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Original Paper

THE EFFECT OF DIFFERENT C:N AND C:P RATIO OF MEDIA ON THE CONTENT OF POLYHYDROXYBUTYRATE IN BIOFLOC INOCULATED WITH BACTERIUM *Bacillus cereus*

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ABSTRACT

Biofloc technology has added values in aquaculture management, both in water quality management and feeding management. As an optional feed, biofloc is capable to enhance growth due to high protein content. Bacteria, main component biofloc, can produce polyhydroxybutyrate (PHB) as reserve of energy and growth accelerator for fish.

The aim of the research were to study the effect of the different C:N and C:P ratio of media on the content of polyhydroxybutyrate in biofloc and to determine optimum media to produce high polyhydroxybutyrate content in biofloc. The experiment was arranged in factorial with completely randomized design in three replications. Treatments were C:N ratio of 15, 20, 25 and C:P ratio of 75, 100, and 125.

The result showed that C:N ratio and C:P ratio of media and their interaction affect the content of polyhydroxybutyrate in biofloc. C:N ratio of 20 and C:P ratio of 125 resulted in most polyhydroxybutyrate (29.25±7.376 mg g^{-1} biofloc dry weight). Ratio of C:N of media gave linier and quadratic responses and C:P ratio of media gave linier one. Optimum polyhydroxybutyrate production was obtained at C:N ratio of 20.9 and C:P ratio of 125 resulting in 29.66 mg g^{-1} biofloc dry weight (2,97%)

Keywords : Biofloc, Polyhydroxybutyrate, C:N ratio, C:P ratio

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INTRODUCTION

Biofloc composed by bacteria, fungi, plankton, and other microorganism that contains high nutrition, mainly protein (> 40%), is organic fibre that rich of cellulose. Each component of biofloc is united due to bacteria producing polymer of polyhydroxyalkanoat (PHA) to form complex matter containing heterogeneous microorganism, organic polymer, and others. The structure of biofloc is similar to structure of bacterial protein, that is $C_5H_7NO_2$ (Avnimelech, 2009).

Bacteria's size is very small, less than 5µ (Volk and Wheeler, 1993, Purwoko, 2007) which is too small to be benefited by fish or shrimp. In biofloc form, the size one could reach 500µ to 2 mm, consequently it could be fed by shrimp or fish (Manser,2006) . As an alternative feed for shrimp, biofloc has huge potency due to high protein content and is available every time in culture media (Avnimelech, 2007).

Bacteria as main component of biofloc is capable to produce polyhydroxybutyrate

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(PHB). Polyhydroxybutyrate is polyester from D(-)-3-*hydroxybutyrate acid* found at first time in bacteria by Lemoigne in 1925 (Dawes, 1988). The formula of PHB generally is :

$$[\text{-O-CH(}CH_3\text{)-CH}_2\text{-C- }]_n$$

Polyhydroxybutyrate is the most dominant polymer and is useful in aquaculture. The advantages of PHB are an energy reserve for fish, digestible in intestine, increasing unsaturated fatty acid, and increasing growth of fish (de Schryver, 2010). According to several researches, PHB is capable to inhibit pathogen in the intestinal tract and to be antimicrobial against *Vibrio, E. coli, and Salmonella* (Boon et al., 2010), to control pathogen of *Vibrio harveyi,* and to enhance survival rate of *Artemia franciscana* larvae (Crab et al., 2010). Several bacteria that capable producing polyhydroxybutyrate are namely: *Bacillus megaterium* (Otari and Ghosh, 2009), *Bacillus cereus* (Nair et al., 2008, Margono, 2011) *Alcaligenes eutrophus* (Shimizu *et al*., 1993), and *Pseudomonas oleovarans* (Santhanam and Sasidharan, 2010).

The aims of this research were to study the effect of C:N and C:P ratio of media on the Content of polyhydroxybutyrate in biofloc and to determine optimum media to produce high polyhydroxybutyrate content in biofloc.

MATERIALS AND METHODS

Experimental design

The experiment was arranged in factorial, comprised of different C/N and C/P ratio with completely randomized design in three replications. The level of C/N ratios were 15, 20, and 25 while C/P ratios were 75, 100, and 125 (Table 1).

Biofloc experiment

Five liters of sterile sea water were filled into the reactors and to each reactor, glucose, $(NH_4)SO_4$, and KH_2PO_4 were added. Ratio of C/N and N/P were adjusted according to the treatments. Each reactor was equipped by aeration and was installed on the reactor bottom to provide sufficient dissolved oxygen (DO) level at min. 4 mg/l. Isolate of *Bacillus cereus* as heterotrophic bacterium was applied to each reactor at beginning experiment at the concentration of 10^6 CFU/ml. Growing biofloc was conducted for ten days before analyzing the content of polyhydroxybutyrate.

Analysis of PHB

The content of polyhydroxybutyrate was analyzed with a method described by Senior et al. (1972) and Yuksekdag et al. (2004). Suspension of biofloc obtained from the experiment was centrifuged at 4000 rpm for 20 minutes, supernatant was discarded. The pellets was dried at 40°C for 24 hours and the dry weight of biofloc was determined. Bacterial cell walls were lysed by adding sodium hypochloride, mixing and incubating at 60°C for 1 hour. The supernatant was obtained by centrifugation and discarded and the pellets were added with 3 ml 96% ethanol and acetone to liberate lipids and other molecules. Polyhydroxybutyrate was extracted by hot chloroform. Chloroform was evaporated to obtain PHB crystals. polyhydroxybutyrate crystals were dissolved in 10 ml of sulfuric acid (98%) to convert into crotonic acid by heating in water bath for 10 minutes. The absorbance of the solution was measured at 235 nm in a UV spectrophotometer against a sulfuric acid blank. The amount of PHB per dry weight of biofloc was determined using a standard curve of PHB.

Data analysis

All data were further analyzed statistically using Two-Way-Anova after testing of normality, homogenety, and linearity using SPSS statistical software. Statistical significance of differences required that $p < 0.05$.

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Table 1. Concentration of C, N, and P of each treatment

No.	Treatments	C (mg/l)	N (mg/l)	P (mg/l)	C:N:P
1	C:N ratio of 15 and C:P ratio of 75	1,200	80	16	75:5:1
2	C:N ratio of 15 and C:P ratio of 100	1,200	80	16	75:5:1
3	C:N ratio of 15 and C:P ratio of 125	1,200	80	16	75:5:1
4	C:N ratio of 20 and C:P ratio of 75	1,600	80	16	100:5:1
5	C:N ratio of 20 and C:P ratio of 100	1,600	80	16	100:5:1
6	C:N ratio of 20 and C:P ratio of 125	1,600	80	16	100:5:1
7	C:N ratio of 25 and C:P ratio of 75	2,000	80	16	125:5:1
8	C:N ratio of 25 and C:P ratio of 100	2,000	80	16	125:5:1
9	C:N ratio of 25 and C:P ratio of 125	2,000	80	16	125:5:1

RESULTS AND DISCUSSION

Polyhydroxybutyrate produced by *Bacillus cereus* on different C:N and C:P ratio of media are shown in Table 2.

Table 2. Polyhydroxybutyrate content of Biofloc for each treatment

No.	Treatments	Biofloc dry weight (mg)	PHB (mg) per sample	Content of PHB (mg g^{-1} biofloc dry weight)	Content of PHB in biofloc (%)
1	C:N ratio of 15 and C:P ratio of 75	20.0±0.00	0.301±0.041	15.05±2.090	1.51
2	C:N ratio of 15 and C:P ratio of 100	20.7±6.81	0.292±0.077	14.56±2.468	1.46
3	C:N ratio of 15 and C:P ratio of 125	21.7±2.08	0.241±0.074	11.33±4.299	1.13
4	C:N ratio of 20 and C:P ratio of 75	20.0+1.00	0.230±0.090	11.50±4.450	1.15
5	C:N ratio of 20 and C:P ratio of 100	20.3±2.31	0.400±0.045	19.98±4.197	2.00
6	C:N ratio of 20 and C:P ratio of 125	14.3±6.03	0.390±0.083	29.25±7.376	2.93
7	C:N ratio of 25 and C:P ratio of 75	23.7±3.21	0.357±0.008	15.33±2.568	1.53
8	C:N ratio of 25 and C:P ratio of 100	\21.0±3.61	0,391±0.036	19.22±5.447	1.92
9	C:N ratio of 25 and C:P ratio of 125	19.7±1.52	0.404±0.023	21.62±1.570	2.16

According to statistical analytic (Anova) showed that C:N and C:P ratio of media affect the content of polyhydroxybutyrate produced in biofloc. C:N ratio, C:P ratio, and their interaction gave an effect significantly on polyhydroxybutyrate production in biofloc. The highest polyhydroxybutyrate content was produced on media with C:N ratio of 20 and C:P ratio of 125 obtaining (±SD) 29.25±7.376 mg g^{-1} biofloc dry weight (Fig.1), followed by C:N ratio of 25 and C:P ratio of 125 (21.62±1.570) , C:N ratio of 20 and C:P ratio of 100 (19.98±4.197), C:N ratio of 25 and C:P ratio of 100 (19.22±5.447), C:N ratio of 25 and C:P ratio of 75 (15.33±2.568), C:N ratio of 15 and C:P ratio of 75 (15.05±2.090), C:N ratio of 15 and C:P ratio of 100 (14.56±2.468), C:N ratio of 20 and C:P ratio of 75 (11.50±4.450), and the lowest C:N ratio of 15 and C:P ratio of 125 (11.33±4.299).

Based on Least significant difference (LSD) test, the treatment of C:N ratio of 15 was significantly different with C:N ratio of 20 and 25, but C:N ratio of 20 wasn't different with C:N ratio of 25. Meanwhile C:P ratio of 75 was

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significantly different with C:P ratio of 125 but C:P ratio of 100 wasn't different with 125. Based on analysis of polynomial orthogonal, both of treatments resulted in different response. Ratio of C:N of media gave linier and quadratic responses, while C:P ratio of media gave linier one. Optimum ratio of C:N and C:P to produce the most polyhydroxybutyrate were 20.9 and 125 obtaining 29.66 mg g^{-1} biofloc dry weight (2,97%), based on equation : Y (PHB) = -0,5282X^2 + 22,045X – 200,363, r = 0,87 (Fig. 2).

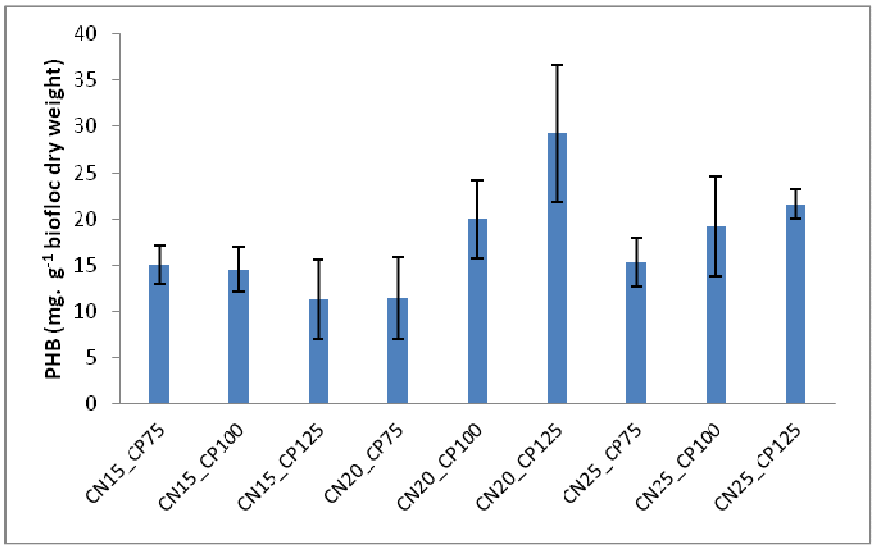

Fig 1. Effect of C:N and C:P of media on the content of polyhydroxybutyrate

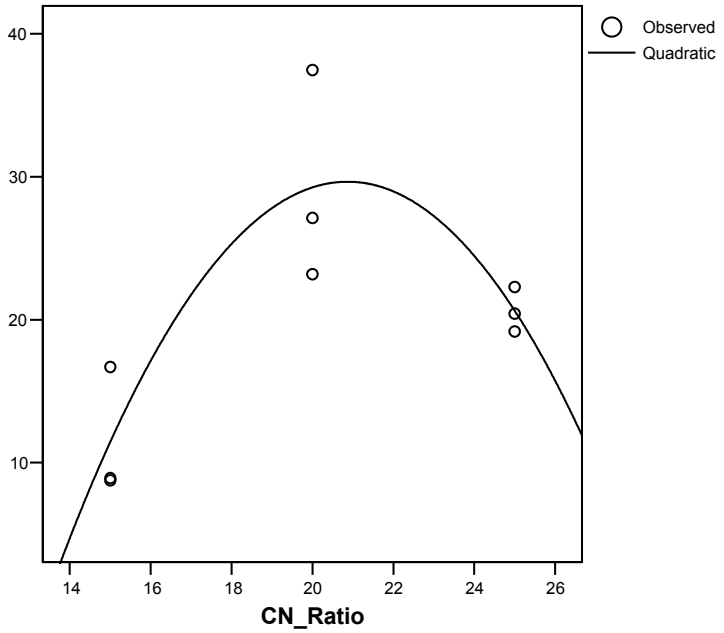

Fig 2. Optimum C:N ratio for producing Polyhydroxybutyrate (mg. g^{-1} biofloc dry weight) at C:P ratio of 125

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Bacillus cereus, useful heterotrophic bacteria in aquaculture, has a capability to produce polyhydroxybutyrate in some kind of carbon source (Margono, 2011). That bacteria, gram-positive one, having peptidoglycan in the cell wall could enhance animal immunity (Volk and Wheeler, 1993).

According to the data analysis, enhancement of C:P ratio resulted in enhancement of polyhydroxybutyrate content in biofloc (linier respon). While enhancement of C:N ratio resulted in enhancement of polyhydroxybutyrate and reached the peak at C:N ratio of 20,9. After that polyhydroxybutyrate in biofloc experienced declining due to lack of nitrogen. The existence of carbon (C), Nitrogen (N), and phosphorus (P) influent development of bacteria. C:N ratio and C:P ratio, such away to promote growth of heterotrophic bacteria. Bacteria use nitrogen to produce cellular protein (Avnimelech, 2009). Hence, excessive of Nitrogen and phosphorus will inhibit growth of bacteria and promote growth of algae. Heterotrophic bacteria will grow well and synthesize polyhydroxybutyrate when the cell become limited an essential nutrient including nitrogen and phosphorus but excess of carbon.

Increasing polyhydroxybutyrate production corresponded increasing C:N and C:P ratio can be explained with further analysis. According to Verlinden et al. (2007), the process of forming PHB by bacteria take place in three phases. Bacteria produce *acetyl- coenzyme-A (acetyl-CoA)* that converted into polyhydroxybutyrate form with supporting by three biosynthetic enzyme . At first phase, *3-ketothiolase* combine two molecule of *acetyl-CoA* to form *acetoacetyl*. At second phase, *acetoacetyl-CoA reductase* assist to form *3-hydroxylbutyryl-CoA*. Third phase, there is polymerization of 3-hydroxylbutyryl-CoA to polyhydroxybutyrate with liberating coenzyme-A. All process is described by Verlinden et al. (2007) as figure below :

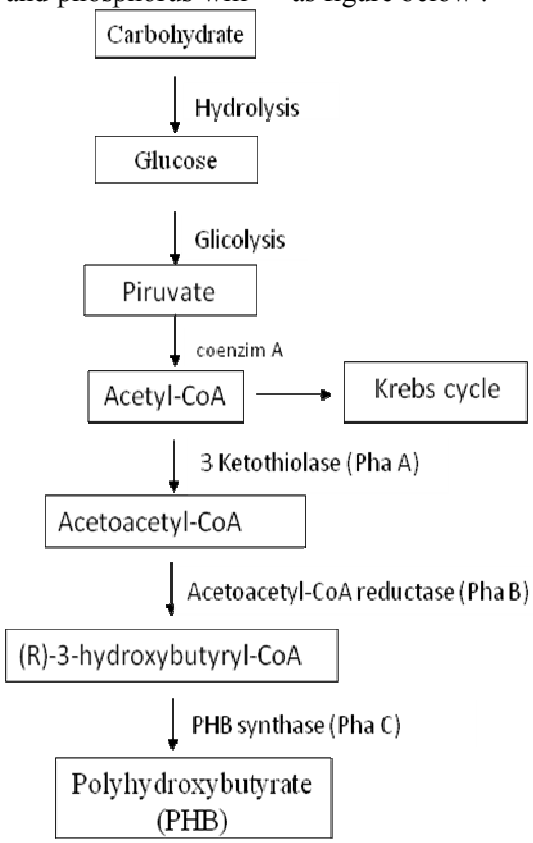

Fig.3. The path way of forming PHB in bacteria (Verlinden *et al*., 2007)

In normal condition, *Acetyl-CoA* will enter to Krebs Cycle, but it will be on the path of forming polyhydroxybutyrate if under nutrient limitation condition to carbon (Verlinden *et al*., 2009), mainly nitrogen (Dawes, 1988).

Optimum biofloc quality for aquaculture could be predicted by content of polyhydroxybutyrate. The content of polyhydroxybutyrate in biofloc could be obtained by administration of C:N and N:P ratio. C:N ratio of 20,9 and C:P ratio of 125 resulted in optimum polyhydroxybutyrate, hence optimum biofloc quality could be performed at these values.

CONCLUSIONS

Producing polyhydroxybutyrate by bacteria in biofloc was affected by C:N and C:P ratio media. Maximal polyhydroxybutyrate resulted in media on ratio C:N of 20 and C:P ratio of 125 (29.25±7.376 mg. g^{-1} biofloc dry weight). Responses of C:N ratio to polyhydroxybutyrate were linier and quadratic, and C:P ratio was linier one. Optimal C:N ratio and C:P ratio for producing polyhydroxybutyrate by bacteria were reached at 20.9 and 125, respectively, obtaining 29,66 mg. g^{-1} biofloc dry weight (2,97%).

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