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Cultivation of Green Microalga, *Chlorella vulgaris* for Biogas Purification

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Abstract— **Algal biomass can be utilized for renewable energy sources, such as hydrogen, biodiesel and biogas. Currently, renewed interest in producing bioenergy from microalgae has arisen because they can grow rapidly and convert solar energy into chemical energy via CO_2 fixation and, thus, are now considered one of the most promising energy sources. Subsequently, microalgae are playing important role in the biological purification of biogas. In this study, samples were collected from fish pond water and samples were enriched and isolated. The strain was identified as species of green microalgae *Chlorella vulgaris*. It was cultivated under open type cement pond systems to produce biomass. The objective of this study was to evaluate the growth of green microalga *C. vulgaris* on low cost artificial medium. The medium was prepared with rice fertilizer, rice bran, fish meal, lime and urea. This medium was named as, Rameshprabu medium. In this research, we investigated the growth, biomass production and biochemical composition of *C. vulgaris* using batch culture. The best biomass in terms of high total carbohydrates, protein and lipid production was obtained through using Rameshprabu medium. Furthermore, algal growth removed nitrogen, phosphorus, and chemical oxygen demand (COD) from the medium.**

Index Terms— **Microalgae, Chlorella vulgaris, Cultivation, Biomass production.**

I. Introduction

The global demand for biomass for food, feed, biofuels, and chemical production is expected to increase in the coming decades. Microalgae are a promising new source of biomass that may complement agricultural crops [1] – [4]. Biofuels productions from microalgae received wide attention recently and have high potential to replace fossil fuels. Although there is much excitement about the potential of algae biofuels such as bioethanol, biodiesel and biogas, much work is still required in the field [5]. The production of biogas via anaerobic digestion (AD) is the most feasible and cost-effective route to an energy product [6]. From an environmental and resource-efficiency perspective biogas has several advantages in comparison to other biofuels.

Biogas is composed of 40-70% methane (CH_4), 20-30% carbon dioxide (CO_2), 100-3000ppmv hydrogen sulfide (H_2S) and water, other trace gas compounds and other impurities. Since the main components of biogas are CH_4, CO_2 and H_2S. To utilize biogas as a transport fuel, CO_2 and H_2S must be removed from the concentration to leave biomethane. Biogas purification is the process where any impurities are removed such as sulphides and ammonia. Biological processes are widely employed for CO_2 and H_2S removal, especially in biogas applications [7]. According to Ramaraj et al. [8] confirmed that microalgae is the best candidate to uptake CO_2 efficiently. In addition, biological methods of CO_2 capture from biogas are potentially useful [9].

Since algae are easy to grow and cultivate anywhere with less energy requirements and using very few of the nutrients. It can be cultivated all year round under autotrophic, mixotrophic or heterotrophic conditions. Mixotrophic and heterotrophic cultures have a place as alternative modes of producing algae biomass. The ideal growth conditions for microalgal cultures are strain specific and the biomass productivity depends upon many factors [5]. These include abiotic factors for example temperature, minerals, CO_2, pH, water quality, light cycle and intensity; biotic factors include cell fragility and cell density. Mechanical factors include continuous mixing, gas bubble size and distribution and mass transfer, all these are of particular concern in photo-bioreactors [5], [6]. Light and temperature are the two most important factors that affect algae biomass productivity. The energy for growing algae is provided by light via photosynthesis. Sufficient light energy must be effectively utilized to achieve higher biomass productivity.

Algae cultivation also depends on pH levels and optimum pH influences the carbon availability, metabolism and biochemical composition of cells [7], [8]. For efficient use of algae as a source of bioenergy, it is very important to focus on the native algal species and to select that algal species which not only has a high growth rate but has greater lipid content. Identification of local algal species, optimization of conditions for native algal species was preferable for further studies. Hence, present study is a significant step forward in utilization and cultivation technique applied as an algae as a source of renewable energy and biological process of biogas purification.

The coccoid green microalgae genus *Chlorella* is one of the most important commercial microalgae [10]. *Chlorella* (Chlorophyta, Trebouxiophyceae), is commercially cultivated by many countries in the world. The annual production of *Chlorella* biomass exceeds 2,000 tonnes, mostly used for dietary supplements and nutraceuticals, with a minor share destined to the cosmetic market and aquaculture. *C. vulgaris* is a robust and fast growing microalgae species commonly cultivated and interesting regarding the production of secondary metabolites with health beneficial properties. The main purpose in this topic is

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to minimize the production costs as low possible. That is main reason in this study open type cement ponds were employed. In this paper, we aim to cultivate the isolated and an identified strain *C. vulgaris* for further establishment of biogas purification.

II. Materials and Methods

A. Isolation and Identification of Microalgae

The methodology of microalgae collection, isolation and identification process were adopted our previous published papers [2], [3], [5], [6]. The sample was collected by plankton net (20-µm pore size) from freshwater fish pond (18° 55′ 4.2″N; 99° 0′ 41.1″E) at a location near Maejo University, Sansai, Thailand. The collected samples were samples of about 5 ml were inoculated into 5-ml autoclaved Bold Basal Medium (BBM) in 20-ml test tubes and cultured at room temperature (30±1°C) under 50 μmol^{-1} m^2 sec^{-1} intensity with 16:8 h photoperiod for 10 days. After incubation, individual colonies were picked and transferred to the same media for purification in 250 mL conical flask.

The culture broth was shaken manually for five to six times a day. The pre-cultured samples were streaked on BBM medium-enriched agar plates and cultured for another 10 days with cool white fluorescent light using the same light intensity. The single colonies on agar were picked up and cultured in liquid BBM medium, and the streaking and inoculation procedure was repeated until pure cultures were obtained. The purity of the culture was monitored by regular observation under microscope. The isolated microalgae were identified microscopically using light microscope with standard manual for algae [11], [12].

B. Maintenance of microalgae cultures

Isolated and purified microalgae were inoculated in 250-ml Erlenmeyer flasks containing 125 ml BBM medium. Flasks were placed on a reciprocating shaker at 120 rpm for 7 d at room temperature of 30±1°C. Light was provided by cool white fluorescent lamps at an intensity of 50 μmol^{-1} m^2 sec^{-1}. The algae culture was then transferred to 500-ml Erlenmeyer flasks containing 450 ml. Algae growth were monitored by measuring the optical density of the algal medium with spectrophotometer (Spectronic Genesys 20, Thermo Fisher Scientific) at a wavelength of 665 nm. Measurements were taken daily and three replicates were measured. .

C. Production of microalgae

Figure 1 showed the open type algal cement pond and detailed descriptions through schematic diagram. Two litters' cultured microalgae were transferred to open type cement pond (total volume 200L) for large amount of biomass production through artificial medium. The medium was prepared with rice fertilizer (100g), rice bran (400g), fish meal (100g), lime (50g) and urea (200g). This medium was named as, Rameshprabu medium. Algal cement pond height was 40 cm and length was 80 cm. Furthermore, pond was filled 150 liters water and medium. It was reached 25 cm height in the pond. All ingredients were filled with 10L water subsequently mixed by stir then transfer to the pond; it associated with air pump. Pond was left for one night to release ammonia and medium dissolve in the water properly. Next day stock algae were transfer to the triplicate cement ponds. Algal growth was measured and stirred the everyday to prevent algae precipitation.

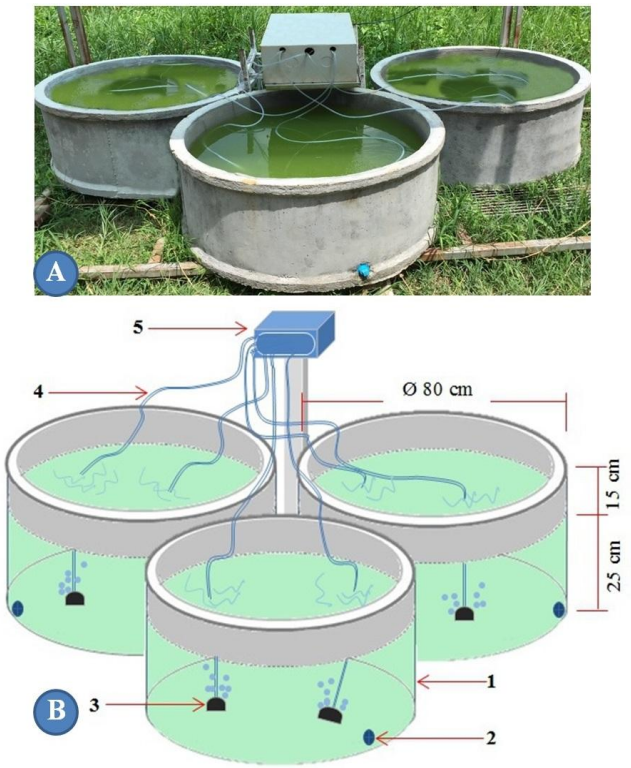

Figure 1 – (A) Open type algal cement pond; (B) - Schematic diagram of algal cement pond: 1. algae growth, 2. outlet of pond, 3. aeration bubble, 4. tube, 5. air pump.

D. Analytical Methods

All the indices including cell count, optical density (OD), pH, alkalinity, chemical oxygen demand (COD), biological oxygen demand (BOD), total nitrogen (TN), total phosphorous (TP), Total suspended solids (TSS), volatile suspended solids (VSS) and fixed suspended solids (FSS) were continuously monitored throughout the study, following the standard protocols of APHA [11]. Fatty acid content was performed by GC-MS analysis. Chlorophylls, protein, carbohydrates, lipids determination procedures were adopted from Tipnee et al. [13]. Elemental composition (C, H, N, O, S) was analyzed using the element analyzer (Perkin–Elmer 2004). Moisture content of raw materials was determined following the procedure given in ASTM Standard D 4442-07. The residual sample in the crucible was heated without lid in a muffle furnace at 700 ± 50 °C for one half hour. The crucible was then taken out, cooled first in air, then in desiccators and weighed. Heating, cooling and weighing was repeated, till a constant weight obtained.

E. Statistical analyses

All experiments were determined in biological triplicate to ensure the reproducibility. Experimental results were obtained as the mean value ± SD. Statistical analyses were performed using the SPSS statistical package (SPSS Inc., Chicago, IL, USA). The statistical significances were achieved when $p<0.05$.

III. Results and Discussion

Species Identification

For many years, strains of *Chlorella* (Chlorophyceae, Chlorococcales) have served as model organisms in plant physiology and biochemical research. Currently available information was positively identifying an environmental strain of the Chlorella genus of freshwater unicellular green algae. The genus Chlorella encompasses spherical or ellipsoidal non-motile green cells that produce autospores, and inhabit freshwater, soil and marine habitats. Its commercial potential has been considered since 1960, being the first microalga to be mass cultured for food, feed and as a source of nutraceuticals. More recently, it has also been suggested that they are good candidates for fuel production and biogas purification [7] , [14].

Since the characteristics traditionally used for taxonomy are sufficient and morphological criteria for the identification of *Chlorella* species. According to taxonomical grouping based on morphology and physiological properties, it belongs to genus Chlorella, family Oocystaceae, order Chlorococcales, class Chlorophyceae, division Chlorophyta of the kingdom Plantae. *C. vulgaris* is a spherical microscopic cell with 2-10 µm diameter and has many structural elements similar to plants [15]. The individual cells of the colonies were in the range of 10 µm. Cells are green color, unicellular, spherical in shape; figure 2 shows the morphology of *C. vulgaris* observed under a light microscope. The cell wall contains hemicelluloses, which accounts for the stability and rigidity of the cells. It has an asexual reproductive cycle, with the production of autospores from the mature large cell, by dividing the cell into smaller units. One mature cell divides into four new ones every 16-20 hours. The algal cells utilize sunlight for photosynthesis. The photosynthetic rate exceeds the respiration rate of Chlorella cells by 10–100 times.

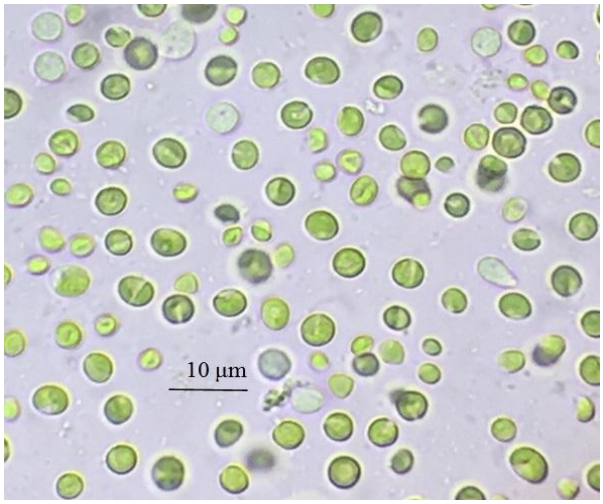

Figure 2 – *Chlorella vulgaris*.

Determination of algae growth and biomass production

Algae growth in this study would be presented by optical density (OD 685) condition and cell densities were determined through cell counts under optical microscope using an improved bright lined haemocytometer. Optical density and cell count clearly indicated that the best growth of *C. vulgaris* was through Rameshprabu medium using open type cement ponds shown in figure 3 and 4. Their growth increased rapidly in the first six days, then slowed down in the next six days, and again from thirteen days growing up, which could be due to the gradual consumption of certain nutrient elements like nitrogen and phosphorus in the medium. *C. vulgaris* have been widely used in numerous field applications for their strong survival abilities and efficient utilization of nitrogen and phosphorus [1]–[4].

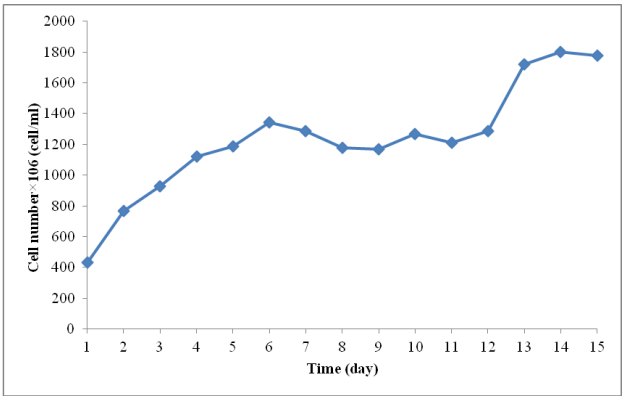

Figure 3 – *C. vulgaris* growth determined through cell count.

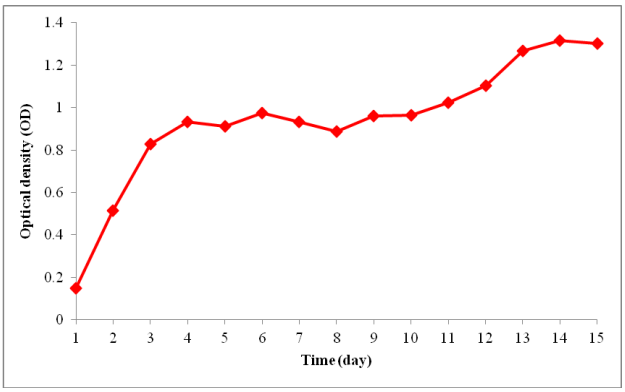

Figure 4 – *C. vulgaris* growth estimated through cell densities.

While CO_2 is available abundantly in the atmosphere as well as from anthropogenic sources, the availability of light is very important for the algae growth [16]. It is known that environmental factors such as light intensity, temperature and nutrients can significantly affect the composition of microalgae [17]. Furthermore, Ramaraj et al. [18] demonstrated that algae's ability to uptake CO_2 from the atmosphere and much greater biomass productivity compared to land plants. This study also proved that the microalga *C. vulgaris* could grow well in open type cement pond utilization atmospheric CO_2 and might be a good candidate microalga for the biogas purification if could apply in the closed system utilizing biogas as a carbon source. Algal biomass by direct measurement of total suspended solids and volatile suspended solids resulted as 474.12±0.15 mg/L and 396.25±0.03 mg/L respectively.

Chlorella vulgaris cultural conditions

The values of measured physicochemical and biological

parameters in the microalgal growth conditions of this study are summarized in table 1 and figure 5. The most important parameters are nutrient quantity/quality, light, temperature and pH [8], [18]. Growth system was setup in the open pond system for utilize solar energy directly. This study was aimed to find a strategy to reduce the costs and environmental impacts of Chlorella biomass production under autotrophic conditions used open type cement ponds. Ramaraj et al. [19] confirmed that photoautotrophic microalgae require several things to grow. Because they are photosynthetic, they need a light source, CO_2 as energy and carbon sources, water, and inorganic salts [19].

The biomass produced absorption of atmospheric CO_2, possibly driven by a decline in the CO_2 partial pressure resulted from photosynthesis. Carbon, phosphorus and nitrogen are considered to be the most important nutrients for algae growth; the results are shown in Table 1. COD measured the nutrition carbon on natural water medium and reactor effluent; the medium was 998±1.16 mg/L, while the reactor was 802.5±0.71 mg/L. After algae growth, the concentration of COD increased significantly. In the medium, nitrogen was 105.5±0.22 mg/L, while in it ponds were 50.59±70.71 mg/L. For alkalinity, the content was reduced medium to ponds. Alkalinity was an important buffering to maintain a fairly optimal growth range in the water body and the changes were consumed by algae growth from its role as one of possible carbon sources. Chlorophyll a production by phytoplankton cells is known to vary with growth conditions; in our experiments, the maximum production of chlorophyll a content of *C. vulgaris* was 28.89 ± 1.33 (mg/g).

Table 1– Physiochemical parameter

Parameters	Rameshprabu medium		Alga cement pond	
	Mean (± SD)	Range	Mean (± SD)	Range
pH	7.53 (±0.15)	7.4-8.2	8.38 (±0.22)	8.2-8.6
Temperature (°C)	-	-	32.56 (±0.78)	31.4-33.7
Light intensity ($\mu mol^{-1}m^{-2}$)	-	-	32.56 (±0.11)	31.4-33.7
DO (mg/L)	8.5 (±1.25)	7-10	13.5 (±0.05)	10-17
TN (mg/L)	105.5 (±0.22)	94-117	50.59 (±70.71)	24.6-84.6
TP (mg/L)	42 (±0.43)	36-48	31.9 (±0.35)	20.7-42.5
Alkalinity ($mgCaCO_3/L$)	29.03 (±1.52)	25-33	9.51 (±2.5)	1.5-20.7
COD (mg/L)	998 (±1.16)	1006-1013	802.5 (±0.71)	560-987
BOD (mg/L)	600 (±2.01)	615-630	427 (±0.66)	190-640
TSS (mg/L)	-	-	474.12 (±0.15)	319-737
VSS (mg/L)	-	-	396.25 (±0.03)	266-537
FSS (mg/L)	-	-	77.63 (±0.02)	60-199

Chlorella vulgaris chemical compositions

Photoautotrophic microalgae can effectively transform the inorganic nutrients, CO_2, H_2O and other substances into organic compounds such as protein, carbohydrate, lipid and other ingredients through photosynthesis. Microalgal biomass, containing lipids, starch, cellulose, proteins, and so on, is considered a promising feedstock for producing a variety of renewable fuels, such as biodiesel, bioethanol, biohydrogen and biogas [1]–[7]. The proximate and ultimate analysis of *C. vulgaris* illustrated in table 2. The major fatty acid composition was determined using GC- MS system (table 3). Green algae have the bulk of their fatty acids as saturated and unsaturated C18 s, a composition similar to that of vegetable oils [19], [20]. In this study, *C. vulgaris*, palmitic acid (C16:0), physetoleic acid (C16:1), and oleic acid (C18:1) were commonly dominant. In particular, lipids with high content of unsaturated fatty acids had been reasonable balance of fuel properties.

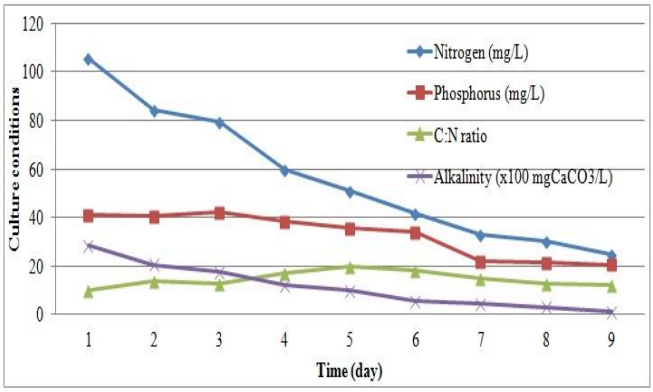

Figure 5 – Culture conditions of *Chlorella vulgaris*.

Table 2 – The proximate and ultimate analysis of *C. vulgaris*

Parameter		*C. vulgaris*
Proximate Analysis (%)	Moisture	9.87
	Ash	14. 87
	Carbohydrate	29.85
	Protein	48.88
	Lipid	13.60
	Fiber	17.06
Ultimate Analysis (%)	Carbon	48.56
	Hydrogen	6.40
	Oxygen	33.71
	Nitrogen	6.26
	Sulphur	0.79

Table 3 – Fatty acids analysis of *C. vulgaris*

Fatty acids	Value
C14:0	3.0± 0.2
C16:0	36.2± 1.5
C16:1	1.8± 0.1
C16:2	1.1± 0.2
C16:3	1.3± 0.1
C18:0	5.46± 0.3
C18:1	18.33± 1.2
C18:2	16.7± 0.6
C18:3	19.8± 0.7
MUFA [a]	20.1± 1.2
PUFA [b]	36.4± 2.2
UFA [c]	57.2± 2.7
DUS [d]	1.16 ± 0.03

Note: [a] percentage of total fatty acids (%); [b] MUFA= monounsaturated fatty acids, [c] PUFA= polyunsaturated fatty

acids, [d] UFA= unsaturated fatty acid, [e] DUS = degree of fatty acid unsaturation.

Biological purification processes for biogas

The main components of biogas are CH_4 and CO_2, but usually biogas also contains H_2S other sulphur compounds, water, other trace gas compounds and other impurities. Purification and upgrading of the gas is necessary because purified biogas provides reductions in green house gas emissions as well as several other environmental benefits when used as a vehicle fuel [7]. Reducing CO_2 and H_2S content will significantly improve the quality of biogas. For biogas impurity removal, biological processes are environmentally friendly and feasible. Biogas purification using algae involved the use of algae's photosynthetic ability in the removal of the impurities present in biogas.

Ramaraj and Dussadee [7] stated that algae application of CO_2 sequestration has developed as a popular topic and the current interests are including: species, power plant flue gas utilization, reactor design, growth condition, growth kinetics and modeling. The most studies in the literature concerned the maximum CO_2 uptake rate by the artificial photo-bioreactors [20]. Among those techs, bio-eco-technology is the most natural and ecological way to accomplish the designed targets by the utilization of "self-designed" bio-functions of nature [8], [18], [19]. Further our establishment study, *C. vulgaris* applicable for biogas purification.

IV. CONCLUSIONS

The cultivation of the microalga, *C. vulgaris* utilized low cost artificial medium which named as Rameshprabu medium. The medium was prepared through rice fertilizer, rice bran, fish meal, lime and urea. This medium was named as, Rameshprabu medium. The aim of the current work was to evaluate the potential of the green alga *C. vulgaris* as a cheap renewable energy source in term of biofuels. The algae grew faster, providing higher productivities of biomass, lipids, carbohydrates and proteins. Furthermore, *C. vulgaris* biomass production and carbohydrate consumption were enhanced by supplementing the inorganic culture. Photoautotrophic conditions cultivation of *C. vulgaris* can be considered as a feasible strategy to reduce the costs of microalgal biomass production, while also contributing to solve the environmental problem.

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List of publications:
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