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The effect of vancomycin on the growth and microbial dynamics of microalgae-bacteria consortium isolated from Glagah Beach, Yogyakarta

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ABSTRACT

The interaction between microalgae and bacteria in aquaculture might hold the key to the increase of biomass production from microalgae culture. With at least 22 phyla having been identified from the phycosphere, bacteria are known for being able to produce essential molecules for microalgae in a consortium culture. A consortium of microalgae and bacteria isolated from Glagah Beach, Yogyakarta Indonesia, named Glagah Isolate, showed to have higher biomass of that a single species cultivation. The effect of Vancomycin antibiotic on bacterial composition and diversity of the consortium culture was investigated using metabarcoding approach to compare between the treated (group A) and untreated samples (group T). The region V6 in the 16S rRNA gene was sequenced by using the MiSeq platform at 2×301 PE, and the library of V6 was eventually pooled according to the Illumina protocol. There were 14 phyla found in the consortium culture where was dominated by phylum Proteobacteria since it had the highest relative abundance on the lag, log, and stationary phase of the group T and the group A. The group T had higher diversity than the group A. The result from the growth imply that bacterial community has dynamically changed due to antibiotic treatment and affect the growth of microalgae.

***Key words**: 16S rRNA region V6, Bacteria composition, Diversity of the bacteria, Metabarcoding, Vancomycin antibiotic*

Introduction

Microbes are one of the most important aspects to pay more attention to it because microbes have played a key role in living condition on earth. Bacteria have a broad distribution especially in the ocean and having control of the biogeochemical cycle, ocean production, and decomposer (Singleton and Sainsbury, 2006; Stocker, 2012; Natrah *et al.,* 2014). This resulted from the interaction amongst microbes that will lead to change of chemical and physical composition of the ocean (Stocker, 2012). The interaction amongst microbes sound complex, but generally classified into mutualism, commensalism, and parasitism (Ramanan *et al.,* 2016; Cho *et al.,* 2017).

There were at least 22 bacterial phyla identified from the phycosphere. These phylum were domi-

nantly consisted of phylum Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria and Verrucomicrobia (Cai *et al.*, 2014). Several bacteria have been known to produce essential molecules for microalgae growth. Sulfitobacter has known to produce Indole Acetic Acid (IAA) to enhance microalgae growth (Ramanan *et al.*, 2016). *Flavobacterium* sp. can produce stimulator compound, such as vitamin B and glycoproteinto enhance the microalgae biomass (Kazamia *et al.*, 2012; Cho *et al.*, 2014). Meanwhile, Azospirilium and Cyanobacteria have known as anitrogenfree fixator (Ramanan *et al.*, 2016). The free nitrogen will be converted into nitrate (NO_3^-) that subsequently be absorbed by the algae (Krustok, 2016). Several bacteria have been known as pathogens and can produce algicidal compound, such as *Microbacterium, Ochrobactrum, Achromobacter, Bdellivibrio, Exophiala* and *Algoriphagus* (Carney *et al.*, 2014; Cho *et al.*, 2014; Gonçalves *et al.*, 2017).

Vancomycin antibiotic has widely used in microalgae axenic culture. Vancomycin inhibits cell wall and RNA synthesis on bacteria and change membrane permeability (Azma *et al.*, 2010). This antibiotic works by inhibiting polymerization of N-acetylmuramic acid and N-acetylglucosamine on bacteria and toward to cell lysis (Stocker, 2012). Vancomycin is less toxic and sensitive to gram-positive bacteria such as *Staphylococcus*, but some are resistant to Vancomycin, for example *Luconostoc* and *Pediococcus* (Azma *et al.*, 2010).

A mixed culture consisted of microalgae and bacteria isolated from Glagah Beach, Yogyakarta, Indonesia has been reported to have higher biomass than a single culture (Suyono *et al.*, 2016a). It highly potential to enhance lipid alongside with the higher biomass of the microalgae (Suyono *et al.*, 2016b). The bacteria of the consortium culture had been identified using streak plate method and six bacteria were identified, which are *Corynebacterium ulcerans, Corynebacterium bovis, Bacillus cereus, Bacillus megaterium, Pediococcusparvulus,* and *Staphylococcus vitulinus* (Suyono *et al.*, 2018). Nevertheless, conventional technique such as the plating method can only identify culturable bacteria, which might not represent the microbiome of the consortium. By utilising metabarcoding approach, we can identify representative microorganisms found from environmental sample, especially unculturable bacteria (Wooley *et al.*, 2010).

The objective of this study is to understand the effect of Vancomycin antibiotic on bacterial composition compared to the normal consortium culture and to know the diversity divergence between the treatedand the untreated consortium culture. The known bacteria composition in theculture was eventually used as basic knowledge to do community manipulation to obtain higher biomass and macromolecules, such as lipid, carbohydrate, and protein.

Materials and Methods

Preparation and cultivation of the consortium culture

This research was conducted at the Laboratory of Biotechnology, Faculty of Biology Universitas Gadjah Mada. In this study, the isolated sample from GlagahBeach was culturedby using Bold's Basal Medium (BBM) as instructed in the protocols by Bischoff and Bold (1963) (Aragaw and Asmare, 2017). The consortium was cultured tothe total volume of 500 mL for each sample, with 1:1 ratio of BBM and stock culture. The experiment consisted of the group without Vancomycin (groupT) and the treatedgroup by Vancomycin (groupA). Group A was given 50 µL L^{-1} of Vancomycin, while group T was not given any antibiotic. The groups of samples T and A were cultivated under 6000 lux at 25! in temperature for 14 days. Each group was divided into three samples based on the day sample was taken. 5 mL of samples were taken in day 0, 6, and 12, respectively called as sample T1H0, T1H6, and T1H12 for group T, while in group A there were A1H0, A1H6, and A1H12.

Sequences preparation and sequencing

The samples are preserved in RNAlater™ and send to PT Genetika Science for library preparation and NovoGene amplicon sequencing using Illumina MiSeq platform. The libraries of the V6 region of SSU rRNA sequences were collected using universal primers 926F (5ˆ–AAACTYAAAKGAATTGRCGG–3') and 1392R (5'–ACGGGCGGTGTGTRC–3 ') (Rinke *et al.*, 2014). The library was pooled according to Illumina protocol adjusted with QIIME2 SILVA database.

QIIME2 workflow

The V6 sequences stored in form of FastQ file. The FastQ file was imported to QIIME2 by Casava 1.8

paired-end demultiplexed. The sequences were paired by VSEARCH then filtered to get the Q-score. Alignment was done by using MAFFT alignment (Katoh *et al.,* 2002). The bacteria community was analysed using Bray Curtis coefficient. Alpha diversity was analysed using Faith's Phylogenetic Diversity. Bacteria classification and composition was adjusted according to Green Genes database gg13899 nb classifier.

The output was consisted of bacteria taxonomy, the relative abundance, and diversity which were showed in the bar chart. The alphadiversity was showed in Observed, Chao1, and Simpson's Diversity Index, while the betadiversity was showed in a Principal Coordinate Analysis (PCoA).

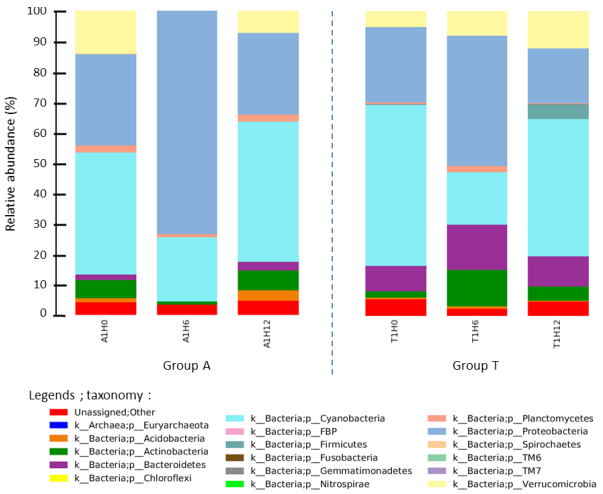

Fig. 1. Bacteria composition on the group of samples without Vancomycin antibiotic (group T) and that given Vancomycin antibiotic (group A).

Result and Discussion

Results

Both groups had the same number of bacteria phyla but differ in the composition. The two groups had 14 bacteria phyla which group T consisted of phylum Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Cyanobacteria, Firmicutes, Fusobacteria, Gemmatimonadetes, Planctomycetes, Proteobacteria, Spirochetes, Verrucomicrobia, and 2 candidate phyla, that were FBP and TM7. On the other hand, group A did not found phylum Fusobacteria and candidate phylum FBP but had Spirochaetes and TM6 (Table S 1). The phylum Cyanobacteria had the highest relative abundance, but in this study, Cyanobacteria referred to plastid organelle which indirectly meant the relative abundance of the microalgae. From the graph, it shows that Phylum Proteobacteria had the highest relative abundane among other phyla.

Fig. 2 shows the dynamics of the microalgae growth for 12 days. The treatedculture (group A) generally had lower microalgae density than the untreated culture by Vancomycin antibiotic (group T). In the early 4 days, the microalgae density of the sample groups seemed tohavethe same pattern of growth. The group T had rapid growth in day 4 to 8,

Phylum	A1H0	A1H6	A1H12	T1H0	T1H6	T1H12
Euryarchaeota	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %
Acidobacteria	1.4 %	0.0 %	3.4 %	0.6 %	0.7 %	0.5 %
Actinobacteria	5.8 %	0.9 %	6.3 %	1.9 %	12.0 %	4.6 %
Bacteroidetes	1.9 %	0.0 %	2.9 %	8.5 %	15.0 %	9.9 %
Chloroflexi	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %
Cyanobacteria	40.2 %	21.2 %	46.1 %	52.8 %	17.0 %	44.9 %
FBP	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %	0.1 %
Firmicutes	0.0 %	0.0 %	0.1 %	0.1 %	0.1 %	4.7 %
Fusobacteria	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %
Gemmatimonadetes	0.0 %	0.0 %	0.0 %	0.2 %	0.0 %	0.1 %
Nitrospirae	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %
Planctomycetes	2.1 %	1.1 %	2.4 %	0.5 %	2.1 %	0.4 %
Proteobacteria	30.0 %	73.3 %	26.9 %	24.8 %	42.7 %	18.0 %
Spirochaetes	0.3 %	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %
TM6	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %
TM7	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %	0.0 %
Verrucomicrobia	14.1 %	0.0 %	7.2 %	5.1 %	8.1 %	12.2 %
Unassigned;Other	4.2 %	3.4 %	4.8 %	5.4 %	2.4 %	4.6 %

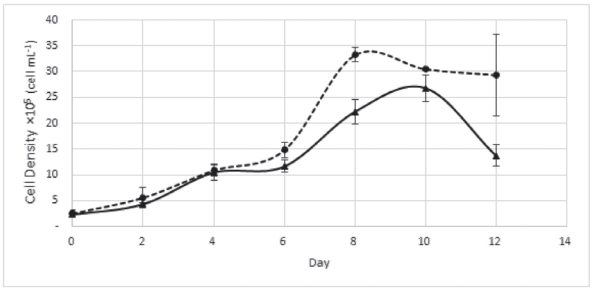

Fig 2. Microalgae growth rate in the consortium culture of isolated culture from Glagah Beach

that was 10,859,375 cell·mL^{-1} to 33, 203, 125 cell·mL^{-1} and then continued decreasing until day 12 (29,375,000 cell·mL^{-1}) (Table 1). Meanwhile, in group A, the rapid growth happened in day 4 to 10, that was 10,479,167 cell·mL^{-1} to 26,791,667 cell·mL^{-1} and then dramatically decreasing in day 12 (13,765,625 cell·mL^{-1}) (Table 1).

Both Fig. 1 and 2 show the relation between bacteria composition and microalgae growth showing by cell density. There were different bacteria composition since day 0. The group A was dominated by Phyla Proteobacteria, Verrucomicrobia, and Actinobacteria, while in the group T was dominated by Proteobacteria, Bacteroidetes, and Verrucomicrobia. In day 6 of the microalgae growth, which the density of group T was higher than group A showed a significant difference inthose bacterial compositions. Phylum Proteobacteria dominated in both groups, but group T had more evenly distributedof the bacteria composition. Both microalgae growth was decreasing in day 12, which group A had dramatically decreasing than group A. Both groups also had different bacteria composition, which group A was dominated by phyla Proteobacteria, Actinobacteria, and Verrucomicrobia, while in group T was dominated by Proteobacteria, Bacteroidetes, Verrucomicrobia,

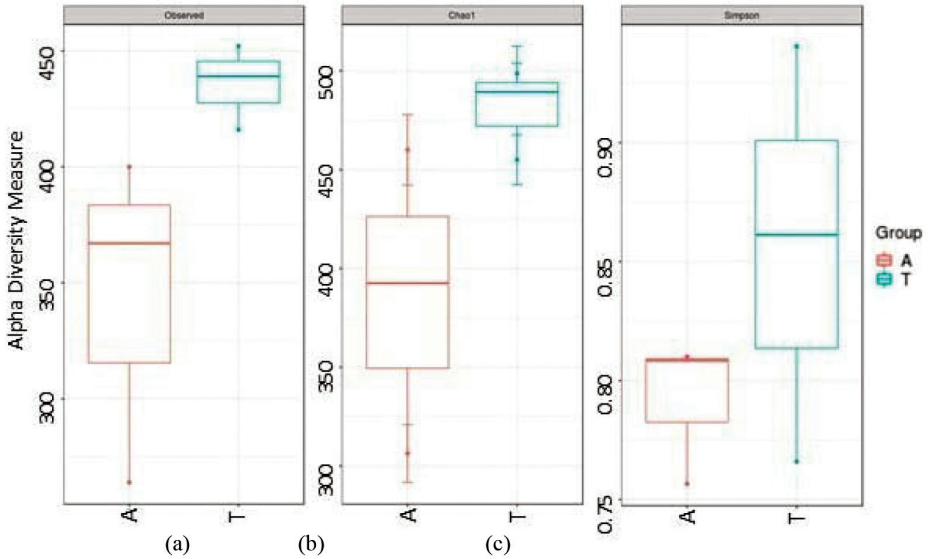

Fig. 3. Alpha diversity of the group of samples without Vancomycin antibiotic (the group of samples T) and by given the Vancomycin antibiotic (the group of samples A) using the isolated consortium culture from Glagah Beach. (a) Observed, (b) Chao1, and (c) Simpson's Diversity Index.

Table 1. Microalga growth in Day 0 to 12 of the consortium culture without Vancomycin antibiotic (group T) and the consortium culture threaten by Vancomycin antibiotic (group A)

	Day 0	Day 2	Day 4	Day 6	Day 8	Day 10	Day 12
Culture without antibiotic (group T)	2,510,417	5,562,500	10,859,375	14,781,250	33,203,125	30,531,250	29,375,000
Deviation standard	714,207	1,949,559	1,171,146	1,458,408	1,392,116	132,583	7,930,792
Culture with antibiotic (group A)	2,385,417	4,385,417	10,479,167	11,656,250	22,177,083	26,791,667	13,765,625
Deviation standard	378,026	154,153	1,496,197	1,135,799	2,342,778	2,613,504	2,010,835

and Firmicutes which drastically increasing.

The diversity of Bacteria in the isolated consortium culture from Glagah Beach

The species diversity of the samples show that every sample had a unique composition of bacteria and niche. The observed total bacteria in group T was 435, while in group A was 389 (Fig. 3). The group T had higher Chao1 than in groupA, that was 481 on the group T and 433.67 in groupA (Fig. 3). The Simpson's Diversity Index on groupT was 0.86, while in groupA was 0.82 (Fig. 3). The higher value of the groupT than on the group A in Observed, Chao1, and Simpson's Diversity Index values indicatedthat the group T had higher species richness and evenness than in the groupA.

The distribution of blue dots (group T) and red dots (groupA) on the plot in PCoA shows that they were gathered in some areas. The shorter distance between two dots implied that they had higher similarity in common(Bassett *et al.*, 2015). According to the PCoA in Fig. 4, sample T1H0 and T1H12 had the closest distance in terms of the group T, while sample A1H0 and A1H12 had the closest distance amongst the group A.Meanwhile, sample T1H6 and A1H6 had fartherdistance than other samples in their group of samples, that indicated that they haddiversein bacteria composition amongst the other samples. The difference in the distribution of the dots was caused bythe presenceof the antibiotic and the microalgae growth, which affected at bacteria composition of the two groups of samples.

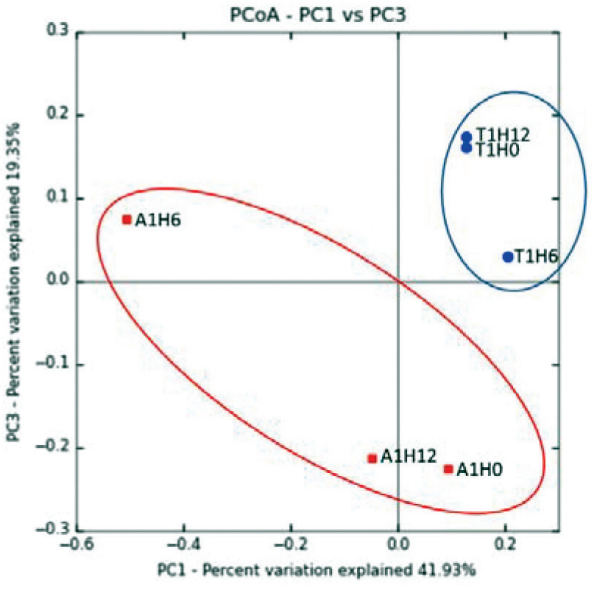

Fig. 4. Principle Coordinate Analysis (PCoA) of betadiversity in the group of samples without Vancomycin antibiotic (group T) and those given the Vancomycin antibiotic (group A)

Discussion

Our study shows that group T and A hadthe same numbers of phyla but slightly different in phyla composition. Even though the group T and A had the same number of phyla, but they had different alpha diversity values and in the density of the microalgae. Alpha diversity value and microalgae density in the group T was higher than the group A. Phylum Fusobacteria and FBP were not found in group A, while phylum Spirochaetes and TM6 were not found in the groupT. The absence of phylum Fusobacteria and FBP on the group (Fig.1 and Table S1) indicated that Fusobacteria and FBP were sensitive toward Vancomycin antibiotic.

On the two groups of the samples, phylum Proteobacteria had the highest relative abundance amongst the other phyla, that was 36% on average. During the growth of microalgae in the consortium culture, the relative abundance of Proteobacteria was increasing to 42.7%at the log phase, then decreased by 24.7% atthe stationary phase of the microalgaegrowth phases (Fig. 1). The high of Proteobacteria relative abundance was caused by the Proteobacteria had the highest numbers of taxa and abundant on the consortium culture that isolated from Glagah Beach, so the universal primers that used could amplify high numbers of V6 region in phylum Proteobacteria. The interesting thing was the relative abundance of Proteobacteria had inversely to Cyanobacteria as in this study the relative abundance of Cyanobacteria indirectly showed microalgae biomass.

Fig.3 shows that the alpha diversity on the group T was higher than on the group A. The high value of Observed and Chao1 on the group T indicated that the group T had higher species richness than on the group A.The higher value of Simpson's Diversity Index means that the higher value of species richness and evenness of a community (Morris *et al.*, 2014). Same with the Observed and Chao1 value, the group T had a higher value of Simpson's Diversity Index than on thegroup A which indicated that the group T had higher species richness and evenness. Based on the difference in the composition of bacteria community and the alpha diversity values, by giving the Vancomycin antibiotic to the consor-

tium culture lead to decrease the bacteria species and abundance in the consortium culture. The decreasing of bacteria species and abundance lead to the decrease of microalga growth and density. It clearly indicated that the beneficial bacteria in the normal consortium culture (group T) had removed and it also affected the absorption of essential nutrients and growth factor by the microalgae.

Beta diversity on the group T and A shows that they gathered based on the presence of the antibiotic. Each of the samples was taken on day 0, 6, and 12 that respectively represented lag, log, and stationary phase of microalgae growth (Fig. 2). Log phase of microalgae growth is where the peak of energy and nutrient consumption as microalgaere produced.As microalgae growin the log phase followed by the bacteria growth, which caused there to be dominated by bacteria that mutualistic and commensals toward the microalgae. Meanwhile at the lag and stationary phase of microalgae that had high similarity amongst the bacteria composition because they had stabilized. The culture which in the stabilized state will be more focused onthe adaptation and photosynthesis

Another effect of Vancomycin antibiotic on the consortium culture was increasing in the relative abundance of phylum Proteobacteria and decreasing of phylum Verrucomicrobia, Planctomycetes, Bacteroidetes, Acidobacteria, and Actinobacteria. Different condition on the group T, which the phylum Verrucomicrobia, Planctomycetes, Bacteroidetes, Acidobacteria, and Actinobacteria had increased in the relative abundance followed by Proteobacteria, still was not much as on the group A. From this study, could be known that the Proteobacteria growth had competed with the growth of Verrucomicrobia, Planctomycetes, Bacteriodetes, Acidobacteria, and Actinobacteria, while these fourth bacteria possibly had associated on each to maintain their relative abundance.

In this study, the relative abundance of phylum Cyanobacteria also recorded in Fig. 1, which referred to the V6 region of the plastid in a microalgae cell. Indeed, each phylum had its own function in the microalgae growth, which indirectly showed by dynamics of bacteria composition in Fig. 1. Until now, the keystone of the bacteria species in the consortium culture of the isolated culture from Glagah Beach has not known, especially on each phase of microalgae growth. On the other hand, the interaction amongst bacteria has not clearly explained, that is why it has to be deeper analysis in the species level of bacteria and further analysis on the bacteria interaction bymath modelling approach.

It can be concluded that by giving Vancomycin antibiotic in a consortium culture will be decreasing bacteria diversity and shifting the bacteria composition compared to the untreated consortium culture

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