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# THE GROWTH KINETICS OF *Bacillus* sp. D2.2 AT DIFFERENT pH AND SALINITY

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**Ringkasan** *Isolate D2.2 is a bacterial isolate with 97% homology level with Bacillus sp. This isolate is a biocontrol bacterium capable of inhibiting the growth of Gram positive bacteria and Gram negative bacteria. The use of biocontrol bacteria is one solution of disease problem in cultivation organism. Prior to being applied to the field, D2.2 isolates need to go through a series of tests, one of which is growth kinetics testing under various environmental conditions, such as different degrees of pH and salinity. This is because environmental factors can affect the rapid growth of bacteria. The purpose of this study was to study the bacterial growth kinetics of D2.2 at different pH and salinity. The growth kinetics was observed by measuring optical density (OD) through a method of turbidimetry using a spectrophotometer at a wavelength of 625 nm to the stage of death. The results showed that the fastest growth rate was found at 20 ppt salinity with 0.179 h-1 and generation time of 5,588 hours. While in all pH treatments, generation time and growth rates achieved all treatments have the same pattern.*

**Keywords** *growth kinetics, Bacillus sp. D2.2, pH, salinity*

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## PENDAHULUAN

The environment acts as an indicator of the state of the organism in it. If the environmental conditions are bad then the organisms inside it become stressed and decrease in the body's resistance. As a result, pathogenic diseases such as bacteria can easily attack aquaculture organisms and cause infectious diseases. In a shrimp pond, Kharisma and Manan (2019) stated that the abundance of *Vibrio* sp. can be used as an early detection of *Vibriosis disease. V. harveyi, V. alginolyticus* and *V. parahaemolitycus* are the types of bacteria that most commonly cause Vibriosis in shrimp. Furthermore, bacteria can also cause infection in fish, such as Gram-positive bacteria *Staphylococcus aureus* which is a cause of food-poisoning

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due to production of enterotoxins therefore the high population of this bacteria indicates the general quality of fish (Ali, 2014), while from Gram negative bacteria such as *Aeromonas hydrophila* can result in a significant reduction in fisheries production due to high mortality (Pachanawan et al., 2008).

Infectious diseases should be treated as early as possible with appropriate treatment. Prevention and treatment are mostly done by giving antibiotics and other chemicals, but the use of antibiotics and chemical ingredients to eradicate pathogenic bacteria in the long term can have a negative effect on the surrounding environment and resistance to pathogenic bacteria. Some weaknesses in the use of antibiotic ingredients include, can make the bacteria resistant to antibiotics, can cause residues in the body of cultivating organisms, damage the environment and can endanger human health to consume aquaculture organisms that have been given antibiotic substances(Kümmerer, 2004; Kesarcodi-Watson et al., 2008). This is what causes developed countries to prohibit the use of antibiotics and fishery products that contain antibiotic residues (Carlet et al., 2012).

The use of biocontrol bacteria is one solution to solving disease problems in aquaculture organisms. In addition, the use of biocontrol bacteria is also environmentally friendly and can suppress the growth of pathogenic bacteria. Biocontrol bacteria utilize the antagonistic relationship between organisms to get space for movement and food. Biocontrols of pathogens have been reported potential in aquaculture such as biocontrol of vibriosis in shrimp (Chythanya et al., 2002; Widanarni et al., 2003; Ravi et al., 2007).

*Bacillus* sp. D2.2 is isolated from traditional shrimp ponds in Mulyosari Village, Pasir Sakti District, East Lampung Regency and able to inhibit the in vitro growth of *Vibrio harveyi* (Setyawan et al., 2014). Moreover, this isolate was proven to be non-pathogenic bacteria (Hardiyani et al., 2016). Before being applied to the field, isolate *Bacillus* sp. D2.2 needs to be tested for the activity of antimicrobial compounds which are influenced by several factors such as pH, salinity, stability of antibacterial compounds, temperature, inoculums of incubation microorganisms, and metabolic activity (Irianto, 2006).

Kinetics of bacterial growth is one way to determine the speed of cell biomass production and the influence of the environment (nutrient restricted) on the speed of cell growth (Schaechter, 2009). Kinetics of bacterial growth is influenced by environmental factors such as temperature, pH and NaCl (Sutherland et al., 1994). The aim of this study was to observe the growth of *Bacillus* sp. D2.2 in media using various ranges of pH and salinity as controlling factors.

## MATERI DAN METODE

### *Bacterial strains and media*

*Bacillus* sp. D2.2 isolated from traditional tiger shrimp in East Lampung (Setyawan et al., 2014) was inoculated on seawater complete-agar (SWC, 5 g bacto peptone (Oxoid, England), 1 g yeast extract (Oxoid, England), 3 ml glycerol, 15 g bacto agar (Oxoid, England), 750 ml seawater, and 250 ml

distilled water) and then transferred to SWC-broth (without agar) with rotary shaker at 140 rpm for 24 h at 30°C (Harpeni et al., 2018). *V. alginolyticus, A. hydrophila,* and *S. aureus* were bacterial collections of the Laboratory of Fisheries Culture, Department of Aquaculture, University of Lampung and cultured on trypticase soy agar (TSA) medium (Oxoid, England).

Bacterial growth stage

Isolates of *Bacillus* sp. D2.2 were cultured on SWC broth media at salinity 0, 10, 20 and 30 ppt; pH 6, 7 and 8 and incubated at 300 C. Cell density measurement (optical density) was carried out every 3 hours using a spectrophotometer at 625 nm wavelength to the death stage. Calculations of *Bacillus* sp. D2.2 growth rate and generation time were calculated based on the following formulas (Augustin et al., 1999):

$$\mu = \frac{\log 10 X_t - \log 10 X_0}{0.301 t} \quad (1)$$

where: μ = Growth rate; $X_t$ = Total density at exponential time; $X_0$ = Number of initial densities at exponential time; and t = Exponential growth time

$$n = \frac{\log N - \log N_0}{0.301} \quad (2)$$

where: n = Number of Generations; Log N = End cell number; and Log $N_0$ = Number of initial cells

$$Generation\,time = t/n \quad (3)$$

where: t = Exponential growth time; and n = Number of Generations

*Production of antibacterial substances*

Antibacterial extraction was a broth of SWC fermentation medium and incubated with agitation at 150 rpm until the death stage at 300 C. After incubation, it was centrifuged at 5000 rpm for 25 minutes to separate the supernatant and bacterial cell pellet (Isnansetyo et al., 2009). Bacterial cell pellet was used in this study based on Setyawan et al. (2014). Bacterial cell pellet was washed using phosphate buffer saline (PBS) and 15 ml of the same buffer was added. The suspension of bacterial cell was then broken down using an ultrasonicator for 5 minutes. The bacterial cell suspension was centrifuged at a speed of 5000 rpm for 25 minutes to obtain the supernatant. The supernatant was divided into two portions. The first portion was added with 50 ml distilled water and then extracted with ethyl acetate twice. The ethyl acetate layer was evaporated using a rotary evaporator at 50ºC, while the second portion was saturated with ammonium sulfate with the same treatment as the first portion.

*Antagonistic spectrum activity of Bacillus sp. D2.2*

Antagonistic spectrum activity of *Bacillus* sp. D2.2 in different range of salinity and pH was carried out using diffusion method. About 24 h cultures of *V. alginolyticus, A. hydrophila*, and *S. aureus* in 10 ml trypticase soy broth (TSB) media (Oxoid, England) were inoculated respectively into a petri dish containing TSA media. Then two paper discs with a diameter of 6 mm were impregnated with the extraction of ethyl acetate cells and saturation of ammonium sulfate cells respectively. Twenty five ppm of Amoxicillin (PT Indo Farma, Bekasi) was used as positive control while distilled water was used as negative control. Those paper discs were placed on agar plate of TSA medi-

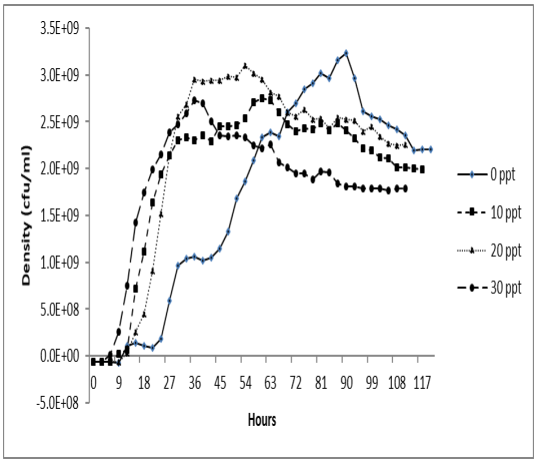
um and then incubated at 300 C for 24 h. After the incubation period, the diameter of the inhibition zone formed around the paper disc was measured.

## HASIL DAN PEMBAHASAN

### *Growth Stages in Different Salinity Treatments*

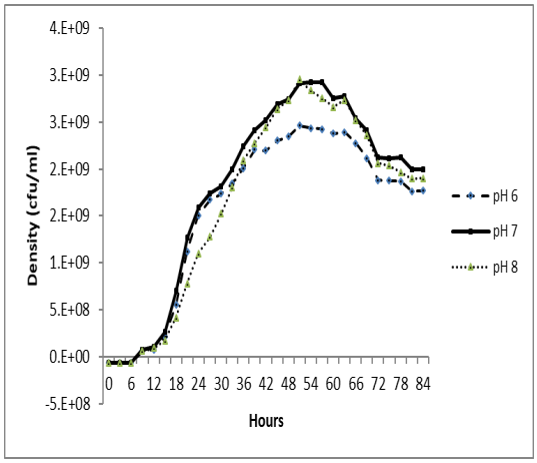
Different salinity treatments showed that these bacteria had fast growth at 30 ppt salinity and had slow growth at 0 ppt salinity. From the graph, it can be seen that the lag stage in all treatments except 0 ppt salinity had the same time period that occured until the 9 hour then entered the exponential stage. The exponential stage in salinity treatment of 10, 20 and 30 ppt showed the similar pattern until around 30 hours. In contrast to the treatment of 0 ppt salinity, the exponential stage occured longer until the 94 hours. There was no stationary stage at 0 ppt salinity recorded, since bacterial cells directly encountered the death stage. Furthermore, salinity 20 and 30 ppt faced the stationary stage at 36 hours while salinity 10 entered the stationary stage in 60 hours then met the death stage (Figure 1). The salinity treatment of 20 ppt has a long stationary stage from 30-54 hours (18 hours stationary stage).

*Bacillus* sp. D2.2 had different growth stages in reaching the death stage. Zwietering et al. (1991)and Buchanan et al. (1997) stated that the growth rate of bacteria shows several stages or phases, a lag stage that is followed by an exponential stage, and finally it shows a decreasing growth rate down to zero stationary stage. If linked between population growth acceleration and a certain time will form a growth curve. Pelczar et al. (1981) states that the lag stage (adaptation) is a stage where there is no population growth, cells only experience changes in chemical composition, increase in size and increase in intracellular substances. The adaptation process includes the synthesis of new enzymes that are appropriate to the media and recovery of toxic metabolites such as acids, alcohols and bases (Purwoko, 2009).




**Gambar 1** The growth of *Bacillus* sp. D2.2 in different range of salinity

Among the treatments carried out at 0 ppt salinity the lag stage had remained until the 27th hour then entered the exponential stage until the 94th hour. Different from 30 ppt salinity treatment, where the stage lag occurs only 6 hours, then went to the exponential stage faster until approximately the 40th hour. While in salinity of 10 ppt and 20 ppt the growth stage was not much different, the lag stage occurs in the 12th hour. *Bacillus* sp. D2.2 reached exponential stage faster in salinity 30 ppt than presumably because the isolate had been taken from traditional shrimp ponds in East Lampung with salinity ranged from 25-30 ppt.




**Gambar 2** The growth of *Bacillus* sp. D2.2 in different range of pH

So that, *Bacillus* sp. D2.2 did not need longer time to adapt to the salinity culture condition.

*Growth Stages in Different pH Treatments*

Similar with those in salinity treatments, the lag stage in the treatment of pH 6, 7 and 8 occured until the 9th hour then entered the exponential stage. While the stationary stage the pH treatment began at the 54th hour before experiencing a decrease to the death stage (Figure 2). Based on the graph of density comparison on pH treatment shows, the highest to lowest density in a row are treatment pH 6, pH 8 and pH 7. The treatment of pH 7 shows the lowest density of 1.23 x 109 CFU/ml.

The growth of *Bacillus* sp. D2.2 in pH treatments exhibited similar pattern. The bacterial exponential stage D2.2 had occured from the 6th hour until the 48th hour. This showed that all three pH treatments did not significantly affect the bacterial growth. It was indicated that the growth of *Bacillus* sp. D2.2 were stable in pH range 6-8.

*Generation Time, Growth Rate and Harvest Time of Bacillus sp. D2.2*

**Tabel 1** Generation time and growth rate of *Bacillus* sp. D2.2

| Treatments | Generation time (hours) | Growth rate (hour-1) | Harvest time (hours) |
|---|---|---|---|
| 0 ppt | 14.783 | 0.069 | 95 |
| 10 ppt | 7.613 | 0.134 | 65 |
| 20 ppt | 5.588 | 0.179 | 60 |
| 30 ppt | 5.976 | 0.173 | 57 |
| pH 6 | 7.311 | 0.136 | 66 |
| pH 7 | 7.395 | 0.136 | 66 |
| pH 8 | 7.048 | 0.156 | 66 |

The treatment of 0 ppt salinity the lowest bacterial growth rate reached 0.069 hour-1 with the longest generation time reaching 14.78 hours, while increasing salinity up to 30 ppt had shorten generation time more than half and had increased generation rate. Harvesing times of *Bacillus* sp. D2.2 isolates were usually produced as cultures reach the end of stationary stage or before the death stage. They also had similar trend to the generation time and the growth rate. They had been harvested faster in higher salinity, range from 57 to 95 hours (Table 1). Compare to salinity treatments, generation time and growth rate of *Bacillus* sp. D2.2 among all pH treatments were alike, just about 7 hours and 0.136 hour-1 sequentially, so that the isolate could be harvested together in 66 hours (Table 1).

The exponential stage of *Bacillus* sp. D2.2 in this study was the longest stage which required one until three days before entering the stationary stage. Based on Pelczar et al. (1981), during the exponential stage, bacterial cells will divide at a constant rate, bacteria double at the same rate, metabolite activity is also constant and the state of growth is balanced.

Entering the static stage, bacteria will produce secondary compounds such as antibiotics. Before entering the death

stage some bacteria are able to survive the static stage from hours to several years before finally entering the stage of death (Purwoko, 2009). *Bacillus* sp. D2.2 had a static stage that lasts for several hours before finally entering the death stage (Figure 1).

Entering the stage of death, all cells will die faster in a few days to several months (Pelczar et al., 1981). *Bacillus* sp. D2.2 utilized glycerol as the main carbon source and other sources in the media used. The provision of carbon sources with different concentrations can also improve the life stage of bacteria (Devianto and Kardena, 2010).

Growth is an increase in mass or total cell number (for example in a culture), the rate of growth is the increase in the number of cells per unit of time, while the generation time is the time interval needed by a cell to divide. The results showed that salinity treatment of 20 and 30 ppt had the fastest growth rate (Table 1), while all of pH treatments exhibited the average growth rate of 0.136 hour-1. This study showed that the higher the salinity accelerated the growth rate and generation time while different range of pH did not result different value of either the growth rate or generation time. Arellano-Carbajal and Olmos-Soto (2002) reported, their isolate, *Bacillus* LMM-12 had the growth rate 0.75 h-1.

Observation of bacterial growth stage D2.2 aimed to determine harvest time to produce antibacterial substances. In this study the production time of antibacterial compounds was carried out at the stationary stage entering the death stage where the time of each treatment was different, at 0 ppt salinity treatment at the 95th hour, 10 ppt salinity at 65 hours, 20 ppt salinity in the 60th hour and 30 ppt salinity in the 57th hour. While at the pH treatment the production time is at the same time which was at 66th hour. The prediction of the harvest time was suspected at the time that *Bacillus* sp. D2.2 had produced secondary metabolites or antibacterial compounds. Isnansetyo et al. (2009) stated in their study that bacteria from the genus Pseudoalteromonas from S2V2 strain produced the highest antimicrobial compounds at 96th hour or in the stationary stage to the death stage. In this stage the highest activity of the bacteria more likely released secondary or antimicrobial metabolites (Tsai and Su, 1999; Arellano-Carbajal and Olmos-Soto, 2002; Liu et al., 2006).

*Antagonistic spectrum activity of Bacillus sp. D2.2*

Antibacterial substance produced by *Bacillus* sp. D2.2 was present in the ethyl acetate extract of bacterial cells. *Bacillus* sp. D2.2 inhibited the growth of *V. alginolyticus, A. hydrophila*, and *S. aureus* in various salinities. The inhibition diameter of *Bacillus* sp. D2.2 against *V. alginolyticus* ranged from 6-14 mm, against *A. hydrophila* ranged from 2-7 mm, and against *S. aureus* ranged from 2-4 mm. On the other hand, the inhibition diameter that had been formed by *Bacillus* sp. D2.2 against the pathogenis bacteria was relatively smaller in different pH, ranged from 2-9 mm (Table 2).

Although smaller, antibacterial substance produced by *Bacillus* sp. D2.2 was also present in the ammonium sulfate precipitate. Mostly the inhibition zone was produced by *Bacillus* sp. D2.2 against *V. alginolyticus* and *A. hydrophila* from different salinities, range from

**Tabel 2** Inhibition diameter (mm) of *Bacillus* sp. D2.2 in different salinity and pH

| Pathogenic bacteria | Salinity/pH | Extraction of ethyl acetate | Saturation of ammonium sulfate | Positive control (antibiotic) | Negatif control (aquadest) |
|---|---|---|---|---|---|
| | 0 ppt | 8 | 6 | 20 | - |
| | 10 ppt | 14 | - | 18 | - |
| | 20 ppt | 6 | 6 | 23 | - |
| *V. alginolyticus* | 30 ppt | 10 | 3 | 20 | - |
| | pH 6 | 4 | - | 20 | - |
| | pH 7 | 9 | 4 | 19 | - |
| | pH 8 | 5 | 3 | 23 | - |
| | 0 ppt | 4 | - | 17 | - |
| | 10 ppt | 3 | 4 | 17 | - |
| | 20 ppt | 2 | 3 | 19 | - |
| *A. hydrophila* | 30 ppt | 7 | 4 | 18 | - |
| | pH 6 | - | - | 17 | - |
| | pH 7 | 2 | - | 17 | - |
| | pH 8 | 3 | - | 19 | - |
| | 0 ppt | 3 | - | 19 | - |
| | 10 ppt | 2 | - | 17 | - |
| | 20 ppt | 4 | - | 19 | - |
| *S. aureus* | 30 ppt | 2 | 2 | 24 | - |
| | pH 6 | - | - | 19 | - |
| | pH 7 | 3 | - | 17 | - |
| | pH 8 | 2 | 3 | 19 | - |

3-6 mm, but was barely detected from different range of pH (Table 2).

This study indicated that antibacterial substance produced by *Bacillus* sp. D2.2 mostly from the ethyl acetate extract. Abraham (2004) in his research on the antibacterial activity of *Alteromonas* P7 stain also showed the similar results, since it was suspected that antibacterial compounds were still stored in the cell wall of the bacteria. Ethyl acetate is a specific solvent that can dissolve water up to 3% and solubility of up to 8% in water at room temperature. Ethyl acetate is a medium polar solvent (semi polar) as well as organic compounds which are esters of ethanol and acetic acid (Daluningrum, 2009). Ethyl acetate was used to obtain secondary metabolites in *Bacillus* sp. D2.2 which function as antibacterial compounds while ammonium sulfate was used to obsrve enzyme activity. The production of antibacterial substances obtained by extraction and cell saturation showed the presence of clear zones in the diffusion test using bacteria *V. alginilyticus, A. hidrophyla* and *S. aureus*. This proved that the *Bacillus* sp. D2.2 had antibacterial compounds that could inhibit the growth of the pathogenic bacteria in the diffusion test. The results showed that the best inhibition zone was found in cell extraction tested on *V. alginolyticus*. This is consistent with research conducted by Setyawan et al. (2014) where cell extracts tested on *V. alginolyticus* obtained the highest inhibition zone.

The study showed that *Bacillus* sp. D2.2 was able to inhibit the growth of pathogenic bacteria from both Gram-positive and Gram-negative, but when compared with the positive control, there was still not enough potential as an antibacterial compound producer. Nevertheless, Pan et al. (2009) scored bacteria that had diameter of the zone between 0 and 3 mm as weak, between 3 and 6 mm as good, and more than 6 mm as a strong inhibition. The best inhibition activity of on types of *Bacillus* sp.

D2.2 was present against Gram-negative bacteria, *V. alginolytcus*. In conclusion, salinities created different growth pattern of *Bacillus* sp. D2.2. The isolate reached the fast growth rate in salinity 20 and 30 ppt with 0.179 and 0.173 h-1 and generation time of 5.59 and 5.97 hours respectively. In contrast, the isolate grew in similar pattern when the pH treatments were applied. The highest antibacterial activity (14 mm inhibition zone) of *Bacillus* sp. D2.2 was found in the antagonistic test against *V. alginolyticus* with ethyl acetate extract.

## SIMPULAN

In conclusion, salinities created different growth pattern of *Bacillus* sp. D2.2. The isolate reached the fast growth rate in salinity 20 and 30 ppt with 0.179 and 0.173 h-1 and generation time of 5.59 and 5.97 hours respectively. In contrast, the isolate grew in similar pattern when the pH treatments were applied. The highest antibacterial activity (14 mm inhibition zone) of *Bacillus* sp. D2.2 was found in the antagonistic test against *V. alginolyticus* with ethyl acetate extract.

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