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Screening of IAA Production on the Interaction of Microalgae and Bacteria in the Glagah Consortium

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The Glagah Consortium is a mixed culture of microalgae and bacteria isolated from Glagah Beach, Yogyakarta. Microalgae and bacteria in the consortium interact with each other. There is an assumed Bacillus bacterium that can produce IAA in the Glagah Consortium. Tryptophan is one of the precursors in IAA biosynthesis. L-tryptophan and antibiotics were given to the Glagah consortium culture medium to determine the role of bacteria in producing IAA. This research used BBM medium by giving three variations of L-tryptophan concentration, namely 0; 0,02; and 0.2 g L-1. Each concentration is given antibiotics Vancomycin and Gentamycin for 100 ppm and 25 ppm. Quantification of IAA by using Salkowski reagents and calculated IAA concentrations with a spectrophotometer. Each treatment consisted of three replications. The other parameters tested were the number of microalgae cells, dry weight, cell productivity, biomass productivity and lipid productivity. The results in this research shows that IAA in all samples with the highest concentration on the second day of observation, they are 0.420 ± μg mL-1; 0.681 μg mL-1; 1,725 ??μg mL-1; 0.261 μg mL-1; 0.565 μg mL-1; and 0.667 μg mL-1. The giving of L-tryptophan in the Glagah consortium culture can increase IAA concentrations, specific growth rates, biomass and lipids. Besides that, the giving of antibiotics led to lower productivity of cells, biomass and lipids of the Glagah consortium.

Keywords: Antibiotic; Glagah Consortium; Interaction; IAA; L-Tryptophan.

Microalgae are micro organisms and has diameter about 1-50 micrometers. Microalgae are photoautotrophic, because they use light and carbon dioxide (CO2) to produce oxygen and carbohydrates. Besides that, microalgae can also produce lipids, proteins, and pigments (ß-carotene), so that the biomass of these microalgae can be utilized in the food and energy industry[1]. Microalgae in nature are known to interact with bacteria in the form of a consortium. Interaction is carried out to achieve growth stability for microalgae and bacteria. The stability of the growth occurs because both of them can exchange nutrients with each other. The relationship between the two is mutually beneficial and forms symbiosis of mutualism[2].

The Glagah Consortium consists of a mixture of 6 species of microalgae and 6 species

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of bacteria isolated from Glagah Coast, Kulon Progo, Yogyakarta. Symbiosis of mutualism was also found in the Glagah consortium 3.Microalgae found in the consortium include Cyclotella polymorpha, Cylindrospermopsis raciborskii, Golenkinia radiata, Turquoise Syracosphaera, Corethron criophilum, and Chlamydomonas sp. The microalgae in their natural habitat symbiosis with bacteria include Corynebacterium ulcerans, Corynebacterium bovis, Bacillus cereus, Bacillus megaterium, Pediococcus parvulus and Staphylococcus vitulinus[3,4]. In tthe Glagah consortium are known to have higher growth rates and biomass compared to microalgae in single cultures[5]. This is supported by the results of a study[6], namely the microalgae of the Glagah consortium had a higher dry weight, lipid content, and cell count than single culture Chlorella zofingiensis, which was 3.42 mg mL-1; 13.58%; and 9.8 x 106 cells mL^{-1}.

The increasing of growth and biomass from the Glagah consortium are thought to be influenced by the presence of growth booster factors released by bacteria, and in return the bacteria obtain growth supporting compounds in the form of organic carbon from microalgae. Growth factors released by bacteria in the form of Cobalamin and Thiamin. In addition, bacteria also produce growth hormones, namely IAA (Indole-3-acetic-acid)[7,8,9]. IAA is a type of auxin found in many microalgae and bacterial consortiums. IAA is used by microalgae for growth and development, such as cell division, cell enlargement and microalgae tissue differentiation[10]. IAA is thought to be produced by bacteria in the Glagah consortium. One of the bacteria that can produce IAA hormone comes from the genus Bacillus7. Bacillus bacteria are also identified in the Glagah consortium. Bacillus bacteria in the Glagah microalgae consortium are thought to be able to supply IAA that is used by microalgae to spur growth and development.

Tryptophan is a precursor used by bacteria to produce IAA. Tryptophan is produced by microalgae and the precursor is used by bacteria to synthesize IAA. L-tryptophan is an effective precursor for increasing IAA production in microalgae cultures[11]. To determine the effect of L-tryptophan on microalgae in the Glagah consortium to produce IAA, in this study additional precursors were conducted in various concentrations. Furthermore, the IAA produced by bacteria in the Glagah microalgae consortium is assumed to affect the growth of microalgae in the consortium. So this research is useful as an effort to increase the productivity of microalgae in the Glagah consortium.

MATERIALS AND METHODS

Study Area

This research was conducted at the Biotechnology Laboratory, FALITMA Laboratory (Joint Facility) of the Faculty of Biology, Universitas Gadjah Mada, Yogyakarta.

Procedures

Culture Cultivation

The sterile bottle is filled with BBM medium (Bold Basal Medium) which is given an additional L-tryptophan with 3 variations of concentration, namely 0; 0.02; and 0.2 L-1, 3 replications each with additional antibiotics (100 ppm Vancomycin and 25 ppm Gentamycin) and without antibiotics. 200 ml of isolates were inoculated into 300 ml growth medium. Then the bottle is closed tightly to prevent contamination and is given CO2 aeration and lighting with 3000 lux lights. For 10 days microalgae was sampled and various tests were carried out. The design of this study is as follows:

Quantification of IAA with Spectrophotometry

Colorimetric methods use supernatants from microalgae and bacterial cultures. This method uses the Salkowski reagent and the supernatant reacted, then measured using a spectrophotometer12. IAA production was identified based on the12 method with several modifications. 1 milliliter of the supernatant was mixed with 2 ml of Salkowski reagent (1 ml of FeCl3 0.5 M into 50 ml of HClO4 35%), incubated for 1 hour. Pink indicates IAA production. Quantification of IAA is read on the UV-Vis spectrophotometer with a wavelength of 530 nm. The amount of IAA content is known in µg mL-1 [13].

Measurement of Growth Parameters

Calculation of Cell Density

Cell counting using Haemocytometer, samples taken as much as 800 µl were inserted into a 2 ml tube that had been added 200 µl of 70%

alcohol, left for about 20 minutes before counting under a microscope[14]. Calculation of cell numbers using formula:

Cells density= (total amount of cells/64) x 160 x 104 x 1.25

Dry Weight Calculation

Calculation of dry biomass is carried out every day on day 0 to day 10. First, 1 ml of sample is inserted into the tube then centrifuged at 8000 rpm for 15 minutes. Supenatan is removed and left with pellets inside the tube. The tube contains pellets then in the oven at 25° C. Calculated the dry weight of the tube with a pellet until the weight is constant.

Dry Weight = Final weight of tube – Dry weight of tube

Total Lipid Measurement

Total lipid levels were analyzed by15. A total of 5 mL of sample was put into a conical tube and then centrifuged for 15 minutes at 4000 rpm. The supernatant which was then removed and then the pellet was added 2 mL of methanol and 1 mL of chloroform. The tube containing pellets, methanol and chloroform is then vortexed until homogeneous. After being homogeneous, the tube was centrifuged for 15 minutes at 4000 rpm. Samples that have been centrifuged will show layers of clear and yellow liquid. The clear liquid layer is carefully removed from the inside of the tube, while the yellow part is pipetted into the flask bottle (the bottle was previously weighed). The glass bottle containing yellow liquid is then evaporated in the fume hood at room temperature. After the liquid in the glass bottle evaporates, the bottle is weighed to determine the total lipid content.

Total Lipid Level = Final bottle weight – dry weight bottle

Data Analysis

The data obtained was analyzed using Statistical Package for Social Science Software (version 20.0; SPSS Inc) with ANOVA test to detect the treatments effects on the IAA production, cell density, biomass, biomass productivity. p value less than 0.05 was considered to be statistically significant.

RESULTS AND DISCUSSION

Quantification of IAA Concentration with a Spectrophotometer

Based on the results (Figure 1) it is known that IAA increased from day 0 and reached a peak on day 2, then declined again on day 4 of sampling. The highest IAA concentration was in the treatment of tryptophan 0.2 g L-1without antibiotics. The IAA concentration in the treatment of tryptophan 0.2 g L-1without antibiotics was 1.72 µg mL-1 while the control with antibiotics had the lowest IAA concentration of 0.26 µg mL-1. Based on the results of ANOVA analysis, it is known that the administration of tryptophan, antibiotics and a combination of both can affect the concentration of IAA present in the culture. The DMRT results showed that there were significant differences between tryptophan 0.2 g L-1and tryptophan 0.02 g L-1and controls. IAA in the culture given by tryptophan concentration is greater when compared with cultures that are not given. In line with this, the concentration of IAA in the medium given antibiotics is lower than without antibiotics. This is because in antibiotic treatment, bacteria can experience partial or complete death, so the production of IAA by bacteria in the Glagah consortium is lower. Based on these results it can be assumed that the administration of L-tryptophan can increase the IAA concentration in the Glagah consortium culture.

Microalgae Growth Parameters

The microalgae growth parameters that

Table 1. Variation of the treatment

Treatment Code	Treatment
Kontrol	BBM (*Bold Basal Medium*)
T 0,02	BBM + Tryptophan 0,02 g L^{-1}
T 0,2	BBM + Tryptophan 0,2 g L^{-1}
A	BBM + Antibiotic (100 ppm *Vancomycin* dan 25 ppm Gentamycin)
A+T 0,02	BBM + Tryptophan 0,02 g L^{-1} + Antibiotic (100 ppm *Vancomycin* dan 25 ppm Gentamycin)
A+T 0,2	BBM + Tryptophan 0,2 g L^{-1} + Antibiotic (100 ppm *Vancomycin* dan 25 ppm Gentamycin)

were calculated included cell density, specific growth rate, cell biomass, and biomass productivity (Table 2). Based on the average results of each treatment it was found that there were significant differences between treatments compared to controls. Cell density, specific growth rate, biomass, and biomass productivity increased on day 7 of the study. The highest value is obtained during the stationary phase of the microalgae. In line with the cell density, the specific growth rate, biomass, and the highest biomass productivity are in that phase.

T 0.2 generally has the highest value if compared with other treatments, it is assumed that the administration of tryptophan 0.2 g L-1can increase growth parameters in microalgae. Treatment given antibiotics in it has a lower yield compared to treatment without antibiotics. Based on the results it can conclude that antibiotics cause bacteria in the Glagah consortium to experience partial or complete death so that the supply of nutrients such as vitamins and phytohormones decreases, this can lead to a decrease in growth of microalgae in the Glagah consortium.

Lipid Total

The Glagah microalgae consortium can produce lipids in various forms. Lipids in the Glagah consortium consist of palmitic acid, linoleic acid, oleic acid and stearic acid. Palmitic acid and stearic acid are SFA (Saturated Fatty Acid) lipids and oleic acid is a type of MUFA (Mono-unsaturated Fatty Acid) lipid. SFA and MUFA are high-quality fatty acids for use as biodiesel fuels14. 16 stated that the lipid content of the Glagah consortium was higher when compared to single culture Chlorella zofingiensis. This is evidenced by the results of his research, the Glagah consortium cultured on a lab scale with a BBM medium which has been modified with a nitrogen content tripled and vitamins (3N BBM +vitamins) produce higher lipids of 1.22 mg L-1.

Another parameter calculated in this study is total lipids. Based on the results of the highest lipid content obtained in the treatment given tryptophan 0.2 g L-1 which is equal to 0.2100 g L-1 (figure 2). Lipid production is influenced by growth environmental factors of microalgae, if the environment is suitable then more lipids will be produced. In this study it was assumed that the administration of tryptophan 0.2 g L-1 in the growth medium could support microalgae to produce lipids. Based on ANOVA analysis it is known that administration of tryptophan can increase lipid content in microalgae. Antibiotics can also affect the lipids produced.

Microalgae given antibiotics produce lower lipids when compared to those not given antibiotics. Likewise with tryptophan, in cultures given tryptophan larger, produce higher lipids when compared with tryptophan in smaller

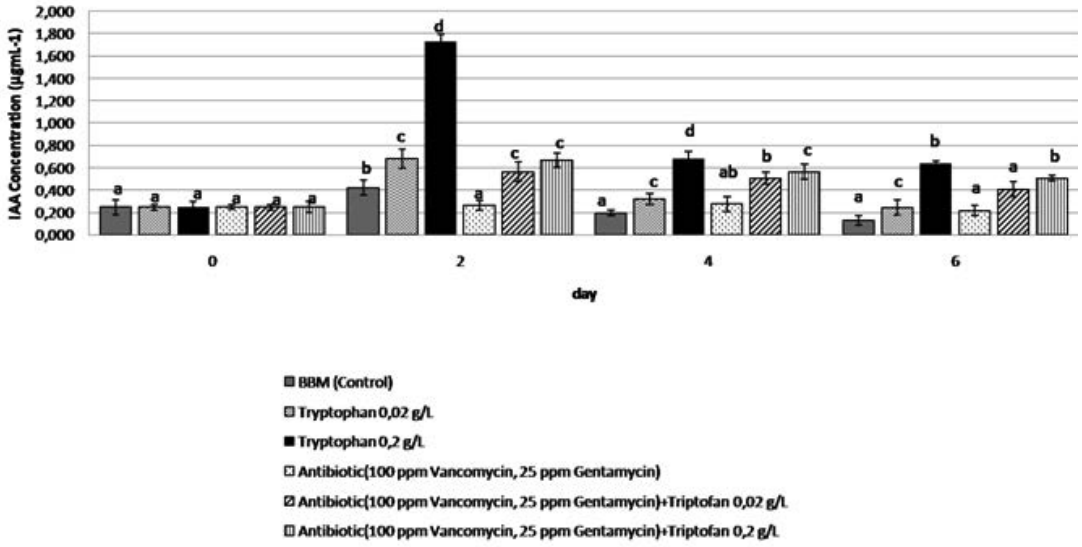

Fig. 1. IAA concentration in the Glagah Consortium

concentrations. From the results of the DMRT test it was found that there were significant differences in lipid yield given tryptophan 0.2 g L-1 with control and tryptophan 0.02 g L-1.

DISCUSSION

This research is an initial study conducted to examine the potential of bacteria in the Glagah consortium in producing IAA with the addition of L-tryptophan and also to determine the effect of IAA produced by these bacteria on the growth of microalgae in the Glagah consortium. The Glagah Consortium is composed of 6 species of microalgae, namely C. polymorpha, C. raciboskii, G. radiata, S. turquoise, C. criophilum, and Chlamydomonas sp.3, and also include bacterial species, namely C. ulcerans, C. bovis, B. cereus, B. megaterium, P. parvulus, and S. vitulinus4. The large number of compilers of the Glagah consortium has caused a complex interaction between microalgae and bacteria in the consortium. Interactions are both known to be mutually beneficial to one another, where the bacteria making up the Glagah consortium thought to produce vitamins (cobalamin, thiamin and biotin) which are beneficial for microalgae growth6. While microalgae in the consortium releases organic carbon compounds that can be absorbed by bacteria and used for growth.

Tryptophan is the main precursor in the biosynthesis of IAA in plants and in microorganisms. The presence of tryptophan precursors in the medium can spur the IAA

Table 2. Average growth parameters of microalgae in the Glagah Consortium

Treatment (Day)	Cell Density	Specific Growth Rate	Biomass	Biomass Productivity
Control (0)	1,63 ± 0[a]	0	0,25±0[a]	0
T 0,02 (0)	1,63± 0[a]	0	0,25±0[a]	0
T 0,2 (0)	1,63± 0[a]	0	0,25±0[a]	0
A (0)	1,63± 0[a]	0	0,25±0[a]	0
A+T 0,02 (0)	1,63± 0[a]	0	0,25±0[a]	0
A+T 0,2 (0)	1,63± 0[a]	0	0,25±0[a]	0
Control (7)	8,84 ± 0, 02127[a]	0,390 ± 0,01092[a]	0,77±0,02886 [a]	0,074±0,0041 [a]
T 0,02 (7)	9,14 ± 0,00828 [a]	0,400 ± 0,02127 [a]	0,77±0,02886 [a]	0,074±0,0041 [a]
T 0,2 (7)	10,57 ± 0,00213 [b]	0,510±0,00283 [b]	1,15±0,05 [b]	0,150±0,008 [b]
A (7)	6,16 ± 0,02907 [c]	0,302±0,02907 [c]	0,57±0,07636 [c]	0,045±0,011 [c]
A+T 0,02 (7)	6,53 ± 0,01092 [c]	0,317±0,00627 [d]	0,67±0,05773 [c]	0,052±0,004 [c]
A+T 0,2 (7)	6, 46 ± 0,00627 [c]	0,370±0,02828 [d]	0,72±0,02886 [a]	0,078±0,004 [a]

Values followed by the same letter(s) in the same column are not significantly different at *P<0,05* based on DMRT

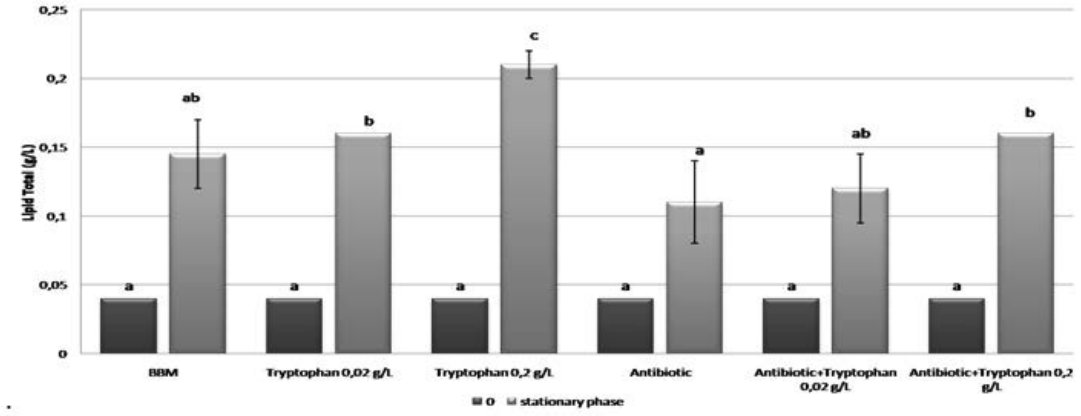

Fig. 2. Total lipids in the Glagah Consortium

production process carried out by bacteria. The type of tryptophan that is known to be better at triggering IAA production is L-tryptophan. In this study L-tryptophan was supplemented into BBM medium. The tryptophan added is from the smallest concentration to the greater concentration which can still be tolerated by microalgae which is 0; 0.02 g L-1and 0.2 g L-1

Groups of bacteria can produce phytohormones such as auxin. This if applied to plants can stimulate growth of plants. Auxin produced at the Glagah consortium is expected to also spur growth in the consortium, so that this research is conducted to determine the IAA produced by the Glagah consortium and to know its effect on microalgae growth. IAA produced by bacteria in the Glagah consortium is used by microalgae for its growth. The highest IAA in tryptophan 0.2 g L-1 treatment increased cell productivity, biomass productivity and lipid productivity of microalgae in the consortium. As is known that IAA plays a role in the growth and development of microalgae. IAA triggers cell division through regulating the expression of genes that function in the cell cycle9, therefore, cell division in the Glagah consortium microalgae becomes faster and produces more cells than cultures with lower IAA levels. More cell density will increase biomass productivity and lipid productivity.

Microalgae and bacteria interact with each other and form symbiosis. At the Glagah consortium there are interactions between microalgae and bacteria and are mutually beneficial (symbiotic mutualism). The mutualism symbiosis that occurs between the two is the exchange of primary metabolites in the form of carbon dioxide and oxygen. Microalgae obtain carbon dioxide released by bacteria, while bacteria can obtain oxygen released by microalgae for metabolism. In addition, microalgae get growth factors released by bacteria in the form of auxin vitamins and fitohormones (indole-3-acetic-acid)[17].

Carbon is released by microalgae into water and then used by bacteria for its growth. The specific carbon source needed by bacteria is glycolate. Glycolate is produced by microalgae during photosynthesis. Other forms of carbon are extracellular polymeric substances (EPS) issued by diatoms. EPS is released by diatoms into biofilms. Based on the research it is known that, in the presence of released EPS, the ?-proteobacteria group bacteria have increased[18,19].

Some microalgae require a combination of vitamins (biotin, cobalamin, and thiamin) as growth factors. But they cannot produce vitamins themselves. Organisms that can have the ability to produce vitamins are from prokaryotic organisms such as bacteria, which are symbionic organisms with microalgae. vitamins can be defined as organic compounds and a metabolite that is needed by an organism, but the organism cannot synthesize it. These organisms are called auxotroph vitamins[17].

Vitamins needed by microalgae come from bacteria that are symbiotic with them. Based on a survey of vitamin needs for microalgae, it was found that out of 326 species studied, 171 species of auxotrophs against vitamin b12. In addition, of the 306 species studied, 61 needed vitamin B1 and 14 requiring vitamin B7[19,20].

The photosynthetic ability of microalgae leads to the perception that algae as autotrophic organisms require light and a mixture of inorganic nutrients. Some members of chlorophyta and cryophyta require thiamin as one of the growth factors in culture. In addition, many studies have described that algae require a combination of three B vitamins, namely Vitamin B12 (cobalamin), vitamin B1 (thiamin) and vitamin B7 (biotin). Nutrients such as vitamins cannot be produced by microalgae, but are obtained from their interactions with bacteria. Vitamins play an important role in microalgae metabolism (Baggesen, 2014). Vitamin B1 (thiamine) is a colorless and water-soluble organosulphur[17]. Thiamin in the form of TPP (thiamine pyrophosphate) acts as a co-factor for various types of enzymatic reactions. Some of these enzymatic reactions include carbohydrate metabolism and amino acid synthesis. In addition, thiamin also functions as a co-factor in the citric acid cycle. In microalgae thiamin also acts as a defense system against cellular oxidative stress which is located inside the chloroplast. However, the enzymes that play a role in the biosynthesis of B1 in microalgae are still not well characterized. Several studies have suggested that thiamin concentration is directly proportional to the increase in microalgae productivity. Microalgae

are cultured together with bacteria, their growth increases 2-6 times. In addition, the lipid content can be doubled[17].

Vitamin B7 (biotin) has a water-soluble and colorless character. Biotin has an important role in carbon dioxide metabolism, acting as a co-factor for various carboxylase enzymes. This enzyme is used in metabolic processes such as gluconeogenesis, citric acid cycle, regulation of gene expression, branched amino acid catabolism and fatty acid biosynthesis17. Vitamin B12 (cyanocobalamin) is the largest vitamin (BM = 1355.4) and the most complex of all vitamins present. Vitamin B12 is soluble in water and can be synthesized by many species of bacteria, especially heterotrophic, cyanobacteria, but cannot be produced by eukaryotic organisms Tandon *et al*. 2017). Vitamin B12 enters two enzymatic reactions, namely DNA synthesis with the help of the enzyme methionine synthase, and assimilation of organic carbon with the help of the methylmalonyl CoA mutase enzyme. Vitamin B12 has been proven to be a co-factor for the enzyme methionine synthase.

Xie *et al*. suggested that the expression of the METE gene in C. reinhardtii decreases with heat stress, therefore when B12 is not available the cell will die19. The bacteria in the Glagah consortium produce thiamin, cobalamin and IAA, therefore, when the culture is given antibiotics, it is possible that the bacteria will be killed partially or completely. This causes reduced availability of vitamin B and IAA for microalgae. Nutrients such as vitamins and IAA are important growth factors for microalgae growth, so that when the concentration is reduced or not available, microalgae growth becomes less optimal. Less optimal growth will lead to lower biomass productivity, cell productivity, and lipid productivity than microalgae compared to cultures that are not given antibiotics.

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