Potential of Secondary Metabolite from Marine Heterotrophic Bacteria against Pathogenic Bacteria in Aquaculture

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Abstract. Marine bacteria including heterotrophic bacteria are the most numerous organisms in nature and are capable of producing secondary metabolites. The secondary metabolite produced is a molecular compound produced from the secondary metabolic process, generally can be in the form of antibiotics, enzyme inhibitors, toxins, growth regulators, hormones, and insecticides. This study aims to examine the inhibitory ability of secondary metabolites of heterotrophic bacteria to the activity of pathogenic bacteria \textit{Aeromonas hydrophilla}, \textit{Pseudomonas aeruginosa}, and \textit{Vibrio alginolyticus}. Around 10 heterotrophic bacterial isolates from the Marine Microbiology Laboratory collection, Fisheries and Marine Sciences Faculty, University of Riau was tested on pathogenic bacteria based on the Kirby-Bauer disk diffusion method. The results showed that all heterotrophic bacterial isolates were capable of inhibiting the growth of pathogenic bacteria such as \textit{A. hydrophilla} (P), \textit{P. aeruginosa} (Q), and \textit{V. alginolyticus} (R). \textit{Vagococcus fluvialis} isolates are the best heterotrophic bacterial isolates compared to 9 other isolates. Where the clear zone formed is wider, that is 12.35 mm with the results of the activity test of 1877.78 mm\textsuperscript{2}/ml. Based on the results, the heterotrophic bacteria are capable of producing secondary metabolites that inhibit the growth of pathogenic bacteria.

1. Introduction
Sungai Kayu Ara Village, located in the Sungai Apit District, Siak Regency, Riau Province is estuary area that connected to the Malacca Strait sea water. Anthropogenic activities around Sungai Kayu Ara are quite high such as shipping, industry, settlement, and others. This condition affects the decline in the quality of marine aquatic ecosystems due to high levels of pollution. Sea pollution can increase the decomposing bacteria activity, while the development and growth of microorganisms and other marine biota are disrupted.

Heterotrophic bacteria are classified as decomposing bacteria with microscopic size and a short life cycle. Heterotrophic bacteria have a role as organic combination decomposers (mineralization) derived from industrial waste, decomposition of food that is not consumed, fecal, fish excretion [1]. These bacteria can degrade complex organic molecules more easily, easily absorbed, and assimilated. This condition is an important factor in the balance of the life cycle of another marine biota. Heterotrophic bacteria produce active and consume sulfides when growing on organic compositions under aerobic conditions [2]. Given its abundance on earth, it is thought that heterotrophic bacteria against the sulfur cycle should not be ignored. Sulfides of heterotrophic bacterial evolution can benefit the bacteria themselves and can protect bacteria from antibiotics through the reaction and transfer of reactive oxygen species [3].
Marine bacteria including heterotrophic bacteria have the ability to produce secondary metabolites [4], for example, the *Sapospira grandis* bacteria which have the ability to produce terpenoid compounds. A secondary metabolite is a molecule of the secondary metabolic process of an organism. The secondary metabolite product is different from the primary need to support the growth process, it refers more to the production of enzyme factors and the body's defense [5]. Secondary metabolites also have the potential to be anti-fungal, neurotogenic, anticancer, anti-algae, anti-malarial and anti-inflammatory [6]. Antibiotic work activities have two functions, namely bacteriostatic which have the role of suppressing the growth of pathogenic bacteria, and bactericidal which has the role of killing pathogenic bacteria.

Naturally, some bacteria have benefits while others do not. Heterotrophic bacteria can inhibit pathogenic bacteria such as *A. hydrophila, P. aeruginosa*, and *V. alginolyticus* by producing bacteriocin possessed [1]. Disease control strategies in fisheries that are always carried out and give good results are the use of probiotic bacteria. These bacteria are safer than chemicals, do not accumulate in the food chain, and control pathogens in their environment [7]. This study can become basic information about secondary metabolites from heterotrophic bacteria for pathogenic bacteria further research.

2. Methodology

This study used an experimental method using 10 heterotrophic bacterial isolates obtained from the Marine Microbiology Laboratory collection, Faculty of Fisheries and Maritime Affairs, University of Riau.

2.1. Preparation of Isolate Culture
A total of 10 selected isolates were biochemically tested, and then cultured for ± 3 days on nutrient broth (NB) medium (37 °C) with salinity ranging from 20 to 27 ppt using an aerator.

2.2. Secondary Metabolite of Isolate
The secondary metabolite compound was extracted on culture media of 10 isolates [8]. The use of ethyl acetate in growth media was done at a ratio of 1: 1, transition using secondary metabolites using Rotary Evaporator (50 °C, 50 rpm), then air-dried. The residue of dried metabolite extract was dissolved using methanol (10 mg: 1 ml). These results were used to test secondary metabolite activity.

2.3. Secondary Metabolite Testing
The results of secondary metabolites from 10 heterotrophic bacterial isolates were tested on 3 types of pathogenic bacteria such as *A. hydrophila* (P), *P. aeruginosa* (Q), *V. alginolyticus* (R). The testing process of each isolate was carried out with 3 repetitions so that a total of 90 experiments were obtained (Table 1).

| Isolate   | Salinity | Strain | ID       | Pathogenic Bacteria | Treatment | Homology |
|-----------|----------|--------|----------|--------------------|-----------|----------|
| *Bacillus cereus* (A) | 20 ppt   | BK4    | KU25828  | P                  | (A-P)     | 96 %     |
|           |          |        | 8.1      | Q                  | (A-Q)     |          |
|           |          |        |          | R                  | (A-R)     |          |
| *Bacillus cereus* (B) | 20 ppt   | SP4    | KC13682  | P                  | (B-P)     | 91 %     |
|           |          |        | 1.1      | Q                  | (B-Q)     |          |
|           |          |        |          | R                  | (B-R)     |          |
| *Bacillus cereus* (C) | 25 ppt   | S5     | KU92749  | P                  | (C-P)     | 97 %     |
|           |          |        | 0.1      | Q                  | (C-Q)     |          |
|           |          |        |          | R                  | (C-R)     |          |
| *Bacillus cereus* (D) | 25 ppt   | Xmb0   | KT98617  | P                  | (D-P)     | 99 %     |
|           |          |        | 51       | Q                  | (D-Q)     |          |

Table 1. Heterotrophic bacterial isolates
Each pathogenic bacterial was tested based on the Kirby-Bauer disk diffusion method. Every paper disks has contained 50 μl of metabolite secondary compounds placed on the pathogenic bacteria growth media (Nutrient Agar, NA) for 24 hours at 37 °C [9]. The clear zone diameter was measured by using calipers.

2.4. Data Analysis
The secondary metabolite test is defined as an AU (Activity Unit). One activity unit in the area of the inhibition zone per unit volume of the sample of the secondary metabolite solution (mm$^2$/ml) [10].

$$\text{AU (mm}^2/\text{ml)} = \frac{\text{Area of clear zone} - \text{Area of disc}}{\text{Volume of secondary metabolite}}$$

**Equation 1.** Activity Unit (AU) from the secondary metabolite solution.

The ability of secondary metabolites against pathogenic bacteria was counted by comparing the average of the clear zone formed with the criteria for antibacterial strength. There are four criteria for inhibition zone, such as Weak (diameter <5 mm), Medium (diameter 5-10 mm), Strong (diameter 10-20 mm), and Very Strong (diameter>20 mm) [11].

3. Results and Discussion
All heterotrophic bacteria in this study were successfully isolated. Every isolate has been identified biochemically and morphologically based on the shape of the colony, edge, elevation, color, gram, catalase, and motility (Table 2).

| Isolate | Color       | Shape             | Edge     | Elevation  | Catalase | Gram | Motility |
|---------|-------------|-------------------|----------|------------|----------|------|----------|
| A       | Milky white | Round edges arise| Smooth   | Like a crater | +       | +    | +        |
| B       | White       | Irregular and spread out | Indention | Flat | +       | +    | +        |
Inhibition zone by secondary metabolites of 10 heterotrophic bacterial isolates against pathogenic bacteria can be seen in Figure 1 below.

| Heterotrophic Bacterial Isolates | A. hydrophila | P. aeruginosa | V. alginolyticus |
|---------------------------------|--------------|--------------|-----------------|
| C                               | Milky white  | Round        | Smooth          |
| D                               | Milky white  | Round        | Irregular       |
| E                               | Milky white  | Irregular and spread out | Smooth |
| F                               | Milky white  | Round        | Smooth          |
| G                               | Milky white  | Round        | Arise           |
| H                               | Milky white  | Round        | Arise           |
| I                               | Milky white  | Round        | Arise           |
| J                               | Milky white  | Round edges arise | Choppy |

**Figure 1.** Inhibition zone by secondary metabolites of 10 heterotrophic bacterial isolates (A-J) against pathogenic bacteria (*A. hydrophila*, *P. aeruginosa*, and *V. alginolyticus*).

In general, the secondary metabolites potency of 10 heterotrophic bacterial against pathogenic bacteria shows the criteria of 'Medium' because it produces inhibition zones ranging from 6.6 - 9.66 mm. There is one heterotrophic bacterial isolate that shows the criteria 'Strong' criteria, that is isolate J (*Vagococcus fluvialis* CT21). It produces an inhibition zone ranging from 10.23-12.35 mm against *V. alginolyticus* and *A. hydrophila*.

The results of research on heterotrophic bacteria *B. cereus* strain Bc7 in producing secondary metabolites, maximum production in the initial stationary phase [12]. In the case of *B. subtilis* 14B strain, the production of secondary metabolites begins after 24 hours of incubation and then reaches a peak within 96 hours of fermentation. This secondary metabolite product produced from marine bacteria has a uniqueness and structure that is different from the others because of the complex conditions and diversity of life, and its bioactivity is very effective. Inhibition zone testing in pathogenic bacteria is carried out to determine the ability of the secondary metabolism used. The secondary metabolic ability is seen by the formation of clear zones around the disk paper in inhibiting
the growth of pathogenic bacteria [13]. Clear zones formed are varied, some are clear, round, and broad clear. This variation factor affects the volume of the concentration of secondary metabolites used in certain types of bacteria.

The secondary metabolite activity of heterotrophic bacteria is carried out on pathogenic bacteria such as *A. hydrophila*, *P. aeruginosa*, and *V. alginolyticus* (Figure 2).

![Figure 2. Heterotrophic bacteria (A-J) secondary metabolite activity test against pathogenic bacteria (*A. hydrophila*, *P. aeruginosa*, and *V. alginolyticus*).](image)

Based on figure 2, Isolate J has the highest activity unit for *A. hydrophila* (1877.78 mm²/ml), and *V. alginolyticus* (1085.02 mm²/ml). While the inhibitory activity of secondary metabolites against the highest *P. aeruginosa* bacteria was seen in isolate C which was 974.13 mm²/ml. The highest activity of this unit forms the ability to inhibit the metabolism produced by isolates C and J in making clear zones of pathogenic bacteria.

Isolate J is a species of *Vagococcus fluvialis*. Bacteria *Vagococcus sp.* is a bacterium isolated from marine corals of the Acropora type. These bacteria change in the Kingdom: Bacteria, Division: Firmicutes, Class: Bacilli, Order: Lactobacillales, Tribe: Enterococccaeae, and Genus: Vagococcus [14]. Most of the representatives of Vagococcus have been isolated from the aquatic environment, suggesting members of this genus have properties that are optimized for life safety in marine habitats [15].

Isolate C is a *Bacillus cereus* species with strain S5. Antimicrobial compounds from the secondary metabolic process of *B. cereus* bacteria have the potential to form clear zones on the growth media of pathogenic bacteria such as *A. hydrophila*, *P. aeruginosa*, and *V. alginolyticus* [16]. Antimicrobial compounds can inhibit the growth of pathogenic bacteria that contain chemicals that act as suppressors in microbial growth proteins [17].

Antimicrobial compounds can inhibit the growth of pathogenic and non-pathogenic microbes, one of which is in the process of genetic engineering of microbes [18]. The use of secondary metabolites as antimicrobial compounds in gram-negative bacteria is known to be more sensitive when compared to gram-positive bacteria. This is caused by differences in the composition of cell membranes such as Lipopolysaccharides (LPS), lipoproteins, and phospholipids in both types of gram bacteria [19].

4. Conclusion
The results of the research can be concluded that 10 heterotrophic bacterial isolates can produce secondary metabolites and can inhibit the growth of pathogenic bacteria *A. hydrophila*, *P. aeruginosa*, and *V. alginolyticus*. The best isolate is isolate J with a clear zone diameter formed at 12.35 mm and inhibitory activity at 1877.78 mm²/ml.
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