Effect of temperature and pH combination on vegetative cell growth of *Bacillus megaterium*

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Abstract. *B. megaterium* is a potential bacterial species to be recommended as a probiotic which has been tested to have beneficial effects for fish. Vegetative cells availability is not only influenced by culture media composition but is also affected by environmental conditions such as temperature and pH. The purpose of this study was to determine the effect of temperature and pH combination on vegetative cell production and growth rate of *B. megaterium*. This study used a combination of temperature treatment (37, 44 and 51 °C) and pH (4, 7 and 10) to produce *B. megaterium* planted in wheat flour (*Triticum aestivum*) media. Results showed that the highest vegetative density was found at 37 °C and pH of 10 as much as 11 x 10⁸ cells mL⁻¹ with a growth rate of 0.65 hour⁻¹. In addition, the morphology of bacteria from this study presented the length and width of *B. megaterium* at 2.44 – 3.82 µm and 1.11 – 1.31 µm, respectively.

1. Introduction

The presence of probiotics with all their advantages in the aquaculture industry provides a new strategy to limit utilization of antibiotics. Probiotics themselves have a positive impact for farmers as an effort to increase fish culture production. Probiotics in the form of vegetative cells are useful not only for improving health status of fish [1, 2, 3], supporting growth performance [4, 5] and inhibiting pathogen bacteria [6, 7], but also reducing ammonia levels in aquaculture organism media [8, 9].

Selection of bacterial species for use as probiotics must complete and pass the standard requirements of both *in vitro* and *in vivo* testing. One of these requirements is the availability of metabolites secreted by bacteria for instance proteins, bacteriocins, extracellular vesicles which have a beneficial effect on the host that uses it [10, 11]. *B. megaterium* is a potential species to be proposed as a probiotic and has been applied to several aquaculture species [12, 13, 14]. The ability of these bacteria to produce extracellular protease enzymes [15] and antibiotic compounds [16] can be an indicator for their sustainable use.

Production of vegetative cells of *B. megaterium* to support its availability as a probiotic candidate must focused on the nutritional composition of the growth medium such as carbon sources, nitrogen sources and C/N ratio [17]. However, environmental factors such as temperature and pH must also be determined for optimal levels to maximize the growth process of bacteria. Temperature will affect cellular processes [18] and enzyme activities of bacteria [19, 20] while pH affects the chemical energy in an environment which is later involved in microbial oxidation reactions [21, 22]. In addition, pH also...
controls microbial activity because it supports regulate microbial metabolism [22]. Combination of temperature and pH treatments has not been confirmed yet to increase the production of B. megaterium grown on low cost media. Thus, this study aims to determine the effect of temperature and pH combination on vegetative cell production and growth rate of B. megaterium.

2. Methods

2.1. Starter of bacteria
B. megaterium isolate was cultured on nutrient agar (NA) media with the addition of 2% Natrium chloride and was inoculated as much as a loop of bacteria into the nutrient broth (NB) media. The bacteria were cultured in an incubator shaker at 37 °C with a speed of 120 rpm for 18 hours. These bacteria were then used as a starter to be planted on the treated media. The initial density of the starter bacteria was calculated before planting on the test medium.

2.2. Culture media
The test media used in this study consisted of wheat flour (T. aestivum) as a carbon source and ammonium chloride (NH₄Cl) as a nitrogen source. The C/N ratio was determined by a ratio of 15:3. These ingredients were mixed and were dissolved with 50 ml of distilled water (Aquadest®) in a 100-ml-Erlenmeyer flask. Afterwards, all the test media were sterilized using an autoclave at 121°C (1 atm pressure) for 15 minutes and were supplemented by several minerals based on research by [17]. Stock culture in NB media transferred to culture media as much 1% (v/v) with initial density of B. megaterium at 2.2 x 10⁸ cells ml⁻¹.

2.3. Temperature and pH treatment
This research used Completely Randomized Factorial Design (CRFD) so there was a combination between temperature and pH treatment. The temperature treatments in this study were 37 °C, 44 °C and 51 °C while the pH treatments were 4, 7 and 10, respectively. The acid and alkaline indicators used were 0.1 N HCL and 0.1 N NaOH to set a pH in media. Vegetative cells were incubated for 24 hours in an incubator shaker at a speed of 120 rpm and the temperature was set according to the combination of treatments given. Each combination of temperature and pH treatment was repeated three times.

2.4. Counting of vegetative cells
Sampling of vegetative cells as much as 2 ml was put into a sterile microtube. Samples were taken once at 0 minutes, 15 minutes, 30 minutes, every 1 hour for the first 6 hour and then every 2 hours for the rest 18 hour of incubation period. Calculation of vegetative cells was done microscopically using the Neubauer chamber. Vegetative cell sample at each sampling time was diluted first using sterile distilled water to ease the calculations.

2.5. Growth rate
Measurement of the growth rate aims to determine the average change in the number of cells in the culture media per unit of time (minutes or hours). Calculation of the growth rate was done by adjusting the exponential growth model to the growth curve.

2.6. Morphologically vegetative cell by SEM
Observation of vegetative cell morphology was carried out using scanning electron microscopy (SEM) after obtaining the optimal combination of temperature and pH treatments. SEM preparation was conducted by culturing vegetative cells on the media with the optimal environmental parameters and had shown an exponential phase. Then 1 ml of the media was centrifuged (2000 x g .4 °C) and incubated with 3% glutaraldehyde. The samples were then observed under a SEM TM 300 microscope.
2.7. Statistical analysis

Data of vegetative cell density and growth rate were analyzed by two-way ANOVA using statistical software. The difference between treatments can be seen from the DMRT (Duncan’s Multiple Range Test) (p<0.05).

3. Result and Discussions

3.1. Vegetative cells of *B. megaterium*

Vegetative cell production of *B. megaterium* cultured at different combination of temperature and pH is shown in Figure 1.

![Figure 1](image_url)

**Figure 1.** Vegetative cells production of *B. megaterium* under different combination of temperature and pH: A) Temperature of 37 °C; B) Temperature of 44 °C; C) Temperature of 51 °C
Production of vegetative cells with a combination of temperature and pH treatments showed a difference in incubation time to reach the bacterial growth phases. Vegetative cell density was recorded based on the growth phase consisting of lag phase, exponential phase, stationary phase and death phase. The number of vegetative cells with various sampling times revealed the results of different vegetative cell densities. Growth patterns of whole treat combinations exhibited almost the same graph trends. Lag phase is a stage of bacterial to adapt in a new growing environment where the fastest time occurred at 7 minutes in the medium with a treatment combination of temperature at 37 °C and pH of 10. The total of vegetative cells gradually increased until it reached an exponential peak at 12 hours for a temperature of 37 °C and 10 hours for a temperature of 44 °C in all given pH differences. However, at a temperature of 51 °C, the exponential peak appeared more rapidly at the 4 hours incubation time at both pH of 7 and 10. Stationary phase in throughout treatments was possible during an interval of 1 hour at temperature of 37 °C and 44 °C while this phase happened on interval of 30 minutes at a temperature of 51 °C which was not observed in this study. After passing through the stationary phase, vegetative cell quantity decreases until the end of the incubation time which was not only due to reduced nutrients in the environment but also influenced by environmental stresses.

Growth phases perceived in each combination of temperature and pH treatments in this research indicated a difference in the duration of time to reach the growth phase. At 37 °C and 44 °C, they take relatively longer to pass through an exponential phase when compared to 51 °C across all given pH differences. [23] reported that the resistance of a bacterial species to heat was highly dependent on the physiological status of bacterial species and nutritional components of growth media. [24] stated that as environmental temperature condition increases, it will raise metabolic rate of bacteria then the metabolism decreases sharply at higher temperature. pH factor also affected vegetative cell population in the culture media. B. megaterium cell used in this research had a limited growth ability at acidic pH. This condition can be reasoned by bacterial periplasm not being able to withstand in low pH conditions so that it can cause cell damage. In addition, the result that occurs from an acidic pH was a decrease in enzyme activity in the cytoplasm of bacteria [25].

Density of vegetative cells as presented in Figure 1 reached highest peak in each medium at temperature of 37 °C and at 44 °C in same pH (pH of 10) which the total production were 11 x 10⁸ cells. mL⁻¹ and 8.5 x 10⁸ cells. mL⁻¹, respectively. While at 51°C accounted in pH of 7 with the value 3.7 x 10⁸ cells. mL⁻¹. Temperature and pH are physical factors that play an important role in influencing the production of biomass from a microorganism [26]. Several studies have shown that there were differences in optimal temperature and pH for growth in other species such as Staphylococcus reaching the shortest lag time at pH of 7 with a temperature of 35 °C, Listeria in pH of 7 at 40 °C [27] and group lactic acid bacteria (LAB) grow well at pH of 6.2 with 37 °C in MRS broth [28]. Differences in species, strains and culture media used will affect the requirement for environmental conditions (temperature and pH) to get the maximum growth of bacteria. Temperature and pH settings in culture media are essential key to be used in identifying a scale of bacteria tolerance to the culture environment.

3.2. Growth rate of B. megaterium
Growth rate of B. megaterium grown at combination of temperature and pH is provided in Figure 2. The maximum vegetative cell growth rate of B. megaterium in whole treatment combinations showed a value of more than 0.35 hour⁻¹. The highest vegetative cell growth rate happened in the combination treatment of temperature at 37 °C and a pH of 10 with a value of 0.65 hour⁻¹. The shortest vegetative cell generation time also occurred in this combination treatment at 63.98 minutes. The higher the temperature given, the lower the bacterial growth rate obtained. This can be seen from Figure 2, the variation in pH at 37 °C has a higher growth rate than the variation in pH at 44 °C and 51 °C. Environmental conditions of the growing media of each bacterial strain will affect the average growth produced [28, 29, 30]. Each species of bacteria has a different optimal temperature for growth such as B. cereus grows optimally at 39.9 °C, Listeria monocytogenes at 41.1 °C and Salmonella at 39.5 °C [31]. Several results from previous studies indicated that bacteria grown at optimal temperatures have various growth rate values, such as Hydrogenophaga pseudoflava CR3/2/10 and Brevisibacterium sp. strain CR3/1/15 presented.
growth rate values of 0.27 hour\(^{-1}\) and 0.11 hour\(^{-1}\), respectively [32]. Even in other study, *Bacillus* sp. cultured at 30 °C can reach a maximum growth rate of 0.98 hours [33].

![Figure 2. Growth rate of *B. megaterium* under different combination of temperature and pH](image)

Apart from temperature factors, growth rate in this study also depends on the variation in pH given. Almost all factors in the treatment combination showed that vegetative cells of *B. megaterium* prefer to live in an alkaline pH condition so that the value of the growth rate obtained was also higher. The same statement with temperature parameters, pH in the environment must also be in an optimal range for bacterial growth [34]. The impact of pH in the culture environment which deviates from the optimal pH level of bacteria is decreasing bacterial growth [35]. Another theory explains that the determination of pH conditions in culture media is not only influenced by the strain of bacteria being planted, but also is initiated by the carbon source in the media used [36]. Combination between temperature and pH is one of the alternative treatments to obtain the maximum growth rate of *B. megaterium* in a suitable environment. This case indicates that the bacteria grown in the optimal conditions will increase the growth rate of these bacteria [32].

### 3.3. Cell vegetative morphology of *B. megaterium* by SEM
Vegetative cell morphology of *B. megaterium* grown at optimal temperature and pH is presented in Figure 3.

In terms of morphology, vegetative cells grown on commercial media did not show any significant difference in shape from those grown in wheat flour (*T. aestivum*) media with a temperature of 37 °C and pH of 10. Size of bacteria growing under a combination of temperature and pH optimal in this study had a length of 2.44 – 3.82 µm and a width of 1.11 – 1.31 µm. Another study of [37] recorded that the size of these bacteria has a length of 3.0 – 4.0 µm. The difference in bacterial size can be influenced by differences in the bacterial strains used, isolation of bacterial samples, media and environmental characteristics of the bacterial growth media. Meanwhile [38] noted that the distribution of bacterial cell size in the growing medium depends on the self-division process, although the size variation that occurs is only up to 10% in cases of enteric rod-shaped bacteria. The difference in bacterial size does not affect the effectiveness of these bacteria in producing extracellular enzymes for application such as in probiotic industry.
Figure 3. Vegetative cell morphology of *B. megaterium* under different culture treatment; A) commercial medium; B) wheat flour (*T. aestivum*) at temperature of 37 °C and pH of 10 (SEM images at 10,000X magnification).

4. Conclusion

Temperature and pH combination affected vegetative cell production and growth rate of *B. megaterium*. The highest vegetative cell (11 x 10⁸ cells. ml⁻¹) and growth rate (0.65 hour⁻¹) of *B. megaterium* were found in temperature of 37 °C and pH of 10. The length and width of *B. megaterium* reached 2.44 – 3.82 µm and 1.11 – 1.31 µm, respectively.

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