Effect of different carbon doses of tapioca (*Manihot esculenta*) flour on vegetative cells and spore production of *Bacillus megaterium*

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Abstract. *Bacillus megaterium* is a spore forming bacteria that is mostly used as a probiotic in aquaculture. Spore formation for probiotic production using carbon source is costly. This research evaluated the effect of different carbon doses of tapioca (*Manihot esculenta*) flour on vegetative cells, spore production, sporulation efficiency and spore germination of *B. megaterium*. Experiments were carried out in Aquaculture Laboratory, University of Brawijaya. In flasks, 50 ml of growth media were used and were inoculated with 1% of *B. megaterium* (2.6 x 10⁸ cells/ml). The cultures were mixed (120 rpm) and incubated at 37 °C for 120 hours with 4 different treatments of carbon doses (5, 10, 15 and 20 g/L, respectively). The results showed that the highest vegetative cells (8.4 x 10⁷ cell/ml) and spore production (4.1 x 10⁷ spore/ml) of *B. megaterium* were found at 15 g/L carbon dose. However, it was not followed by high sporulation efficiency (only 49.01%). The high spore germination was observed in more than 5 g/L carbon dose. Thus, 5-15 g/L carbon doses of tapioca flour could positively enhance vegetative cells, spore production, sporulation efficiency and spore germination of *B. megaterium* and could be used as a potential source of probiotics in aquaculture.

1. Introduction

*Bacillus* is a genus that commonly used as a probiotic in the aquaculture industry [1,2]. Several advantages of using it for the aquatic animal are supporting digestive fish function through an essential enzyme production [3], reducing ammonia and nitrite concentration [4] and improving immune system and health status of fish [5]. On the other hand, in aquaculture practice, there are some problems of probiotic in the vegetative cell such as inactivated bacteria in an acid environment (stomach) [6] and stressed in limited of nutrition [7]. The impact of this case is bacterial cell damage [8] consequently it will not work optimally in the digestive tract of an organism. One of the solutions to repair the problems is by using spore bacillus as probiotic [9]. The spore is dormant, resistant to lack of nutrition, heat temperature and organic chemicals [10,11]. The spores can survive in a long time even hundreds of years [12]. One of *Bacillus* species that produce spore is *B. megaterium* which has exosporium as a special characteristic in the outer membrane [13].
Production of *B. megaterium* spore requires medium that consisted of carbon source. Glucose and lactate as commonly commercial carbon source but they have costly [14,15]. Alternative carbon source that could be used in medium to produce spore comes from local farming product such as tubers and beans. Tapioca (*Manihot esculenta*) flour is a carbon source with the composition of organic carbon more than 40% based on experimental study (data unpublished). In Indonesia, tapioca flour is cheap, easily found and contained high carbon content. Development of low-cost medium is needed to reduce the cost of spore production which can be used as a probiotic in fish farming. Thus, this study evaluated the effect of different carbon levels of tapioca flour on vegetative cells, spore production, sporulation efficiency and spore germination of *B. megaterium*.

2. Materials and methods

2.1 Strain of bacteria

*B. megaterium* used in this study was isolated from shrimp pond in Tuban East Java which had confirmed by molecular analysis 16SrRNA.

2.2 Culture Media

Tapioca flour was used as the carbon source and ammonium chloride (NH₄Cl) served as nitrogen source. The doses of the carbon were divided by four treatments (5, 10, 15 and 20 grams per liter of distilled water (Aquadest®), respectively), while the nitrogen doses were adjusted to the carbon doses to achieve C:N ratio 5:1. Culture media consisted of tapioca flour and ammonium chloride based on treatments were dissolved with Aquadest® and enhanced with some minerals such as CaCO₃ (0.3 g), MgSO₄ (0.00033), MnSO₄ (0.12 g), FeSO₄·(0.084 g), CaCl₂ (0.09 g) [16], with little modifications. All treatments were replicated three times.

2.3 Inoculum preparation

Bacteria from the frozen stock were inoculated by a loop needle to a 100 mL shake flask containing 20 mL of nutrient broth as an inoculum medium. The culture was incubated in rotary shaker at 37 °C and 120 rpm for 18 hours. Bacterial cells were counted as an initial density that would be the stock culture.

2.4 Cultivation condition

The research used 100-ml-erlenmeyer flasks with 50 ml medium and were inoculated 1% (v/v) stock culture of *B. megaterium* (2.6 x 10⁸ cells. ml⁻¹) in each flask. The culture conditions were similar to inoculum preparation in rotary shaker (at 37 °C, 120 rpm) for 24 hours of vegetative cells and continued to 120 hours for sporulation. In continuum, the spore germinations were carried out in the 100-ml-erlenmeyer flasks with 50 ml nutrient broth medium and were inoculated for high spore production in all treatments. The desired density of spore germination in each of treatments was 10⁶ cfu/ml.

2.5 Determination of vegetative cells, spore count, sporulation efficiency and spore germination

Characteristics of vegetative cells and spore were observed and determined by their shapes. Vegetative cell has a rod-shaped and spore has a circle one. They were diluted with Aquadest® for sterilization to simplify counting before counting in the Neubauer chamber. Sporulation efficiency (%) was determined based on the ratio of maximum spore production and vegetative cells [17]. Spore germination was calculated by the number of spores that have changed to the vegetative cell.

2.6 Statistical analysis

All data were analyzed by one-way ANOVA in statistical software. The differences of all treatments were tested using Duncan’s Multiple Range Test (P<0.05).

3. Result and discussion

3.1 Vegetative Cells of *B. megaterium*
Vegetative cells production of *B. megaterium* cultured at different carbon (tapioca flour) levels are presented in Figure 1.

The highest vegetative cells were obtained at carbon dose 15 g/L with a density of $8.4 \times 10^7$ cells/ml. Overall, result shows that the higher carbon doses, the higher vegetative cells produced. However, at 20 g/L carbon dose, the peak of production was reached of vegetative cells and were found lower than carbon dose 15 g/L. This condition is caused by the amount of carbon available in the environment exceeds the limits of the bacterial ability to use carbon as a nutrient that supports their lives. In giving too much carbon, the bacteria could not utilize and absorb all the carbon available optimally, although incubation time before 10 hours has the highest density when given 20 g/L carbon doses. Carbon is a limiting factor for bacterial growth [18,19]. The main principle of carbon as a limiting factor occurs when the bacterial growth rate increases with an increasing C/N ratio [20]. Excessive of acetate concentration as the carbon source in the environment causes unbalanced carbon metabolism, therefore, it can inhibit the bacterial cellular growth [21].

![Figure 1. Time Series of vegetative cells production of *B. megaterium* under different carbon doses of tapioca flour (▲: 5 g/L; ■: 10 g/L; ●: 15 g/L; ○: 20 g/L)](image)

In all treatments, the highest vegetative cell was found at the incubation time of 10 hours, then the stationary phase occurred until the 12 hours. Afterwards, a phase of decreased vegetative cells occurred until 24 hours of incubation time. Vegetative cells decline is caused by the longer incubation time affect the availability of the nutrients in the culture media decreases. Nutrients derived from carbon source (tapioca flour) and nitrogen source (ammonium chloride) are used as food sources for the lives of these bacteria. Bacteria require carbon as constituents of cells and energy source while the nitrogen as the main element in amino acids and protein [22]. Decreasing of vegetative cells is influenced by inappropriate culture media such as lack of nutrients, pH, oxygen and temperature [23]. Apart from cell death, this research reveals that a declining vegetative cell population is also indicated by the formation of *Bacillus* spores to survive in the environmental conditions that are less supportive of life.

### 3.2 Spore production of *B. megaterium*
Spore-forming bacteria can change themselves from vegetative cells to spores when an unfavourable environmental condition (nutrients decline) [24]. The production of *B. megaterium* spores in the culture media with different carbon doses are presented in Figure 2. *B. megaterium* spore initially appeared 14 hours of incubation in almost all treatments of carbon doses of tapioca flour. The beginning of the spore formation was after the growth of vegetative cell of *B. megaterium* reached a peak. Initial spores continued to increase in line with a decrease of vegetative cell density [25]. At 14–24 hours of incubation, the spores in all treatments increased gradually and reached a stationary phase at 120 hours. The highest spore production was with dose level of 15 g/L dose (4.1 x 10⁷ spore/ml) and showed almost similar results when given lower dose of 5-10 g/L carbon dose. On the other hand, 20 g/L carbon dose showed a lower number of spores compared to other treatments (3.5 x 10⁷ spore/ml).

![Figure 2. Time series of spores production of *B. megaterium* under different carbon doses of tapioca flour (▲: 5 g/L; ■: 10 g/L; ●: 15 g/L; ◆: 20 g/L)](image)

The high vegetative cell was not an indicator of the high spore production. Given a carbon dose of 15 g/L, the vegetative cells count was 8.4 x 10⁷ cell/ml but the spore production was recorded only at 4.1 x 10⁷ spore/ml. Increasing amount of carbon doses affects the amount of carbon available in the culture media that impacted to bacterial lives. Other studies concurred with the recent result that the higher carbon doses given, the lower the spores produced. Glucose concentration as a carbon source showed that glucose with a dose of 3.5 g/L produces 4.3 x 10⁹ spores/ml. Contrastingly, a dose of 20 g/L carbon produced 3.4 x 10⁹ spores/ml. This condition inhibited the induction of several enzymes involved in the sporulation process [26]. Likewise, *Trichoderma harzianum* when given carbohydrate source (glucose) 30 g/L, smaller spores count was recorded at 1.6 x 10⁹ spore [27]. This case indicated that the dose of carbon depends on bacterial strain and carbon source that used in spore production.

3.3 Sporulation efficiency of *B. megaterium*

Sporulation efficiency is the percentage maximum vegetative cell that converts the highest spore production [17]. The sporulation efficiency of *B. megaterium* was seen in Figure 3.

The sporulation efficiency of *B. megaterium* was affected by carbon dose of tapioca flour. The highest of sporulation efficiency of *B. megaterium* was showed in the media with the carbon dose of 5 g/L with 80.97% sporulation efficiency. However, results showed no significant differences when
given with a higher dose of 15 and 20 g/L carbon levels. The high counts of vegetative cells and spores did not follow a high sporulation efficiency. Increasing the sporulation efficiency of \textit{B. coagulans} can be expected through the addition of carbohydrates, mineral salts, pH in culture media [28]. In addition, the concentration of glucose as a carbon source should be reduced because it can decrease the results of sporulation efficiency [17]. In fact, the usage of low glucose content (2.0 g/L) increases spore production between $0.51 \times 10^9$ cfu/ml and $1.87 \times 10^9$ cfu/ml with sporulation efficiency 50.7% and 93.2%, respectively [29]. Another carbon source (acetate) only requires $\leq 0.04\%$ to increase the sporulation efficiency of \textit{Saccharomyces cerevisiae} [30]. The results shown by some of these researches indicated that each carbon source has an optimal dose to achieve the highest sporulation efficiency.

![Figure 3. Sporulation efficiency of \textit{B. megaterium} under different carbon doses of tapioca flour](image)

### 3.4 Germination of \textit{B. megaterium}

Germination process is the proliferation of spore to vegetative cell when the environmental supporting condition ensues [31]. The spore germination of \textit{B. megaterium} was presented in Figure 4.
Figure 4. Time series of spore germination of \textit{B. megaterium} under different carbon doses of tapioca flour (\textbullet{} : 5 g/L; \textblacksquare{} : 10 g/L; \textblacktriangle{} : 15 g/L; \textblacktriangleleft{} : 20 g/L)

In this study, the spore germination of \textit{B. megaterium} was recorded at five (5) hours since there was no significant difference between the different treatment means. The highest vegetative cell from spore germination was found more than 5 g/L of carbon dose. The entire treatments achieved 100% spore germination before 0.5 hours in the nutrient broth medium due to the presence of new nutrients that stimulated bacteria cell division. Other reasons from some studies showed that spore germination influenced by the heat activation of spore. The percentage spore germination reached 100% after 0.5 hours when heat activation of spore at 65 °C [32] different to this study did not use heat activation. Another study showed that \textit{B. subtilis} produce lower spore germination at 52.9%, than when spore was not heated treatment with 94% spore germination rate [31]. The difference of this germination percentage was influenced by concentration, density of spore, incubation temperature, heat activation and oxygen [33].

4. Conclusion
Carbon doses of tapioca flour affected the vegetative cells, spore production, sporulation efficiency and germination of \textit{B. megaterium}. The highest vegetative cells (8.4 x 10^7 cell/ml) and spore production (4.1 x 10^7 spore/ml) of \textit{B. megaterium} were found in the carbon dose of 15 g/L with sporulation efficiency 49.01%. The spore germination optimally was observed in more than 5 g/L of carbon dose.

5. References
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