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Research Article

Culture of Macroalgae *Spirogyra ellipsospora* for Long-Term Experiments, Stock Maintenance and Biogas Production

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Abstract

The freshwater alga *Spirogyra ellipsospora*, a filamentous charophyte, collected from the stream, was identified on the basis of morpho-anatomical characters. In this study, we tried to utilize the natural water resource to develop the algae growth system by ecological engineering concept to develop a low cost medium for macroalgae growth. The outdoor photo-reactor was used to grow macroalgae through using natural water as medium. The results showed that the reactor had good performance on algae growth. Culture media for growth of this study species have not yet been tested for long-term experiments, stock maintenance and biogas production. Here we tested the *S. ellipsospora* growth with natural water medium in a 6-weeks laboratory experiment. Consequently, the study consists of laboratory tests showing *S. ellipsospora* growth, harvesting, chlorophyll extraction, biomass analysis and anaerobic fermentation for biogas production.

Keywords Bio-methane, Biogas, Macroalgae, Stream, *Spirogyra* culture

Introduction

Algae are the dominant primary producers in aquatic ecosystems. Since algae are highly varied group organisms, which have important functions in ecosystem; they are widely distributed around the world and closely connected with human life (Ramaraj et al. 2015a; 2015b). Furthermore algae biomass is an essential biological resource. Algal biomass has been recently investigated as a possible and complementary alternative to lignocellulosic substrates to produce biofuels/biotechnological products, due to several advantages, such as (1) a higher productivity yields, (2) they do not require arable lands for growth and therefore do not outcompete food resources, (3) they can grow in a variety of environments including fresh water, salt water and municipal wastewaters, (4) many species of algae can be induced to produce particularly high concentrations of chosen compounds – proteins, carbohydrates, lipids and pigments - that are of commercial value, and (5) the ability to produce non-toxic and biodegradable biofuels (Ramaraj 2013; Ramaraj et al. 2014a; 2014b).

Recently, macroalgae are receiving a considerable attention due to their ability to synthesize valuable compounds, accumulate high energy compounds and sequester carbon (Lawton et al. 2013). They are therefore considered as a third generation feedstock for biofuel production and have a great potential as renewable feedstock (Hughes et al. 2012). The genus *Spirogyra* has recently drawn attention to researchers due to its various biotechnological and industrial applications. *Spirogyra*, one of the commonest green filamentous freshwater macroalgae, is named because of the helical or spiral arrangement of the chloroplasts (Krupek et al. 2014). There are more than 400 species of *Spirogyra* in the world. This genus is

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photosynthetic, with long bright grass-green filaments having spiral-shaped chloroplasts. It is bright green in the spring, when it is most abundant, but deteriorates to yellow. In nature, *Spirogyra* grows in running streams of cool fresh water, and secretes a coating of mucous that makes it feel slippery. This freshwater alga is found in shallow ponds, ditches amongst vegetation at the edges of large lakes, small stagnant water bodies, rivers, and streams. Under favorable conditions, Spirogyra forms dense mats that float on or just beneath the surface of the water. Blooms cause a grassy odour and clog filters, especially at water treatment facilities.

Spirogyra sp. contains about 11–21% of lipids and a high content of sugar, about 33–64% (Becker 2007). *Spirogyra* sp. contains Chlorophyll-a *and* Chlorophyll-b which are responsible for its green color. However, in some culture/stress conditions the macroalga appears yellow or orange due to the presence of secondary pigments (carotenoids). *Spirogyra* sp. are promising source of novel biochemically active compounds like fatty acids, steroids, carotenoids, polysaccharides, lectins, vitamins and phyco-proteins, amino acids, dietary minerals, halogenated compounds, polyketides, diverse antioxidants, antibiotic, antiviral, anti-inflammatory and other positive biological activities (Kumar et al. 2015). The high productivity of the macroalga *Spirogyra* and its capacity to accumulate high amounts of sugar, make this biomass also attractive as substrate for bioenergy production. Biofuel is a renewable energy, which may be instead of the fossil fuel resources in the future with decreasing of the fossil fuel on a daily basis (Unpaprom et al. 2015). The application of anaerobic digestion (AD) technology is growing worldwide because of its economic and environmental benefits (Dussadee et al. 2014). As a consequence, a number of studies and research activities dealing with the determination of the biogas potential of solid organic substrates have been carrying out in the recent years.

The biogas production by this macroalga is still in development and so far, there are no references in the literature related to biomethane gas production from *Spirogyra* using natural water medium. Consequently, this study was to examine the *Spirogyra ellipsospora* growth conditions, biomass and biogas potential through natural water medium also long-term experiments, stock maintenance.

Methodology

Algal sample collection, cultivation and experiment setup

The freshwater macroalgae, *Spirogyra* was collected from the slow running fresh water stream at Tumbon Pang Yang (19° 18'42.41" N; 98° 48'44.11" and elevation 722 m), Mae Taeng district, Chiang Mai province, Thailand and transport to the Energy Research Laboratory at Maejo University, Chiang Mai, Thailand. The methodology was illustrated in (Figure 1). This investigation is to simulate the ecosystem in natural water body with macroalgae growth ecological engineering concepts. For the macroalgae cultivation, the nearby water was screened by 1x1 mm sieve (mesh No. 18) to remove macro particles.

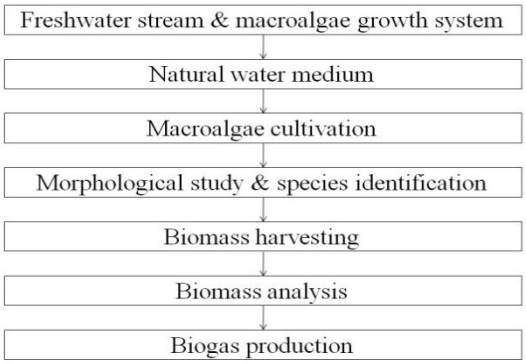

Figure 1. A flow chart of study methodology

According to Ramaraj et al. (2015a; 2015b) the stream water was used as medium. Water collected from the same sampling zone afore mentioned and the water was filtrated by 0.45 µm filter paper as feed. *Spirogyra* Sp. were grown in autotrophic conditions of 10 L open type outdoor jar. The jar containing 5 L working volume and 5 L base filled with sterilized white sand and growth system was demonstrated in Figure 2.

Identification of alga

The algal samples were observed under light microscope and were then visualized with a Nikon Eclipse 80i microscope and photographs were taken with attached digital camera.

Figure 2. Macroalgae growth system

Relevant publication of Prescott (1951) was referred for the identification of algal taxa and taxonomically determined with the help of authentic literature (Randhawa 1959; Transeau 1951; Vidyavati 1995; Kargupta and Jh 2004, Taft 2009). For the taxonomic description of taxa, dimensions were given in micrometer (µm). The measuring scales given for algae photographs were equal to 20 µm. The morphological characters including length, width, number of spiral chloroplasts, and number of granules were recorded for species confirmation.

Chlorophylls estimation

Ten ml of sample was taken and centrifuged at 6000 rpm for 15 minutes. Supernatants were discarded and re-suspended in a known volume of methanol, while pellets extracted with 5 ml of 96% methanol extraction. The tubes were wraped with aluminum foil and kept in dark. The samples were centrifuged again and the supernatants were used for measuring the optical density at 663 nm and 645 nm against 96% methanol as a blank by spectrophotometer (Spectronic Genesys 20, Thermo Fisher Scientific). After extraction chlorophyll concentration was determined spectrophotometrically and calculated Chlorophyll content (Chlorophyll *a*, chlorophyll *b* and total chlorophyll) were computed using the following equations:

Chlorophyll-a (µg/ml) = {(15.65xA_{666} – 7.340xA_{653}) x V/ 50 x W} x dilution

Chlorophyll-b (µg/ml) ={(27.05xA_{653} – 11.21xA_{666}) x V/ 50 x W} x dilution

Total chlorophyll = chlorophyll-a + chlorophyll-b

Inoculum

Anaerobic sludge was obtained from a leachate recirculation digester (Napier grass biogas fermenter), located in the Energy Research Center at Maejo University, Chiang Mai, Thailand, was used as inoculum in all biodegradability assays.

Anaerobic digestion batch tests

The anaerobic assays were conducted in 500 mL bottles (triplicate reactor) containing 40 mL of inoculum and 200 g of fresh *S. ellipsospora and* remaining make up with double distilled water. The total working volume is 400 mL. The biochemical methane potential (BMP) assay was used to determine the methane productivity of *S. ellipsospora*. The bottles were closed with a septum and flushed with N_2 to remove oxygen. Triplicate, 500 mL fermenters were incubated in the room temperature. Production of biogas was monitored by measuring the overpressure in the bottle headspace at time intervals depending on the production of biogas.

Analytical methods

Table 1 Methods employed for determination of physico-chemical parameters. The solids contents, including total solids (TS) and volatile solids (VS), chemical oxygen demand (COD) were characterized using the Standard Methods for the Examination of Water and Wastewater (method # 2540) (APHA-AWWA-WEF, 2005). Metrohm 774 pH-meter was used in all pH measurements. The entire experiments were done in triplicate. Biogas estimation method was adopted from literature (Pavlostathis and Giraldo-Gomez 1991; von Sperling and Chernicharo 2009).

Statistical analysis

All analytical results were conducted at least in triplicate. Values of different parameters were expressed as the mean ± standard deviation. The standard deviations were analyzed by using Microsoft Excel 2003 for Windows.

Results and Discussion

Morphological study of Spirogyra

Spirogyra is a genus of filamentous green algae in the order Zygnematales. The name indicates the helical or spiral arrangement of the chloroplasts, which is the main diagnostic characteristic of the genus. The *Spirogyra* species typically develops unbranched filaments and is one cell thick, which grows longer through normal cell division. There are more than 400 species of *Spirogyra* in the world. Vegetative growth of *Spirogyra* can be recognized by three characteristics: (1) type of cross walls (plane, replicate, semi-replicate or colligate), (2) cell length and width and (3) chloroplast numbers.

Table 1. Physicochemical parameters

Parameter	Method	Reference
TS	Method 2540 C	APHA-AWWA-WEF, 2005
VS	Method 2540 E	
COD	Method 5220	
Chlorophylls	spectrophotometric method	-
pH	Metrohm 774 pH-meter	-
Biomethane estimation	via COD	Pavlostathis and Giraldo-Gomez, 1991; Ramaraj et al. 2014
Percentage of CH_4, CO_2 and H_2S	BMP analysis	

There are classical and standard morphological methods that were used in the identification of the *Spirogyra* specimens with help of specific literatures (Randhawa 1959; Transeau 1951; Vidyavati 1995; Kargupta and Jh 2004; Taft 2009). The morphological characteristics of each sample were recorded via cell dimensions, along with the number and arrangement of chloroplast spirals/pyrenoids. The morphological characteristics of biological parameters were also studied and presented in Table 2. The classical morphologically based methods are used for the identification of *Spirogyra* specimens. The structure of species from this study demonstrated definitive identity matches in the range of 99% for the agreement of *S. ellipsospora*. Light microscopic pictures of *Spirogyra ellipsospora* is presented in Figure 3.

S. ellipsospora growth and biomass measurement

Biomass was a critical measurement in the algal harvesting process for applications. A number of methods had been developed to estimate and quantify, which were useful in different cases (Ramaraj 2013; Ramaraj et al. 2015c).

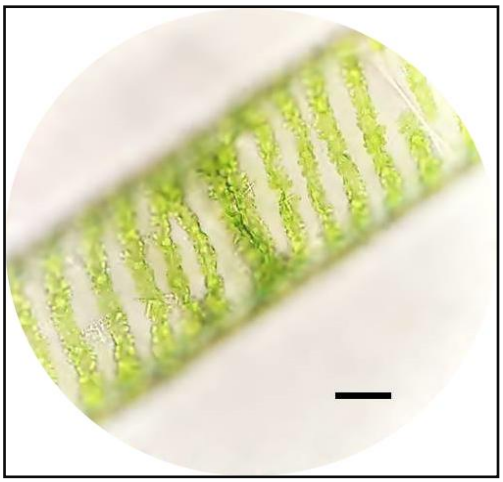

Figure 3. Light microscopic pictures of macroalgae *Spirogyra ellipsospora*

Different methods were available such as dry weight: Total suspended solids, volatile suspended solids and fixed suspended solids; wet weight method; chlorophyll (Chl) method: Chl-a, Chl-b and Chl-a+b), epifluorescence microscopy, bioluminescence, photometric, turbidity, packed cell volume and cell count etc (Unpaprom et al. 2015). According to Ramaraj et al. (2013), algal biomass measurement and roughly we could classify into two groups, (1) direct index such as dry weight and (2) indirect index such as chlorophyll, so-called proxy index.

Chlorophyll is the most widely used proxy measurement of algae or phytoplankton and their determination is relatively simple and straightforward. In this study, we used chlorophylls measurement to analysis biomass. Chl-a as an algal biomass measurements in natural systems was very popular. Chl-b is used to calculate pigment concentrations. The total Chl-(a + b) is used to measure algal growth (Ramaraj et al. 2010). Growth system was setup outdoor conditions. Algae

biomass measured by Chl-a, Chl-b and total chlorophyll results were average as 9.36 μgmL^{-1}, 3.88 μgmL^{-1} and 13.24 μgmL^{-1}, respectively. Accordingly, this study presents results to produce algae biomass using the natural water and result was encouraging.

Table 2. Morphological characteristic of *S. ellipsospora*

Type of parameters	Parameters	Equipments and methods	Charac teristics
Biological	Width of cell	Light microscope	120–150
	Length of cell		90–280
	Chloroplasts per cell		5–8
	Vegetative cell width (μm)		35–85
	Vegetative cell length(μm)		80–190
	L/W ratio vegetative cell		2.0–3.4
	Number of chloroplasts		2–5
	Shape of zoospore		Ellipsoid
	Zoospore width		60–73
	Zoospore length		75–95

The potential of natural water medium for S. ellipsospora long-term experiments and stock maintenance

The utilization of natural water medium which came from water body directly without any extra nutrition addition, demonstrated the potential to adopt the algal function for natural growth and long time surveillance. The study confirmed that macroalgae can get essential nutrition from natural water body (natural water medium). Utilizing this growth uptake function we could apply the natural medium in controlled environments such as lab (outdoor lab scale) or field scale growth units or even further applied in natural environment, but nowadays most of researchers and algae manufactures are using artificial medium which is expensive to produce algal biomass. Our study could take advantage of nutrients available in natural water to reduce the total cost, long-term experiments and stock maintenance.

The productivity of macroalgae cultured for 6 cycles of 6 days using with outdoor lab environment to imitate the natural system. The culture that is continuously provided natural water medium each of cycle ends. Macroalgae were placed in 10 L cylindrical tanks in an outdoor system to be cultured for 36 days. Biomass was initially stocked at 2 g/L fresh weight (fw) for *S. ellipsospora*. The algae were cultivated in a batch culture system, described in detail previously (in methodology part). Biomass was harvested every 6 days (6 cycles of 6 days each in total) using a net , spun to a constant fresh weight, weighed and subsequently re-stocked at initial stocking densities for a new cycle. Stock maintenance, long time experiment and growth of *S. ellipsospora* are an essential for its subsequent use in biotechnology. For this purpose, we tested the suitability of the stock maintenance and growth of algal species is essential for their use in biotechnology. Therefore, natural water medium is the most suitable culture media and ease of laboratory culture is relevant topics. This environmental friendly process offers a substantial potential source of algae biomass to provide bioenergy and to reduce the greenhouse gas, carbon dioxide.

Theoretical analysis of S. ellipsospora biogas production and biochemical methane potential

The well known use of the microbiological process of anaerobic digestion (AD) to generate biogas (mixture of methane and carbon dioxide) is now widely implemented for the production of renewable energy worldwide; bio-methane potential (BMP) tests are commonly used in studies concerning the AD of organic solids (Ramaraj et al. 2015c). Macroalgae can be converted to biofuels by various processes including thermal processes and fermentation. The most direct route to obtaining biofuel from macroalgae is via AD to biogas (Hughes et al. 2012; Montingelli et al. 2015). Algal biomass contains considerable amount of biodegradable components such as carbohydrates, lipids and proteins. This makes it a favorable substrate for anaerobic microbial flora and can be converted into methane rich biogas (Sialve et al. 2009). In spite of the fact that macroalgae have high potential for biogas production, there are some studies on anaerobic digestion of macroalgal biomass utilizing *Chaetomorpha linum*, *Saccharina latissima*, *Gracillaria vermiculophylla* and *Ulva lactuca* biomass.

Apparently, the BMP of algae depends mainly on its composition, which itself depends on

the growth conditions and and is specific species. The concentration of substrate in the BMP assay also impacts on the final biodegradability and methane productivity (Sialve et al. 2009). When the C, H, O and N composition of a wastewater or substrate is known, the stoichiometric relationship repo rted by Buswell and Boruff (1932) and Angelidaki and Sanders (2004), and can be used to estimate the theoretical gas composition on a percentage molar basis.

Elements	Percent	Mol[a]	CH_4[b]	CO_2	NH_3	Biogas (L/kg)
C	41.87	3.49				
H	6.00	6.00				
O	35.77	2.24	48.00 (%)	43.96 (%)	8.04 (%)	910.10
N	4.27	0.31				
S	0.43					

Table 3. Composition of methane and biogas production from *S. ellipsospora*

In this equation, the organic matter is stoichiometrically converted to methane, carbon dioxide and ammonia. The specific methane yield expressed in liters of CH_4 per gram of volatile solids (VS) can thus be calculated as:

$$C_aH_bO_cN_d + \left(\frac{4a\text{-}b\text{-}2c+3d}{4}\right) H_2O \rightarrow \left(\frac{4a+b\text{-}2c\text{-}3d}{8}\right) CH_4 + \left(\frac{4a\text{-}b+2c+3d}{8}\right) CO_2 + dNH_3 \quad \text{..... Eq. (1)}$$

Eq. (1) is a theoretical approach that allows estimation of the maximum potential yields. Using Eq. (1), it is possible to compute a theoretical specific methane yield. Composition of methane and biogas production from *S. ellipsospora* details were presented in Table 3 (by dry weight basis). The biogas composition of carbon dioxide (44%) and methane (48%) of was estimated from the biogas. The carbon, hydrogen, nitrogen, oxygen and sulphur contents of were in *S. ellipsospora*, 41.87%, 6%, 35.77%, 4.27% and 0.43 respectively. Consequently *S. ellipsospora* has plenty of nutrients for biogas production process; it is suitable to be used as energy crops for biogas production.

Laboratory analysis of S. ellipsospora biogas production and biochemical methane potential

Gross composition of several algae species were presented by Becker (2007). As expected, the species that can reach higher lipid content have a higher methane yield. COD is commonly used in the water and wastewater industry to measure the organic strength of liquid effluents. It is a chemical procedure using strong acid oxidation. The strength is expressed in 'oxygen equivalents' i.e. the mg O_2 required to oxidise the C to CO_2. However, the COD concept could be estimate the methane yield (Pavlostathis and Giraldogomez 1991; von Sperling and Oliveira 2009; Than et al. 2014). One mole of methane requires 2 moles of oxygen to oxidise it to CO_2 and water, so each gram of methane produced corresponds to the removal of 4 grams of COD.

$$\underset{16}{CH_4} + 2O_2 \rightarrow \underset{64}{CO_2} + 2H_2O \quad \text{..... Eq. 2}$$

Or

1kg COD is equivalent to 250g of methane.

1kg COD ⇒ 250g of CH_4

250g of CH_4 is equivalent to 250/16 moles of gas = 15.62 moles

1 mole of gas at STP = 22.4 liters

Therefore 15.62 x 22.4 = 349.8 liters = 0.35 m^3.

In our study, the sample content of total solids (TS) and volatile solids (VS) was measured; the results were average as 16622 mg/kg and 13959 mg/kg, respectively. The average pH was 7.4 and average COD 14236 mg/L. Methane formation takes place within a relatively narrow pH interval, from about 6.5 to 8.5 with an optimum interval between 7.0 and 8.0. The process is severely inhibited if the pH decreases below 6.0 or rises above 8.5. The pH value increases by ammonia accumulation during degradation of proteins, while the accumulation of VFA decreases the pH value. The accumulation of VFA will often not always result in a pH drop, due to the buffer capacity of the substrate (Mösche and Jördening 1999; Wang et al. 1999; Weiland 2010). According to the COD estimation, our study shows the mixed culture microalgal biomass is a potentially valuable fermentation substrate, and produce 1.9930 L (0.002 m^3) of methane gas.

In conclusions, production of biofuels is undoubtedly one of the best solutions for declining the crude oil reserves and global warming due to excessive greenhouse gasses emissions. As fossil fuel prices increase and environmental concerns gain prominence, the development of alternative

fuels from biomass has become more important. Biogas is considered a renewable energy carrier. As demonstrated here, macroalgal biogas is technically feasible. Macroalgae have several advantages over terrestrial plants such as higher photosynthetic efficiencies, lower need for cultivation area, higher growth rates, more continuous biomass production, no direct competition with food production, and possibility to use artificial medium, natural water medium (freshwater/marine water) and wastewater for biomass production. The algae biomass thus produced constitute an additional source of organic substrate in the installation for biogas production. The biogas production was 910.10 L/kg. Therefore, fast growing, high-yielding and rich in organic matter of *S. ellipsospora* was promising energy crops for biogas production. This suggested that it is possible to achieve stable operation using *S. ellipsospora*, as a substrate for biogas production in pilot or large scale biogas plant in the future.

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