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A newly isolated green alga, *Pediastrum duplex* Meyen, from Thailand with efficient hydrogen production

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Abstract: Biofuels are gaining attention worldwide as a way to reduce the dependence on fossil fuels. Biological Hydrogen (H_2) production is considered the most environmentally friendly route of producing H_2, fulfilling the goals of recycling renewable resources and producing clean energy. It has attracted global attention because of its potential to become an inexhaustible, low cost, renewable source of clean energy and appears as an alternative fuel. H_2 production processes offer a technique through which renewable energy sources like biomass can be utilized for the generation of the cleanest energy carrier for the use of mankind. This paper presents laboratory results of biological production of hydrogen by green alga was isolated from fresh water fish pond in Sansai, Chiang Mai province, Thailand. Under light microscope, this green alga was identified as belonging to the genus *Pediastrum* and species *P. duplex* Meyen. The successful culture was established and grown in poultry litter effluent medium (PLEM) under a light intensity of 37.5 $\mu mol^{-1}m^2$ sec^{-1} and a temperature of 25°C. The nutrient requirements and process conditions that encourage the growth of dense and healthy algal cultures were explored. The highest H_2 was produced when cultivated cells in PLEM for 21 hours under light and then incubated under anaerobic adaptation for 4 hours.

Keywords: Freshwater Algae, *Pediastrum Duplex* Meyen, Poultry Litter Effluent, Biohydrogen

1. Introduction

Today environmental pollution is a great concern to the world, mainly due to rapid industrialization and urbanization. And the utilization of fossil fuels is the main contributing factor to global climate change, mainly due to the emission of pollutant, especially carbon dioxide (CO_2) released into the atmosphere upon their combustion. Algae, the major biomass of living organisms in marine and freshwater [1], are the most important CO_2 fixer, the primary producer in aquatic ecosystem through the photosynthesis into biomass to fulfill the responsible duty of CO_2 [2, 3], and play a crucial niche of CO_2 bio-fixation of the ecosystem [3].

At present algae is broadly recognized as one of the best strategies of CO_2 fixation and biomass production [4]. There are several reasons for this approach: (i) the best growth rate among the plants, (ii) low impacts on world's food supply, (iii) specificity for CO_2 sequestration without gas separation to save over 70% of total cost, (iv) excellent treatment for combustion gas exhausted with NO_x and SO_x, (v) high value of algae biomass including of feed, food, nutrition, pharmaceutical chemicals, fertilizer, aquaculture, biofuel, etc [5–7].

The current boom in microalgae biotechnology has led to a further strong increase in the expectation that the production of biofuel from microalgae will be sustainable both energetically and financially. As efficient photosynthetic organisms, microalgae have unique advantages in capturing solar energy to generate reducing equivalents and converting atmospheric CO_2 to organic molecules [9]. Microalgae also have special advantages in ability to adapt to various stressful environments, non-requirement of agricultural land and so on [4]. Microalgae's high ability to use inorganic nutrients (nitrogen and phosphorous) from wastewater makes them a useful bioremediation tool in waste water treatment process. Many green microalgae species are commonly used in the wastewater treatment system due to their high tolerance to soluble organic compounds [10–12]. Algae can be successfully cultivated in wastewaters. It can be potentially a sustainable growth medium for the algal feed stock [12, 13].

Use of microalgae in treatment and recycling of waste water has attracted a great deal of interest because of excessive biomass generation at cheaper cost without extra input of nutrients such as inorganic fertilizer (chemical medium) [14]. The selection of carbon source is important for microalgae cultivation also. Microalgae can use inorganic carbon (CO_2) and organic carbon source (glucose, mannitol, acetate, sucrose). Cultivation of microalgae in swine wastes, dairy manure, and other animal residues has been reported by several authors [14–16].

More importantly, there were no bacteria and pathogens found on aerated manure when algae are grown. The algae culture carried out in different types of reactors, flasks and plastic bags are common practice with mineral medium as nutrient sources. Kumar et al. [17] stated digested piggery effluent could be an alternative nutrient source for mass algal production since anaerobically treated animal waste contains nutrients such as phosphorous, nitrogen species including, ammonium nitrogen, nitrate, nitrite and which are suitable for growing algae. In addition, pathogens in the effluent were eliminated by anaerobic digestion particular after two-stage (thermophilic and mesophilic) digestion [17]. Therefore, digested effluent as medium of algal culture could be an alternative to using costly mineral medium which is not environmentally sustainable.

In addition, microalgae grown under heterotrophic condition (using organic carbon) could have more potential to produce hydrogen than in autotrophic condition (inorganic carbon). In heterotrophic condition, high biomass is achieved. Moreover, in heterotrophic mode of cultivation organic wastes, as cheaper carbon sources can be used. Unlike autotrophic condition, light is also not required in heterotrophic cultivation [18]. Therefore, heterotrophic cultivation can be cheaper than autotrophic cultivation. One of the advantages of green algae for producing hydrogen is their ability to grow under photoautotrophic and photoheterotrophic condition. There is little information available for undertaking intensive algal production by using digested poultry effluent as a nutrient re-source. The present article reports simultaneous algae growth, biomass production and waste recycling with the green microalga *P. duplex* Meyen from the poultry litter waste water.

At present, the potential of microalgae as a source of renewable energy has received considerable interested in biofuel such as biodiesel, bioethanol, biogas, biohydrogen and bio-oils [17, 18]. Hydrogen gas (H_2) is a valuable energy carrier, an important feedstock to the chemical industry, and useful in detoxifying a wide range of water pollutants. As an energy carrier, it is especially attractive due to its potential to be used to power chemical fuel cells. It is considered an ideal energy carrier for the future. Compared to fossil fuels as traditional energy sources, hydrogen is a promising candidate as a clean energy carrier in the future because of its higher heating values 141.6 MJ kg^{-1}, or 12.6 MJ m^{-3} [8,9]. Heat and water are the only products of combustion of H_2 with no releasing of CO_2 into the atmosphere [19, 20]. Thus, hydrogen is a clean, renewable, and non-polluting fuel. Previous reports showed that photosynthetic microorganisms, *cyanobacteria* and *green algae*, can generate hydrogen from solar energy and water [17–20].

Green algae are act as the pioneer photosynthetic organism or producer in the world of ecosystem. The genus *Pediastrum* Mayen (Chlorophyceae) is a free floating, coenobial, green algae occurs commonly in natural freshwater lentic environments like ponds, lakes, reservoirs etc [21]. In this study, we examined a newly isolated species of green microalga *P. duplex* Meyen for biohydrogen production applied with inexpensive poultry litter effluent medium (PLEM).

2. Materials and Methods

2.1. Isolation and Identification of Microalgae

Microalgae were 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) [22] in 20-ml test tubes and cultured at room temperature (25 °C) under 37.5 $\mu mol^{-1}m^2$ 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 [23,24].

2.2. Inoculums Preparation

Isolated and purified microalgae (*P. duplex* Meyen) were inoculated in 250-ml Erlenmeyer flasks containing 125 ml culture medium (BBM). Flasks were placed on a reciprocating shaker at 120 rpm for 7 d at room temperature of 25±1 °C. Light was provided by cool white fluorescent lamps at an intensity of 37.5 $\mu mol^{-1}m^2$ sec^{-1}. The algae culture was then transferred to 500-ml Erlenmeyer flasks containing 450 ml.

2.3. Preparation of Poultry Litter Effluent Medium

The anaerobically digested poultry litter effluent (PLEM) raw was collected from the chicken farm at Mae Fak Mai, Chiang Mai province, Thailand (18°97'20.01"N; 98° 97'99.41"E) and transported to the Maejo University laboratories in 10 L plastic containers and stored in a cold room maintained at 4°C. Pre-treatment was carried out by sedimentation and filtration with a filter cloth to remove large, non-soluble particulate solids. After filtration the substrate

was autoclaved for 20 min at 121°C, after which the liquid was stored at 4 °C for 2 days for settling any visible particulate solids and the supernatant was used for microalgae growth studies.

2.4. Growth Conditions and Measurements

The isolated green alga was grown in 450 ml Erlenmeyer flasks each containing 450 ml of poultry litter effluent medium (PLEM) for the heterotrophic growth condition. It was cultivated at room temperature for 3 days under fluorescent light illumination for 18 hours per day with a shaking speed of 120 rpm. All experiments were carried out in triplicate. After obtaining the optimal growth medium, cultures were selected with an initial optical density of 0.01, *P. duplex* Meyen in PLEM under continuous light illumination at various light intensities and temperatures (same as before mentioned). Optical density at 730 nm was measured using a spectrophotometer (HACH, DR/4000U) every 3 days interval for 18 days.

The cell density and growth was determined by measuring the chlorophyll concentration; the algal culture was measured every 3 hours of cultivation by optical density measurement at wavelength 750 nm. The chlorophyll of cell suspension was extracted with 90% methanol and the total chlorophyll concentration was calculated by the method of Kosourov et al. [25].

2.5. Analytical Methods

All the indices including pH, chemical oxygen demand (COD), total nitrogen (TN), total phosphorous (TP) were continuously monitored throughout the study, following the standard protocols of APHA [20].

2.6. Measurement of H_2 Production

One hundred ml of cell culture was harvested by centrifugation at 5000xg for 10 min at 4°C. The cell pellet was washed with PLEM medium and the cell resuspension was transferred into a 10 ml glass vessel and sealed with a rubber stopper. The anaerobic adaptation was performed by purging Argon gas to the cell suspension for 5 min under dark condition and the cells were incubated at room temperature for 2h. Hydrogen was determined by analyzing gas phase by a gas chromatography (Agilent 6890 gas chromatography coupled to electron impact, EI, 70 eV with HP 5973 mass selective detector and a molecular sieve 5A 60/80 mesh packed column using a thermal conductivity detector). The injector and detector temperatures were kept at 100^{o}C whereas the oven temperature was maintained at 50^{o}C. Argon gas was used as a carrier gas during hydrogen analysis.

Hydrogen production was calculated as a term of hydrogen evolved per chlorophyll content per time (nmolH_2/µg chl/h). Hydrogen evolution of cells at 6, 12, 18, 24 and 36 hours of cultivation was measured under light and dark conditions. In addition, algae cells were harvested and incubated under anaerobic condition for 2, 4, 6, 8 and 24 hours in darkness before measuring hydrogen evolution.

2.7. Statistical Analysis

Data are reported as mean ± SE from triplicate observations. Significant differences between means were analyzed. All statistical analyses were performed using SPSS Version 20.0.

3. Results and Discussion

3.1. Species Identification

Pediastrum is green algae occur frequently in lentic environment like pond, puddles, lakes, mostly in warm and humid terai region [21]. The green alga isolated from a fresh water fish pond, was identified as *P. duplex* Meyen. Under light microscope, single cells, four cells surrounded by transparent sheath, and truncated transparent sheaths can be observed. Figure 1 shows the morphology of *P. duplex* Meyen observed under a light microscope.

Systematic classification:
Phylum: Chlorophyta
Subphylum: Tetraphytina
Class: Chlorophyceae
Order: Sphaeropleales
Family: Hydrodictyaceae
Genus: Pediastrum
Species: *Pediastrum duplex* Meyen

Colonies free floating, disc-shaped to stellate, flat, monostromatic with 4-8-16-32-64 or more polygonal cells, compact or perforate; cells coenocytic, smooth or rough walls, marginal cells with or without process and usually differently shaped than interior cells; chloroplast parietal, disc shaped, in later stages filling entire cell, with 1-4 pyrenoids; reproduction by formation of zoospores, aplanospores, isogametes and zygotes [21].

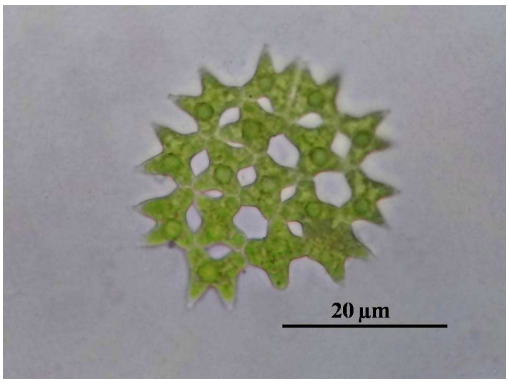

Figure 1. *Cell morphology of cells of P. duplex* Meyen *observed under a Light Microscope*

3.2. Algae Growth Medium, Biomass Production and Characterization

Algal growth was monitored by utilizing the optical properties of the culture to measure either its chlorophyll content / optical density. Growth of *P. duplex* Meyen was examined when grown in PLM medium, *P. duplex* Meyen showed a fast growth. PLM medium was identified as the most suitable for *P. duplex* Meyen since high cells density. The

nutritious characteristics of PLEM presented in Table 1. The PLEM had high concentrations of nitrogen (1890 mg L^{-1}), phosphorus (187 mg L^{-1}), potassium (11749 mg L^{-1}) and other micronutrients (Table 1), which were significantly higher than recommended for algae cultivation. Algae growth was presented in Figure 2. It was found that the cells stayed in the lag phase period for the first 9 hours of cultivation. After that cells grew rapidly and entered the log phase period until reaching the stationary phase at about 15-27 hours of growth. Consequently, anaerobically digested poultry litter effluent medium was revealed that feasible algae biomass production. And the biomass is projected as a virtually eternal raw material for H_2 production.

Table 1. *Elemental composition of PLEM (after removing TSS).*

Elemental analysis	PLEM (mg^{L-1})
Aluminum (Al)	10.55
Boron (B)	2.33
Cadmium (Cd)	<0.1
Calcium (Ca)	210.2
Chromium (Cr)	<0.1
Copper (Cu)	14.7
Iron (Fe)	28.71
Lead (Pb)	1.05
Magnesium (Mg)	57.35
Manganese (Mn)	5.99
Molybdenum (Mo)	1.16
Nickel (Ni)	1.02
Phosphorus (P)	187
Potassium (K)	1749
Silicon (Si)	48.07
Sodium (Na)	359
Sulfur (S)	124
Zinc (Zn)	10.27
Nitrate nitrogen (NO_3-N)	4.811
Ammonia nitrogen (NH_4-N)	1384
Total nitrogen	1890
Total organic carbon	881

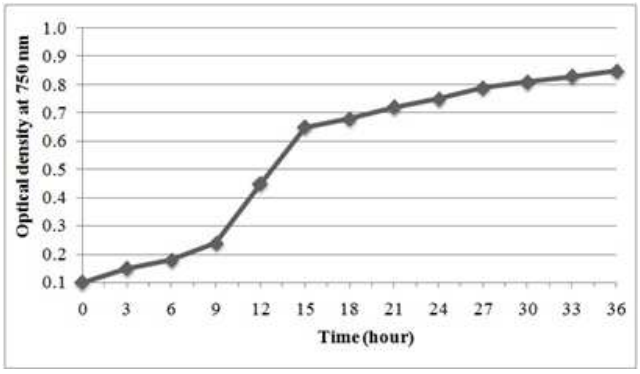

Figure 2. *Algae growth under photoheterotrophic condition*

3.3. Hydrogen Production P. Duplex Meyen *in Photoheterotrophic Condition*

Biohydrogen is a renewable biofuel produced from biorenewable feedstocks by a variety of methods, including chemical, thermochemical, biological, biochemical, and biophotolytical methods [26]. Biological hydrogen production processes are found to be more environment friendly and less energy intensive as compared to thermochemical and electrochemical processes. Furthermore, biohydrogen produced from biological processes has the potential for renewable biofuel to replace current unsustainable hydrogen production technologies, which rely on nonrenewable fossil fuels through thermochemical processes [26]. The basic advantages of biological hydrogen production over other "green" energy sources are that it does not compete for agricultural land use, and it does not pollute, as water is the only by-product of the combustion. These characteristics make hydrogen a suitable fuel for the future. Among several biotechnological approaches, photobiological hydrogen production carried out by green microalgae has been intensively investigated in recent years. One of the promising biohydrogen production approaches is conversion from microalgae, which is abundant, clean, and renewable [26, 27].

The ability of green algae to photosynthetically generate molecular H_2 has captivated the fascination and interest of the scientific community because of the fundamental and practical importance of the process. Below is an itemized list of the properties and promise of photosynthetic H_2-production [28]:

- Photosynthesis in green algae can operate with a photon conversion efficiency of > 80%.
- Microalgae can evolve H_2 photosynthetically, with a photon conversion efficiency of > 80%.
- Molecular O_2 acts as a powerful and effective switch by which the H_2-production activity is turned off.

Figure 3 exhibited the H_2 evaluation which was measured in cells at different time of cultivation period. The result showed that H_2 production was highest with 1.82 nmolH_2 /µg chl/h in cells grown for 21 hours (late-log phase cells) in PLEM. After 21 hours of cultivation, hydrogen production of cells decreased very sharply.

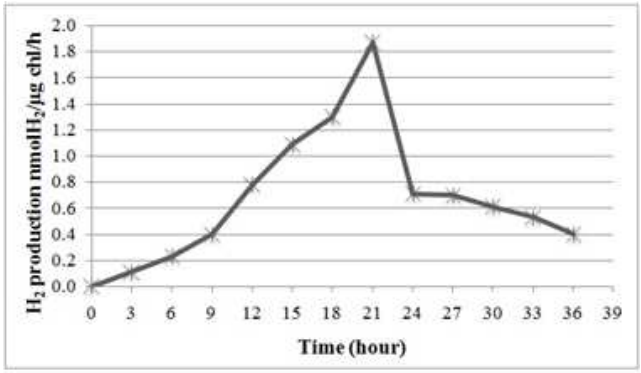

Figure 3. *Hydrogen production during cultivation periods*

It was found that the late-log phase cells or 21-hours old cells could produce the highest hydrogen yield due to the enough accumulation of glycogen from the fermentation process in the PLEM. In the lag-phase cells or 18-hours old cells, under photoheterotrophic condition they need acetic acid for generating energy utilized in the cellular metabolism and for dividing cells. The generated energy and reducing powers are necessarily used for cell growth instead of

producing hydrogen. In case of stationary phase cells (24-and 36-hours old cells), they were not fit and began to die because of carbon source starvation. Hence, the results from P. *duplex* Meyen verified that 18 to 21 hours are suitable time for H_2 production using biological method.

3.4. Hydrogen Production P. Duplex Meyen *in Anaerobic Adaptation Time, under Light and Dark Condition*

P. duplex Meyen was heterotrophically grown in PLEM at room temperature with same the shaking speed and time as before mentioned. Algae cells were incubated under anaerobic condition for 2 to 36 hours in darkness before hydrogen production measurement. It was found that hydrogen production was highest, with 0.137 nmolH_2/µg chl/h, in cells incubated under anaerobic adaptation for 4 hours (Figure 4). After that hydrogen production of cells was obviously decreased. It might be explained that during anaerobic adaptation, oxygen, an inhibitor of hydrogenase enzyme, was decreased resulting in an increase of hydrogen production in the first 4 hours after anaerobic adaptation. Incubation under anaerobic condition for more than 4 hours did not promote the higher hydrogen production because of the decrease of electron and proton donors in cells as well as the limitation of hydrogenase enzyme.

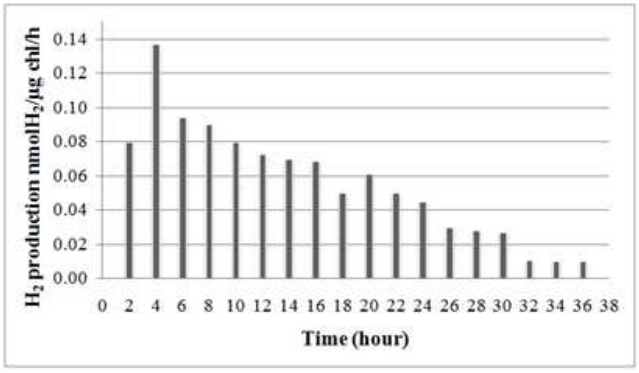

Figure 4. *Hydrogen production under different anaerobic adaptation time*

P. duplex Meyen cells were separately grown in PLEM either providing light illumination of 37.5 µmol^{-1}m^2 sec^{-1} intensity or under dark condition. The result showed that cells grown under dark condition have higher optical density than those under light condition (data not mentioned). It might be explained that cells grown under dark condition used only acetic acid as carbon source for growing and dividing cells whereas cells grown under light condition could fix CO_2 from the atmosphere via photosynthetic process, therefore requiring more time for the initial growth.

The result showed those cells grown under light produced hydrogen about 4 times higher than cells grown under dark condition (1.82 and 0.37 nmolH_2/µg chl/h, respectively); the results presented in Figure 5. It was suggested that under light condition the energy in form of ATP (adenosine triphosphate) and the reducing powers NADPH (nicotinamide adenine dinucleotide phosphate) or NADH (nicotinamide adenine dinucleotide) were obtained from the light reaction of photosynthesis, giving their electrons to excess protons for hydrogen production. Under dark condition cells produced less ATP and reducing powers, resulting in less hydrogen production. Consequently, the isolated green alga *P. duplex* Meyen produced the highest hydrogen when cultivated cells for 21 hours in PLEM under light and then incubated cells under anaerobic adaptation for 4 hours.

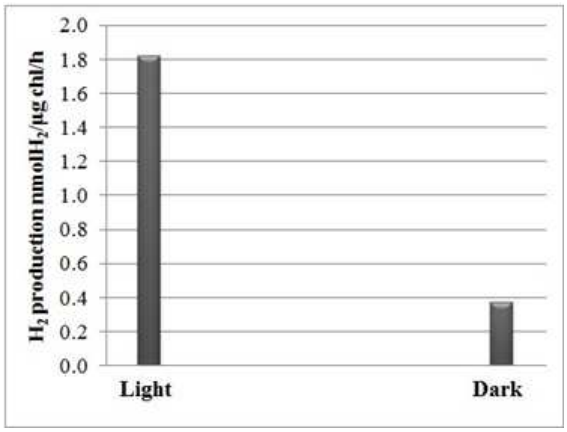

Figure 5. *Hydrogen production under light and dark condition*

4. Conclusions

Biological hydrogen production from biomass is considered one of the most promising alternatives for sustainable green energy production. One of the promising hydrogen production approaches is conversion from biomass, which is abundant, clean and renewable. We identified and isolated a new green alga, *Pediastrum duplex* Meyen, from freshwater fish pond at a location near Maejo University, Sansai, Thailand with a capacity of efficient biohydrogen production *using with* poultry litter medium (PLM). *P. duplex* Meyen can be produced by using anerobically digested poultry effluent without any chemical supplementation. This method may reduce the cost of commercial algal production and biofuel applications. *P. duplex* Meyen produced the highest hydrogen at 21 hours in PLEM under light and then incubated cells under anaerobic adaptation process. This study results revealed that biological dark fermentation is also a promising hydrogen production method for commercial use in the future.

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