14

Effect of the Presence of Subtituted Urea and also Ammonia as Nitrogen Source in Cultivied Medium on *Chlorella*'s Lipid Content

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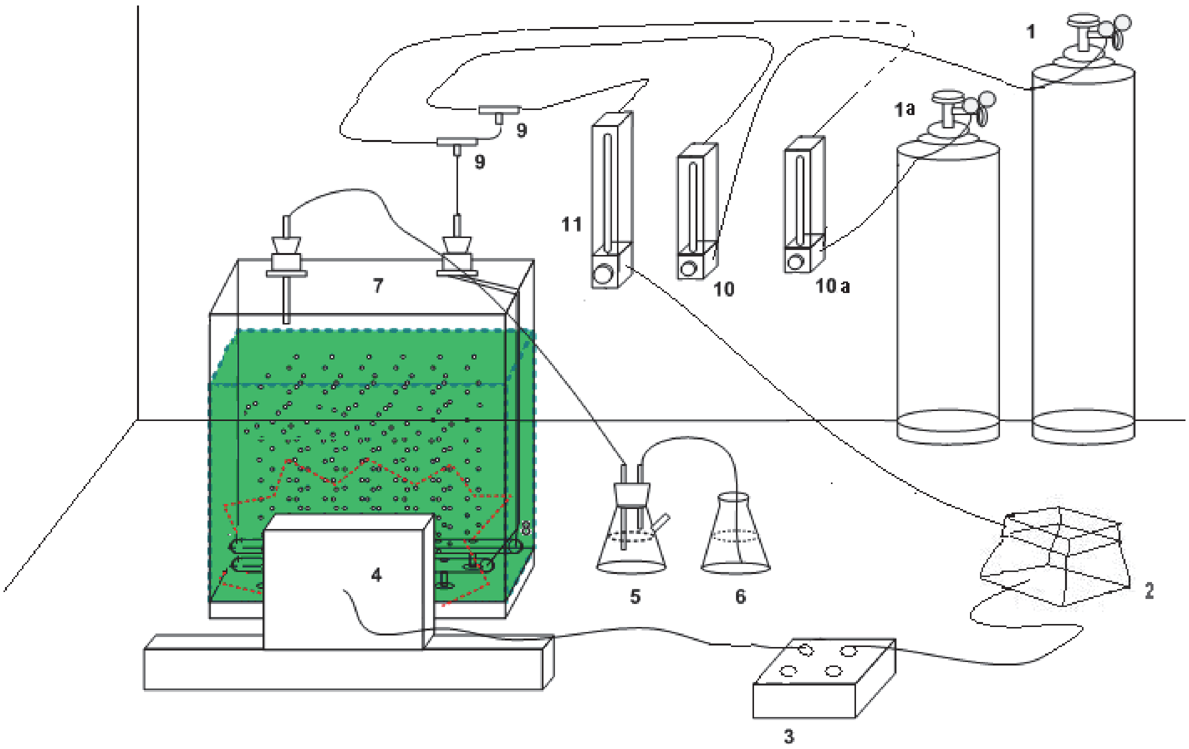
1. Introduction

Global warming has become one of the most serious environment problems. The main cause of this is because of the increasing of CO_2 level in the atmosphere. In recent years, many attempts have been done to reduce the quantity of CO_2 in the atmosphere. Studies on photosynthesis, CO_2 fixation and utilization of micro algae biomass has been carried out. Similar to another *Chlorella* strain, *Chlorella vulgaris* Buitenzorg is known widely of its high valued potential substances such as chlorophyll, CGF, carotene, and protein, and it can be used as potential biomass albeit the function of CO_2 fixation and also possible content long chain un–saturated fatty acid potencies biodiesel as a renewable fuel stock. These characteristics suggest that *Chlorella* is potential for removal and utilization of CO_2 to minimize the accumulation carbon dioxide emitted from industrial plant as a solution to GHG problem.

For its growth, CO_2 that was also enriched by a little content of unburned hydro carbon (PAH), NO_x, SO_x, CO in flue gas (Wijanarko & Dianursanti, 2009; Dianursanti et al, 2010), *Chlorella* needs light energy that was converted to chemical energy in the form of ATP to be used in photosynthesis, metabolism, growth and cell division. It also need substrates such bi-phosphoric salt as phosphor source that was functioned in phosphoric linkage of RNA and DNA structure; urea, nitrate salt or mono ethanol amine as nitrogen source that is an important factor for protein synthesis and cellular growth (Ohtaguchi & Wijanarko, 2002). Based on previous work using *Chlorella*, this work uses a large flat surface photo bioreactors as a part of scale up design for large scale biomass production by using NO_x enriched flue gas utilization as carbon source and also using ammonia or urea as substitution nitrate salt content in its substrate medium as simulated waste contaminated water.

2. Materials & methods

Chlorella vulgaris Buitenzorg is taken from Depok Fresh Water Fishery Research Center that was grown in Benneck medium. This strain grows in 18.0 dm^3 of culture medium in bubble column photo bioreactor that have sizing of (38.5 cm x 10 cm x 60 cm). Experimental apparatus used in the experiment is shown on **Figure 1**.



1. CO_2 storage bomb; 1a. N_2O storage bomb; 2. Air blower; 3. Electric switch; 4. Light source; 5. CO_2 trapped erlenmeyer; 6. CO_2 Erlenmeyer discharge; 7. Flat bubble column photo – bioreactor; 8. Gas bubbler; 9. T – Septum; 10. CO_2 flow-meter; 10a. N_2O flow-meter; 11. Air Flow – meter.

Fig. 1. Experimental apparatus

Conditions were defined as following. Temperature (T) was set at 29.0 ^oC (302 K), Pressure (P) was set at ambient pressure (1 atm.; 101 kPa), Light intensity (I) was set at 3.0 Klx, superficial gas velocity (U_G) was set at 15.7 m/h and CO_2 concentration (y_{CO2i}) in blown bubble air was set around 5.0%. Before cultivation, this strain was grown with pre-culture condition that was set by blowing bubble fresh air with U_G 1.0 vvm with similar operation condition. These photo bioreactors are illuminated by 4 (four) lamps [*Philips Halogen* lamp 20W/12V/50Hz].

Culture biomass content (OD_{600} method) was measured at 600 nm using UV-Vis Spectrophotometer (Labo-Med Inc.); Ammonia was measured at 425 nm using Spectrofotometer and calculated by Nessler method; Lipid content is analysis by Bligh-Dyer Method [Manirakizal et al, 2001); extracted fatty acid content is analyzed using GCMS; protein was measured by Lowry method; elemental analysis is done by XRD and CHNS analyzer; CO_2 inlet and outlet is measured using TCD Gas Chromatography; Chlorophyl a and carotene contents are assayed and calculated by pigment assay procedure (Richmond, 2004; Wijanarko et al, 2006a. 2006b).

3. Results & discussion

For industrial application purposes, utilization of waste water that was analyzed rich of nitrogen source such as urea CO $(NH_2)_2$, ammonia NH_3 or other excess nitrogen substance make biomass production more economically and important cause of a prediction of it's biomass contain more un-saturated fatty acid.

Figure 2 tend a determination of proper diluted nitrogen nutrients for *Chlorella* growth that it varied into control experiment that existed at the Benneck Medium (500 mg/L $NaNO_3$), deficiency diluted nitrogen (250 mg/L $NaNO_3$), excess diluted nitrogen (750 mg/L $NaNO_3$), and different diluted nitrogen sources (500 mg/L $CO(NH_2)_2$). At excess diluted nitrogen source that was shown at medium content 750 mg/L $NaNO_3$ and 500 mg/L $CO(NH_2)_2$, *Chlorella*'s growth result tend lower although growth result in medium content urea more higher than result on excess nitrate salt.

Based on our previous result that was known $CH_{3.3}N_{0.203}O_{0.322}P_{0.041}$ as biomass compound and was constructed from elemental analysis result of dry biomass of *Chlorell vulgaris* Buitenzorg, in presence of nitrate salt in cultivation media, whole chemical reaction of biomass cultivation (Dianursanti et al, 2010) could be shown as below:

$$CH_{3.3}N_{0.203}O_{0.322}P_{0.041} + 1.11\ H_2O + HCO_3^- + 0.041\ H_2PO_4^- + 0.203\ NO_3^- \rightarrow 2CH_{3.3}N_{0.203}O_{0.322}P_{0.041} + 2.03\ O_2 \quad (1)$$

Meanwhile, in case of presence of different diluted nitrogen sources such as $CO(NH_2)_2$), whole chemical reaction of biomass cultivation could be changed as below:

$$CH_{3.3}N_{0.203}O_{0.322}P_{0.041} + 0.984\ H_2O + 0.898\ HCO_3^- + 0.041\ H_2PO_4^- + 0.102\ CO(NH_2)_2 \rightarrow 2CH_{3.3}N_{0.203}O_{0.322}P_{0.041} + 1.81\ O_2 \quad (2)$$

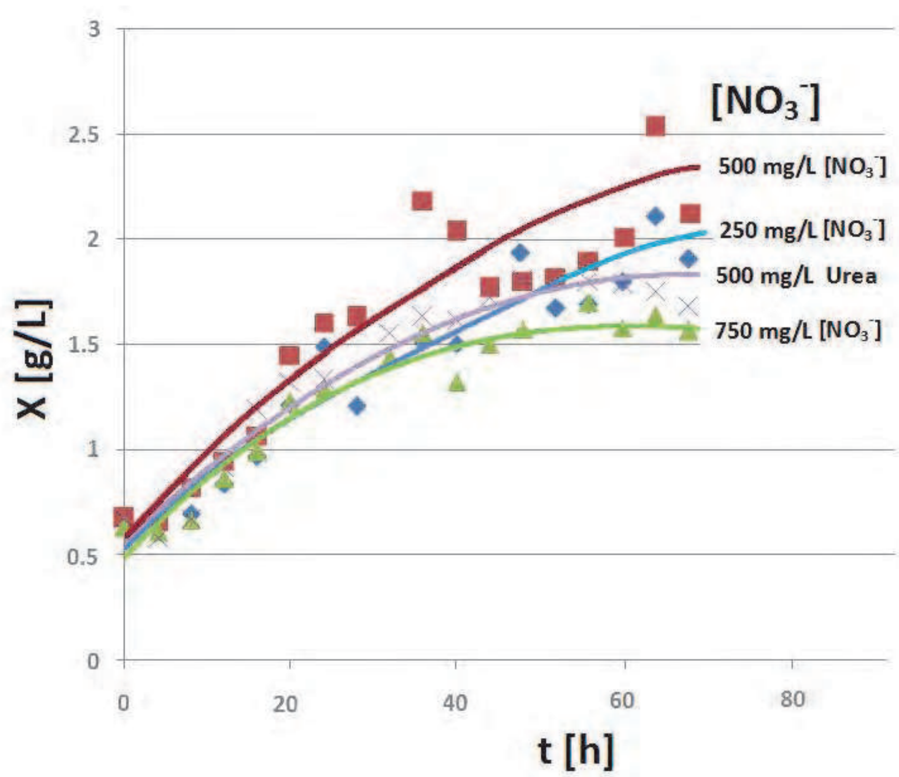


Fig. 2. Effect of composition nitrogen source on *Chlorella*'s growth at beginning 72 hours cultivation

It could be understood, presence of 500 mg/L CO $(NH_2)_2$ that was equivalent to two times concentration compare to diluted nitrate salt in cultivation media making nitrogen source concentration excess around 40% and then it change to form ammonium ion that was easily and freely to metabolize for making essential amino acid, protein and chlorophyll, cause of intracellular conversion of urea could be change to ammonium ion easily using urease (urea amidohydrolase) or urea amidolyase that was reacted together with ATP. Both of enzymes was commonly present in unicellular algae (Leftley & Syrett, 1973).

urea amidohydrolase pathway

$$CO(NH_2)_2 + H_2O \rightarrow CO_2 + 2NH_3 \quad (3)$$

urea amidolyase pathway

$$CO(NH_2)_2 + ATP + HCO_3^- \xleftrightarrow{Mg^{2+}K^+} \text{allophanate} + ADP + P_i \quad (4)$$

$$\text{allophanate} \rightarrow 2NH_3 + 2CO_2 \quad (5)$$

In case of nitrate assimilate reaction, intercellular conversion of nitrate ion was performed via nitrate reduction pathway need NADH that was also needed for intracellular lipid, protein and chlorophyll formation and it directly influence to cellular growth.

Nitrate Reduction pathway

$$NO_3^- + NADH + H^+ \xrightarrow{NR} NO_2^- + NAD^+ + H_2O \quad (6)$$

$$NO_2^- + 3H_2O + 2H^+ + hv \rightarrow NH_4^+ + 1.5O_2 + 2H_2O \quad (7)$$

Meanwhile, excess of intracellular ammonium ion or ammonia could be inhibited formation ATP in chloroplast [9] and it could be understood that optimum condition for *Chlorella*'s growth was around 500 mg/L $NaNO_3$ that existed at the Benneck Medium. This phenomenon could be impressed that Chlorella's growth was followed subtrate activation and inhibition model (Sallisbury & Ross, 1992).

Determination of proper diluted nitrogen nutrients for *Chlorella* growth shown that diluted nitrogen concentration in the Benneck medium (control) there is the most optimal nutrition to produce lipids up to 0.42 g / g biomass for biodiesel utilizing purpose [**Figure 3**].

Cause of intracellular conversion of urea could be change to ammonium ion more easily using both of intracellular algal's urease (urea amidohydrolase) or urea amidolyase, it could be understood why algal's lipid content of alga that was cultivated in diluted urea tend more high [0.3 g/g biomass] at beginning and hereafter shown relatively constant. Urea metabolism was not consumed NADH which was also necessary for intracellular lipid formation. In the meantime, composition of diluted nitrate ion as nitrogen source, at excess diluted nitrogen source that was shown at medium content 750 mg/L $NaNO_3$, algal's cellular produce lipid up to 0.40 g/g biomass but similar to experimental result that was held by Yanqun, as consequence of substrate activation and inhibition growth model, this lipid formation could be happen only at stationer phase of cellular growth (Bailey & Ollis, 1986).

Although cellular growth was decrease around 30%, presence of urea as nitrogen source, diluted urea in cultivation media is the most appropriate nutrients to produce protein until it reaches 0.54 g / g biomass [**Figure 4**]. This protein content is attractable for food supplement development purpose and it was around one and half times increasing compare to result on control experiment. The evidence of intracellular protein formation was closed similar to the reason of lipid formation. Urea metabolism was not consumed NADH which was also necessary for intracellular protein formation and produced ammonium was easily to metabolize for making essential amino acid and also protein (Leftley & Syrett, 1973; Sallisbury & Ross, 1992)

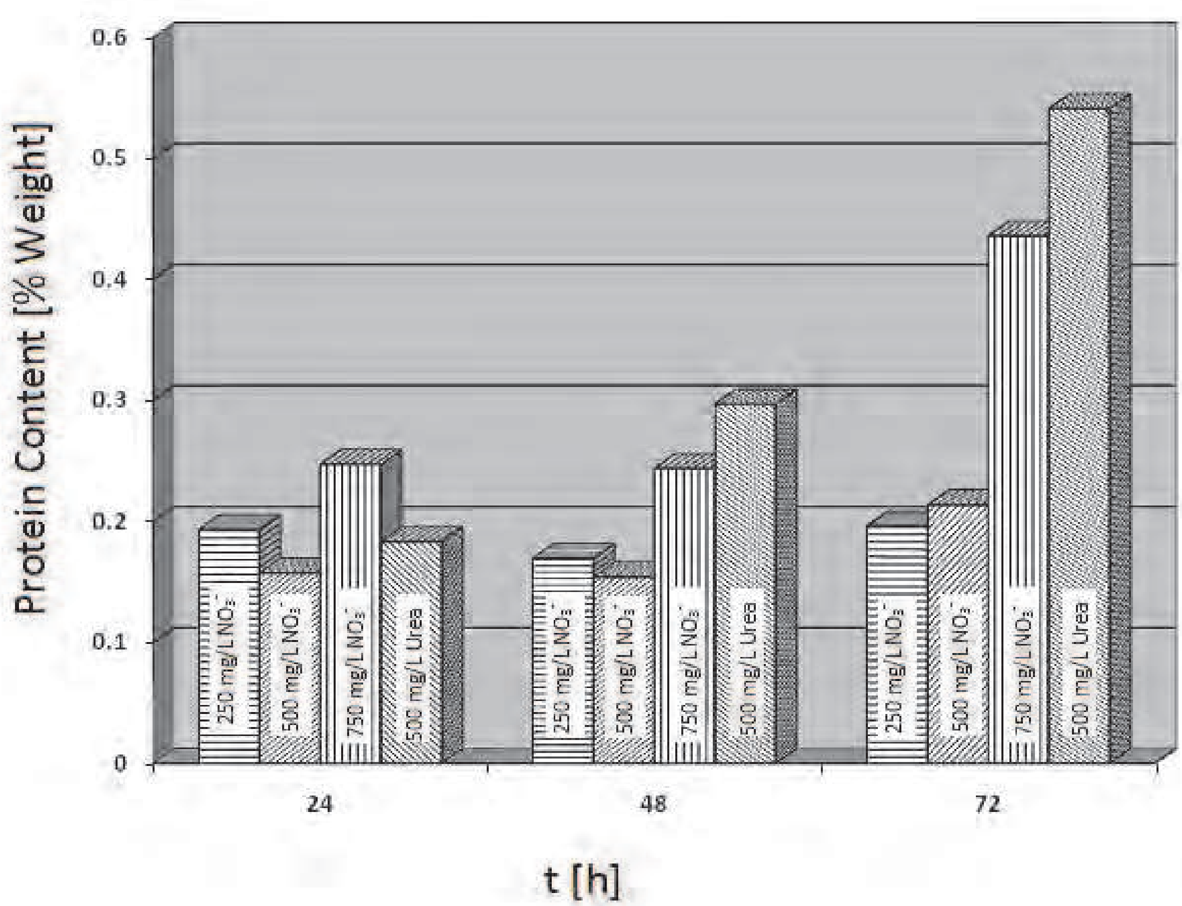


Fig. 3. Effect of composition nitrogen source on *Chlorella*'s lipid content at beginning 72 hours cultivation

Whereas, in excess diluted nitrogen (750 mg/L $NaNO_3$), cell growth produced relatively high protein content on its intracellular around 0.24 g / g biomass at the beginning and increasing to 0.43 g / g biomass at 72 h cultivation and it was closed to result in media contain urea as nitrogen source [**Figure 5**]. Cause of growth relatively lower than both of control experiment that existed at the Benneck Medium (500 mg/L $NaNO_3$) and deficiency diluted nitrogen (250 mg/L $NaNO_3$), increasing of ammonium as conversion produced of excess nitrate via nitrate reduction pathway, together with carbon metabolite product spontaneously could be metabolize for making essential amino acid and then also protein (Sallisbury & Ross, 1992).

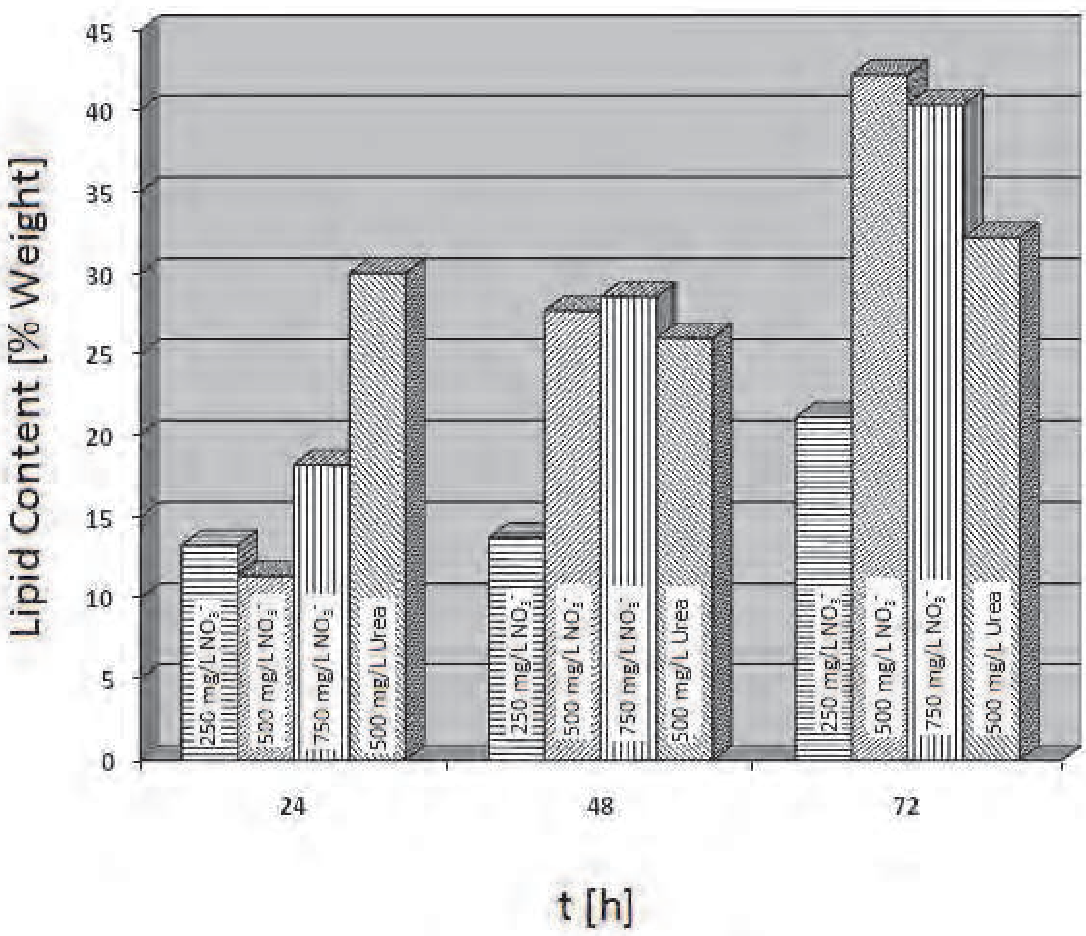


Fig. 4. Effect of composition nitrogen source on *Chlorella*'s protein content at beginning 72 hours cultivation

Furthermore, medium that excess diluted nitrogen is the most appropriate nutrients to produce chlorophyll and it reach 4.9 g/100g biomass at beginning 48 hours [**Figure 6**]. Similar to explanation in above, increasing of ammonium as conversion product from media contain excess nitrate via nitrate reduction pathway, beside making essential amino acid and then also protein, together with carbon metabolite product spontaneously could be metabolize for intracellular chlorophyll (Sallisbury & Ross, 1992). Meanwhile, presence of urea as nitrogen source, as consequence of its high cellular protein producing, algal's growth produce small amount of cellular chlorophyll.

Henceforth, presence of urea as nitrogen source, drastically change intracellular fatty acid content [**Table 1**]. It is shown that presence of urea as substitution species of nitrate salt in Benneck medium, was converted fatty acid C_{16} species (around 30.4 % C_{16} in Benneck) to be fatty acid C_{18} species significantly (around 77.0 % C_{18} in presence of urea) that was guessed by presence of additional carbonyl group in urea structure that was already absorbed into cytoplasm and carry out in cellular metabolizing and converting significantly 16:0 fatty acid to be 18:0 fatty acid and also other species un-significantly 18:1, 18:2 fatty acids.

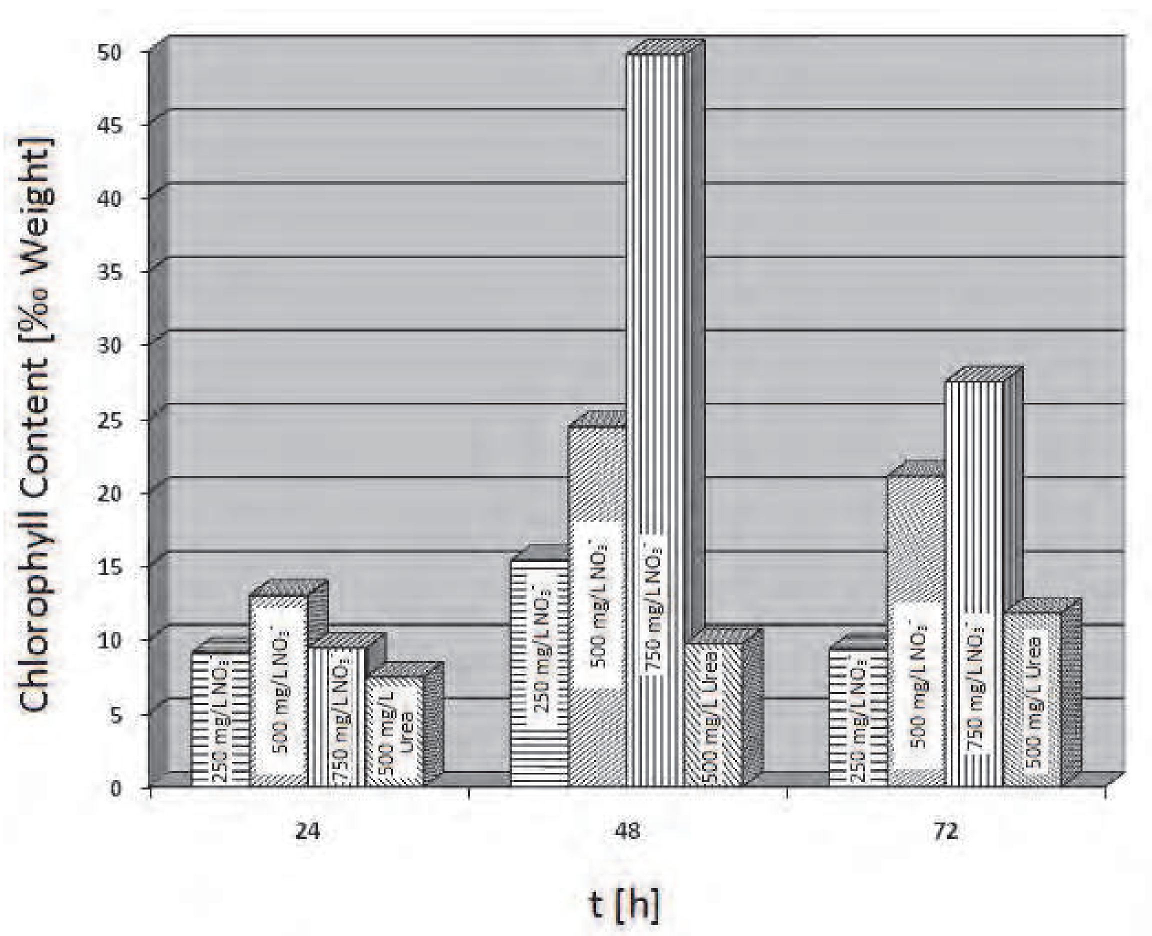


Fig. 5. Effect of composition nitrogen source on *Chlorella*'s chlorophyll content at beginning 72 hours cultivation

Fatty Acid	% Content	
	Appropriate diluted Nitrate Salt (Benneck)	Diluted Urea Media
08 : 0	0.48	0.65
12 : 0	5.50	4.93
14 : 0	3.15	8.60
16 : 0	30.04	0.55
16 : 1	0.33	1.63
18 : 0	9.53	18.04
18 : 1	34.23	40.91
18 : 2	16.74	18.04
20 : 0	0.0	0.60

Table 1. *Chlorella*'s fatty acid content that was cultivated in media contain urea or nitrate salt as nitrogen source.

Determination of proper ammonia nutrients from diluted domestic waste water by 1 : 15 for *Chlorella* growth and compare to appropriate nitrate ion concentration in the Benneck medium (control, 500 mg/L $NaNO_3$) was shown in **Figure 6.** This diluted domestic waste water contain 4.7 mg/L NH3, 330.8 Chemical Oxygen Demand, 78.8 mg/L phosphate salt and pH 8.67. This comparison was done for elaborate effect of substitution nitrate salt in cultivation media with more cheaply and acceptable consumed chemical substance which was contained in waste water such as ammonia to maximize producing of cellular lipids for biodiesel development purpose.

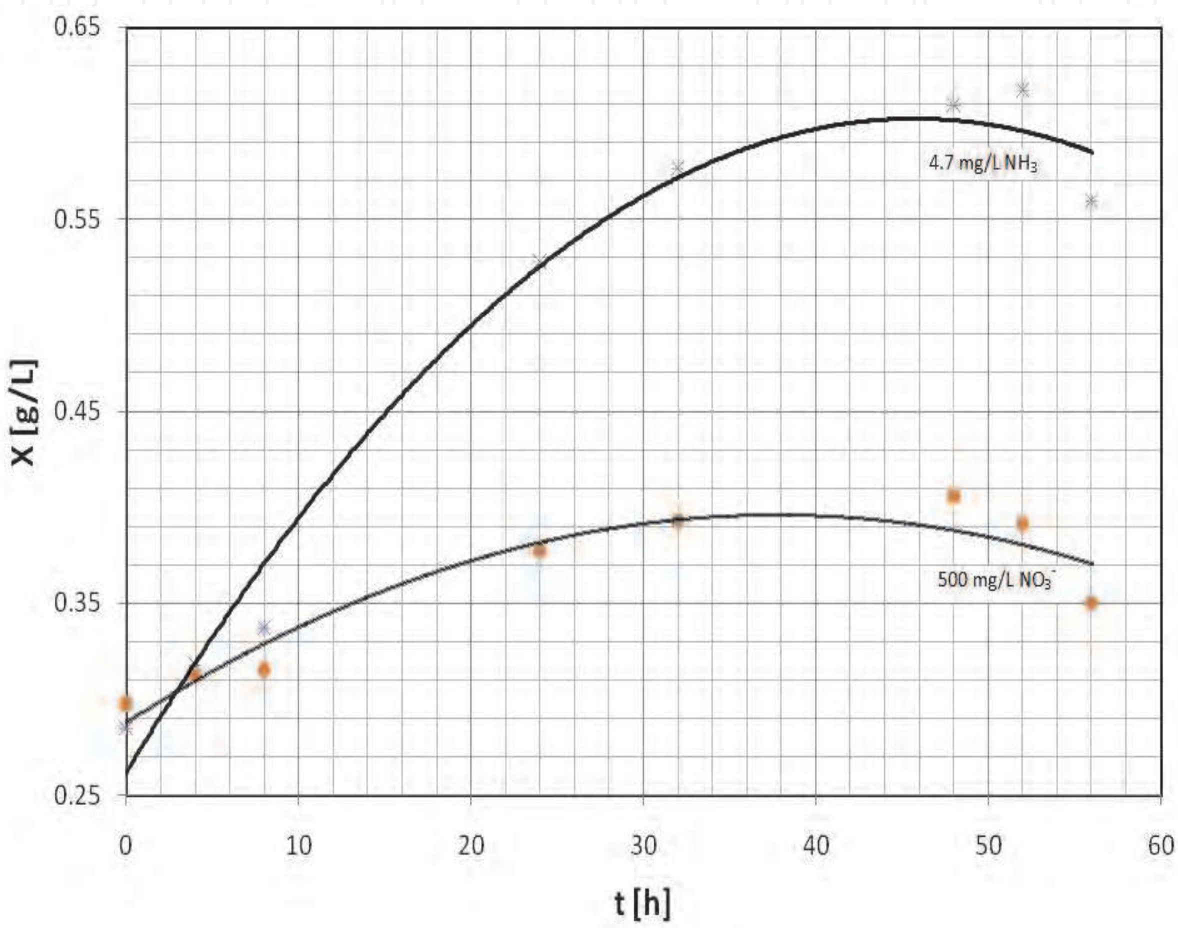


Fig. 6. Effect of replacement diluted domestic waste water 1 : 15 which contained NH_3 as nitrogen source on *Chlorella*'s growth at beginning 56 hours cultivation

At diluted domestic waste water that was measured 4.7 mg/L NH_3 as nitrogen source shown that chlorella's growth result tend near 60% higher than cultivated biomass production in commonly growth media contained appropriate nitrate salt content. It could be understood, in diluted waste water, contained ammonium ion could be directly metabolized for making essential amino acid, protein and chlorophyll that directly related to microbial growth. Composition of free ammonia and ammonium ion in diluted waste water was found 1.05 and 3.65 g/L, as a notification, presence free ammonia could be inhibited cellular growth. Although free ammonia in cultivation media was inhibited algal's growth but in this waste water, presence only 1.05 g/L free ammonia and it was lower than *Chlorella*'s tolerance limit that was found around 6 g/L free ammonia(Strauss et al, 2010).

Compare to intercellular growth in nitrate salt contained media that must be converted to ammonium species at beginning step, presence of ammonium ion in this waste water make it more quickly utilized and of course increasing its biomass production significantly. This phenomenon was similar to previous result on cellular growth of *Chlorella pyrenesoide* which was already done (Ogbonna & Tanaka, 1996). During 48 hours hours cultivation in waste water, ammonia could be decreased to 1.6 mg/L and it is around 66% ammonia nitrogen removal. Furthermore, intracellular lipid formation in algal's growth in waste water, was un-significantly higher than in appropriate nitrate content in Benneck media. **Table 2** shown that change nitrate salt to ammonia as nitrogen source could be increased around 15% in algal's lipid formation. Beside it, chlorophyll formation was also increasing significantly, it was around 55% increasing.

Media	Lipid Content (% weight)	Chlorophyill content (mg/L)
Diluted waste water	57.1	12.1
Benneck	48.7	7.8

Table 2. *Chlorella*'s fatty acid content that was cultivated in diluted waste water and Benneck media

Finally, as a conclusion remarks, compare to result on utilization urea as nitrogen source, substitution nitrate salt in cultivation media with ammonia that was more cheaply cause it presence in domestic waste water, is more significantly for maximizing producing of cellular lipids for biodiesel development purpose.

4. Conclusion

For biodiesel utilizing purpose, diluted nitrogen concentration in the Benneck medium (control) is the most optimal nutrition to produce lipids up to 0.42 g / g biomass. In another case, although cellular growth was decreased around 30%, presence of urea as substituted nitrogen source is the most appropriate nutrients to produce protein up to 0.54 g / g biomass that is necessary for food supplement purpose. Beside that, for producing chlorophyll, medium that excess diluted nitrogen is the most appropriate nutrients to reach up to 49 $^{o}/_{oo}$ weight. Furthermore, presence of urea, drastically change intracellular fatty acid content and it is shown that presence of urea as substitution species of nitrate salt in Benneck medium, was converted fatty acid C_{16} species (around 30.4 % C_{16} in Benneck) to be fatty acid C_{18} species significantly (around 77.0 % C_{18} in presence of urea) that was guessed by presence of additional carbonyl group in urea structure that was already absorbed into cytoplasm and carry out in cellular metabolizing. Finally, compared to result on utilization urea as nitrogen source, substitution nitrate salt in cultivation media with ammonia which was used to minimizing operation cost cause it more cheaply and commonly presence in domestic waste water. Utilization of ammonia for maximizing producing of biomass and cellular lipids is more interesting for biodiesel development purpose. It makes around 55 – 60 % increasing in both *Chlorella*'s growth and cellular lipid formation.

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6. References

Wijanarko, A. & Dianursanti. 2009. Simulated flue gas fixation for large-scale biomass production of *Chlorella vulgaris Buitenzorg*. *International Journal for Algae*, 11: 351-358

Dianursanti; Nasikin, M. & Wijanarko, A. 2010. NOx enriched flue gas fixation for biomass production of *Chlorella vulgaris Buitenzorg*. *Asian Journal of Chemical Engineering*, 10: 24-30

Ohtaguchi, K. and Wijanarko, A. 2002. Elevation of the efficiency of cyanobacterial carbon dioxide removal by mono ethanol amine solution. *Technology*, 8:. 267 – 286

Manirakizal, P.; Covaci, A. & Schepens, P. 2001. *Comparative* Study on Total Lipid Determination using Soxhlet, Roese Gottlieb, Bligh Dyer, and Modified Bligh Dyer Extraction Method. *Journal of Food Composition and Analysis*, 14: 93 – 100

Richmond A. [Ed.]. 2004. *Handbook of Microalgal Culture: Biotechnology and Applied Phycology.* Jhon Wiley & Son, New York: 40 – 54

Wijanarko, A.; Dianursanti; Heidi; Soemantojo, R W. and Ohtaguchi, K. 2006. *Effect* of Light Illumination alteration on *Chlorella vulgaris* Buitenzorg's CO_2 fixation in bubble column photobioreactor. *International Journal for Algae*, 8: 53-60

Wijanarko, A.; Dianursanti; Gozan, M.; Andika, S. M. K.; Widiastuti, P.; Hermansyah, H.; Witarto, A. B.; Asami, K.; Soemantojo, R. W.; Ohtaguchi, K. & Song, S. K. 2006. Enhancement of carbon dioxide fixation by alteration of illumination during *Chlorella vulgaris* Buitenzorg's growth. *Biotechnology and Bioprocess Engineering*, 11: 484-488

Leftley, J.W. & Syrett, P.J. (1973). Urease and ATP: Urea Amidolyase Activity in Unicellular Algae. *Journal of General Microbiology*, 77: 109-115

Salisbury, F. B. & Ross. C. W. (1992). *Plant Physiology*, 4th ed., Wadsworth Publishing Co., Colorado

Bailey, J. E. & Ollis, D. F. (1986). *Biochemical Engineering Fundamentals*, 2nd Ed., McGraw Hill Book Co., Singapore

Yanqun, L.; Horsman, M.; Wang, B.; Wu, N. & Lan, C. Q. (2008). Effects of nitrogen sources on cell growth and lipid accumulation of green alga *Neochloris oleoabundans*. *Applied Microbiology and Biotechnology*, 81, pp:629-636

Strauss, M.; Larmie, S. A. & Montenegro, H. A. (2010). Treating Faecal Sludges in Ponds. Available from: *www.eawag.ch/forschung/sandec/.../treating_FS_in_Ponds_Strauss_IWA.pdf*

Ogbonna, J. C. & Tanaka, H. (1996). Night biomass loss and changes in biochemical composition of cells during light/dark cycle culture of *Chlorella pyrenesoide. Journal of Fermentation and Bioengineering*, 82: 558 – 564