The Effect of Starting Shocking Time on Carbon, Nitrogen, and Organic Matter Absorption of *Bacillus megaterium* BM1 in Vegetative and Sporulation Phases

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Abstract. The application of shock induction is concerned with the sporulation. The essential indicator of the vegetative cell and spore quantities is influenced by the absorption of the organic compound components. The principal objective of this experiment was to establish the impact of starting shocking time on organic carbon, total nitrogen, and organic matter absorbed by *B. megaterium* BM1 in vegetative and sporulation phases. This research was conducted in 250 ml Erlenmeyer flasks with 200 ml media and was added with 1% (v/v) bacterial starter (1.7 x 10⁸ cells/ml⁻¹). The test media were incubated at 37 °C and shocking started at 5, 10, and 15 hours after inoculation, respectively. The sample from each test medium was measured as much as 15 ml to calculate organic compound uptake. The results showed that the carbon and nitrogen concentrations in the culture media are decreasing from the vegetative phase to the sporulation period throughout the given treatments. The highest organic carbon and total nitrogen absorption in vegetative phase occurred at the starting shocking at 10th hour and 15th hour with a value of more than 30%. However, in the sporulation, the highest of three parameters happened in the starting shocking time at the 5th hour.

1. Introduction

The use of microorganisms as probiotics exhibits extremely potential to be developed continuously to support increased productivity of fish farming. The bacterial genus that is widely encountered providing a beneficial effect on the aquaculture organism is *Bacillus* [1, 2, 3, 4]. The advantages of *Bacillus* are not only in the form of vegetative cells by extracellular enzymes secretion [5, 6] but also in the form of spores that can survive in harsh environmental conditions such as desiccation, toxic compounds, lack of nutrients and heat [7, 8]. *B. megaterium* is a species of *Bacillus* that can produce spores with an exosporium as special physiological properties on the outer membrane [9, 10]. The stability of spores in extreme environments can be a strategy in the development of biotechnology products [11].

Vegetative cell mechanism converts into spore through several stages known as a sporulation process. Spore production is affected by the content of the test media used and environmental conditions
[12, 13, 14]. Media components that must be available in producing spores are sources of carbon, nitrogen sources, and minerals with appropriate doses [15]. Furthermore, the condition of the cultivated environment for instance pH scale, temperature, and activity of water must also be considered to stimulate the appearance and characteristics of spores [16, 17].

An essential indicator for achieving high spore production and efficient sporulation can be seen from the activity of vegetative cells to absorb readily metabolizable organic compounds in the cultivated media [18]. Elements (C, N) available in the media are macromolecules used as nutrients that have an important role in both structurally and functionally in bacteria cells [19, 20, 21]. Based on previous research from Mahariawan et al. [22], bacteria cultured on a preferred medium with high carbon levels (20 gram. litre⁻¹) and suitable environmental conditions could not absorb all the available nutrients optimally and impacted to sporulation process and low spore levels generated. An alternative method that can be applied to support sporulation processes by inducing environmental shock treatment has been carried out by several researchers [23, 24, 25]. However, the optimal time to initiate environmental shock has not been confirmed yet to affect the vegetative cell in absorbing chemical elements in culture media until the sporulation period. Thus, this research provides a principal objective to establish the impact of starting shocking time on organic carbon, total nitrogen and organic matter absorption by B. megaterium BM1 in vegetative and sporulation phases.

2. Methods

2.1 Bacterial culture
Isolates of B. megaterium BM1 utilized in this study had been confirmed by 16SrRNA analyses [26]. These bacteria that have grown on nutrient agar (NA) as a pure culture were inoculated by taking a single loop of isolate into 20 ml of sterile broth medium (NB). Fermentation of liquid medium was carried out in an incubator shaker for 18 hours using a temperature of 37°C and stirring speed at 120 rpm. This culture was then used as a starter to be transferred to the culture medium.

2.2 Preparation of tested media
Vegetative and spore growing media in this study consisted of essential compounds (C, N) and several trace minerals that support spore initiation. The C/N ratio between wheat (T. aestivum) and ammonium chloride (NH₄Cl) was set at 15:3. All components of salt materials were mixed by 200 ml of sterile distilled water (Aquadest®) in 250 ml Erlenmeyer flasks and then were carried out sterilization using an autoclave (at a temperature of 121°C during 0.25 hours). Mineral supplementations added to the test media were CaCO₃, MgSO₄, CaCl₂, MnSO₄ and FeSO₄ with an optimal dose [14, 22]. Bacterial starter grown in broth medium was put into each test medium as a bacterial starter as much as 1% (v/v) of the total volume [15]. The initial density of bacterial stock used was 1.7 x 10⁸ cells.ml⁻¹.

2.3 Initiation of shocking time treatments
The experimental design of this research was a completely randomized design (CRD) with three different treatments and five repetitions. Shock initiation time treatments were conducted at 5th, 10th, and 15th hours of vegetative cell culture. The starter of culture that had been planted on each test medium was incubated in an incubator shaker using a temperature of 37°C and a speed of 120 rpm during the period before the shock treatment began. Starting shocking time was prepared according to each treatment by inducing a change in an initial environmental temperature to 51°C and adjusting the media to be a pH of 10. This environmental change condition was maintained until the end of the sporulation period.

2.4 Calculation of organic carbon, nitrogen and organic matter uptake by B. megaterium
Samples to test the uptake of carbon, nitrogen and organic matter by bacteria were taken in three growth phases namely adaptation, vegetative exponential and sporulation phase. The sample was measured as much as 15 ml from the culture media and stored in a 15 ml sterile falcon bottles for each growth phase to be tested for the uptake of the component of the organic compound. Determination of the organic
carbon and organic matter used the Walkley and Black method [27]. This method oxidizes the sample using a concentrated of K$_2$Cr$_2$O$_7$ and H$_2$SO$_4$ extractors. Furthermore, the percentage of total nitrogen was measured by the Kjeldahl method.

2.5 Data analysis
The results of all parameters during this study were statistically tested by one-way ANOVA applying SPSS 25. The distinctions between the whole treatments can be seen from DMRT Test at 95% of confidence level.

3. Result and Discussion
3.1 Organic carbon absorption
Organic carbon uptake in the log cell vegetative phase and also transition process to the spore phase of 
*B. megaterium* BM1 is shown in Figure 1.

![Figure 1. Organic Carbon Absorption by *B. megaterium* BM1](image)

The percentage of organic carbon uptake by *B. megaterium* BM1 showed a difference in value between the vegetative phase and the sporulation phase. The highest organic carbon uptake occurred in the log vegetative phase with the treatment starting shocking time at the 10th hour and the 15th hour, which had an absorption percentage of more than 30%. This value showed 1.2 times greater than the 5th hour of shocking time treatment in the vegetative phase. The use of appropriate initiation shocking time in the maintenance of vegetative cells will affect the carbon uptake obtained in both the vegetative and sporulation phases. The results of this study indicated that in the vegetative phase, the faster the shock time starts, the less percentage of carbon uptake by vegetative was produced. This condition was suspected by vegetative cells at the 5th hour had not reached their peak growth. The shock treatment has given an impact on the suboptimal vegetative ability to absorb carbon in the culture environment. The utilization of nutrients such as carbon is an important indicator that affects bacterial growth in culture media, especially when it enters the exponential phase [28]. Blagodatskaya et al. [29] reported that the efficiency of carbon use by microbes can reach 35% in the exponential phase and depends on the availability of substrates in the environment. The absorption of carbon substrates directly by bacteria is used as nutrition for cellular biosynthesis and metabolic functions [30, 31]. The carbon which absorbed by the bacteria can be a source of energy both in the form of ATP and biosynthetic precursors such as acetyl-CoA, glucose-6-phosphate and fructose-6-phosphate [32].

The sporulation phase that occurred during culture in all treatments showed lower carbon absorption values when compared to that at the exponential growth phase of vegetative cells. In contrast to the
results in the vegetative phase, the highest organic carbon uptake in the sporulation phase was obtained at the 5th hour of shock with a value of 23.25%. The low carbon uptake in the sporulation phase in the 10th and 15th hour of starting shocking time treatments was due to the fact that carbon available in the environment decreased significantly when compared to the 5th hour of starting shocking time. This condition is caused by bacteria utilized carbon sources to grow. In consequences, the high carbon absorption at the final of the logarithmic period of vegetative cells would have an impact on decreasing carbon levels in the environment [33]. When cells approach starvation conditions, it can result in decreased nutrient uptake so that bacterial growth will slow down [34]. In addition, limiting carbon levels in the environment can affect bacteria to become stressed and cause bacteria to enter a sporulation phase to defend themselves [35]. The spore is a dormant form that is not metabolically active so that in the sporulation phase the carbon uptake produced by bacteria will be lower.

3.2 Total nitrogen absorption
The total nitrogen uptake by *B. megaterium* BM1 revealed the same trend as the organic carbon uptake parameter. The absorption percentage of nitrogen in the vegetative phase provided a greater value than that in the sporulation phase (Figure 2). The timing of initial shock indicated that there was a difference in the total nitrogen uptake between treatments. In the vegetative cell phase, the highest total nitrogen uptake was shown at the 15th hour of shock initiation treatment with a value of 31.28%. The environmental shock of temperature and pH at the right time can trigger vegetative cells to take up nitrogen in the culture medium. Organic nitrogen available in the environment will be absorbed by bacteria in the biosynthesis process, especially for the growth stages [36]. Bacteria utilize nitrogen through various kinds of transport enzymes to absorb nitrogen both molecules and monomers available in media for instance some amino forms (amino acids and amino sugars). The process of accepting amino acids into cells was promoted by a particular useful transport system set within the living substance membrane [37]. Zhang et al. [38] reported that the efficiency of nitrogen uptake by microbes determined the balance between anabolic and catabolic nitrogen processes.

The transformation phase of vegetative cells into spores indicated that the nutrient condition of the culture environment was limited, especially after passing the stationary phase of vegetative cells [39, 40]. The decrease in nutrient content in culture media such as nitrogen causes the total nitrogen absorption by bacteria to decrease so that the sporulation phase shows a lower absorption percentage compared to the vegetative phase. The high nitrogen uptake in the exponential phase of the vegetative cells at the shocking of 15th hour resulted in the proportion of nitrogen decrease in the environment after

![Figure 2. Total Nitrogen Absorption by *B. megaterium* BM1](image)
the log phase ended so that the vegetative cells were stressed and transformed into spores to survive. Besides being influenced by limited nutritional conditions, the sporulation phase was also influenced by environmental shocks. The timely application of environmental shocks will trigger the emergence and production of the resulting spores. Jafari et al. [25] noted that giving a temperature shock of 68 °C within 20 minutes produced 102% more spores of *B. coagulans* than without shock. Furthermore, providing extreme temperatures can stimulate *Bacillus* to protect itself through the sporulation mechanism. Heat shock genes (*htrA* and *htrB*) played a role in controlling regulators and sensors against vegetative cell stress before deciding on the sporulation phase [41].

### 3.3 Organic matter absorption

The uptake of organic matter in the sporulation phase is presented in Figure 3. The uptake of organic matter by *B. megaterium* in the sporulation phase had a value of less than 25% in all treatments with the lowest absorption percentage at the start of the 15th hour shocking, reaching 13.09%. The lowest percentage of carbon, nitrogen, and organic matter uptake in the sporulation phase occurred in the initiation shocking time at the 15th hour. This condition was caused by the bacteria utilize organic material available in the environment to grow in the vegetative phase [42]. On the other hand, the implementation of shocks and decreased nutritional conditions in the culture media might cause the bacteria to become stressed and break off sporulation. Bacteria have an important role in decomposing organic matter. Bacterial abundance depends on temperature and the availability of organic matter in the decomposition process [43]. Bhattarai [44] stated that the low density of microorganisms was influenced by the low percentage of organic matter available in the environment. Enzyme activity provided a significant role especially in determining the mechanism of organic matter decomposition and the transformation of the nutrient cycle [45]. In addition, this study also showed that giving a suitable environmental shock time will have an impact on the absorption cycle of organic matter by bacteria from the vegetative phase to the sporulation phase.

![Figure 3. Organic Matter Absorption by *B. megaterium* BM1](image)

4. **Conclusion**

From the results of this study, it can conclude that the longer the initiation time of the shock, the higher the absorption of carbon and nitrogen by *B. megaterium* BM1 in the vegetative phase. However, the sporulation phase showed less absorption of the component of the organic compound.
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