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Promoting the Growth of Chlorella vulgaris in Secondary Wastewater Treatment Effluent of Tofu Industry using Azospirillum sp

Wahyunanto A. Nugroho[#], Musthofa Lutfi[#], Bambang Susilo[#]

[#] *Department of Agricultural Engineering, Brawijaya University, Malang, East Java, Indonesia 65145*
E-mail: wahyunanto@ub.ac.id

***Abstract*— The objective of this research is to investigate the influence of growth promoting bacteria (GPB) Azospirillum sp on the growth of microalgae Chlorella sp in wastewater of tofu industry as a medium. To observe the influence, about 106 cells/mL of Chlorella sp was cultivated in 1 L of wastewater of tofu industry. The wastewater was an effluent of an aerobic treatment. Six different treatments were set regarding to the Azospirillum sp added to the medium. The glass was marked as A0 as no GPB inoculants added to the medium, and A2, A4, A6, A8 and A10 for 2 mL, 4mL, 6mL, 8 mL and 10 mL of GPB added to the medium respectively. The concentration of GPB inoculant was 108 cfu per mL. The result showed that the highest number of Chlorella population was achieved by addition of 6 mL GPB Azospirillum sp in the day 10, while the highest maximum growth rate was achieved by addition of 10 mL GPB Azospirillum sp to the medium.**

***Keywords*— microalgae; growth promoting bacteria; wastewater**

I. INTRODUCTION

Microalgae is a potential organism for bio-fuel feedstock production [1]. Lipid production by microalgae is in the range of 20% to 70% of its dry weight, depending on its species [2], [3], [4]. With an advanced culturing technique, the potential oil production of microalgae could reach 136,000 L/Ha/y, which is much higher than oil production of terrestrial plants [5]. Another estimation, microalgae can produce about 120,000 kg biodiesel/ha year, or 20 times as much as the productivity of palm oil (5,800 kg biodiesel / ha year) and 80 times higher than that of castor oil, which was about 1,500 kg biodiesel/ ha year [6]. Another benefit is this source of alternative energy does not compete with food crop for the land use.

Microalgae require an adequate nutrient in order to grow well. Carbon and inorganic nitrogen and phosphor are major element needed by the microalgae, besides some trace elements. To reduce the production cost due to the use of fertilizer, cultivation of microalgae in nutrient rich wastewater can be an alternative. Many microalgae have been reported to be feasible to grow well in wastewater [7], [8, 9]. While most of the research was done in laboratory and pilot scale treatment [10, 11], a few of them have been practiced for industrial scale [12]. When it is cultured in the wastewater, microalgae have dual purposes, i.e. producing oil and other useful chemical substances, and also cleaning the wastewater by nutrient uptake, which is typically done in tertiary treatment, [13, 14, 15].

Wastewater of food industries contains complex organic materials, such as carbohydrates, protein and lipids, besides several microelements [16]. Tofu industries are widely spread in areas of Indonesia, and consumes a large amount of water per kg of raw material. The protein in its raw wastewater occupies about 60% of all solid fractions. Through amino acids fermentation in anaerobic treatment process, protein can be degraded into various organic compounds [17], which some of them can be used either for macronutrient or micronutrient for the autothrophic organism, such as microalgae. It is expected that this rich-nutrients wastewater is able to suppress the use of fertilizers that commonly practiced in the microalgae cultivation. A study by Nugroho et al. [18] shows that microalgae Chlorella vulgaris could grow well in nutrient-rich tofu industrial wastewater.

Microalgae have various productivities in wastewater, regarding to its growth and lipid production. So do its efficiency in nutrient uptake. Nutrient sources [1, 19], pH [20], temperature of medium [21], light intensity [22, 23], and size of the population [24] are several factors that affect microalgae productivity as well as its nutrient uptake efficiency. Studies of Chlorella sp cultivated in the municipal wastewater effluent revealed that the average growth rates of the microalgae were range from 0.343day-1 to 0.498day^{-1} [25]. Higher growth rate showed when

microalgae were cultivated in more concentrated wastewater [25, 26].

To support the microalgae growth without any additional nutrient, Azospirillum sp as a growth promoting bacteria has been employed for the process [27]. Using alginate immobilization technique, about 25% increase for the growth rate was revealed when Chlorella vulgaris was coupled with Azospirillum sp, while less growth rate was occurred when Chlorella sorokiniana was cultivated instead of Chlorella vulgaris [28]. Indole Acetic Acid (IAA), a growth hormone secreted by Azospirillum sp is expected to be responsible for this significant increase of the microalgae growth. Azospirillum sp is a non-symbiotic nitrogen-fixing bacterium in terrestrial ecosystem. These bacteria lives freely in the soil and is employed as a soil fertilizer [29]. The growth hormone produced by this bacteria was about 285.51 mg / liter of total culture medium, and is likely able to improve the efficiency of fertilization [30]. In a separate study on the microalgae-bacteria interaction, an increase of the growth rate occurred when two species of green microalgae, Scenedesmus bicellularis and Chlorella sp, was cultured in laboratory-based experiment with two bacteria, Pseudomonas diminuta and P. vesiculari. was

A few has been reported about the use of non-native aquatic microorganism to support the growth of microalgae, and to further help purifying the water. Since it is water intensive process, tofu wastewater effluent contains less concentrated nutrient compared to wastewater from other food industries. It is expected that the addition of this bacterium could increase the growth rate of the bacterium during its culture. The objective of this research is to investigate how Azospirillum sp would promote the growth of microalgae Chlorella vulgaris in the tofu processing wastewater. Its performance in nutrient removal was also been investigated.

II. Materials and Method

A. Culture and Growth Medium

Chlorella vulgaris and Azospirillum sp was used for the microalgae and the growth promoting bacteria respectively. Chlorella vulgaris used in this experiment was a culture that was developed by Indonesian Bureau for Aquaculture Research (BBAP) Situbondo, East Java, Indonesia, while the bacterium was developed by the Laboratory of Soil Microbiology, Faculty of Agriculture, Brawijaya University, Indonesia.

The growth medium for the experiment was wastewater of tofu industry, which is located in Batu City, East Java, Indonesia. The wastewater was an effluent of an anaerobic treatment process. To avoid some gross fragment in the wastewater, a filtration screen was used prior to the sample storing in a cool box with maximum temperature of about 4^{o}C. As soon as it arrived in the laboratory, it was stored in under 4^{o}C refrigerator and was used one day later. About 10 ml of the wastewater sample was analysed to observe its initial chemical compounds.

B. Analysis of wastewater and microorganism

The ammonium analysis was performed using standard nessler test using spectrophotometer Genesys 10uv with 425 nm of wavelength. Colorimetric nitrate analysis was done using Genesys 10uv with 410 nm of wavelength. The orthophosphate concentration was analysed using stannous chloride method and quantified using spectrophotometer Genesys 10uv with wavelength of 620nm. The medium temperature and pH was measured periodically to ensure that those two parameters were in the allowed condition for the microalgae and the bacterium to grow. The number of Chlorella vulgaris and Azospirilum sp were counted using haemocytometer under microscope. The microalgae growth rate was calculated using the equation below

$$\mu = \frac{\ln X_{\max} - \ln X_0}{T_{\max} - T_0}$$

The same formula was also used to calculate the growth rate of the *Azospirillum sp*.

C. Experimental set up and statistical analysis

The experiment was carried out in laboratory using 1000mL Erlenmeyer glasses, and each glass contained about 500mL of the medium. To give the aeration, an air pump was used with its air flow rate was 6 litre per min per bottle. A 40 W of fluorescence lamp was employed for 24 hours a day for the light source.

Each bioreactor was labelled differently, i.e. A0, A2, A4, A6, A8, and A10. All bioreactors was added with similar number of *Chlorella vulgaris* and mixed slowly. The targeted initial concentration of the *Chlorella vulgaris* in the medium was 10^6 cells/ml. A different volume of *Azospirillum sp* inoculums was added to each reactor, except for reactor A0, which was acted as a control. The concentration of the *Azospirillum sp* inoculum was 10^8 cfu/mL. Reactors A2, A4, A6, A8, and A10 were added with 2 ml, 4 ml, 6 ml, 8 ml and 10 ml of the inoculum respectively. The number of microalgae was counted every day at 4 pm, while the number of bacteria was counted every four days. Each experiment was run for twelve days in triple replication.

The significance of the bacteria influence for the microalgae growth rate was analysed together using ANOVA in SPSS, then were followed by Tukey post-hoc analysis at $P \leq 0.05$.

III. Result and Discussion

A. Medium of Culture

The quality of tofu industrial wastewater that was used as the culture medium was presented in Table 1. This wastewater was provided by taking the effluent of anaerobic treatment of tofu industrial wastewater. The wastewater was filtered with clean cloth filter before being stored in glass bottles. The wastewater was kept in the refrigerator under 4^{o}C prior to be used in the following day. While the dissolved oxygen and temperature of the whole process showed the optimum condition for the growth, the pH of the medium showed a slightly higher than the optimum condition. Table 1 show that the concentration of ammonium and phosphate, which are two macro nutrients for the microalgae, was about 0.5 mg/l and 1.66 mg/l respectively. It is typical that the phosphorus concentration in the tofu

processing wastewater is higher than that of the ammonium concentration [18]. The ammonium concentration in the wastewater is quite low compared to the ammonium concentration of municipal wastewater. Several papers have reported that a common ammonium concentration of municipal wastewater was about 30 mg/l [31, 32]. Chiu [33] reported that higher concentration of ammonium was found in the dairy raw wastewater. However they suggested that it was not suitable for being used to cultivate microalgae directly.

TABLE I
INITIAL WATER QUALITY OF THE GROWTH MEDIUM

Component	Concentration
Ammonium (mg/l)	0.5
Nitrate (mg/l)	0.12
Phosphate (mg/l)	1.66
DO (mg/l)*	7.1-7.6
pH*	8.1-8.9
Temperature (^oC)*	25.1-26.9

B. *Effect Of Azospirillum sp Concentration on the Chlorella Vulgaris Growth Rate*

As shown in Figure 1, all treatments show the growth of microalgae until they reached their peak concentration, followed by dead phase. The peak concentration of microalgae and the time to reach the peak point vary for each treatment. A dead phase that was following the peak point indicates that the medium undergo substrate or nutrient depletion for the microbial growth. Since the substrate (CO_2) is supplied continuously by aeration, the depletion of macronutrient seems to take the dominant role on the dead of the microalgae. Among all treatments, A10 is the first treatment to reach the peak concentration (in day 7) with about 5.56 x 10^7 cells/ mL, followed by A8 and A2, which reached its peak concentration in the following day. The earliest decrease of the microalgae in A10 is due to the earliest shortage of the nutrients. The earliest depletion of nutrients in A10 might due to the existence of the highest population of microalgae and GBP (Figure 2) in the medium that had highest consumption rates of nutrients.

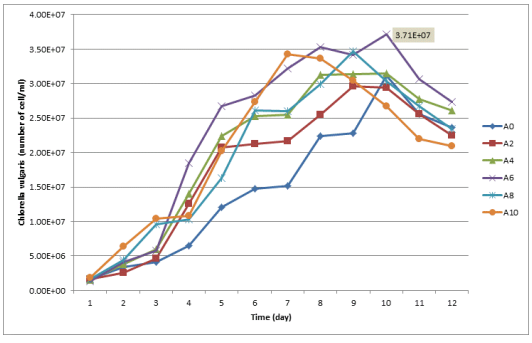


Fig.1 Population of *Chlorella vulgaris*

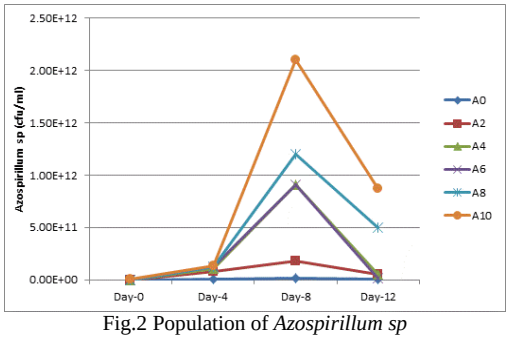


Fig.2 Population of *Azospirillum sp*

The highest concentration in peak point was achieved by A6 with its cell concentration of about 3.7 10^7 in the tenth day. For treatment A2 and A4, it reached the peak point in day-8 and day-9, respectively. For A2 and A4, the peak point was continued by two and three days of stationary phase, respectively. This stationary phase was last until day-10, which was the time where A0 and A6 achieved the peak point. A longer time to reach peak point for several treatments might due to their lower microbes' concentration.

The result of the experiment also shows that *Azospirillum sp* has significantly promoted the growth of the microalgae *Chlorella vulgaris.* Figure 1 illustrate that all bioreactors that had *Azospirillum sp* addition in it showed higher population of *Chlorella vulgaris* than that of without *Azospirillum sp*. While in the bioreactors that had no GPB in it shows its final population increase for about thirteen fold, a higher final population was demonstrated by the *Chlorella sp* in the treatment with *Azospirillum sp*, i.e from 18 to 27 times higher as much as its initial population. The increase of the *Azospirillum sp* also occurred in this experiment (Figure 2). Among all treatments, the highest population was achieved by A6 with 37.13 x 10^7 cells/ml, and it was achieved in the tenth day. This finding is higher than the result reported by Bashan et al [27] where co-cultured of microalgae and GBP was immobilized in alginate beads for ten days, which was only about 5 times higher than the initial population. This might due to in this experiment; the microalgae live freely in the wastewater compared to the immobilized microalgae. From the day-1 until the day-10, all the treatment shows an increase on the microalgae population, except for A8 and A10. The peak population for A8 and A10 occurred in the ninth and seventh day respectively, and then started to decline. This decrease might be because of the ammonium shortage in the ninth day (for A8) and seventh day (for A10), or even in several days before (Figure 4a). It could also be said that ammonium was the limiting factor for this culture.

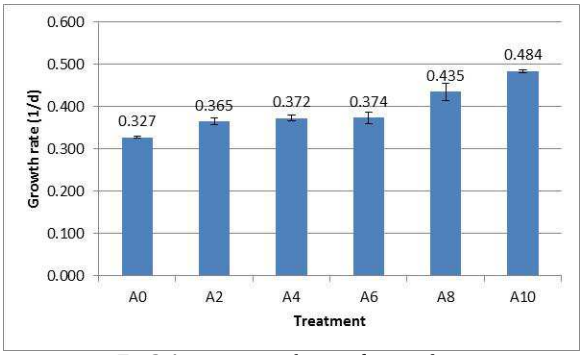


Fig.3 Average growth rate of microalgae

By computing the experiment result using formula 1, it shows that the highest average growth rate was achieved by A10 followed by A8, which were $0.484d^{-1}$ and $0.435d^{-1}$ respectively (Figure 3). Even though they achieved the highest growth rate, the highest peak population of microalgae among all treatments was achieved by A6. The rapid ammonium utilization rate by both microalgae and GPB in A10 and A8 might have caused they underwent higher rate but only in a shorter period due to nutrient shortage.

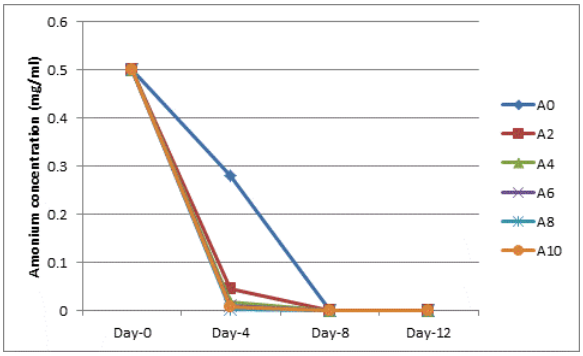
TABLE II
STATISTICAL ANALYSIS OF THE GROWTH RATE

Treatment	N	Subset for alpha = .05			
		1	2	3	4
A0	3	.327467			
A2	3		.364647		
A4	3		.372329		
A6	3		.373500		
A8	3			.434527	
A10	3				.483642
Sig.		1.000	.928	1.000	1.000

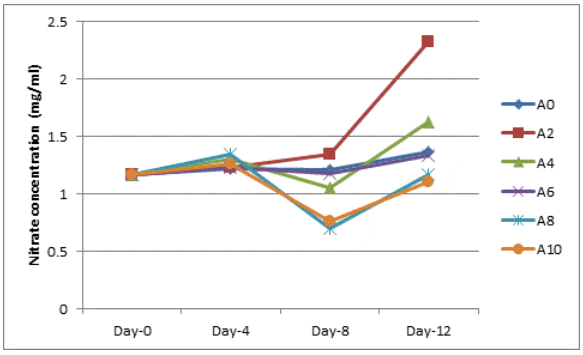
The significance of the GPB concentration on the growth rate was analysed using one-way Anova and followed by Tukey post-hoc analysis (Table 2). In general, the result shows that GPB had significant impact on the growth rate of microalgae. By comparing the growth rate of A2, A4 and A6 descriptively, there was a slight difference on the growth rate of the microalgae among the treatments. However, there was no significant difference in the growth rate of those three treatments statistically. Treatment with initial concentration of GPB higher than 1.5×10^6 cfu/ ml (A8 and A10) had significance difference compared to other treatments.

C. Effect of Azospirillum sp on the Nutrient Removal of the Medium

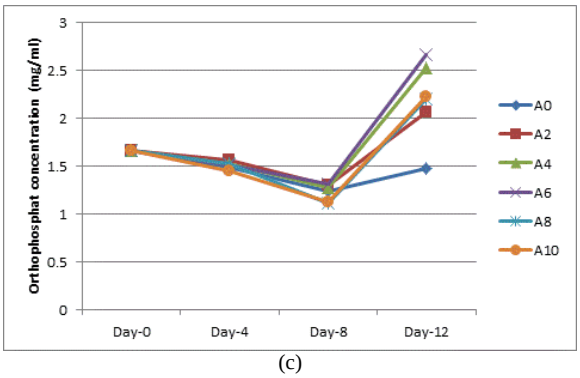
To analyse how the process could also performed nutrients removal, the water quality was measured every four day. The initial concentration of the nutrient was reported in Table 1. Except for A0, all the ammonium concentration was below 0.01 mg/ml in the fourth day (Figure 4A). In the day-8, no ammonium concentration was detected in all treatment. It was the reason why all the treatment experienced growth rate decrease after eight day. A complete removal of ammonium was also demonstrated by de-Bashan et al [28], when co-cultured of microalgae and GBP was applied into nutrient-containing wastewater. Less removal of ammonium was demonstrated when microalgae was employ alone in the semi continuous reactor [34] and [35]. A different condition occurred in the nitrate concentration that the final concentration was higher that its initial concentration in all treatment, but A8 and A10 where its concentrations were slightly lower than its initial concentration (Figure 4B). The rising of nitrate concentration was also reported by Gonzalez et al [34] when the wastewater was used as a medium for microalgae culture. The increase of the nitrate in an aerated treatment might be the result of nitrification when the ammonium was biologically converted into nitrate by the nitrifying bacteria. This typical conversion is occurred in aerobic section of suspended growth process for nitrogen removal.



(a)



(b)



(c)

Fig.4 Nutrient concentration of the medium. (a) ammonium, (b) nitrate, (c) orthophosphate

In the first eight day, all the phosphate showed a removal, which is between 21% to 32%. The highest 8 day-removal achieved by A10 (32%) while the lowest removal achieved by A6 (21%). A better removal (36%) showed by de-Bashan [28] when the microalgae co-immobilized in alginate beds with *Azospirillum brasilliensis*, but lower removal when the microalgae was immobilised alone (19%). The best phosphate removal (85%) was reported when an advanced coupled treatment of attached microalgae biofilm and membrane bioreactor was applied for wastewater [36].

In this research, unexpected final concentration occurs in the final phosphate concentration. All the treatment show higher phosphate concentration than the initial. The increase of the phosphate concentration might have relation to the decay of the microalgae as well as the *Azospirillum sp*. Stopping the process before the decay phase of microalgae likely needs to be done to avoid the increase of phosphate due to the microalgae decay.

IV. CONCLUSIONS

This study demonstrated that GPB has significantly promoted the growth of microalgae. While in the lower GPB addition it did not show the significance of the GPB concentration in their growth rate, their significant influences were demonstrated in higher GPB concentration. Removal of ammonium was also demonstrated by the microalgae, either alone or co-cultured with GBP. Nitrate and phosphate concentration increased during the process. Since the nitrate and phosphate removal was not well performed by the process, an additional work has to be done to remove both nutrients.

NOMENCLATURE

μ	specific growth rate	day^{-1}
X_{max}	maximum cell concentration	cell/mL
X_0	initial cell concentration	cell/mL
T_{max}	time when the cell reach its maximum concentration	day-n
T_0	initial time	day-0

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