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Effect of C:N ratio on the spore production of *Bacillus* sp. indigenous shrimp pond

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Abstract. *Bacillus* sp SB4 was isolated from shrimp pond in East Java and screened to be a potential probiotic. Yet, this bacteria experienced decrease in number during storage in the form of vegetative cells. As *Bacillus* species can produce spore which able to survive in harsh condition, production of those spore will be favourable. The objective of this research was to evaluate the effect of C:N ratio on vegetative cells, spore production and sporulation efficacy of *Bacillus* sp. The results showed that C:N ratio influenced the vegetative, spore production and sporulation efficacy of *Bacillus* sp. SB4. Increase of vegetative cells of *Bacillus sp* was observed with the increase of C:N ratio on the media. In all treatments, the first spore could be identified after hour of 30. The highest spore production of *Bacillus* sp. SB4 was found in the medium with C:N ratio of 10 (5.13×10^7 cells. ml^{-1}) with sporulation efficacy of 42.8%. High vegetative cells production did not always followed by the high spore production. The different source and concentration of C and N and may become the reasons. Optimization of C:N ratio in the growing media for *Bacillus* sp. SB4 is still needed for higher spore production.

1. Introduction

Generally antibiotics are used to control bacterial diseasesAlmost 75% of fish and shrimp farmers use antibiotics to control several bacterial diseases they found during the culture [1]. However, as reported in several studies, the excessive use of antibiotics caused antibiotic resistances [2–5]. Furthermore, antibiotic resistances in the aquaculture industry has been transmitted horizontally through gene transfer to bacteria in the environment including pathogenic bacteria in humans. Therefore, the use of antibiotics in animal feed had been banned in Europe since 2006 [6]. Similar experince also had implemented in fish and shrimp industry which then alternative measures are urgently needed. Several usages of Bacillus as alternatives to antibiotics had recorded in aquculture industry [7–9].

Bacillus species are sprore-forming gram-positive bacteria which common in water, soil, dust and air [10]. These species are considered allochthonous bacteria that enter the digestive tract together with the food [6]. Bacillus spores also provide advantages over vegetative ones because spores are more resistant to extreme temperatures, desiccation, radiation and toxic materials [11]. Furthermore, spore of Bacillus are uneffected by condition within the digestive tract and make this possible to produce more stable probions[6,12]. Some Bacillus were isolated from the aquatic environment in East Java Indonesia and screened to be a potential probiotics for aquaculture industry [13]. Production of Bacillus sp SB4 in the form of vegetative cells experienced extreme decline in number during the storage. Therefore, the production of spore of this species will be promising. Some factors such as selection of carbon and nitrogen sources, ratio of C:N and mineral supplementation affected the spore

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production of *Bacillus* species [14]. Therefore, this study was aimed to evaluate the effect of C:N ratio on the spore production and sporulation efficacy of *Bacillus* sp SB 4.

2. Methodology

2.1. Strain.

Bacillus strain used in this study was *Bacillus* sp. SB4 which had already confirmed by molecular study of its 16SrRNA [13]. This strain was isolated from shrimp pond in Situbondo East Java. This bacterial stock was stored on Nutrient Broth (NB] with 20% of glycerol in -80°C.

2.2. Culture media.

In this carbon:nitrogen ratio study, glucose was used as the source of carbon and yeast extract was served as the source of nitrogen. The amount of yeast extract used was similar for all treatments about 14 g.l-1. On the other hand, the total glucose was adjusted to desired C:N ratio of 4:1, 7:1 and 10:1, i.e 10,20, 30 $g.l^{-1}$. Nutrient Broth (NB) ($8g.l^{-1}$) enhanced with minerals as commercial spore medium was considered as control. Several trace mineral were added to all media (1 litre) as much as: $CaCO_3$ 0.3 gr, $MgSO_4$. $7H_2O$ 0.00033 g, $MnSO_4$ H_2O 0.12 g, $FeSO_4$.$7H_2O$ 0.084 g, $CaCl_2$. $2H_2O$ 0.09 g. During this study, the pH was set to the value of 7. All treatments were repeated three times.

2.3. Cultivation condition

Experiments were carried out in 250-ml-erlemeyer flask with 100 ml of medium inoculated with 2 ml of stock culture (1×10^7 $cells.ml^{-1}$) in each flask. The culture were incubated in rotary shaker (120 rpm) at 30˚C for 70 hours.

2.4. Determination of vegetative cells, spore concentrations and sporulatian efficacy

Calculation of vegetative cells and spore of *Bacillus sp*. SB4 were performed microscopically with Neubauer chamber which had previously been diluted to facilitate the calculation process. The difference between vegetative and spore cells was based on the shape. The vegetative cell is in the shape of rod, while the spore cell is a cicle one. The value of vegetative bacterial cell density from the treatment was averaged and then plotted on the non-linear equation of the Logistics model $y(t) = a / (1 + b\ e^{-ct}$, where y = bacterial density, t = incubation time, e = 2.718 and a, b and c = estimated coefficient (Zar, 1984) using LAB Fit software [15]. The models was rewritten according to [16], who replace the mathematical parameters to bilogical ones to calculate the maximum specific growth rate (μ) and generation time. The sporulation efficacy (%) was determined as the ratio of spore production and the maximum of vegetative cells [14]

2.5 Statistical analysis

Statistically, all data were analyzed using one-way ANOVA using SPSS 15.0. Differences among treatments were compared using Duncan's Multiple range test ($p<0.05$).

3. Result and Discussion

3.1. Vegetative Cells of Bacillus sp.

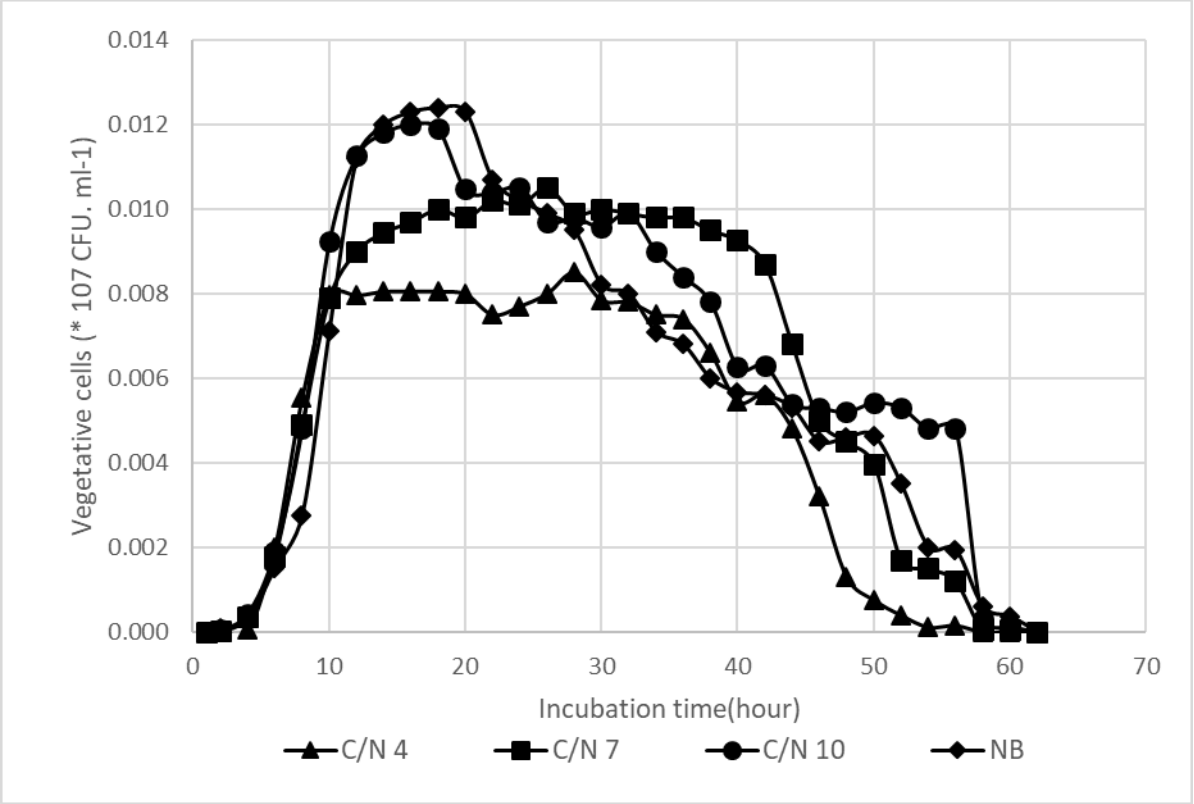

Figure 1. Time course of vegetative cells of *Bacillus sp* SB4 under different C:N ratio in the growing media

The production of vegetatif cells of *Bacillus* sp. SB4 cultured under different C:N ratio were pictured in Figure1. The highest vegetative cells production of Bacillus sp was recorded in the media NB with the C:N ratio of 11.9. The higher the C:N ratio, the higher the production of vegetative cells of *Bacillus sp*. In all treatments, there were significant increases during the first ten hours. After that, in the medium with C:N of 4, the number of vegetative cells tended to be in stationary phase. In contrast, in the medium with higher C:N ratio, increase in vegetative cells number were still noted until the hour of 18. *Bacillus* cultured in the medium with C:N ratio lower than 10 had longer stationary phase. In the stationary phase, the cell division proccess declined as the nutrient available reduced. The phase of death was marked by an increase of death rate that exceeds the growth rate of this species. The higher the growth rate, the sooner the death phase. The death phase of *Bacillus* with C:N ratio of 10 and 11.9 were recorded after the hour of 20, while those of *Bacillus* with lower C:N ratio were observed after hour 30. The vegetative cells of *Bacillus* sp. could not be detected anymore at the hour 58 in all treatments.

Data of vegetative cells of *Bacillus* sp. SB 4 in each treatment were plotted in logistic equation (Table 1) with the R^2 were more than 0.90. The maximum growth rate of vegetative cells of *Bacillus* sp. were influenced by ratio of C:N in the growing media. Significantly, increase of vegetative cells of *Bacillus* sp. SB4 was observed with the increase of C:N ratio. When nutrients in the growing media are abundant and freely available, microorganism will grow fast. There was a positive correlation between maximum growth rate and generation time. The higher the growth rate, the shorter the generation time. Generally the generation time for bacteria was between 20 minutes and 20 hours (17]. The variation of bacterial growth rate was strongly influenced by the genetic traits. In addition, the growth rate is also influenced by nutrient levels in the media, incubation temperature, pH conditions and aeration. The maximum population of vegetative cells *Bacillus* sp. *SB4* was also

influenced by the C:N ratio of growing media. In this study, the time needed for reaching the maximum population varied between hour of 10 to hour of 20.

Table 1. Growth character of *Bacillus* sp. vegetative cells cultured in various C:N ratio.

C:N ratio	Logistic Equation	Max growth rate (hour^{-1})	Generation time (minutes)	Max population (x10^7 CFU. ml^{-1})
4	$\frac{7,90}{1+1,3e^{-0,4t}}$ (R^2 = 0.94)	0.80 a	52.64	8.50 ± 0,21
7	$\frac{8,00}{1+1,44e^{-0,48t}}$ (R^2 = 0.99)	0.96 b	43,21	10.50 ± 0,31
10	$\frac{8,02}{1+1,41e^{-0,49t}}$ (R^2 = 0.95)	0.98 b	42.33	12.00 ± 0,15
11.9	$\frac{7,82}{1+1,31e^{-0,510t}}$($R^2$ = 0.96)	1.00 c	41.71	12.40 ± 0,32

3.2 Spore Production of *Bacillus* sp.

Ussually, when all effort to compete, grow and survive had been done, sporulation is considered to become the last choice for spore-forming bacteria [18]. The production of *Bacillus* sp. spores in the growing media with different C/N ratio are presented in Figure 2.

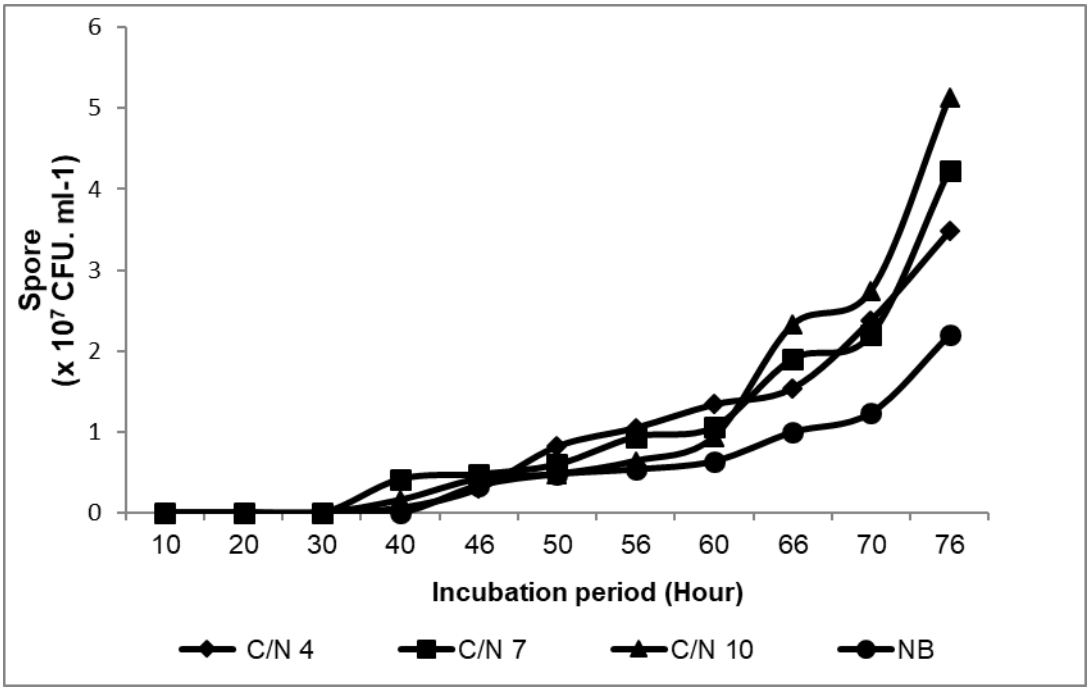

Figure 2. Time series of *Bacillus* sp. spore production under various C:N ratio in the growing media

In all treatments, *Bacillus* sp. SB4 spore production was recorded after hour of 30. At the same time the growth of vegetative cells of *Bacillus sp* began to decrease. The formation of spores begins when *Bacillus* reaches the highest concentration and its concentration will increase as the concentration of vegetative cells decreases [19]. The C: N ratio is one of the factors that influence bacterial growth. C/N ratio is a key factor in spore production of bacteria and fungi [14,18,20]. Furthermore, the balance between carbon and nitrogen was needed to avoid decrease of pH below 5.6 [21]. Later, this low of pH would affect the cell growth and spore production. The spore production

reported in several research extremely varied with the highest value of 3.8 x 10^{11} cells.ml^{-1} of *B. coagulans* and 3.6 x 10^{10} cells. ml^{-1} of *B. subtilis* [14,22]. In this study, the maximum spore production was only 5.13 x 10^{7} cells. ml^{-1}. Higher C:N ratio and higher carbon concentration will be needed for improving spore production.

It can be seen from the graph that the higher the C:N ratio of the media, the higher the production of *Bacillus sp* SB 4 spore. In average, there were an increase about 21.41% for every three (3) unit increase of C:N ratio in the media. However, high vegetative cells production did not always followed by the high spore production. This was true for the spore production of *Bacillus* sp. in the media of NB. There was a decrease about 57.11% of *Bacillus* sp. spore production in NB compared to that in the C:N ratio of 10. The different source and concentration of C and N and may become a reason of this. In this study, the source of N in the NB were beef extract, yeast extract and peptone. Meanwhile, in other treatments the source of N was only yeast extract. Furthermore, the concentration of glucose also effected the spore production. In NB media, the amount of glucose was considered limited as the source of C came also from the source of N. Some research showed that the certain concentration of glucose was required for the sporulation process for preventing lytic process of the cell at the end of exponential phase [14,23,24]. In bacteria, the concentration of carbon source was important in spore production. When glucose concentrations are more than 200g / l, *B. thuringiensis* cannot produce spores [25]. On the contrary, the concentration of carbon did not significantly influence the spore production of fungi. The concentration of carbon source had no significant effect on spore production of *Colletotrichum coccodes* [20]. Furthermore, they said that in a low C:N ratio, nutrient run out more quickly therefore it had positive effect on spore production of *C. coccodes*.

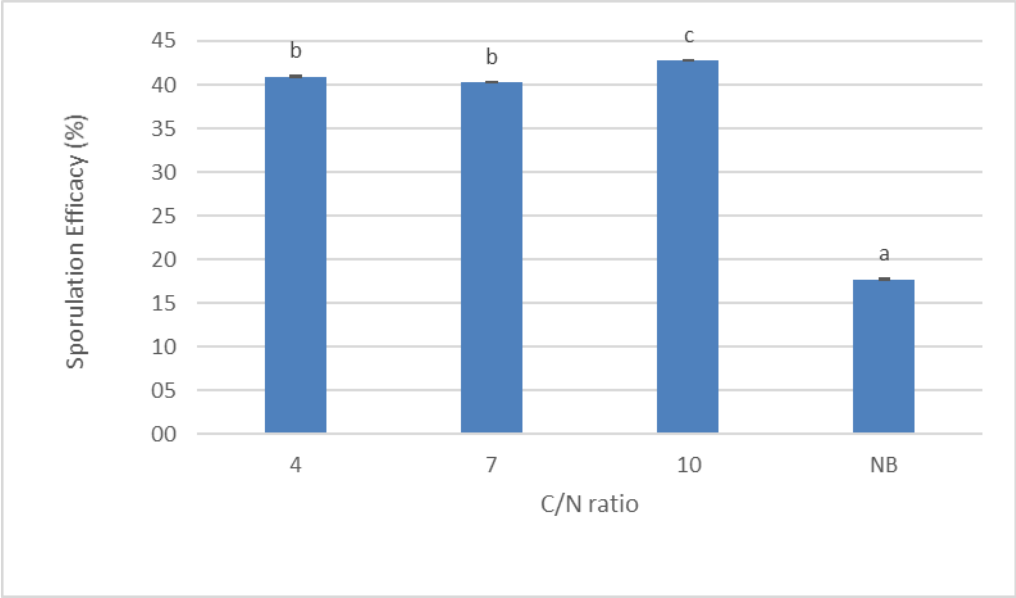

Figure 3. The sporulation efficacy of *Bacillus* sp. cultured in the media with various C:N ratio

Sporulation efficacy is percentage of the vegetative cells which experience a complete sporulation [14]. The sporulation efficacy of *Bacillus* sp. (Figure 3) was affected by the C:N ratio in which this bacterium grew. The highest sporulation efficacy of *Bacillus* sp was recorded in the media with C:N ratio of 10 (42.8%). It can be seen in this study that the higher the C:N ratio, the higher the sporulation efficacy. However, even the C:N ratio in NB was quite high (about 12), the sporulation efficay was the lowest. The highest sporulation efficacy (81%) of *B. coagulans* was reached in the medium of C:N ratio of 30 [22]. That result was correlated to 3.8 x 10^{11} cells. ml^{-1} of *B. coagulans* spore. The different results of *B. thuringiensis* spore production were recorded in several studies. While [26] found that the

highest spore production was recorded in the C:N ratio of 4, [19] noted those in the ratio C:N of 10. This showed that the origin of species was an important factor. In this study, optimization of C:N ratio for *Bacillus sp* SB4 is still needed for higher spore production.

4. Conclusion

Ratio of C:N in the media effected the vegetative, spore production and sporulation efficacy of *Bacillus sp* SB4. The highest spore production of *Bacillus* sp SB4 was found in the medium with C:N ratio of 10 (5.13×10^7 cells. ml^{-1}) with sporulation efficacy of 42.8%

5. References

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