Effect Of Different C:N Ratio Media On The Spore Production Of Bacillus Firmus

Kartika Septiana Kusumaningrum, Sri Andayani, Ating Yuniarti

Abstract: Bacillus is bacteria which can produce a single spore called endospore. In this recent years a lot of research on Bacillus has been carried out. In aquaculture, bacterial spores can be used as additional ingredients in feed, namely probiotics. Bacillus spore have many advantages compared to vegetative cells. To support the growth of Bacillus spores that have many advantages in aquaculture, this research was using media with glucose as carbon resource and extract yeast as nitrogen source. This works evaluated the effect of different C:N Ratio media on vegetative cells, spore production, and sporulation efficiency of B. firmus. By using media which contain adjustable C:N ratio hopefuly could minimalized the cost of growth medium for B. firmus spores. The result showed that the highest vegetative cells of B. firmus (1,95 x 10^8 cell/ml) was reach in the media with C:N ratio 11, the highest spore production of B. firmus (1,46 x10^8 cells/ml) was reach in the media with C:N ratio 5 with 91% sporulation efficiency and it could be used as a potential source of probiotics for aquaculture.

Index Terms: Bacillus, Carbon, C:N Ratio, Aquaculture, Media, Nitrogen, Probiotic, Sporulation.

1 INTRODUCTION

Bacillus is a Gram positive bacteria with a rod shape which have a single spore form (endospore), generally it can be used as probiotic [1]. This bacteria is one of biologic agent in aquaculture which can produce spore that useful for aquaculture [2]. Bacillus is bacteria which have many advantages needed from biologic agent in aquaculture because they can be found anywhere, [3];[4]. Bacilllus is one of gram-positive bacteria that able to increase the fish health digestibility. This bacteria have a characteristic that could excreting enzymes such as protease, lipase and amilase, so that it can be used in a probiotic form [5]. Bacillus can produce the endospore that can be survive in an extreme environment [6]. Bacillus in a vegetative form has been widely used, but not in the spore form probiotic. Bacillus spores are round, oval elliptical or cylindrical formed in vegetative cells [7]. Bacillus spores has many advantages than its vegetative form. It is more resistant to heat, chemicals, irradiation and desiccation [8]. [9] also stated that the use of spores as probiotics has survival and levels of inoculation that will be higher than vegetative cells. By knowing the advantages of Bacillus spores, it is necessary to culture with the right method and develop a spore production system, and determine the optimum conditions for these spore production. The existance of Carbon (C) and Nitrogen (N) inside media can affect the development of bacteria [10]. It is also said that the concentration of C:N ratio can affect to microorganism growth and its sporulation. When the C:N ratio were maintained constantly, the production of spore will increase by increasing carbon concentration [11]. The objective of this research was to evaluate the effect of C:N ratio in the media to B.firmus sporulation.

2 PROCEDURE OF RESEARCH

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2.1 Strain of Bacteria

Strain of Bacillus that used in this research was B. firmus isolate which cultured in Fish Disease and Health Laboratory of Brawijaya University. The bacterial stock was stored in Nutrient Broth (NB) with 20% of glycerin in -80°C.

2.2 Media Culture Preparation

As the source of carbon for C:N ratio medium is glucose and as the source of nitrogen is extract yeast. The doses of glucose as carbon source that used in this research were divided into three treatment (10, 20, and 30 grams per liter of distilled water (Aquadest®), respectively) and the doses of yeast extract as nitrogen source was 14 grams per liter, so that the medium could achieved C:N ratio 5:1, 8:1 and 11:1. Each treatments of media was dissolved with 1000 ml distilled water (Aquadest®) and mixed with minerals added in the medium as much as 0,3 mg $CaCO_3$; 0,00033 g $MgSO_4.7H_2O$; 0,12 g $Mn.SO_4.H_2O$; 0,084 g $FeSO_4.7H_2O$; 0,09 g $CaCl_2.2H_2O$. The treatments were repeated three times.

2.3 Cultivation Condition

The research was used 250 ml erlenmeyer flask, with 100 ml of medium that innoculated with stock culture as much as 2 ml in each flask (the density was about 1x10^7 cells per mililiters). The culture condition were incubated in rotary shaker at 30°C, 120 rpm for 70 hours.

2.4 Determination of Vegetative Cells, Spore Concentrations and Sporulation Efficacy

Vegetative cells and spores of B. firmus were calculated microscopically using Neubauer chamber and diluted using Aquadest® to simplify the counting process. The observation of vegetative cells and spores were determined by their shape. The shape of vegetative cells of B. firmus is a rod-shape but the spore is circle.

2.5 Statistical Analysis

The research data's was analyzed with one-way ANOVA using SPSS 16.0. If there is any differences among all repetition, the Duncan's Multiple Range Test ($p<0,05$) was used to compared each data.

3 RESULT AND DISCUSSION

3.1 Vegetative Cells of B. firmus

The production of B. firmus vegetative cells were cultured at different C:N ratio are showed in Figure 1.

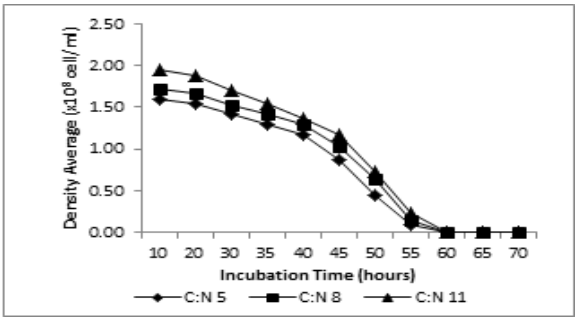

Fig.1 *Incubation time of the vegetative cells of B. firmus using different C:N ratio media*

The vegetative cells was started to grow at the first 10 hours. After that it showed declined phase until the 60th hours. It can be caused of the nutrition inside the media were decreased. [12] said that nutrition was one of the factor that can affect the bacterial growth pattern. The phase was not showed as bacterial growth that commonly seen such as adaptation phase, exponential and stationer phase. It could be happened because of the sampling time was held every 10 hours so that those phase can not be detailed observed. On the 60th hours to 70th hours, the vegetative cells was not produced because the cell was changed to be sporulated. [13] said that spore can be formed if the medium has decreased to empty nutrition. The result of the treatment of different C:N ratio which analyzed using ANOVA were showed that it give the different effect. The result showed that the highest of vegetative cells was produced in the medium with C:N ratio 11, then C:N ratio 8 and the lowest of vegetative cells was produced in the medium with C:N ratio 5. The lowest production of vegetative cells of B. firmus were meant that in this medium the vegetative cells has changed to another form that is called sporulation. The density of this vegetative cells showed at the Table 1. The result showed with the average ± SD with 3 treatment and 3 repetition.

Table 1.
The density average of the vegetative cells of B. Firmus

Fermentation time (hours)	*Density (x10⁸ cell/ml)*		
	C:N 5	*C:N 8*	*C:N 11*
10	*1,6±0,07[a]*	*1,72±0,05[b]*	*1,95±0,04[c]*
20	*1,54±0,04[a]*	*1,67±0,02[b]*	*1,88±0,04[c]*
30	*1,42±0,06[a]*	*1,53±0,05[b]*	*1,70±0,06[c]*

3.2 B. firmus Spore Production

Sporulation can be happened as a form of adaptation of microorganism body in the environmental that less of nutrition and give them time to survive until they encounter conditions acceptable to vegetative growth [14]. It also could happened if there is a lack of nutrients, high minerals, neutral pH, temperature and high cell density can make the vegetative cells changed into endospore or become spore formed [15]. The used of media with different C:N ratio on B. firmus would produced complete and uncomplete spores. In order to make it clearly seen, we can use staining method using malachite green and safranin. Malachite green function is to make spore form seen clearly, and safranin function is to make vegetative cells form seen clearly.

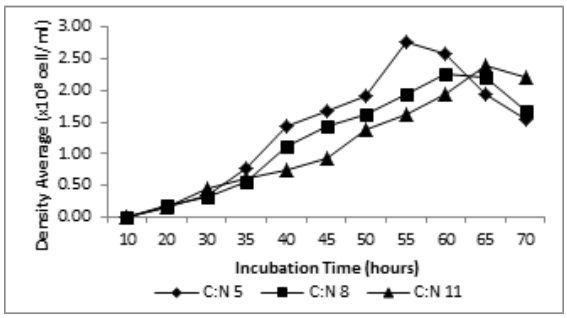

Fig.2 *Fermentation time of Uncomplete Spores of B. firmus using different C:N ratio media.*

Production of B. firmus uncompleted spore was cultured inside the medium that used different C:N ratio can be seen in Figure 2. Uncomplete spore production of B. firmus that used different C:N ratio was showed that it produced in its first 20th hours. The increased of uncompleted spores was started at the first 20th hours up to 55th hours and it decreased in media with a C:N ratio 5, whereas in media with C:N 8 and 11 ratio still continued. It because of the media with C:N 5 ratio has less nutrient, so that when nutrients begin to run out incomplete spores at the 60th hour have started to run into complete spores. It because of the media with C:N ratio 5 has the lowest nutrient than two other C:N ratios, so when the nutriens begin to run out incompleted spores at the 60th hour, it started to turn into completed spores form. This media has the decreasing uncompleted spore production significantly started at 20th hours to the 55th hours, compared to C:N 8 and C:N 11, this media was the fastest media which produce uncompleted spore. Because it contained the lowest nutrient so that it can produce the highest uncompleted spores. According to [16] B. thuringiensis produced the highest uncompleted spores on the first 40th hours. Beside the nutrient availability that can affect the production of uncompleted spores, the other thing that can affect it too was the type of Bacillus that used in the research. The result of ANOVA showed that the treatment of different C:N ratio give the real differences to uncompleted spore production. It showed in Table 2. The result showed with the average ± SD with 3 treatments and 3 repetitions.

Table 2.
The density average of uncompleted spores production of B.firmus

Fermentation time (hours)	*Density (x10⁸ cell/ml)*		
	C:N 5	*C:N 8*	*C:N 11*
50	*1,92±0,06[c]*	*1,61±0,16[b]*	*1,38±0,07[a]*
55	*2,76±0,16[b]*	*1,93±0,40[a]*	*1,62±0,03[a]*
60	*2,59±0,02[c]*	*2,26±0,10[b]*	*1,93±0,08[a]*

The highest uncompleted spore production was reach on the medium with C:N ratio 5, because it has the lowest nutrient. According to [11] glucose composition as the carbon source

can affect spore production. With the lowest C:N ratio, spore production will be fastest to form, because the nutrient inside will be run out fast. It suitable with [16] that the lowest C:N ratio, the fastest production of spore.

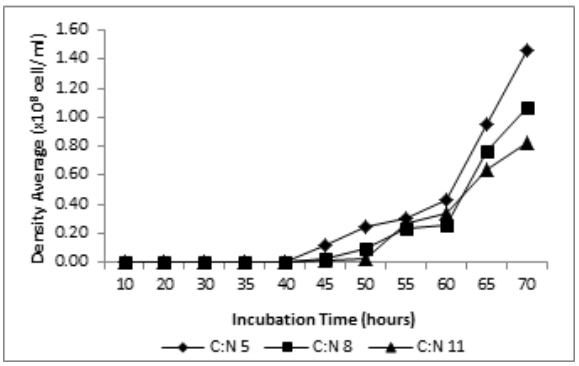

Fig 3. *Fermentation time of completed spores of B. firmus using different C:N ratio media.*

As it said according to [5] stated that the completed spore production will happened when the vegetative cells is not produced anymore. The spore production of B. firmus in this research can be seen in Figure 3. The result of those three treatments has significant differences. Media with C:N ratio 5 and C:N ratio 8 was increased from 45th hours until 70th hours. In medium with C:N ratio 11 was increased on the 50th hours. In this research, we got the result that medium with C:N ratio 5 was produced the highest spore than two other medium. It suitable with [17],[18],[19] who stated that maximal spore production is affected by the compositions of C:N ratio in the media. Source of carbon is glucose, which the concentration of glucose has ability to support the spore growth and conidia, and generally there is a tendency to increase spore production with an increase in glucose levels between 10-30 g/l. The result of ANOVA showed that treatment with different C:N ratio affected B. firmus spore production. It showed in Table 3. The result showed with the average ± SD with 3 treatments and 3 repetitions.

Table 3.
The density average of completed spore production of B. firmus

Fermentation time (hours)	*Density (x10^8 sel/ml)*		
	C:N 5	*C:N 8*	*C:N 11*
60	*0,43±0,01^c*	*0,25±0,02^b*	*0,34±0,04^a*
65	*0,96±0,02^c*	*0,76±0,03^b*	*0,64±0,01^a*
70	*1,46±0,03^c*	*1,07±0,02^b*	*0,82±0,02^a*

According to the research, the result showed that medium with C:N ratio 5 produce the highest completed spore than C:N ratio 8 and 11. It because of the nutrition in the medium with the lowest of C:N ratio was less and it made the sporulation growth higher and faster. So it can produce the highest completed spore. This result is suitable with [11] that the medium which has C:N ratio 5 to 10 can make spore grow faster.

3.2.3 Sporulation Efficiency of B. firmus

Sporulation efficacy counting was used to inform how many percentage the complete spore was formed. It comes from the percentage of maximum number of spore divided by the maximum number of vegetative cells [20]. The sporulation efficiency of B. firmus can be seen in figure 4.

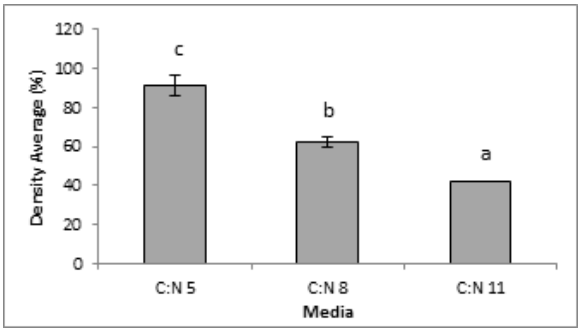

Fig 4. *Sporulation efficiency of B. firmus cultured in different C:N ratio media.*

Under the favorable conditions, vegetative cells in media that are rich in nutrients will produce high enough number of vegetative cells and grow faster. But as vegetative cells increased, the available nutrients also become limited. This affects the value of sporulation efficiency in B. subtilis [21]. The result of sporulation efficiency in this research can be seen in the picture that the highest efficacy is in the lowest C:N ratio media. The medium with C:N ratio 5 produce the highest sporulation efficacy as much as 91%, while the medium with C:N ratio 11 produce the lowest sporulation efficiency as much as 42%. This result is related to [17],[20] stated that the higher C:N ratio, the lower sporulation efficacy of B. thuringiensis will produced, it also found that the medium with C:N ratio 4 has the highest spore production. On the other research found that the highest sporulation efficacy of B. coagulans was reached in the medium with C:N ratio 30 as much as 81% [22]. The difference result of each study to another was influenced by the different strain of Bacillus, the different source of carbon and nitrogen, and the different ratio of C:N that used in the research.

4 CONCLUSION

The conclusion of this research is that the medium with different C:N ratios affected vegetative cells, spore production and sporulation efficiency of B. firmus. The highest number of vegetative cells of B. firmus (1,95 x 10^8 cell/ml) reached in the media with C:N ratio 11. The highest number of spore production of B. firmus (1,46 x 10^8 cell/ml), reached in the medium with C:N ratio 5. The sporulation efficacy of B. firmus was reached in media with C:N ratio 5 as much as 91%.

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