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Research Article

Spore production and sporulation efficacy of *Bacillus subtilis* under different source of manganese supplementation

Produksi Spora dan Efisiensi Sporulasi *Bacillus subtilis* dengan Suplementasi Mangan dari Sumber yang Berbeda

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Abstract

Bacillus is a species widely used as a probiotic in the aquaculture industry. The *Bacillus* spores have more advantages than their vegetative ones, and an addition of minerals such as calcium, magnesium, and manganese can improve the spore production. The purpose of this study was to determine the effect of different sources of manganese on the production and sporulation efficacy of *B. subtilis* SB3. The sources of manganese used in this study were manganese chloride ($MnCl_2$) and manganese sulfate ($MnSO_4$) at the concentration of 10 mM. Media without manganese supplementation was used as a control. The results showed that there was a significant effect of different manganese sources on the spore production of *B. subtilis* SB3. The highest spore production was found in media with $MnCl_2$ supplementation with the total spore of 8.77 x 10^7 spores. mL^{-1}. However, spore production with $MnSO_4$ supplementation was still higher (22.7%) compared to that without manganese supplementation. The decrease in spore production with $MnSO_4$ supplementation was possible due to the sulfate inhibition. The high spore production in media with $MnCl_2$ supplementation was also preceded by the high production of vegetative cells from *B. subtilis* SB3 (2.54 x 10^8 cells. mL^{-1}). The results indicated that manganese could stimulate both vegetative cell growth and its spores. The highest sporulation efficacy (35%) was also achieved in media with $MnCl_2$ supplementation. On the other hand, the germination rate of *B. subtilis* SB3 spores was not influenced by manganese supplementation.

Abstrak

Bacillus adalah species yang banyak digunakan sebagai probiotik pada industri akuakultur. Dalam bentuk spora, species ini lebih banyak mempunyai kelebihan dibandingkan dalam bentuk vegetatifnya dan peningkatan produksi sporanya dapat dilakukan dengan penambahan mineral seperti kalsium, magnesium dan mangan. Tujuan penelitian ini adalah untuk mengetahui pengaruh sumber mangan yang berbeda terhadap produksi dan efisiensi sporulasi *B. subtilis* SB3 indigenous akuatik. Sumber mangan yang dipakai dalam penelitian ini adalah mangan klorida ($MnCl_2$) dan mangan sulfat ($MnSO_4$) sebanyak 10 mM dan sebagai kontrol digunakan media tanpa suplementasi mangan. Hasil penelitian menunjukkan bahwa terdapat pengaruh yang nyata penggunaan sumber mangan yang berbeda terhadap produksi spora. Produksi spora tertinggi didapatkan pada media dengan suplementasi $MnCl_2$ sebanyak 8,77 x 10^7 spora. mL^{-1}. Sedangkan produksi spora dengan suplementasi $MnSO_4$ juga masih lebih tinggi (22,7%) dibandingkan tanpa suplementasi magan. Penurunan produksi spora pada media dengan penambahan mangan sulfat diduga karena adanya penghambatan oleh sulfat. Tingginya produksi spora pada media dengan suplementasi $MnCl_2$ sebelumnya juga didahului dengan tingginya produksi sel vegetatif dari *B. subtilis* SB3 (2,54 x 10^8sel. mL^{-1}). Hal ini menunjukkan bahwa mangan dapat menstimulasi baik pertumbuhan sel vegetatif dan sporanya. Efisiensi sporulasi tertinggi juga dicapai pada media dengan suplementasi $MnCl_2$ sebesar 35%. Di sisi lain, kemampuan germinasi spora *B. subtilis* SB3 tercatat sama dan tidak dipengaruhi oleh suplementasi mangan.

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1. Introduction

In the aquaculture industry, probiotic is an alternative to reduce the use of antibiotics in treating bacterial diseases. Probiotics can also stimulate immune responses, increase feed efficiency and improve water quality (Kesarcodi-Watson *at al*., 2008). The use of probiotics had proven to work effectively in several species of fishes and shrimps (Lara-Flores, 2011; Boonthai *et al*, 2011; Villamil and Reyes, 2012; Yuniarti *et al*., 2013; Yuniarti *et al*., 2015).

Bacillus spp is the most widely used probiotic species in aquaculture. Bacillus are ubiquitous in sediments and therefore they are naturally consumed by fish or shrimps which fed in and on sediment (Moriarty, 1999). *Bacillus* is able to form endospores which have several advantages over their vegetative ones. *Bacillus* spores can survive in extreme temperatures, desiccation, radiation, and toxic materials (Wolken *et al*., 2003). These advantages indicated that it is possible to formulate more stable probiotics in the form of spores (Hong *et al*., 2005; Ugoji and Hunter, 2006)

Bacillus spores are greatly influenced by the production media. Different *Bacillus* species required different components of media to achieve high efficacy in spore production (Buhr *et al*., 2008). Some studies were conducted to find optimal media to produce spores from several *Bacillus* species (Levinson and Hyatt, 1964; Cauble *et al*., 1984; Monteiro *et al*., 2005; Verma *et al*., 2013; Wangka-Orm *et al*., 2014; Monteiro *et al*., 2014; Posada-Uriber *et al*., 2015). As reported in several studies, the supplementation of minerals, such as calcium, magnesium, and manganese, can increase the production of *Bacillus* spores (Monteiro *et al*., 2005; Ghosh *et al*., 2011; Omer, 2010). Technically, manganese is available in the form of manganese chloride ($MnCl_2$) and manganese sulfate ($MnSO_4$). To date, the effectiveness those kinds of manganese for the production of *Bacillus* spores has not been reported yet. Therefore, this study was aimed to determine the influence of different sources of manganese on the production and sporulation efficacy of *B. subtilis* SB3 indigenous aquatic.

2. Materials and Methods

2.1 Materials

The bacteria used in this study were *B. subtilis* SB3 which had been identified by 16SrRNA (Yuniarti *et al*., 2015b). The basic media used for all media in this study was Nutrient Broth (NB) (5 gr peptone, 3 gr meat extract per liter). The supplementation of various minerals as treatments in this study is presented in Table 1. In this study, supplementation of manganese was in the form of manganese chloride and manganese sulfat. Medium with no manganese supplementation was used as control. All teatments was repeated four (4) times to ensure the reproducibility.

Table 1. Mineral composition added for each media

No.	Composition	Dosage	
1.	Media 1:		
	KCl	1	gr. L^{-1}
	$MgSO_4$	0,25	gr. L^{-1}
	$FeSO_4$	1,0	mM
	$CaCl_2$	1	M
	$MnCl_2 .4H_2O$	10	mM
2.	Media 2:		
	KCl	1	gr. L^{-1}
	$MgSO_4$	0,25	gr. L^{-1}
	$FeSO_4$	1,0	mM
	$CaCl_2$	1	M
	$MnSO_4.7H_2O$	10	mM
3.	Media 3:		
	KCl	1	gr. L^{-1}
	$MgSO_4$	0,25	gr. L^{-1}
	$FeSO_4$	1,0	mM
	$CaCl_2$	1	M

2.2 Methods

2.2.1 B. subtilis SB3 Culture

Reculture of *B. subtilis* SB3 isolates was conducted by taking one dose of the Nutrient Agar (NA) pure culture and inoculating it into NB media. Fermentation were then performed in incubator shaker (100 rpm) at 37°C for 24 hours. This culture was then used as a stock culture. The basic culture media for *B. subtilis* SB3 was NB with the supplementation of several minerals (Table 1). All components was dissolved in distilled water in a 100 mL Erlenmeyer. The media was then sterilized in an autoclave for 15 minutes at a pressure of 1 atm and a temperature of 121°C. The inoculum (1 x 10^6 cells. mL^{-1}) was inoculated into media at a concentration of 2% (v/v) of the total volume. The fermentation was conducted in incubator shaker 37°C for 70 hours.

2.2.2 Calculation of vegetative cells and spores of B. subtilis SB3.

The calculation of vegetative cells and spores was conducted with the support of the microscope by using a Neubauer chamber. To facilitate the calculation, *B. subtilis* SB3 culture was serially diluted. The difference between vegetative cells and spores of the bacteria were distinguished by their shape. Vegetative cells of *B. subtilis* SB3 are rod-shaped while the spores are round-shaped. The calculation of spores was done by heating the suspension at a temperature of 80°C for 15 minutes (to eliminate vegetative cells of the bacteria, but not the spores). Sporulation efficacy is the percentage of vegetative cells that have undergone complete sporulation. It was calculated as the ratio between the number of spores and the maximum number of vegetative cells achieved (Monteiro *et al*., 2014). The spore fraction was the ratio between the concentration of vegetative cells and spores at the same time. The germination rate can be calculated based on the percentage of the highest number of cells germinated which divided by the number of spores. The calculation of germination percentage was conducted by plating them on NA after it was heated at a temperature of 80°C for 15 minutes.

2.2.3 Statistical Analysis

The data obtained were analyzed statistically by one-way ANOVA using SPSS 20.0. The differences between treatments were analyzed using Duncan's Multiple Range Test at a confidence level of 95%.

3. Results and Discussion

3.1 Production of B. subtilis SB3 spores

Both members of the genus *Clostridium* and genus *Bacillus* are capable to produce spores. The production of *B. subtilis* SB3 spores in media with different sources of manganese (Mn) is presented in Figure 1. The production of spores in all treatment media was recorded after 40 hours. In contrast, on the 40th hour, the vegetative cells of *B. subtilis* from all treatments entered the death phase. In the following hours,

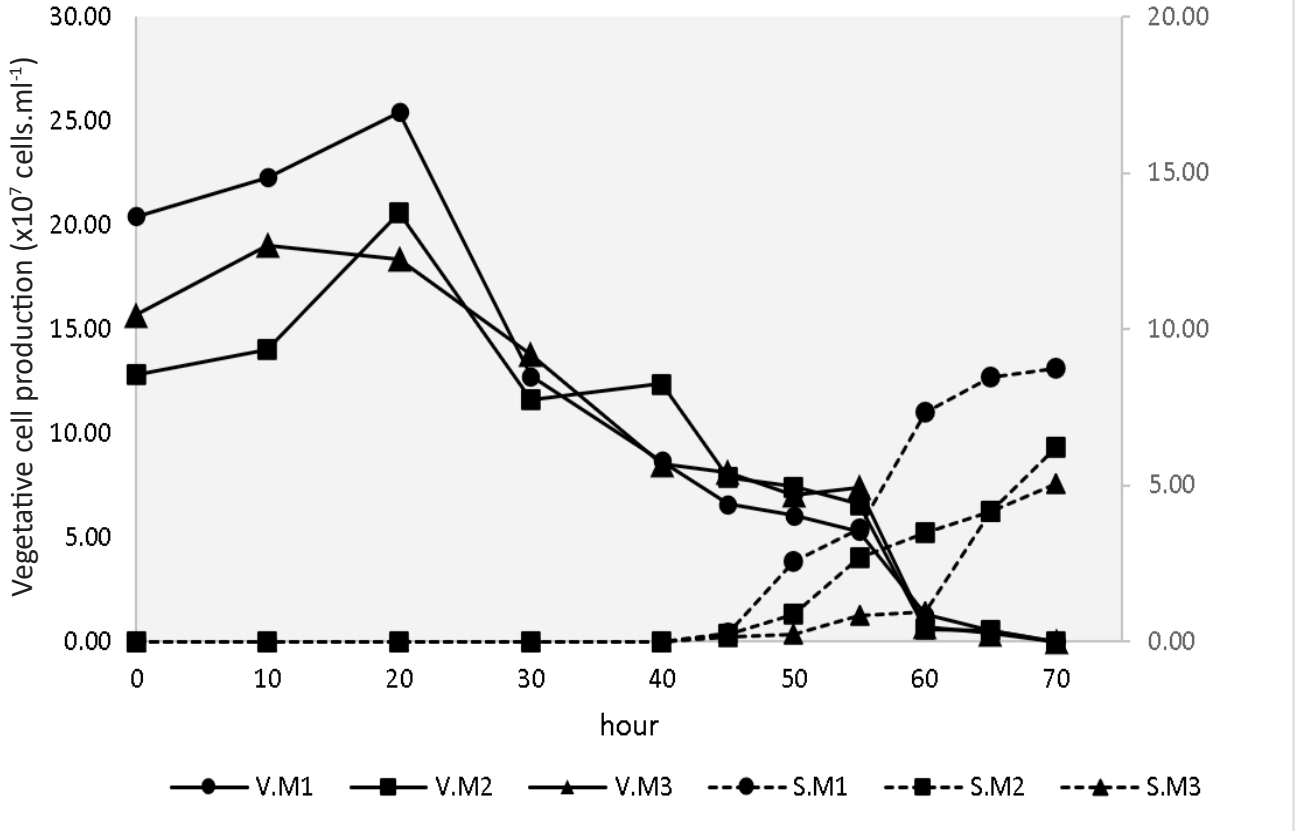

Figure 1. Production of *B. subtilis* SB3 vegetative cells and spores in different media.

Note: V = vegetative cells, M = media, V.M1 = vegetative cells grown on media 1, etc.

the spore production in all treatments increased along with the decrease of vegetative cells. Thus, the spore production process in this study occurred after the stationary phase or in the death phase. The spore production process in *Bacillus* species can be occured in different phases, exponential phase (Dawes and Mandelstam, 1970) and after the stationary phase (Ryu *et al*., 2016).

In the 70th hour, vegetative cells of *B. subtilis* no longer could be detected in all treatments. The decrease in vegetative cells of *B. subtilis* was due to the limited availability of nutrients in the media. Furthermore, the limited availability of nutrients caused an increase in spore production. The increase in spore production was recorded in all treatments up to the 70th hour. Some studies showed that the main stimulus for sporulation was nutrients (carbon, nitrogen, phosphate, and micronutrients) deficiency (Errington, 2003, Nicholson *et al*., 2000).

It can be seen from Figure 1 and Table 2, that manganese supplementation affected the production of *B. subtilis* SB3 spores. The spore production in manganese-supplemented media (SM1 and SM2) was higher than that of the non-supplemented media (SM3). Furthermore, there was different spore production with different sources of manganese ($MnCl_2$ and $MnSO_4$). The same pattern was obtained by Sinnelä *et al*., (2019) and Ryu *et al*., (2016) with the production of *B. cereus* spores which increased by manganese supplementation. Several studies showed that manganese was important mineral for the Bacillus sporulation process (Charney *et al*., 1951; Sinnelä *et al*., 2019; Weinberg, 1964) . Moreover, the need for manganese in the Bacillus spore production was related to its role in the formation of proteases or protein-breaking enzymes. Processes of Bacillus spore production was related to its role in the formation

Table 2. The number of vegetative cells, maximum spores and growth rate of *B. subtilis* SB3 spores

Treatment	Maximum vegetative cells (cells. mL-1)	Maximum spores (spores. mL-1)	Spores growth rate (Hour $^{-1}$)
M1	2.54×10^8 c	8.77×10^7 c	0.77c
M2	2.06×10^8 b	6.21×10^7 b	0.56b
M3	1.88×10^8 a	5.06×10^7 a	0.21a

The same notation behind the numbers indicated insignificantly different (p-value>0.05)

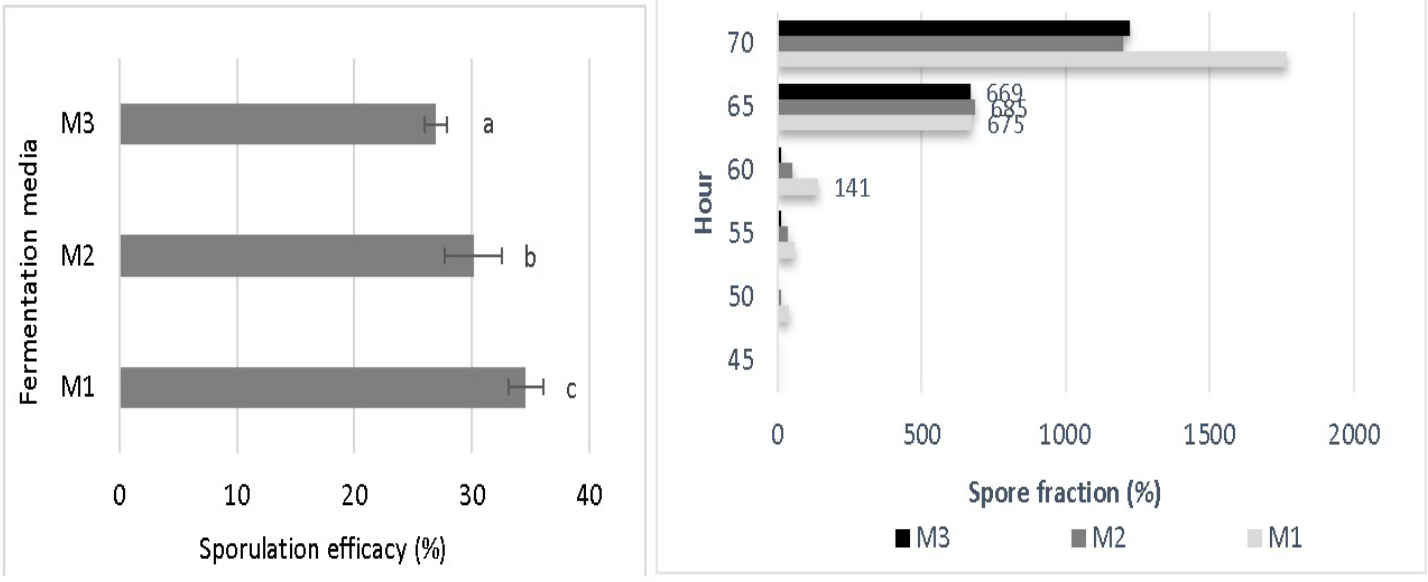

Figure 2. The sporulation efficacy (2A) and spore fraction (2B) of *B. subtilis* SB3 with different sources of manganese

of proteases or protein-breaking enzymes. Manganese was needed to produce proteases in *B. subtilis,* where the proteases can increases the function of enzymes in the process of *B. subtilis* spore formation.

The highest spore production of *B. subtilis* SB3 (8,77 x 10^7 spores. mL^{-1}) was observed in manganese chloride-supplemented media (SM1). Compared to the control (no manganese), the increase of spore production in manganese-supplemented media, in the form of manganese chloride and manganese sulfate, were accounted for 73.2% and 22.7%, respectively. The decrease *B. subtilis* SB3 spore production with supplementation of manganese sulfate occured as there was an inhibitory effect by sulfate (SO_4) in certain concentrations. In this study, the concentration of sulfate given in the form of manganese sulfate was 0.96 g. L^{-1}. The same result was noted by Monteiro *et al*. (2014a) that there was a decrease in the production of the spores in *B*. *subtilis* when ammonium in the form of $(NH_4)_2SO4$ was used at the concentration of more than 0.4 g. L^{-1}.

Spores are formed in vegetative cells and released to the media when mother cells undergo lyses. The spores undergoes physical and chemical changes along with its development. According to Setlow (2014), the stages of spore formation have started with the synthesis of low molecular weight proteins functioned to protect the DNA from damage. With divalent cation (Ca^{2+}), a large amount of dipicolinic acid (DPA) synthesized in stem cells have been taken by pre-spores. This then would cause a dehydration and mineralization in the spores. Meanwhile, the spore cortex (modification of the cell wall) has been synthesized outside the protoplast membrane of the spores. The outermost layer of spores have formed outside the cortex. Along with the spore formation, several abilities such as the ability to respond to certain germinants, to release the spore protective layer and to continue their vegetative cells would be also developed on the spore structure.

The high production of *B. subtilis* spores in media with manganese supplementations were initiated with the high production of their vegetative cell (Table 2). This indicated that in addition to its role in spore production, manganese also played a vital role in the growth of the vegetative cells. The same thingsalso happened with *B. brevis* that manganese can also stimulate the growth of its vegetative cells and spores (Charney *et al*., 1951). On the other hand, addition of manganese had no role in *B. pumilis* spore production eventhough it stimulated their vegetative one. This phenomenon indicated that a higher concentration of manganese was needed to stimulate sporulation in the *Bacillus* species. The need for manganese for normal vegetative cell growth was $5x10^{-8}$- $5x10^{-7}$M (Weinberg, 1964). Thus, the supplementation of manganese in the form of manganese sulfate and manganese chloride in this study has exceeded the manganese requirement for their vegetative cell growth.

3.2 Sporulation Efficacy

Sporulation efficacy is the percentage of vegetative cells that undergo a complete sporulation process. The Bacillus spores produced were heat-resistant up to 80°C. The efficacy of *B. subtilis* SB3 sporulation in this study is presented in Figure 2. It can be seen in Figure 2 that the efficacy of *B. subtilis* SB3 sporulation was affected by manganese supplementation. The non-supplemented media showed lower efficacy compared to that of manganese-supplemented media both in the form manganese chloride and manganese sulfate. Furthermore, the highest sporulation efficacy (35%) was recorded in media 1 (supplemented with manganese chloride) with the total spore production of 8.77 x 10^7 spores. mL^{-1}. Monteiro *et al*. (2014a) added glucose to the media during the exponential phase of *B. subtilis* and found a sporulation efficacy of 31.3%. In an ideal conditions, sporulation occured when the bacterial population reaches a bacterial cell density of about 10^8 cells. mL^{-1} with the sporulation efficacy ranged from 30-100% (Nicholson and Setlow, 1990). In this study, it was found that the higher growth, the higher sporulation efficacy of *B. subtilis* SB3 . It indicated that manganese was an important element, both for the process of vegetative cell growth and for the spore development in *B. subtilis* SB3.

Spore fraction showed the percentage of the number of spores compared to the vegetative cells at a certain time. It can be seen from Figure 2B that in manganese chloride-supplemented media, spore production was faster than that of manganese sulfate-supplemented and control media. It was detected that the number of spore cells in the manganese chloride-supplemented media was higher than that of their vegetative cells after hour 55. In the manganese sulfate-supplemented and control media, the *Bacillus* were able produce spore in similar number with their vegetative cells after hour 60. Yet, the number of spores produced in the manganese sulfate-supplemented media (3x 10^7 cells. mL^{-1}) and non-supplemented media (1x 10^7 cells. mL^{-1}) was significantly different.

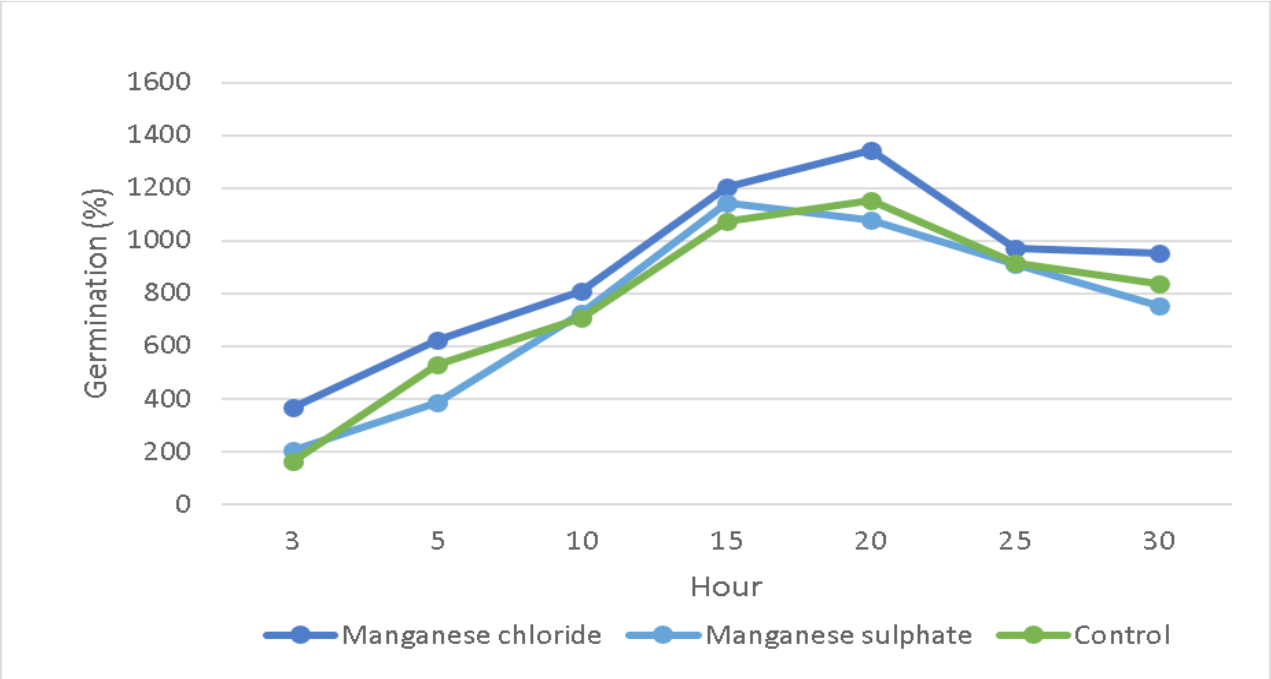

Figure 3. Germination rate of *B. subtilis* SB3 spores in different source of manganese suplement media

3.3 Germination

Spores can survive for years in dormant conditions. However, once there is a stimulus, the spores will lose their dormant and some resistance features (Setlow, 2014). The germination process will be the followed by the development of their vegetative cells. *B. subtilis* SB3 spores produced by supplementing different manganese sources in this study were re-grown in NB media. This germination rate showed the quality of spores produced in the media with different sources of manganese. Beside it can be produced in large quantities, the high-quality of spores have to be able to germinate in to vegetative cells. The result of the germination process in this study is depicted in Figure 3. The germination rate of *B. subtilis* SB3 spores was not affected by the supplementation of manganese from different sources. The supplemented media with manganese (both manganese sulfate and manganese chloride) as well as non-supplemented media, produced statistically similar germination rate. It showed that manganese affected the process of *B. subtilis* SB3 sporulation. However, it didn't affect its ability to germinate. The concentration of manganese ions that binds to dipicolinic acid (DPA) in the spore core affects the spore's resistance to various effects, including heat and ultraviolet light (Granger *et al*., 2011). Furthermore, the accumulation of Mn^{2+} in the cells protected the active enzyme spot from oxidative damage.

In the first 3 hours, it can be seen that all spores had germinated completely and even their vegetative cells grew more. The germination process is usually began with the adaptation phase or lag time. In this study, the lag time of the germination process can not be observed as the first observation was carried out on the 3rd hour. The adaptation phase for germination proccess varied for *Bacillus* species ranging from a few minutes to 24 hours (Setlow, 2013). The lag time of the germination process of the spores of *Bacillus* and *Clostridium perfringens* were influenced by several factors and it decreased along with (a) increase in the nutrient concentrations to saturated concentrations, (b) heat activation, (c) increase in the level of germination receptors.

The germination process can be stimulated by various agents which it called as germinant. The germinant was divided into nutrient and non nutrient germinants. Some non nutrient germinant were identified such as CaDPA, dodecylamine, and hydrostatic pressure (Setlow, 2013). Generally, the nutrient germinant will allow vegetative cell growth. An important phase in the germination process are the release of single cations and CaDPA, hydrolysis of peptidoglycan from the spore cortex, and expansion of the spore core. It will cause the protoplast of spores contains a lot of water, where metabolism and synthesis of macromolecules will be taken place. The difference in germination rate from spores is affected by the level of the germinant receptor (RG) functioned to recognize and respond to the nutrient germinant. Spores with low GM levels will germinate slowly.

4. Conclusion

Spore production and sporulation efficacy of *B. subtilis* SB3 are affected by different sources of manganese. It was recorded that the highest spore production and sporulation efficacy was in the media supplemented with 10mM of manganese choride.

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Author‘s Contribution

All authors have contributed to the final manuscript. The contribution of each author as follow, ATY: Data analysis and article writing, NBA: data collection, MFH: data collection, AMH: literature review.

Conflict of Interest

The authors declare that they have no competing interest

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