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The Influence of Environmental Shock Initiation on Vegetative and Spore Production of *Bacillus megaterium*

I Made Dedi Mahariawan[1], Wahyu Endra Kusuma[2], Ating Yuniarti[2] and Anik Martinah Hariati[2*]

[1] Universitas Brawijaya, Fisheries and Marine Science Faculty, Postgraduate Program, Malang, 65145, East Java, Indonesia
[2] Universitas Brawijaya, Fisheries and Marine Science Faculty, Aquaculture Department, Malang, 65145, East Java, Indonesia

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ABSTRACT

Production of *B. megaterium* spores as probiotic candidates is not only influenced by the nutrient composition in the culture media but also initiated by environmental factors. One of the efforts to reduce spore production costs is by utilizing wheat flour as a medium for spore growth. Large quantities of *B. megaterium* spore production in a short time on wheat flour media can be carried out by implementing environmental shocks. The objective of this study was to evaluate the influence of environmental shock initiation on the spore production of *B. megaterium*. The culture media consisted of carbon source (wheat flour) and nitrogen (ammonium chloride) at the C/N ratio of 15:3. Environmental shock conducted at 51 °C and pH of 10 in vegetative maintenance. The treatments of shock were started at the 5^{th}, 10^{th}, and 15^{th} hour after inoculation, respectively. The parameters of this study were vegetative cells, spore production, sporulation efficiency and spore fraction. The highest vegetative cell was revealed in the shock initiated at 10^{th} hour (3.5×10^8 cells. ml^{-1}). The high density of vegetative cells did not indicate high spore production. The highest spore production in this study was found in the shock started at 15^{th} hour (1.73×10^8 spores. ml^{-1}) with sporulation efficiency (79%) and spore fraction was 57.28%. The target of high spore production can be achieved in the shock initiation at the 15^{th} hour in wheat flour media.

Key words: *Heat-shock, Probiotic, Shock-initiation, Spore-forming bacteria, Wheat-flour.*

Introduction

The ability of bacteria to produce extracellular activity is an important indicator to be used in the field of biotechnology especially as a probiotic candidate in the aquaculture industry (Doroteo *et al.,* 2018). For instance, protease as extracellular enzyme has been proven the resistance of bacteria at low pH level (Sunu *et al.,* 2019). Recently, several specific properties of bacteria are also of great potential to be applied (Palkova, 2004; Gray *et al.,* 2019). Bacteria can also maintain stability in order to avoid harsh environments such as the influence of extreme temperatures, desiccation, UV and ã-radiation, toxic chemical and other environmental shocks (Nicholson *et al.,* 2000; Setlow, 2006). Some Gram-positive bacteria are known to produce spores such as *Bacillus* and *Clostridium* (Swick *et al.,* 2016; Andryukov *et al.,* 2019).

Application of spore utilization as probiotics has

been studied which provides beneficial effects on cultivated organism such as shrimp and fish (Wangka-Orm *et al.,* 2014; Thurlow *et al.,* 2019). One of the bacteria from the genus Bacillus that can produce spores is *B. megaterium* which presents its own uniqueness in its outer membrane known as *exosporium* (Zhou *et al.,* 2017). This particular characteristic causes *B. megaterium* spores when applied *in vivo* test to have the advantage of attaching to the intestinal epithelium (Barra-Carrasco *et al.,* 2013; Mora-Uribe *et al.,* 2016). Production of *B. megaterium* spores is influenced by several factors such as growth medium (Gopinathan *et al.,* 2016) and environment (Trunet *et al.,* 2015). Commercial media frequently used to obtain spores have been evaluated as providing high production costs (Kabore *et al.,* 2019). This condition means that the cost efficiency of *B. megaterium* spore production as a probiotic candidate needs to be considered.

According to Movahedi and Wales (2002) and Minh *et al.* (2011) the initiation of spore formation can be conducted by giving an environmental shock. Providing environmental shocks has been studied to increase the response of vegetative cells to transform into spore through sporulation mechanism (Melly and Setlow, 2001; Noor *et al.,* 2019). Byun *et al.,* (2011) reported that environmental shock induction can increase spore production by up to 102%, while Lee *et al.* (2003) explained that shock treatment can also improve spore resistance at high temperatures. However, the initiation of environmental shocks to produce spores is still limited to commercial growth media, thus the information about environmental shock and the initiation shocking time to increase spore production in low-cost media needs to be confirmed.

Furthermore, objective of this study was to evaluate the influence of environmental shock initiation on spore production of *B. megaterium*.

Materials and Methods

Bacterial preparation

Species of *B. megaterium* in this study were cultured on the four-quadrant agar medium (NA) and were transferred to 30 ml of sterile nutrient broth (NB) media with an inoculating loop. Afterwards, this broth culture was incubated at 37 ˚C using a speed of 120 rpm for 18 hours in an incubator shaker. The starter bacteria used in this study had an initial density at 1.7×10^8 cells ml^{-1}.

Culture media

The composition and preparation of test media were carried out based on Mahariawan *et al.* (2021).

Environmental shock initiation treatments in vegetative cell maintenance

All test media were incubated at 37 ˚C and were treated with environmental shock at different times. This research used 3 treatments in variations of the initiation of environmental shock, namely at the 5^{th}, 10^{th}, and 15^{th} hours after inoculation of the vegetative cells and each treatment was repeated 5 times. Environmental shock was given using a pH of 10 and a temperature of 51 ˚C which was set until the sporulation period ended (84 hours). The alkaline conditioning on the test media used 0.1 N of sodium hydroxide. The vegetative incubation temperature which was initially at 37 ˚C was increased to 51 ˚C with different times based on the variation of the shock initiation used.

Measurements of vegetative cell, spore density, sporulation efficiency and spore fraction

Calculation of vegetative cells and spores of *B. megaterium* was carried out as per Yuniarti *et al.* (2019). The growth rate of vegetative cell was measured by establishing an exponential growth model and revealed linear regression in vegetative curve shape. Sporulation efficiency and spore fraction were determined by the method of Monteiro *et al.* (2014).

Statistical analysis

Statistical analysis in this study used SPSS 20 for windows software which was applied to analyze data among treatments. The level of significance was defined at 95%.

Results and Discussion

Vegetative cell density and growth rate

The highest vegetative cell density of *B. megaterium* was obtained in the environmental shock started at the 10^{th} hour with a value of 3.53×10^8 cells. ml^{-1}. Other treatments reached the highest cell density of less than 2.5×10^8 cells ml^{-1} (Table 1). The growth rate of vegetative cells recorded a significant difference in all treatments ($p<0.05$). The highest vegetative cell

Table 1. Vegetative and Spore of *B. megaterium* under different environmental shock initiation

Environmental shock initiation	Maximum Vegetative Production (x10^8 cells. ml^{-1})	Spore Density at 84 hours after Inoculation (10^8 spores. ml^{-1})	Spore fraction (%)
at the 5th hour	0.56±0.01a	0.30±0.01a	53.92
at the 10th hour	3.53±0.11c	0.74±0.04b	22.31
at the 15th hour	2.20±0.11b	1.73±0.05c	57.28

The different letter behind the numbers indicated significantly different ($p<0.05$)

growth rate was obtained at the shocking started in the 10th hour with a value of 0.48 $hour^{-1}$ (Figure 1). The application of environmental shocks at different times in this study revealed that there was a different density and growth rate of vegetative cell. This difference was caused by the effect of giving shocks on proteins which had an impact on internal and external changes in bacterial cellular function (Shahriar *et al.,* 2019). The environmental shock should be carried out at the right time therefore it does not cause major physiological changes to the vegetative cells.

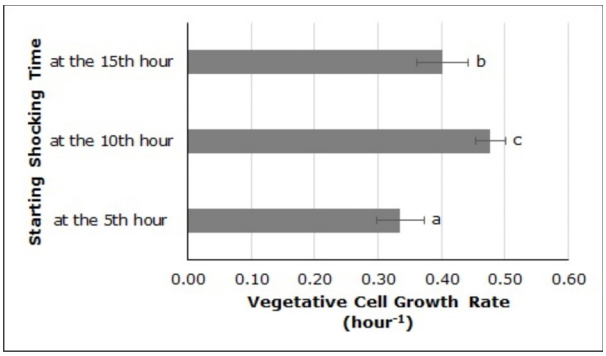

Fig. 1. Growth rate of *B. megaterium* under different environmental shock initiation.

The appropriate timing of shock will affect the production of acquired vegetative cells. Based on research by McMahon *et al.* (2001) showed that applying heat shock at 48 ˚C for 10 minutes in the exponential phase resulted in a higher number of cells compared to 60 minutes. Alo *et al.* (2019) explained that the sigma factor σ*32* plays a role in mediating the environmental shock response given. The stress condition due to high ambient temperature causes a transient increase in the transcription of σ32 and stimulates the transcription of RNA polymerase (RNAP) from the heat shock promoter resulting in the induction of HSPs.

Spore production

Spore production at the initiation of different shocks showed the difference trend as the vegetative cell (Table 1). The highest vegetative cells in the treatment of shock initiated at the 10th hour did not show the highest spore density. Spore production which occurred in the environmental shock started at the 15th hour after inoculation reached the density at 1.73 x 10^8 spores ml^{-1}. The initiation of different shock time can affect the metabolism of bacteria in utilizing the nutrients available in the culture environment. Moreover, giving an environmental shock of temperature and pH can trigger physiological bacteria to transform into spores due to environmental stress.

The emergence of spores is caused by the decreasing availability of nutrients in the environment which impacted to the vegetative cells to defend themselves by forming spores. In addition, environmental shock treatment can stimulate the rate at which spores appear due to environmental stress (Nicholson *et al.,* 2002). When vegetative cells enter the stationary phase, an increase in the production of *sigB* and *sigF* expressions will occur, which will also increase *sigA*transcription (de Vries *et al.,* 2005). Cell conditions under stress can affect carbon metabolism from bacteria (Tarrant *et al.,* 2019).

Sporulation efficiency and spore fraction

Sporulation efficiency showed a significant difference in all the treatments ($p<0.05$) (Figure 2). The highest sporulation efficiency was in the shockstarted at 15th hour with a value of 79% and showed a percentage three times greater than the shock initiated at the 10th hour. Furthermore, the high spore fraction also happened at the shock started at 15th hour (57.28%) (Table 1). The high concentration of vegetative cells and spores obtained is not an indicator of the high efficiency of the resulting sporulation. Another study showed that the sporulation efficiency value of *B. anthracis R"spoVG* strain with heat resistance of 70 ˚C for 30 minutes showed 3 times the lower sporulation efficiency (20%) when compared to the *A16R* strain (70%). The different values of the two strains were due to the *SpoVG* factor, which is a regulatory pleiotropic fac-

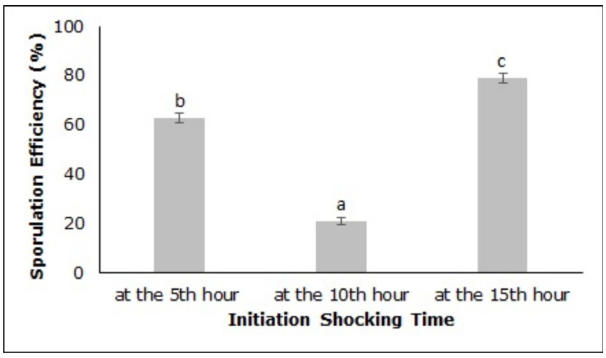

Fig. 2. Sporulation efficiency of *B. megaterium* under dif ferent environmental shock initiation.

tor that plays a role in the sporulation process (Chen *et al.,* 2020).

The sporulation efficiency is not only influenced by the timing of initiation of the shock but also other factors such as nutritional composition and environmental factors. Sporulation efficiency is influenced by the availability of spores promoted by several factors for instance temperature, pH and water activity (Nguyen Thi Minh *et al.,* 2008). Garcia *et al.* (2010) showed that *B. weihenstephanensis* KBAB4 produced efficiency values>99% at 12 °C and 30 °C. Baril *et al.* (2012) also stated that temperature and pH affect the specific growth rate and sporulation rate of the genus Bacillus.

Conclusion

This study has successfully concluded that the target of high spore production of *B. megaterium* can be achieved optimally in the shock initiation at the 15th hour in wheat flour media.

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