Vinasse as Cultivation Medium of *Chlorella* sp. to Produce Poly–Hydroxy Butyrate in Various Limited Low–Cost Primary Nutrient

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Abstract. Vinasse is ethanol wastewater that still contains nutrients. It can be medium cultivation for C*hlorella* sp. Cultivation *Chlorella* sp. in vinasse did not only minimize its COD content but also potentially produce Poly–Hydroxy Butyrate (PHB) in a limited nutrient. This paper presents a cultivation process of *Chlorella* sp. in vinasse on the various limited nutrient. *Chlorella* sp. was cultivated in vinasse by adding complete nutrient (urea and TSP), TSP (limited N), and urea (limited P). Experimental data was optimized by a mathematical model to predict the behavior of the *Chlorella* sp. in limited nutrient systematically. The study confirmed that the best condition of the cultivation medium of *Chlorella* sp. to minimize COD content in vinasse by addition phosphate into the reactor. However, PHB could be best accumulated in the cell on limited phosphate.

Key words: Biological plastic**,** cultivation medium**,** environment friendly, stillage, wastewater utilization

1 Introduction

The cultivation process of microalgae (*Chlorella* sp.) is a costly process when developed by a specific medium. Alternatively, the cultivation process can be done in wastewater. One wastewater that has the potential to cultivate C*hlorella* sp. is vinasse. The contents of vinasse are simple nutrients such as total carbon (COD), total nitrogen, and PO_4^{3-} [1, 2]. The utilization of vinasse as a cultivation medium does not only decrease production cost but also promise to remove the COD [3]. Besides, it is potential to produce algae–based plastics in a specific condition. The characteristic of algae–based plastics is degradable in nature due to containing Poly–Hydroxy Butyrate (PHB). PHB is produced in a microalgae cell as a stress response of limited nutrients [4, 5]. In addition, to improve productivity,

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C*hlorella* sp. has a special characteristic; it can grow in autotroph and heterotroph condition [6]. In this case, CO_2 in the air as an inorganic carbon source to support autotroph condition and vinasse as an organic carbon source to support heterotroph condition.

The aim of this research is to analyze the influence of limited primary nutrient difference into kinetic parameters on *Chlorella* sp. cultivation process in vinasse, especially on PHB production. The quantitative analysis developed to predict the rate of COD degradation (k_L), biomass productivity (μ), lag phase (λ), and yield PHB to COD ($Y_{PHB/COD}$). The assumptions taken to quantify the rate of substrate degradation is quasi single substrate expressed as COD. The rate of COD degradation was approached by Equation (1):

$$-\frac{dS}{dt} = k_L S^n \tag{1}$$

where k_L is the rate of COD degradation constant (L mg^{-1} d^{-1}), S is concentration COD at t time (mg L^{-1}) and n is kinetic order (suitable value was obtained by trial–error method).

The rate of PHB forming, modeled by Yield concept, as depict in Equation (2)

$$-\frac{dS}{dt} = \frac{1}{Y_{PHB/COD}}\frac{dP}{dt} \tag{2}$$

where $Y_{PHB/COD}$ is yield of PHB formation per unit COD consumed.

Cultivation C*hlorella* sp. in vinasse is approached by Modified Gompertz to know the correlation between biomass productivity and lag phase [7], as depict in Equation (3)

$$A(t) = A_\infty \exp\left\{-\exp\left[\frac{2.718282\,\mu}{A_\infty}(\lambda - t) + 1\right]\right\} \tag{3}$$

where, $A(t)$ is dry mass at t time (mg L^{-1}), A_∞ is dry mass at stationer phase (mg L^{-1}), μ is biomass productivity (mg L^{-1} d^{-1}) and λ lag phase (d).

The data (COD, PHB, and dry mass) is fitted to Equation (1) (2) and (3), respectively, and the coefficient determination (R^2) is used as a fitting validation. The obtained constants (k_L, $Y_{PHB/COD}$, μ and λ) are used to quantitative analysis the performance of the reactor in the different limited primary nutrients.

2 Materials and methods

2.1 Materials

Vinasse was used for cultivation medium. It was collected from one of the ethanol industry in Sukoharjo, Central Java, Indonesia. Vinasse was sterilized to eliminate bacterial content then diluted in tap water until 5 mg L^{-1} (COD = 1 435 mg L^{-1}). *Chlorella* sp. was provided by CV. Algae Park, Sukoharjo, Central Java, Indonesia. Both of them were mixed and added low–cost primary nutrients. Four variations were tested at low–cost primary nutrient of (R1) no nutrient added, (R2) 40 mg L^{-1} urea and 10 mg L^{-1} TSP, (R3) 10 mg L^{-1} TSP, and (R4) 40 mg L^{-1} urea.

2.2 Experimental setup

Reactor artificial with a fluid capacity of 1 000 mL used 1 L glass. The reactors were equipped by 54 Watt neon lighting and conventional aerator (4 L min^{-1}). The process was conducted in 10 d on batch mode. A sample was taken every day to be assayed for its dry mass, COD, and PHB contents. Dry mass and COD followed by APHA Method [8] while the PHB contents followed by Senior Method [9].

3 Results and discussion

Vinasse is potential to utilize as microalgae cultivation medium especially *Chlorellla* sp. because vinasse contains 33.60 mg L^{-1} N and 0.028 mg L^{-1} P. However, vinasse also contains toxic material (0.146 mg L^{-1} phenol) so it needs intensified in utilization as cultivation medium. Based on the calculation, kinetic parameter was obtained and it is shown in Table 1. These parameters are use to compare the performance among the reactors.

Table 1. The value of k_L, $Y_{PHB/COD}$, μ and λ in each reactor

Reactor	COD		Dry mass			PHB	
	k_L*	R^2	μ	λ	R^2	$Y_{PHB/COD}$	R^2
R1	0.493E–4	0.965	0.643	13.843 0	0.976	0.022 2	0.944
R2	1.847E–4	0.865	0.158	0.105 8	0.999	0.204 7	0.804
R3	0.865E–4	0.912	0.343	9.655 7	0.971	0.030 3	0.646
R4	1.491E–4	0.825	0.096	0.691 0	0.991	0.265 5	0.824

***** The suitable parameter n (Equation 2) that gave the best–fitting of the data was n = 2 for all reactors

3.1 COD degradation

COD is a suitable parameter to approach amount of organic material utilized by *Chlorella* sp. for growing. COD degradation in each reactor best fitted by mathematical model (R^2 closer to 1) as shown in Figure 1, it was also proved that nutrient content in vinasse could support *Chlorella* sp. to degrade COD.

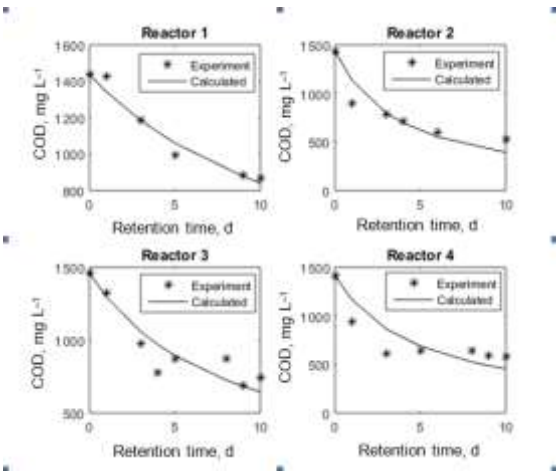

Fig. 1. COD best–fitting on the model proposed in Equation (1)

In vinasse treatment by microalgae (*Chlorella* sp.), the efficiency of COD removal becomes a primary parameter to measure the performance of process, the best performance can be reached when the highest of COD efficiency. Figure 1 and Table 2 showed that the nutrient addition could make the process of COD degradation faster than without nutrient addition. This phenomenon was correlated with the growth of *Chlorella* sp. can grow in a mixotroph condition. In the autotroph growth, *Chlorella* sp. could make its own nutrient by photosynthesis process. The other way, the heterotroph growth force C*hlorella* sp. convert organic material for growing. However, to obtain value products especially PHB, the limited nutrient should be treated as well as the previous research.

Limiting nutrients, especially limiting nitrogen (N), made *Chlorella* sp. consume organic material as their nutrient in heterotroph condition. On the other hand, limited N was triggered by organic material degradation to be faster [3, 10]. However, in this research was obtained the rate of COD degradation (*kL*) in the reactor without N and P addition (R1) on the lowest value was 0.493E–4, followed by reactor without N addition (R3), reactor without P addition (R4) and reactor with N and P addition (R2) with the value 0.865E–4 1.491E–4 dan 1.847E–4 respectively. It was because of constant lighting since the process. Based on the previous research, the heterotrophic condition occurs without lighting, and the function of aeration was just to help C*hlorella* sp. consumed organic material [11]. The lighting was only needed by C*hlorella* sp. as a catalyst in the photosynthesis process (autotrophic condition). In this condition, C*hlorella* sp. needed macronutrient such as C, N and P in big amount [12], so R1 and R3 were in situation when heterotrophic condition could not be reached, but losing big amount of nutrient since the process as effect the presence of aeration and lighting. It was equal to the previous research. The weakness of the heterotrophic condition was lighting, organic material, and CO_2 [13]. On the other hand, in R2 and R4 could grow well because the need of nutrient and environment condition support to photosynthesis process (autotrophic condition) and could obtain the high efficiency of COD removal because of constant aeration since the process. The higher oxygen in the system can give an advantage in COD degradation [14].

Table 2. Percentage of COD removal in each reactor

COD	Reactor			
	R1	**R2**	**R3**	**R4**
Influent (mg L^{-1})	1 437	1 435	1 459	1 413
Effluent (mg L^{-1})	868	533	746	578
Efficiency removal (%)	39.60	62.86	48.87	59.09

Chlorella sp. was expected to grow well in a mixotroph condition compared to either autotrophic or heterotrophic. It was proven by k_d parameter from previous research which had equal meaning with k_L in this research, but the value of k_d was higher than k_L. It was due to a lighting scheme so that *Chlorella* sp. could make its own nutrient by autotrophic condition (light) and degraded vinasse in heterotroph condition (dark) [15].

3.2 Biomass productivity and lag phase

The other parameter in order to approach the biological treatment process was the lag phase. The lag phase is the first phase of the growth curve that reflects the adaptation of *Chlorella* sp. in their environment. In general, the microorganism growth curve was sigmoidally shaped (Figure 2), which had similarity to microorganism dry mass [7].

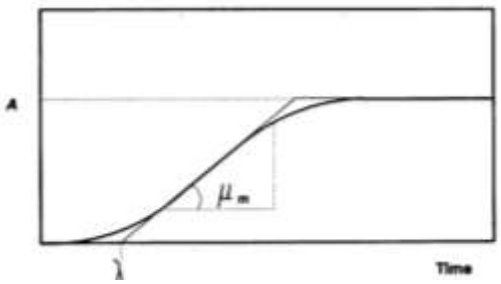

Fig. 2. Microorganism growth curve

In Figure 3 showed that the profile of dry mass in each reactor. R2 and R4 curves had a similar shape to the sigmoidal curve. It was due to the short lag phase. Based on the microorganism growth curve (Figure 2), the shorter lag phase showed that *Chlorella* sp. already grew well (exponential phase).

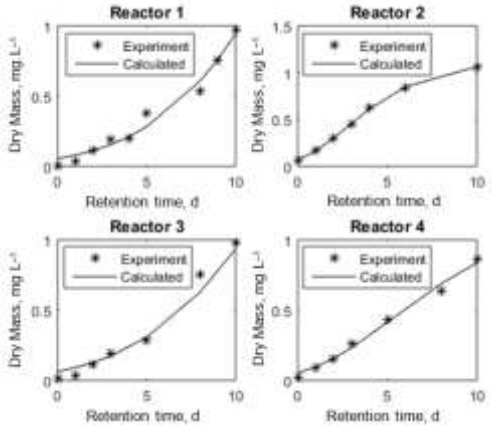

Fig. 3. Dry Mass best–fitting on the model proposed in Equation (3)

Quantitatively, the value of lag phase in each reactor was shown by Table 1, it was shown that *Chlorella* sp. in R1 and R3 undergo longer adaptation phase compared by R2 and R4. It was equal with previous research, which said that in limited N condition made the lag phase of *Chlorella* sp. to be longer [3]. On the other hand, the addition of nitrogen in the cultivation process of *Chlorella* sp. in vinasse could cut the lag phase to be shorter.

Limited N also affected on biomass productivity. Theoretically, biomass productivity is linear, with the rate of COD degradation in heterotrophic conditions [16]. In this research was obtained different scheme where the profile of biomass productivity (μ) in each reactor is R1 > R3 > R2 > R4 and the rate of COD degradation (kL) in each reactor is R2 > R4 > R3 > R1. It was shown that the lower N condition, the higher biomass productivity. However, the high biomass productivity in R1 and R3 were not accompanied by the rate of COD degradation and only occurred during the lag phase, so it could be called the biomass productivity during lag phase. It was just a response of *Chlorella* sp. self–defense.

3.3 Poly–Hydroxy Butyrate (PHB) formation

The profile of PHB in each reactor was shown in Figure 4. The R1 could form PHB. It was pointed out that vinasse could be utilized as a cultivation medium, especially in order to form PHB without nutrient addition. However, the yield of PHB to COD ($Y_{PHB/COD}$) was low, so that it needed nutrient addition to increasing the $Y_{PHB/COD}$. Based on previous research, limiting primary nutrient (N or P) could increase $Y_{PHB/COD}$ [17]. Quantitatively, biomass productivity (μ) and $Y_{PHB/COD}$ in each reactor was shown in Table 1.

Biomass productivity represents the rate of growth of *Chlorella* sp. in vinasse, and the higher value of biomass productivity reflects the higher value of PHB. The same idea with previous research that said accumulated PHB reaches 80 % by their dry mass [18]. In contrary to these statements, in this research, especially in R1 and R3, which obtained higher biomass productivity but the lower yield of PHB. It was due to *Chlorella* sp. undergo deficiency of N, which affect to the lack of metabolic energy so that the *Chlorella* sp. cell decrease in endurance. On the other hand, vinasse contains a phenolic compound, which was inhibitor substance for *Chlorella* sp. [19]. The impact of decreasing cell endurance and the presence of phenolic compounds caused R1 and R3 poisoned. Meanwhile, R2 and R4 could form PHB well because *Chlorella* sp. could grow well in autotroph condition. Nevertheless, to obtain the PHB, one of the efforts was limiting P [17].

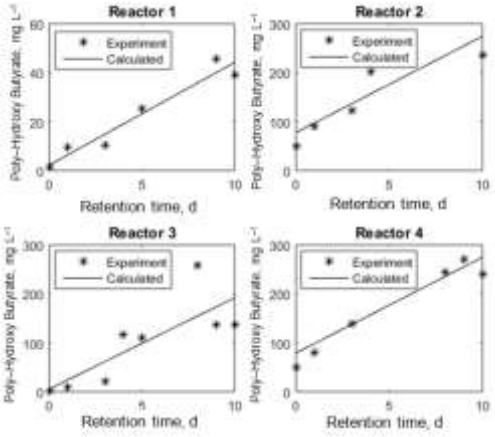

Fig. 4. PHB best–fitting on the model proposed in Equation (2)

4 Conclusions

The reactor with complete nutrients can give the best performance in treating COD (efficiency COD removal = 62.86 %). On the other hand, the reactor without nutrient N addition was undergone N deficiency and toxicity as an impact on the presence of phenol. It was indicated from the highest value of biomass productivity (μ = 0.643 mg L^{-1} d^{-1}). The longest lag phase (λ = 13.8430 d) and the shortest value of the rate of COD degrade (k_L = 0.493E–4 L mg^{-1} d^{-1}). N nutrient addition into the cultivation process of *Chlorella* sp. in vinasse could cut the lag phase and made it shorter (λ = 0.158 d). However, the yield of PHB formation in the reactor with limited phosphate medium was the highest ($Y_{PHB/COD}$ = 0.2655). The insignificant difference among the parameters (k_L and $Y_{PHB/COD}$) in each reactor show that the cultivation of *Chlorella* sp. in vinasse especially to produce PHB was not only influenced by limited nutrient, but also there was some aspect such as substrate ratio, lighting scheme and aeration correlated by CO_2 existence.

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