obtained[53].

Phytochemical test and Fourier-transform infrared spectroscopy (FT-IR)

Phytochemical test was conducted on the secondary metabolite extracts of heterotrophic bacteria, which included tests for alkaloid, terpenoid, flavonoid, phenolic, and saponin compounds[54].

Mayer reagent was prepared by adding 1.36 g $HgCl_2$ (Merck, 1.04419. 0050) to 60 mL distillled water and 5 g Ki (Meck 1.05043.1000) to 10 mL distilled water. Both solutions were then mixed with a further 20 mL distilled water. The Liebermen – burchad reagent was prepared by mixing 97% H_2SO_4 (Merck 1.00731.2500) and 100% CH_3COOH (Merck 1.00063.2500).

Alkaloids were tested for using using 10 mg heterotrophic bacteria extract and 250 µL Mayer reagent.

The terpenoid was tested using 10 mg heterotrophic bacteria extract, 10 drops of CH_3COOH, and 3 drops of H_2SO_4.

Flavonoid tests were performed using 10 mg heterotrophic bacteria extract added to 5 mL distilled water. This was then boiled before adding 0.05 g Mg (Merck 1.05815.1000) and 10 drops of 37% HCl (Merck 1.00317. 2500), the mixture was then shaken for one minute.

Phenolic compounds were tested by using 10 mg heterotrophic bacteria extract combined with 500 µL 5% $FeCl_3$ (Merck 1.03943.0250).

Saponin compounds were tested for using 10 mg heterotrophic bacteria extract added to 5 mL distilled water which was then shaken for 1 minute. 150 µL 1N HCl (Merck 1.00317. 2500) was then added, and shaken for another minute.

A positive alkaloid test was indicated by the formation of a white precipitate after adding Mayer regent. A positive terpenoid test was indicated by the formation of a red colour. A positive flavonoid test was indicated by a red colour change. Phenolic compounds were indicated by a blue colour change. Saponin compounds were indicated by a foam forming.

Meanwhile, to determine the functional groups in secondary metabolite extracts, FT-IR (Shimadzu, IR prestige-21, IR solution software ver. 1.1) spectroscopy analysis was performed. This was conducted by crushing 1 mg of each extract, added to KBr (Merck-1.04950.0500), and mixed vigorously until homogenized. This mixture was then measured for infrared absorbance at 4500–450 cm wavelength.

Inhibitory activity of heterotrophic bacterial extract

The secondary metabolite extracts of heterotrophic bacteria obtained were tested on pathogenic bacteria namely, *V. algynolyticus, A. hydrophila,* and *P. aeruginosa* using agar diffusion method, and 6 mm disc paper (Macherey-nagel, MN827 ATD)[55]. The procedure is as follows, 1 ml of pathogenic inoculants (OD_{600nm} = 0.08–0.1) (OD measured with Thermo scientific, Genesys 10S UV-Vis) added to 15 ml liquid nutrient agar media at 50°C, then homogenized, and poured into a petri dish to solidify. Furthermore, Oxytetracycline antibiotic disc paper (Oxoid, CT0041B, OT30 mcg) was used as the positive control, while methanol disc paper was the negative control. The metabolite extracts were then dissolved in 1 mg / mL methanol (P.a) and incubated at 30°C for 24 hours. The inhibitory power of heterotrophic bacterial extracts was measured from the diameter of clear zone formed around the disc.

Data analysis

The data were subjected to one-way analysis of variance followed by the Post Hoc Tukey multiple range test using R 4.0 software (GNU General Public License), $p<0.05$ is considered to indicate a statistically significant difference.

Results

Phytochemical test and functional groups

Phytochemical test results of the metabolite extracts when added to Lieberman-Burchard reagents produced a red colour indicating the presence of terpenoids in the seven isolates. Meanwhile, the test for alkaloid, flavonoid, phenolic, and saponin compounds gave negative results.

Based on infrared spectrum analysis, the secondary metabolite extracts of *Bacillus* sp. strain JS04 contained O-H alcohol, C-H aldehyde, O-H carboxylic acid, and C=C alkene groups. *Bacillus toyonensis* JS08 contained C-H alkanes, C=N nitriles, C=O carbonyl, and C-N amine groups. *Bacillus cereus* JS10 contained C-H alkanes, O-H carboxylic acid, C=C alkenes, and C-H alkanes groups. *Bacillus* sp. JS11 contained O-H alcohol, C-H alkanes, O-H carboxylic acids, and C=O carbonyl groups. *Pseudoalteromonas sp.* JS19 contained alcohol O-H, C-H alkanes, C=O carbonyl, and C=C alkenes groups. *Bacillus cereus* JS22 contain O-H alcohol, C-H alkane, C=O carbonyl, and C=C alkene groups. *Bacillus sp*. JS25 contain C-H alkanes, O-H carboxylic acids, O-H alcohols, and C=C alkenes groups (Table 1[56]).

Inhibitory activity

The results showed that the seven heterotrophic bacterial isolates inhibited the growth of pathogenic bacteria. The extracts inhibitory activity against pathogenic bacteria are shown in Table 2[56]. The average inhibition zone diameter of the extracts against pathogenic bacteria namely, *V. alginolyticus, A. hydrophila,* and *P. aeruginosa* ranges from 9.3 to 17.5 mm, 9.3 to 16.8 mm, and 8.5 to 17.3 mm, respectively. This inhibitory zone activity was indicated by the presence of clear zones formed around the disc paper. The largest inhibition zone diameter of the extracts against the growth of pathogenic bacteria was derived from isolates of *Bacillus sp*. strain JS04 (17.5 mm) on

Table 1. Infrared spectrum of secondary metabolite extracts of heterotrophic bacteria.

Secondary metabolite extracts	Spectrum (cm^{-1})	Functional groups
Bacillus sp. strain JS04	3148 2732 2535 827	O-H C-H O-H C=C
Bacillus toyonensis strain JS08	2925 2361 1722 1229	C-H C=N C=O C-N
Bacillus cereus strain JS10	2925 2735 1669 1459	C-H O-H C=C C-H
Bacillus sp. strain JS11	3330 2925 2527 1721	O-H C-H O-H C=O
Pseudoalteromonas sp. strain JS19	2930 2732 1720 1455	C-H O-H C=O C-H
Bacillus cereus strain JS22	3567 2925 1710 827	O-H C-H C=O C=C
Bacillus sp. strain JS25	2895 2602 1364 830	C-H O-H O-H C=C

Table 2. Inhibitory activity in the secondary metabolite extracts of heterotrophic bacteria against pathogenic bacteria. Mean values with different superscripts in the same columns were significantly different ($p < 0.05$).

Secondary metabolite extracts	Average of inhibition zone diameter (mm)		
	V. alginolyticus	***A. hydrophila***	***P. aeruginosa***
Bacillus sp. strain JS04	17.5^{a}	16.8^{a}	17.3^{a}
Bacillus toyonensis strain JS08	11.0^{b}	9.5^{b}	10.0^{b}
Bacillus cereus strain JS10	10.8^{b}	8.5^{b}	9.5^{b}
Bacillus sp. strain JS11	10.8^{b}	9.0^{b}	9.5^{b}
Pseudoalteromonas sp. strain JS19	10.8^{b}	9.8^{b}	9.8^{b}
Bacillus cereus strain JS22	9.3^{b}	9.3^{b}	8.5^{b}
Bacillus sp. strain JS25	15.8^{a}	15.5^{a}	16.3^{a}

V. alginolyticus, 17.3 mm on *P. aeruginosa,* and 16.8 mm on *A. hydrophila*.

Discussion

Phytochemical test results showed that the seven heterotrophic bacterial isolates produced terpenoids, which consist 5 carbon atoms or isoprene (C5) units. Microbes carry out biosynthesis by producing isopentyl pyrophosphate and dimethyl allyl pyrophosphate for terpenoid formation[57]. A significant relationship between terpenoids gene expression and isoprene production in *Bacillus subtilis* has previously been reported[58].

Infrared spectrum analysis provided information about the detected compounds in the mixture[59]. Metabolite extracts showed the presence of hydroxyl, aldehyde, carboxylic acid, alkene, alkane, carbonyl, and amine functional groups in these extracts. This indicated that the seven bacterial isolates produced terpenoids, while the functional groups contained in the terpenoids were namely, O-H hydroxyl, C-H aliphatic, carbonyl, C-H cyclic, and carboxylic acid[60].

The result of inhibitory activity in the secondary metabolite extracts of *Bacillus* sp. strain JS04 showed the largest inhibition zone against the growth of pathogenic bacteria. The formation of clear zones on culture media indicated that heterotrophic bacteria produced terpenoid compounds for antibacterial purposes.

The terpenoid compounds contained several phytochemicals that possess antimicrobial activity[61]. For example, Terpenes and terpenoids have been reported to exert antimicrobial activity against a wide variety of bacteria, both Gram-positive and Gram-negative[62]. Terpenes cause membrane disruption through acting on lipophilic compound in the membrane[63]. Therefore, terpenoid compounds were able to prevent the forming of biofilm cell in the bacterium *Streptococcus mutans*[60,64].

There are many antimicrobial compounds produced by sea bacteria especially from the *Bacillus* and *Pseudoaltreromonas* genus. For instance, *B. pumilus* produces antimicrobial compound against *V. algynolyticus, V. anguillarum, Listeria monocytogenes* and *Staphylococcus aureus* pathogens[48]. The *Bacillus* sp. from sea water produced chemical compound effective at preventing motility of *V. Algynolyticus*[47]. *Bacillus subtilis* produced antibacterial compound against *Aeromonas hydrophila* and *Vibrio parahemolyticus* pathogens[49]. The genus *Pseudoalteromonas* hosts 16 antimicrobial metabolite producers. To date, a total of 69 antimicrobial compounds are classified into alkaloids, polyketides, and peptides[45]. Furthermore, the bacterium *Pseudoalteromonas rubra* which was symbiotic with soft coral *Sarcophyton* sp. produced carotenoid pigments with antibacterial activity against *Staphylococcus aureus*[65] and *V. algynolyticus* pathogens[66].

Conclusion

The secondary metabolite extracts produced by the seven isolates of haterotrophic bacteria can inhibit the growth of pathogenic bacteria, namely *V. alginolyticus, A. hydrophila,* and *P. aeruginosa*. The secondary metabolite extracts of *Bacillus sp*. strain JS04 has the highest inhibitory activity against the growth of these three pathogenic bacteria.

Data availability

Underlying data

Figshare: Antibacterial activity in secondary metabolite extracts of heterotrophic bacteria against Vibrio alginolyticus, Aeromonas hydrophila, and Pseudomonas aeruginosad Item. https://doi.org/10.6084/m9.figshare.12818798.v3[56]

This project contains the following underlying data:

- Data FT-IR activity in the secondary metabolite. Jarod Setiaji.pdf (Infrared spectrum of secondary metabolite extracts of heterotrophic bacteria)

- Data Inhibitory activity in the secondary metabolite. Jarod Setiaji.xlsx (Inhibitory activity in the secondary metabolite extracts of heterotrophic bacteria against pathogenic bacteria)

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Open Peer Review

Current Peer Review Status: ✔ ✔

Version 1

Reviewer Report 26 January 2021

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✔ **Yuhanis Mhd Bakri**
Department of Chemistry, Faculty of Science and Mathematics, Universiti Pendidikan Sultan Idris, Tanjong Malim, Malaysia

Overall the authors have presented sound article with sufficient findings. Suggestion: Title could be amended to ethyl acetate extracts instead of secondary metabolite extracts. The word inhibitory power in method of inhibitory activity shall be amended appropriately. Isolate tests subtopic shall be amended to extraction. Functional groups investigation is rather general, although acceptable, a further study is needed to verify in length the chemical constituents of secondary metabolites extracts.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
I cannot comment. A qualified statistician is required.

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Natural product chemistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 14 January 2021

https://doi.org/10.5256/f1000research.28932.r76449

Agung Damar Syakti
Environmental Sciences, Marine Science and Fisheries Faculty, Raja Ali Haji Maritime University (UMRAH), Tanjung Pinang, Indonesia

Evidence of Antibacterial activity in secondary metabolite extracts of heterotrophic bacteria against *Vibrio alginolyticus, Aeromonas hydrophila,* and *Pseudomonas aeruginosa* could be interesting for many applications in aquatic science. The finding may be useful for Indonesia's fish farming activities. The study scientifically detailed enough. However, the author(s) should develop much better the occurrence of the specific spectrum thon FT-IR spectra related to the metabolite extracts. The authors should present the FTIR spectra. Thus, the manuscript could be accepted after such minor revision of the spectra figures addition showing the relative response of the functional groups in regard to the spectrum for target compound validation.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Analytical Environmental Chemistry

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.