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Alternative media for the culture of the Cyanobacteria *Nostoc paludosum* and conditions for optimizing biomass and lipid production

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Abstract. The objective of the present study was to evaluate the possibility of using pond water as an alternative media for culturing *Nostoc paludosum*. Lipid content of *N. paludosum* using BG II media was investigated. Lipid content was measured by using soxhlet method with petroleum ether as solvent. The highest lipid content (7% of dry weight) was obtained after 11 days, when nitrate concentration is low (451 ppm). Biomass was measured by gravimetric method using Whatman filter paper No. 42. Dry weight of algae was measured after drying in oven at 60°C for 24 hours. Chlorophyll-a was analyzed by using spectrophotometric method. The limiting nutrients of *N. paludosum* were investigated by using the bioassay method and urea as nitrogen source, K_2HPO_4 as phosphorus source and $CaCO_3$ as carbon source. Nitrogen is the limiting nutrient. Pond water can be alternative media for culturing *N. paludosum* although it can produce contamination.

Key Words: *Nostoc paludosum*, pond water, nutrients, lipid content.

Introduction. The renewable source is necessary for environmental and economic sustainability. Biofuel processes are capable to provide a carbon neutral for fuel production (Chisti 2007; Schenk et al 2008), in order to replace of fossil fuels over the long term and reduce Greenhouse Gas Emissions. Biodiesel and bioethanol are the most common biofuels. Biodiesel can substitute diesel and bioethanol as a substitute gasoline (Mata et al 2010).

Some microalgae are capable to collect huge quantities of lipid inside their cells, then it is transformed to biodiesel (Chisti 2007). Microalgae is recognized as the reliable choice for fuel production because it has high growth rate compared with oil crops and higher photosynthetic efficiency, higher biomass production, low land requirement, and possible high (30–50 wt.%) oil content (Gonzalez et al 2015; Mata et al 2010; Miao & Wu 2004; Minowa et al 1995). Cyanobacteria are increasingly recognized as promising cell factories for the production of renewable biofuels (Erdrich et al 2014). Algal biodiesel is obtained by converting fatty acid methyl esters and other lipids contained in the cells (Beal et al 2010). The primary determinants to produce biofuel are lipid content and fatty acid compositions of algae species (Anandarajah et al 2012).

Algae growth performance relies on many factors among which major macronutrients carbon, nitrogen, and phosphorus availability and balance play a critical role (Christenson & Sims 2011). Nutrition can influence the biomass of cyanobacteria (Markou & Georgakakis 2011), and several studies reported contradicting results on the effect of changing the nutrient concentrations on biomass lipid content.

Piorreck et al (1984) revealed that lipid composition of cyanobacteria did not change significantly, whereas Walach et al (1987) pointed out that lipid content increased under nitrate limitation, in contradiction with the findings of Sassano et al (2010) who reported that lipid content decreased under nitrate limitation.

The microalgal population growth rate influences lipid accumulation rate where the lipid productivity and concentration are affected by the nutrient condition. Mostly, microalgae are made of protein, carbohydrate and lipid. The cell division is under environmental determinism and the lipid production is source of energy storage. This occurs when environmental condition become favorable and results in accumulation of lipid in microalgal biomass increases (Li et al 2010).

The critical parameters for the profitability of biodiesel production using algae are the algal yield and the lipid content of cells, which result in overall lipid productivity that determines the production costs and economic benefits of the process (Li et al 2008).

Nostoc spp. are irregular filamentous organisms (Teaumroong et al 2002). Several *Nostoc* algae aggregate in colonies (called *thalli*) that are visible to human eyes. Their shape is spherical at the initial stages, but can become hollow or flat (Mollenhauer et al 1999). Hence, the objectives of the present study weres to identify the possibility of pond water as an alternative medium for cultivating *Nostoc paludosum* and identify the limiting nutrients (urea, K_2HPO_4 or $CaCO_3$) of *N. paludosum*.

Material and Method. The experiment was conducted at the Aquaculture and Aquatic Resources Management (AARM) facilities of the Asian Institute of Technology (AIT) from January 2013 to March 2013.

Stock culture. The research investigated the cyanobacteria *N. paludosum,* obtained from the algae culture collection of Thailand Institute of Scientific and Technological Research. A stock culture was maintained for inoculation of experimental cultures. *N. paludosum* was preserved in BG II medium as described in Table 1. For the stock culture, *N. paludosum* was inoculated at 10% (v/v) in BG II medium with 2000 lx continuous fluorescent light illumination and air input by aeration system. Stock culture was renewed every 2 weeks. All the glassware and media were sterilized in autoclave at 121^{o}C, 15 psi for 15 minutes prior to inoculation.

Table 1

Compositions of BG II medium

Chemical	*Stock solution (quantity / mL distilled water)*	*Culture solution (required per L)*
$NaNO_3$	75 g / 500 mL	10 mL
$K_2HPO_4.H_2O$	8 g / 200 mL	1 mL
$MgSO_4.H_2O$	15 g / 200 mL	1 mL
$CaCl_2.H_2O$	7.2 g / 200 mL	1 mL
Na_2CO_3	4 g / 200 mL	1 mL
Citric acid	1.2 g / 200 mL	1 mL
$Fe_2SO_4.H_2O$	1.2 g / 200 mL	1 mL
EDTA	0.2 g / 200 mL	1 mL
Trace elements		1 mL
H_3BO_3	2.68 g / 200 mL	1 mL
$MnCl_2.H_2O$	1.81 g / 200 mL	1 mL
$ZnSO_4.H_2O$	0.22 g / 200 mL	1 mL
$Na_2MoO_4.H_2O$	0.39 g / 200 mL	1 mL
$CuSO_4.H_2O$	0.079 g / 200 mL	1 mL
$Co(NO_3)_2.H_2O$	0.049 g / 200 mL	1 mL

N. paludosum lipid composition using BG II medium as culture media. *N. paludosum* was cultured in 30 L aquarium, at 28°C providing 2000 lx continuous fluorescent light illumination. Experiment was carried out with 3 replications during 14 days. Biomass, lipid content, chlorophyll-a, water quality was measured daily during 14 days. A total of 10% of *N. paludosum* was inoculated in 20 L BG II medium. Experiment was conducted in 3 replications.

Identifying the limiting nutrients of N. paludosum. *N. paludosum* was cultured in 250 mL flasks, at 28°C providing 2000 lx continuous fluorescent light illumination. Experiment was divided by 7 treatments. Experiment was carried out with 3 replications during 7 days. Biomass and chlorophyll-a were measured daily. *N. paludosum* was inoculated at 20% in 200 mL pond water. The 7 treatments are listed below:

- Control : pond water only;
- Treatment 1: pond water + nitrogen (N) source;
- Treatment 2: pond water + phosphorus (P) source;
- Treatment 3: pond water + carbon (C) source;
- Treatment 4: pond water + nitrogen (N) source + phosphorus (P) source;
- Treatment 5: pond water + nitrogen (N) source + carbon (C) source;
- Treatment 6: pond water + phosphorus (P) source + carbon (C) source;
- Treatment 7: pond water + nitrogen (N) source + phosphorus (P) source + carbon (C) source.

Biomass and chlorophyll-a measurements. Subsample was collected for determining biomass. It was measured by gravimetric method using Whatman filter paper No. 42. Dry weight of algae was measured after drying in oven at 60°C for 24 hours. A total of 10 mL of each treatment sample was collected for determining chlorophyll-a. By using a vacuum filter apparatus and a GF/C filter paper, 10 mL sample water was filtered, 1 mL $MgCO_3$ suspension was pipetted to the sample water during filtration. Furthermore, the filter was folded and placed in a tissue grinder. Approximately 2 mL of 90% acetone solution was added and grinded well. Next, the 8 mL of 90% acetone solution was added and grinded for 30 seconds. The contents of tissue grinder was transferred to a 15-mL glass centrifuge tube with screw cap. The tube was covered tightly and shaked vigorously. The tube was kept at 4°C in the dark refrigerator for at least 3 hours. The chlorophyll extract was centrifuged at 4000 rpm for 5 min and the supernatant obtained for spectrophotometric analysis. The extract was filled in 1-cm cuvette, absorbance was read at wavelength of 750 nm, and 663 nm. Then 2 drops of 1N HCl solution was added into chlorophyll extract and mix. Absorbance was read at 750 nm and 665 nm.

Lipid, nitrate and nitrite measurements. Lipid content was measured by using soxhlet method with petroleum ether as solvent. Cups were placed into an oven for 15 minutes, and cooled in desiccators and dry cups were weighted. Dried biomass has been used to determine the lipid content. Samples were wrapped and inserted into extraction thimble. Thimbles were hooked into thimble holder. Heating unit of Soxtec was switched on, turn cool water supply to condenser unit. Extraction cups were filled with petroleum ether (30-60 Ml). The control lever in the Soxtec is lowered into “rinsing” position, thimbles were inserted and the lever raised to “boiling” position. The lever is lowered into controlling position of hot plate and cups were inserted into soxtec. Lipids were extracted for 30 minutes in “boiling” position and 45 minutes in “rinsing” position. Valve was turned off, cups were released and dried at 100°C for 30-60 minutes. Cups were cooled in a desiccators and weighed. The nitrate and nitrite were analyzed using standard procedures proposed by Boyd (1992).

Results and Discussion

Nostoc paludosum lipid composition using BG II medium as culture media. Biomass of *N. paludosum* cultured in BG II medium slightly increased until at the end of culture. The highest biomass occurred on day 12 and 14 culture (Figure 1). Lipid content of *N. paludosum* was between 1.4% and 7.4 % of dry weight from day 0 to day 14 (Figure 2). Lipid content sharply increased on day 11 to 0.0090 g or 7.4% of dry weight. It could have occurred because of stress condition such as nutrient depletion. *N. paludosum* accumulates lipid because their cellular composition change under nutrient depletion, leading to a decrease in protein content and increase in carbohydrates and lipid storage. Another study found similar results in marine diatom *Phaeodactylum tricornutum* (Xue et al 2015) and *Chlorella* strains (Feng et al 2012). Xue et al (2015) pointed out that nitrogen induce intracellular lipid turnover and promote neutral lipid accumulation. Moreover, optimizing stress conditions to obtain the highest possible lipid yields in the cells may also be important (Bhattacharjee & Siemann 2015).

The total amount of nitrate at the beginning was 600 ppm and it was measured throughout the 14 days culture (Figure 3). The highest lipid content of *N. paludosum* was obtained when nitrate concentration was 451 ppm. Whereas, nitrite did not increase significantly (Figure 3).

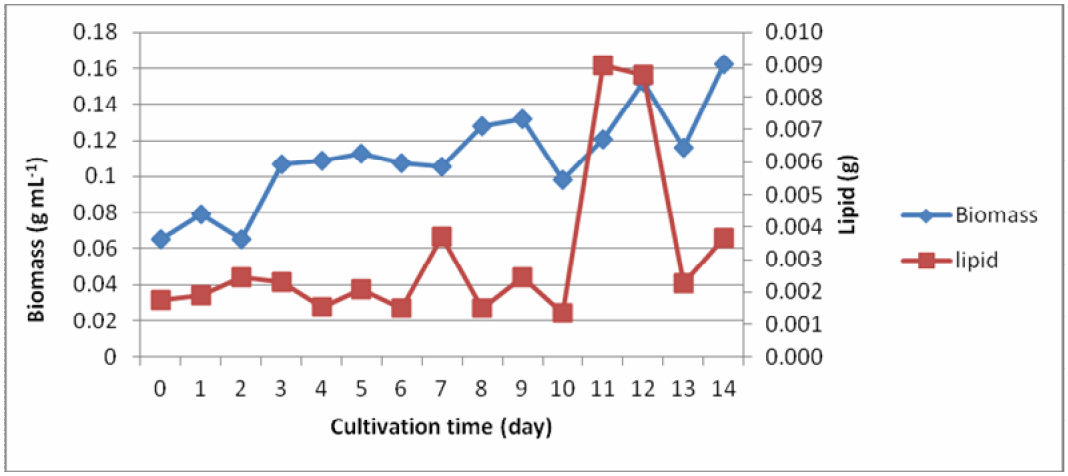

Figure 1. Biomass and lipid content of *N. paludosum* in BG II medium.

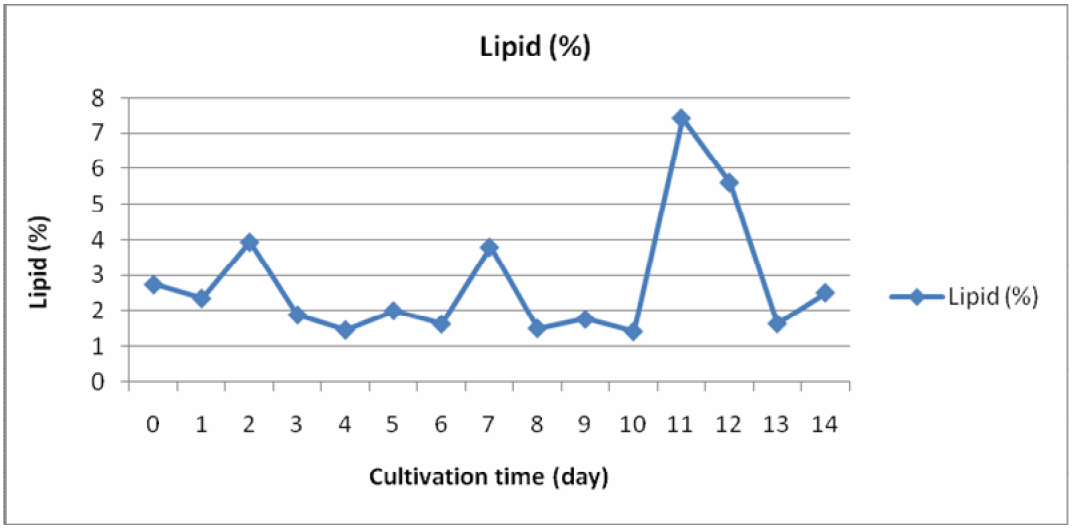

Figure 2. Percent lipid content of *N. paludosum* in BG II medium.

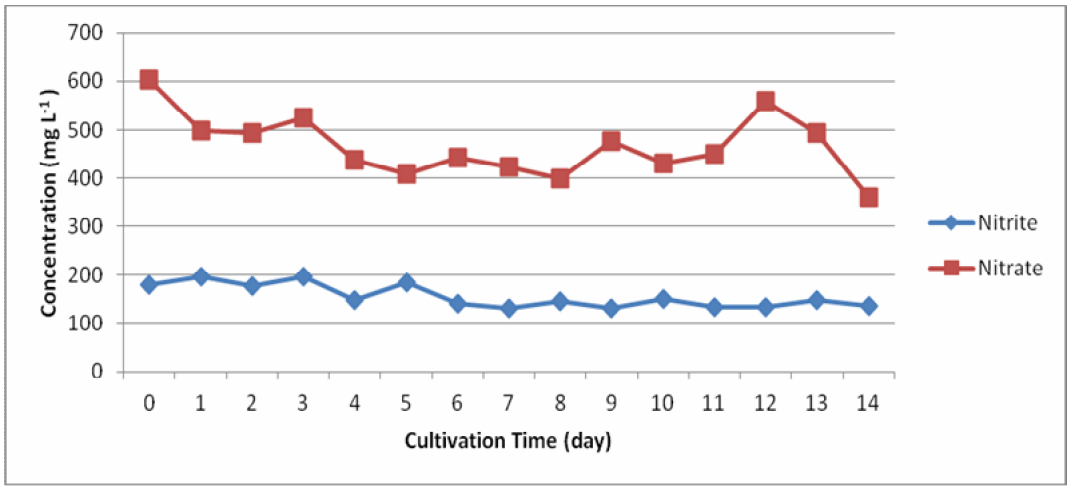

Figure 3. Nitrite and nitrate concentration in culture using BG II medium.

Limiting nutrients to N. paludosum growth. Biomass of *N. paludosum* for all treatments and control increased from the beginning of culture until day 3. It dropped drastically on day 4 for control and all treatments and increased afterward. Biomass of *N. paludosum* increased on day 3 for control and treatments N, P, C, N+P, and P+C. Treatment using N+C reached the highest biomass on day 6.

Biomass of *N. paludosum* was highest on day 3 in treatment with addition of N (urea) with 0.0028 g mL^{-1} (Figure 4). In this study, nitrogen seems to be the limiting nutrient for *N. paludosum* (Figure 5) during short culture period (3 days culture). According to Becker (1994), ammonia or urea is the best source of nitrogen in terms of profitability. In addition, Berman & Chava (1999) studied other species of cyanobacteria such as *Aphanizomenon ovalisporum, Microcystis aeruginosa,* and *Synecochoccus* sp. that grew well on urea. In contrast, results (Figure 4) show that biomass of *N. paludosum* was low in presence of urea in treatment using combination N+P+C and biomass of *N. paludosum* on day 3 with treatments using N+P, N+C and N+P+C is lower than other treatments. Chlorophyll-a content in control and all treatments changed during experiment (Figure 6). The increasing trend was only obtained by treatment 4 with addition of N and P.

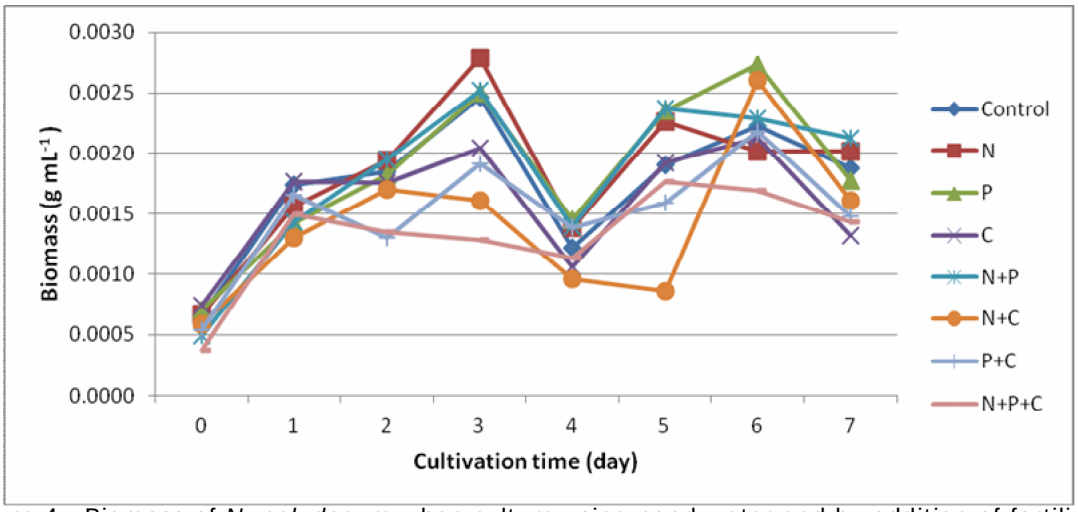

Figure 4. Biomass of *N. paludosum* when culture using pond water and by addition of fertilizer.

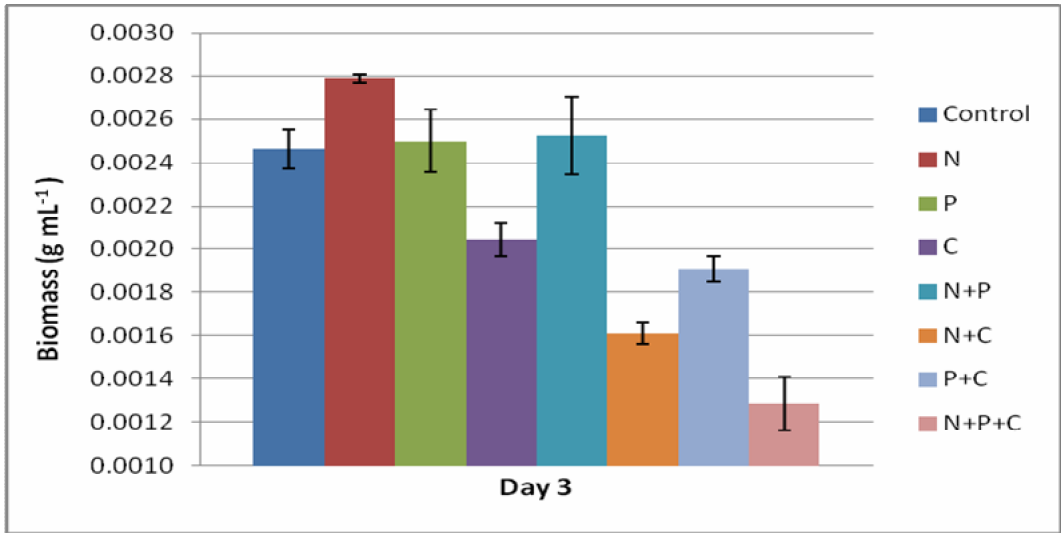

Figure 5. Biomass of *N. paludosum* on day 3 when culture using pond water and by addition of fertilizer.

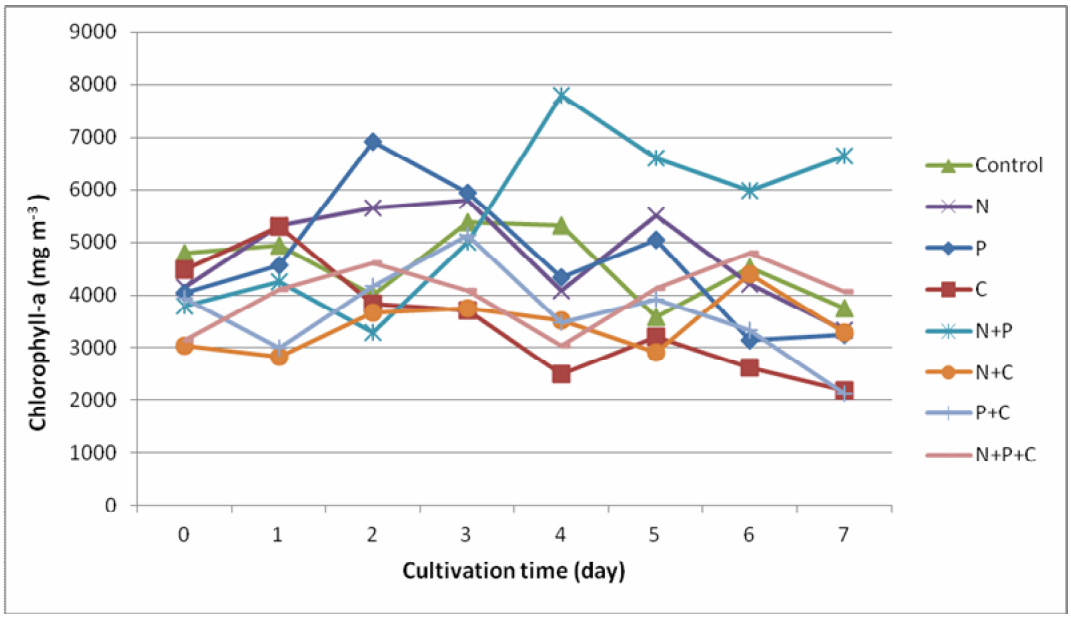

Figure 6. Chlorophyll-a content of *N. paludosum* when cultured using pond water and addition of fertilizer.

Conclusions. Maximum lipid content was achieved after 11 days of culture using BG II medium (7% of dry weight). It occurred with a low nitrate concentration and it is assumed that it results from stress condition such as as nutrient depletion. *N. paludosum* accumulates lipid because their cellular composition change under nutrient depletion, leading to a decrease in protein content and increase in carbohydrates and lipid storage. The limiting factor for *N. paludosum* growth has been identified as being nitrogen during experiment. However, further studies are needed to understand the reason why treatment, such as N+P, N+P+C did not produce expected results.

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