The effect of temperature, salinity and antimicrobial agent on growth and viability of *Aeromonas hydrophila*

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Abstract. *Aeromonas hydrophila* bacteria have been pathogen of Motile Aeromonads Septicemia (MAS) disease that infects all of the freshwater fish. Control of microbe growth can be done by manipulating environmental factors and utilizing antimicrobial agents. The purpose of this study was to investigate the effect of temperature, salinity, and antimicrobial on the growth and viability of *A. hydrophila*. The treatments of the study consisted of temperature, salinity, and antimicrobial tests. Temperature treatment used Trypticase Soy Broth (TSB) medium incubated at 4°C, 28°C, 37°C, and 70°C. Trypticase Soy Agar (TSA) added with NaCl of 0%, 3%, and 10% in salinity treatment. Antimicrobial treatment was performed by disc diffusion method using chloramphenicol (25 ppm and 50 ppm), *Phyllanthus niruri* extract (3 ppt and 30 ppt), formaldehyde (0.4% and 4%), penicillin (25 ppm and 50 ppm), and NaCl 0.9% (control). All treatments were incubated for 24 hours at 28°C and were observed for bacterial growth. The study showed that *A. hydrophila* bacteria were growth optimally in medium with salinity 0% and 3% after incubated in temperatures of 4°C, 28°C, and 37°C. Antimicrobial that inhibits the growth of *A. hydrophila* was Chloramphenicol 25 and 50 ppm; *Phyllanthus niruri* extract 30 ppt and formaldehyde 0.4% and 4%.

1. Introduction

*A. hydrophila* bacteria have been known as the cause of Motile Aeromonads Septicemia (MAS) disease infecting various kinds of tropical freshwater fish including those of Siluridae, Ictaluridae, Claridae, and Cyprinidae families with high mortality rate [1]; [2]. The rapid widespread of the *A. hydrophila* follows the distribution of fish for either fresh or living fish [3]; [4]. Moreover, changes in environmental conditions, including a high density of fish, low dissolved oxygen, excess feeding, and fertilizer, as well as algae booming and upwelling are often associated with this epidemic [5].

*A. hydrophila* are facultative anaerobic bacteria belonging to the Aeromonadaceae family, moving with flagella and not forming spores [6]. *A. hydrophila* colony is round in shape, has curved elevation and flat edge, and displays yellow color in Rhimler-Shotts medium [7], white color in Blood Agar medium and blue-green color in Istratii-Meitert medium [8]. Microscopic observation shows that *A. hydrophila* has cocobacillus shape, Gram-negative, and no capsule [9]. The *Aeromonas* genus is opportunistic bacteria capable of isolation from the water environment, including groundwater, surface water, drinking water, and wastewater. The bacteria can also be detected in food, cheese and milk [10]. *Aeromonas* secretes some kinds of extracellular products, including amylase, chitinase, elastase, arolysine, nuclease, gelatinase, lecithinase, lipase and protease. Such extracellular products are responsible for causing *A. hydrophila* a pathogen in fish [11].

Growth and activities of bacteria can be controlled in a number of factors. Control of over microbial activities can be conducted by controlling environmental factors, such as temperature and
salinity. The study of [12] and [13] showed that temperature and salinity can be a limited factor for bacterial growth. Bacteria growth, especially disadvantageous bacteria, can be inhibited with the antimicrobial agent, which will disturb microbial growth and metabolism [14]. Accordingly, investigation on the effect of temperature and salinity parameters, as well as antimicrobial material on A. hydrophila bacteria viability is necessary to control the bacteria population to the amount of not causing disease that may bring loss to fish culture. A study by [15] shows that Phyllanthus niruri extract can inhibit A. hydrophila growth in vitro. Another study reports that formaldehyde can reduce the number of bacteria in Rainbow Trout eggs [16]. Therefore, the purpose of the present study is to determine the effect of temperature, salinity and antimicrobial agent on the growth and viability of A. hydrophila as well as to identify the effective antimicrobial agent to inhibit its growth.

2. Material and method

2.1. Salinity Treatment

The A. hydrophila culture medium used in this study was TSA (Tripticase Soy Agar). TSA medium was prepared by adding NaCl to obtain different salinity. Salinity used in this study comprised 0%, 3%, and 10% preparation. The media was incubated for 24 hours at 28°C temperature and was observed for the bacteria colony growth.

2.2. Temperature Treatment

TSA medium was used for A. hydrophila bacteria growth. A. hydrophila bacteria culture on TSB (Tripticase Soy Broth) medium was incubated at room temperature (28°C), 4°C, 37°C, and 70°C. The bacteria were then inoculated and striated on TSA medium mount and incubated in room temperature. The growing colony was observed after 24 hours.

2.3. Growth Inhibition with Antimicrobial Agent

Antimicrobial agent used in this study was chloramphenicol (25 ppm and 50 ppm), Phyllanthus niruri extract (3 ppt and 30 ppt), formaldehyde (0.4% and 4%), penicillin (25 ppm and 50 ppm), and NaCl 0.9% as control. Petri dish with the medium was divided into four areas (Figure 1). The amount of 0.1 ml bacteria culture suspension was aseptically taken with a serologic pipette and dropped on TSA medium. The drop was placed on some points and then equally spread. Tweezers were heated on a bunsen burner, and then a dish-shape sterile disc diffusion paper was dipped into the antimicrobial agent for experimentation and was left for a while to make the paper slightly wet, and the solution dripped. The disc diffusion paper was then placed at the center of each area divided according to the antimicrobial agent. The petri dish was incubated in an incubator (28°C) for approximately 24 hours, then observation was conducted by the diameters on the clear zones on each disc diffusion paper were measured. Afterward, the data were analyzed using two-way ANOVA with SPSS software (P>0.05).

Figure 1. Patterns of disc diffusion paper placement
3. Result and discussion

3.1. The effect of salinity and temperature on A. hydrophila bacteria growth

The result of the observation of A. hydrophila growth on culture medium with different salinity and incubation temperature is presented in Table 1. The result of the study shows that A. hydrophila cannot grow on 10% salinity and at an incubation temperature of 70°C, whereas its optimum growth is reached in media with 0% and 3% salinity, and incubation temperature of 4°C, 28°C, and 37°C.

The environmental factors affecting bacterial growth and viability include temperature, pH, light intensity, DO and salinity [14]. Based on temperature endurance, bacteria can be classified into three groups: psychrophilic (growing at a temperature of 0-20°C), mesophilic (growing at temperature of 10-50°C), and thermophilic (growing at a temperature of 40-110°C) [17]. Based on adaptation to salinity, bacteria can be classified into two groups: obligate halophile (growing only in high salinity environment), and facultative halophile (capable of growing in both low and high salinity) [18]. The result of observation shows that A. hydrophila is only capable of growing upon incubation at temperature of 4°C, 28°C and 37°C. This signifies that the bacteria are characteristically psychrophilic as they can grow within wide temperature range. This result corresponds with the previous study in various strains of A. hydrophila, A. hydrophila can growing within temperature range of 0-55°C and can optimally live at temperature range of 15-35°C [19].

The temperature has greatly affected the rates of microbial growth, enzyme synthesis, and inactivation. Consequently, the rate of bacterial growth gradually increases as temperature rises until it reaches its maximum growth rate. Then, above the maximum temperature, the bacterial growth rate reduces rapidly along with the increase in temperature [18]. Moreover, the temperature is the physical factor affecting growth by means of chemical reaction rate and protein molecule structure stability [20]. The chemical reaction will increase as temperature rises since increasing the temperature increases the kinetic energy of the reactants.

The result of observation on A. hydrophila bacteria growth in medium with different salinity reveals that the bacteria can only grow in 0% and 3% salinity. Correspondingly, [21] stated that A. hydrophila bacteria can only grow in 0-3% salinity. The effect of the salinity may result in hyperosmotic stresses limiting microbial growth and activity. High salinity reduces bacterial activity so that the energy produced is low since bacteria use the energy to maintain the permeability of the cell membrane to prevent lysis [22].

| Replication | Salinity | Temperature |
|-------------|----------|-------------|
|             | 0%  | 3%  | 10% | 4°C | 28°C | 37°C | 70°C |
| 1           | +   | +   | -   | +   | +   | +   | -   |
| 2           | +   | +   | -   | +   | +   | +   | -   |
| 3           | +   | +   | -   | +   | +   | +   | -   |
| 4           | +   | +   | -   | +   | +   | +   | -   |
| 5           | +   | +   | -   | +   | +   | +   | -   |
| 6           | +   | +   | -   | +   | +   | +   | -   |
| 7           | +   | +   | -   | +   | +   | +   | -   |
| 8           | +   | +   | -   | +   | +   | +   | -   |
| 9           | +   | +   | -   | +   | +   | +   | -   |
3.2. Inhibition of growth by the antimicrobial agent
The observation upon incubation (Table 2) reveals that the most effective antimicrobial agent to inhibit *A. hydrophila* growth is formaldehyde 4% as demonstrated in the inhibition zone diameter upon incubation. Moreover, Penicillin was not capable of inhibiting the growth of *A. hydrophila*. Thus, it confirms that *A. hydrophila* has already been resistant to antibiotics.

**Table 2.** Clear zone diameter on *A. hydrophila* bacteria medium culture with diffusion disc given antimicrobial agents

| No. | Agent                        | Clear zone diameter (cm)* |
|-----|------------------------------|---------------------------|
| 1   | Chloramphenicol 50 ppm       | 1.50 ± 0.09b              |
| 2   | Chloramphenicol 25 ppm       | 1.25 ± 0.13c              |
| 3   | *Phyllanthus niruri*30 ppt   | 0.08±0.03d                |
| 4   | *Phyllanthus niruri*3 ppt    | (-) (no clear zone)       |
| 5   | Formaldehyde 0.4%            | 1.52 ± 0.06b              |
| 6   | Formaldehyde 4%              | 2.62 ± 0.10a              |
| 7   | Penicillin 50 ppm            | (-) (no clear zone)       |
| 8   | Penicillin 25 ppm            | (-) (no clear zone)       |
| 9   | Control                      | (-) (no clear zone)       |

One-Way ANOVA  
P < 0.000  
Standard Error 0.004

*Different superscript letters in the same column indicate significant difference at P < 0.05 based on Duncan's multiple range test.

The highest clear zone diameter in the surround of disc diffusion paper has resulted from formaldehyde 4% that is 2.62 ± 0.10 cm compared to the other treatments. Formaldehyde is highly toxic to microbes, and it has wide application as a disinfectant for sterilization. Formaldehyde exposure has been shown to result in DNA and protein damage, including the formation of irreversible formaldehyde adducts as well as formaldehyde-catalyzed DNA-DNA, DNA-protein, and protein-protein cross-links[23]. [24] also asserts that formaldehyde works by denaturalizing nucleic acid through the alkylation process. This process works optimally in alkaline pH and less in neutral or acidic pH. The result of this study supports the previous study by [25] confirming that formaldehyde can kill such pathogenic bacteria as *Salmonella typhi*, *Shigella dysenteriae*, *Staphylococcus aureus*, *Klebsiella sp.*, and *Escherichia coli*. [26] claimed that formaldehyde is an effective disinfectant to kill *E. coli* and *Proteus sp.*

Penicillin as antibiotics is ineffective in killing *A. hydrophila* bacteria as evidenced from the absence of a clear zone. Therefore, it is assumed that *A. hydrophila* bacteria were resistant to penicillin antibiotics. The bacteria developed mechanisms of resistance to penicillin by producing beta-lactamase enzymes. This enzyme can break beta-lactam ring functioning to destroy and inhibit bacterial cell wall synthesis [27]. A similar study conducted by [28] reveals that 100% of *A. hydrophila* isolated from India is resistant to antibiotics of the penicillin group. [29] further asserts that *A. hydrophila* isolated from 53 areas in Malaysia develop antibiotic resistance to the penicillin group.
4. Conclusion
This study concludes that *A. hydrophila* bacteria demonstrate optimum growth in medium with 0% and 3% salinity and at incubating temperature of 4°C, 28°C, and 37°C. Formaldehyde is the most effective agent to kill *A. hydrophila* bacteria in comparison to antibiotics (Chloramphenicol and Penicillin) and natural agents (*Phyllanthus niruri*). Hence, further studies are required to identify the effectiveness of formaldehyde to other fish pathogenic bacteria.

5. Reference
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