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Hydrogen Production by Unicellular Green Alga *Chlorella* sp. LSD-W2 Isolated from Seawater in Thailand

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Abstract

Green algae are able to convert the unlimited sunlight energy to produce hydrogen via photosynthesis. In seawater, several kinds of marine microalgae are widespread and abundant and have been shown to tolerate and survive under the extreme salt concentrations. This work aimed to study the screening of high H_2 producing marine green algal strains isolated from the Gulf of Thailand and the Andaman Sea, and the selection of the highest H_2 producing strain. Its H_2 production was investigated under photoheterotrophic cultivation. The result revealed that among 20 marine green algal strains, the green alga *Chlorella* sp. LSD-W2 gave the highest H_2 production rate in both light and dark anaerobic conditions. During photoheterotrophic cultivation *Chlorella* sp. LSD-W2 was rapidly grown in TAP (Tris-Acetate-Phosphate) medium and reached the stationary growth phase after 36 h of cultivation. The highest photohydrogen production rate was found in cells incubated in NH_4Cl-deprived TAP medium. It was approximately 20-fold higher than H_2 production rate of cells in a normal TAP medium.

Keywords : *Hydrogen production, marine green algae, heterotrophic cultivation*

1. Introduction

Biohydrogen is one of alternative interesting energy sources. Its combustion provides a high energy and causes less air-pollution than the combustion of other energy sources. H_2 can be produced by several kinds of microorganisms such as bacteria, cyanobacteria and green algae. Among these organisms, H_2 production by green algae shows a good advantage in using unlimited sunlight energy to evolve hydrogen via photosynthesis. At photosystemII (PSII) reaction center, the sunlight energy is absorbed, and then water splitting occurs. This leads to the O_2 production at PSII and the series of electron transfer. The electrons are transferred from

PSII to PSI, and finally to a soluble carrier protein ferredoxin (Fd). Ferredoxin transfers photosynthetic reductants to an [FeFe]-hydrogenase, where the electrons are recombined with protons, yielding H_2 (1, 2). Many green algae have been shown to produce H_2 under the light such as *Chlamydomonas reinhardtii* (3), *Chlorella protothecoides* (4) and *Scenedesmus obliquus* (5). The green alga *C. reinhardtii* is the model organism for studying the H_2 metabolism in greenmicro algae (6).

In Thailand, many studies has focused on investigating the production of H_2 by natural freshwater green algae such as *Chlorella vulgaris var. vulgaris* TISTR8261 (7), *Tetraspora* sp. CU2551 (8), *Scenedesmus* sp. KMITL-O1 (9) and *Pediastrum duplex* Meyen (10). Therefore, this work focused on studying H_2 production by marine green algae isolated from seawater in Thailand. Marine green algae are one type of green algae that are widespread and abundant in the ocean. They can tolerate and survive under the extreme salt concentrations resulting that some marine green algae can grow in the natural seawater.

In green algae, several environmental factors play key roles in stimulation of H_2 production such as growth conditions, anaerobic condition, nutrient and mineral compositions, etc. A previous report showed that *C. reinhardtii*, a model green algal organism for studying H_2 metabolism, was able to improve H_2 production when cultivated in nitrogen- and sulfur-deprived medium (3,11). In addition, the deprivation of phosphorus could increase the H_2 production in *Chlorella* sp. (12) and *C. reinhardtii* (13).

The aims of this work were to screen high H_2 producing marine green algae isolated from the Gulf of Thailand and the Andaman Sea and to investigate the influences of N-, S- and P-deprivation on H_2 production by the highest H_2 producing strain under the photoheterotrophic cultivation.

2. Materials and methods

2.1 Green algal isolation and cultivation

Twenty green algal strains were isolated from seawater, stones, sands and shells in the Gulf of Thailand and Andaman Sea. They were purified using a single cell isolation technique (14). For screening high H_2 producing strains, the purified green algal strains were cultivated in a 250-mL Erlenmeyer flask containing 100 mL of ASN III medium (15) and shaken at 120 rpm under white-light illumination of 30 μmol photons $m^{-2}s^{-1}$ at 30 °C for 14 days. Under phototrophic condition they were cultivated in TAP (Tris-Acetate-Phosphate) medium (16) and shaken at 120 rpm under white-light illumination of 30 μmol photons m^{-2} s^{-1} at 30 °C for 3 days.

2.2 Growth measurement

Growth of green algae was determined by measuring the optical density at 750 nm of cell culture using a spectrophotometer (Shimadzu UV-1601, Japan) every 6 h of cultivation for 3 days.

2.3 Hydrogen measurement

The green algal cultures were harvested by centrifugation at 7,000×g at 4 °C for 10 min. The cell pellet was subsequently washed twice and resuspended in 100 mL of N-deprived medium and further incubated for 1 day

before harvesting cells to determine H_2 production. The cell pellet was resuspended in 5 mL of N-deprived medium and transferred to a gas-tight vial. H_2 production was measured under four conditions; (1) anaerobic/dark condition, (2) anaerobic/light condition, (3) aerobic/dark condition and (4) aerobic/light condition. In case of an anaerobic condition, the cell suspension was bubbled with argon gas for 5 min to eliminate O_2 in a vial. This process was ignored in an aerobic condition. The vial was incubated at 30 °C either under the light intensity of 30 µmol photons $m^{-2}s^{-1}$ (light condition) or in the dark (dark condition) for 24 h before analyzing H_2 production. H_2 production was determined by analyzing the gas phase by Gas Chromatograph (Perichrom, PR2100, France) using a thermal conductivity detector. The injector and detector temperature were kept at 100 °C whereas the oven temperature was maintained at 50 °C. Argon gas was used as a carrier gas during H_2 analysis.

2.4 Dry cell weight and chlorophyll measurements

For dry cell weight measurement, 10 mL of culture was filtered through a filter paper No. 1 (55 mm diameter) (Whatman, UK). The filter containing cells was washed twice by 10 mL of distilled water and dried at 60 °C in an oven for 3 days. The filter was put in a desiccator for 1 h before weighting. Dry cell weight was calculated from weight of filter containing cells compared with that of filter without cells. The chlorophyll measurement was performed by extracting the cell culture with 90% (v/v) methanol. The chlorophyll concentration was determined by measuring the chlorophyll extracts at absorbance 665 and 660 nm by a spectrophotometer (17).

3. Results

3.1 Screening of high H_2 producing green algal strains

Twenty marine green algal strains were isolated from the Gulf of Thailand and the Andaman Sea in Thailand. They were grown in ASN III medium for 14 days, harvested by centrifugation, washed twice and resuspended in 100 mL of N-deprived ASN III medium. The cultures were shaken at 30 °C under the light for 24 h, subsequently harvested and resuspended in 5 mL of the same medium. The cell suspensions were transferred to a glass vial and incubated under four conditions. Their H_2 production was measured after incubating for 24 h. The result revealed that H_2 yield was detected in 13 marine green algal strains when incubating cells under the dark anaerobic condition for 24 h (Table 1). H_2 yield could be detected at a low level or hardly detected in most green algal strains under aerobic condition (Table 1). Among them, the green alga *Chlorella* sp. LSD-W2 gave the highest H_2 production with 66.47±5.44 and 67.64±1.77 nmolH_2 mg dry $weight^{-1}$ under dark and light anaerobic conditions, respectively (Table 1).

Table 1. H_2 production by 20 marine green algal strains isolated from the Gulf of Thailand and the Andaman Sea in Thailand

Strains	Origin	Habitat	H_2 production yields (nmolH_2 mg dry wt.$^{-1}$)			
			Anaerobic		Aerobic	
			darkness	light	darkness	light
JM-W	Jaomai, Trang	seawater	0	0	0	0
JT-W	Jomtien, Chonburi	seawater	0	0	0	0
KT-W2	Krating, Chanthaburi	seawater	0	0	0	0
KT-W3	Krating, Chanthaburi	seawater	64.13±3.20	2.84±0.07	0.71±0.04	0
KVM-ST4	Kungviman, Chanthaburi	stone	0	0	0	0
KVM-ST4.2	Kungviman, Chanthaburi	stone	0.08±0.01	0	0	0
LS-W1	Laemsing, Chanthaburi	seawater	1.33±0.01	0	0.42±0.04	0
LSD-W2	Laemsadet, Chanthaburi	seawater	66.47±5.44	67.69±1.77	0	0
N-W	Nang, Krabi	seawater	0.58±0.03	0	0	0
NE-W	Neun,Chonburi	seawater	0.50±0.04	0.11±0.01	0	0
NR-ST2.2	Nangram, Chonburi	stone	0	0	0	0
NTR-W	Nopparattara, Krabi	seawater	18.23±0.80	16.72±0.81	1.98±0.18	0.40±0.03
P-ST2.3	Phla, Rayong	stone	24.75±0.11	1.70±0.07	0	0
PM-SH14	Pakarang, Trang	shell	14.45±0.29	1.05±0.04	0	0
SK-W2.2	Saikeaw, Chonburi	seawater	0.70±0.02	0	0	0
SKR-SA1	Saikeaw, Rayong	sand	0	0	0	0
SR-W	Samran, Trang	seawater	0.68±0.03	0	0	0
TG-W2.3	Toeingam, Chonburi	seawater	14.12±0.35	27.50±0.69	0	0
TL-SH4	Talan, Krabi	shell	0	0	0	0
VD-ST2.1	Vongdeuan, Rayong	stone	64.00±1.15	1.00±0.05	0	0

3.2 Growth of *Chlorella* sp. LSD-W2 under photoheterotrophic condition

Only green alga LSD-W2 was classified by morphological and genetic characteristics using 18S rRNA sequence analysis. It was identified as *Chlorella* sp. and named as *Chlorella* sp. LSD-W2. The *Chlorella* sp. LSD-W2 is a unicellular green alga. It has a round shape with an approximate 5-10 µm cell diameter. No motile flagella were found (Fig. 1). To determine growth, *Chlorella* sp. LSD-W2 was grown in 100 mL of TAP medium and shaken under the light for 3 days. TAP medium contains 0.2 mM Tris, 7 mM NH_4Cl, 0.45 mM $CaCl_2.2H_2O$, 0.83 mM $MgSO_4.7H_2O$, 1.65 mM K_2HPO_4, 1.05 mM KH_2PO_4, 0.134 mM Na_2EDTA, 0.136 mM $ZnSO_4.7H_2O$, 0.184 mM of H_3BO_4, 40 µM $MnCl_2.4H_2O$, 32.9 µM $FeSO_4.7H_2O$, 12.3 µM $CoCl_2.6H_2O$, 10 µM $CuSO_4.5H_2O$, 4.44 µM $(NH_4)_6MoO_3$ and 1 mL L^{-1} conc. acetic acid. Optical density at 750 nm of cell culture was measured using a spectrophotometer. It was shown that during photoheterotrophic condition *Chlorella* sp. LSD-W2 was rapidly grown in TAP medium and reached the stationary growth phase after 36 h of cultivation (Fig. 2).

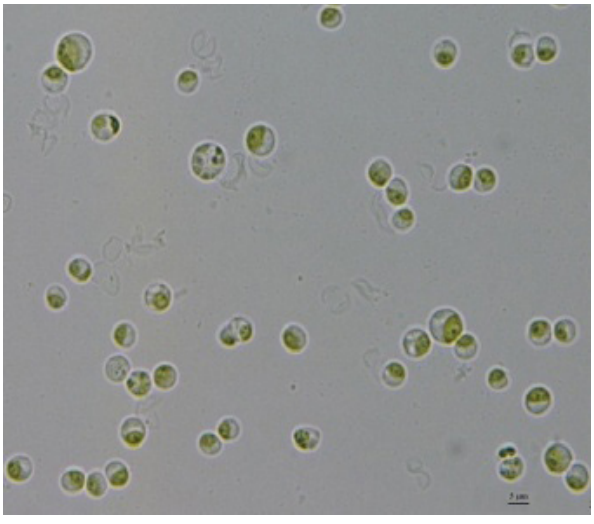

Figure 1. Cell morphology of *Chlorella* sp. LSD-W2 observed under a light microscope

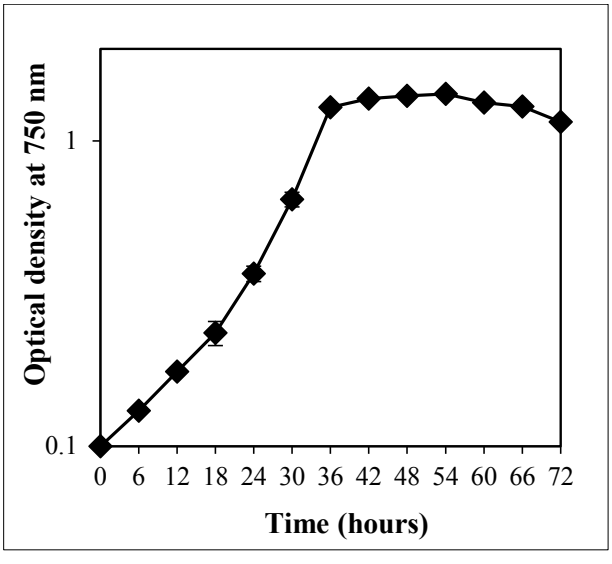

Figure 2. Growth by OD_{750} measurement of *Chlorella* sp. LSD-W2 cultivated in TAP medium for 72 h

3.3 Effect of nutrient deprivation on H_2 production by *Chlorella* sp. LSD-W2

Chlorella sp. LSD-W2 was grown in TAP medium for 36 h, then harvested, washed and resuspended in medium with a deprivation of either nitrogen or sulfur or phosphorus. In N-deprived medium, NH_4Cl was removed from TAP medium. In S-deprived medium, $MgSO_4$, $FeSO_4$, $ZnSO_4$ and $CuSO_4$ were removed from the medium but Mg^{2+},Fe^{2+}, Zn^{2+} and Cu^{2+} were replaced with $MgCl_2$, $FeCl_2$, $ZnCl_2$ and $CuCl_2$. In P-deprived medium, KH_2PO_4 was removed from the medium and K^+ was replaced with KCl. Cells were incubated for 24 h before harvesting. The cell pellet was resuspended in a fresh medium and

transferred to a glass vial. The culture was purged with argon, and incubated under the light at 30°C for 24 h before analyzing H_2 production. It was shown that lack of nitrogen and sulfur in the medium resulted in an increase of photohydrogen production rate in *Chlorella* sp. LSD-W2 but lack of phosphorus did not show much significant effect on its H_2 production (Fig. 3). Under N-deprived condition, *Chlorella* sp. LSD-W2 gave the highest photohydrogen production rate with 1.52±0.09 μmolH_2 mg $chl^{-1}h^{-1}$. It was approximately 20-fold higher than that of cells incubated in a normal TAP medium (Fig. 3).

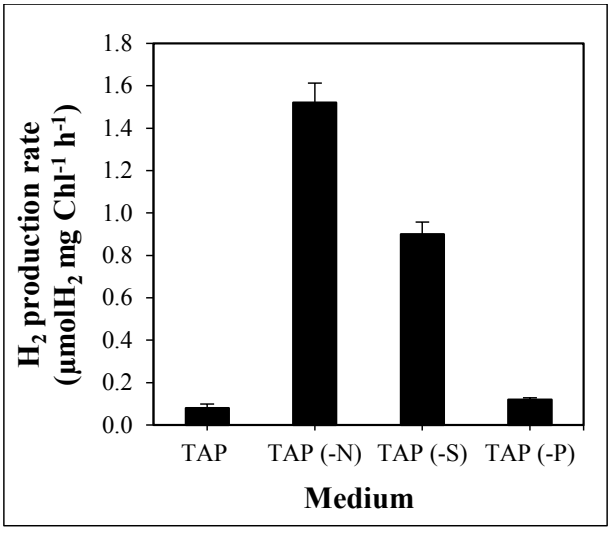

Figure 3. Photohydrogen production rate by *Chlorella* sp. LSD-W2 in N-, S- and P-deprived medium (Data are means±SD (n=3))

4. Discussions

In this study, twenty marine green algal strains were isolated from seawater, stones, sands and shells in the Gulf of Thailand and the Andaman Sea. Most of them (twelve strains) were isolated from seawater. They are single-cell microalgae with a green color and round in shape, and did not contain flagella for their movement. Some marine green algal strains isolated from shells and stones were larger and longer than those isolated from seawater. It is possible that they use these structures to attach on the wall of the adhesives.

To screen the high H_2 producing green algae from 20 marine green algal isolates, they were grown in ASN III medium and their H_2 production was determined under four conditions as previously described (18). Table 1 showed that most green algae were not capable of producing H_2 under aerobic condition whereas only few green algal strains produced a low H_2 yield. It could be explained that under aerobic condition, O_2 existing in the vial gas phase interfered the metabolism of H_2 production because O_2 is an inhibitor of iron [Fe]-hydrogenase, a key enzyme for H_2

production in green algae (19). However, iron hydrogenase in green algae was more oxygen-tolerant than cyanobacterial hydrogenase (20).

In the study, *Chlorella* sp. LSD-W2 isolated from seawater in Laemsadet, Chanthaburi province gave the highest H_2 production yield with 66.47±5.44 and 67.64±1.77 nmolH_2 mg dry weight^{-1} under both light and dark anaerobic conditions, respectively (Table 1). It was suggested that *Chlorella* sp. LSD-W2 produced H_2 via both photosynthetic and light-independent fermentative pathways (1, 2). In addition, it is possible that iron hydrogenase in *Chlorella* sp. LSD-W2 was oxygen-tolerant during photosynthesis in the light whereas that of other strains was inactive. Some green algae were shown to produce high H_2 yield under both light and dark conditions such as *C. reinhardtii* (21), *Tetraspora* sp. CU2551 (8) and *C. protothecoides* (4).

The growth of *Chlorella* sp. LSD-W2 was investigated under photoheterotrophic condition. The result revealed that *Chlorella* sp. LSD-W2 was rapidly grown in TAP medium and reached the stationary phase after 36 h of cultivation (Fig. 2), indicating that it can use acetic acid or acetate in TAP medium as a carbon source. Tris-acetate-phosphate (TAP) medium was a suitable medium for green algal cultivation and for studying many metabolisms in green algae including H_2 production (22).

Under N-deprived condition, *Chlorella* sp. LSD-W2 gave the highest H_2 production rate with 1.52±0.09 μmolH_2 mg chl^{-1}h^{-1}. It was approximately 20-fold higher than that in normal TAP medium (Fig. 3). In this study, lack of nitrogen was shown to give the most influence on H_2 production in *Chlorella* sp. LSD-W2, whereas lack of sulfur induced an increase of H_2 production in *C. reinhardtii* (11). Under nitrogen deprivation *Chlorella* sp. LSD-W2 produced H_2 from electrons obtained by the photosynthetic pathway and the degradation of accumulated carbohydrate via light-independent fermentative pathway (1, 2).

Under S-deprived condition, *Chlorella* sp. LSD-W2 gave H_2 production rate with 0.90±0.06 μmolH_2mg chl^{-1}h^{-1} (Fig. 3). Sulfur is one the amino acid constituents; therefore sulfur deprivation affected protein synthesis, especially D1 and D2 protein in photosystem II. When the photosystem II activity of *Chlorella* sp. LSD-W2 was inhibited, O_2 evolution activity was declined in the chloroplast, resulting in the higher H_2 production.

The P-deprivation did not show much effect on H_2 production in *Chlorella* sp. LSD-W2 (Fig. 3). Like sulfur deprivation, phosphorus deprivation limits O_2 evolving activity in algal cells and causes other metabolic changes that are favorable for H_2 photoproduction (12, 13). However, the inhibition process by phosphorus deprivation is slower than that by sulfur deprivation and the cultures establish anaerobiosis later (23). This explained the lower H_2 production rate obtained from *Chlorella* sp. LSD-W2 incubated under P-deprivation compared to that under S-deprivation.

In this study *Chlorella* sp. LSD-W2 gave the highest H_2 production rate of 1.52±0.09 μmolH_2 mgchl^{-1} h^{-1} or 102 mL L^{-1} or 4.25 mLL^{-1} h^{-1} under N-deprived condition. When compared the maximum H_2 production rate with other marine green algae, it was shown that our studied green alga *Chlorella* sp. LSD-W2 gave the highest H_2 production rate (Table 2). However it

could not be compared with the H_2 production rate of *Chlamydomonas* sp. MGA161 due to the use of different units of H_2 production rate (Table 2). From our result, the marine green alga *Chlorella* sp. LSD-W2 showed relatively high H_2 photohydrogen production rate, making it to be one of the potential strains for photobiological hydrogen production.

Table 2. The maximum H_2 production rate by *Chlorella* sp. compared with other marine green alga strains.

Marine green algae	Maximum H_2 production rate	H_2 evolution assay conditions	References
Chlorella sp. LSD-W2	1.52±0.09 µmolH_2 mg chl^{-1} h^{-1} or 4.25 mLH_2 L^{-1} h^{-1} or 102 mL H_2 L^{-1}	N-free TAP medium, light intensity of 30 µmol photons m^{-2} s^{-1}, temperature at 30 °C	This study
Chlamydomonas sp. MGA161	0.22 µmol H_2 mg dry wt.$^{-1}$ h^{-1}	Okamoto medium (pH 8.0), temperature at 20 °C	24
Platymonas subcordiformis	0.189 mL H_2 h^{-1}	Seawater medium supplemented with micronutrients (-S) (pH 8.0), 25 mM acetate and 37.5 mM glucose	25
Chlorella autotrophica	0.11 mL L^{-1} h^{-1}	S-free L1medium with acetic acid (L1+HAc-S), temperature at 26 °C, darkness	4
Chlorella capsulate	0.019 mL L^{-1} h^{-1}	S-free L1medium with acetic acid (L1+HAc-S), temperature at 26 °C, darkness	4
Nannochloropsis sp.	0.018 mL L^{-1} h^{-1}	S-free L1medium with acetic acid (L1+HAc-S), temperature at 26 °C, darkness	4
Tetraselmis helgolandica	0.04 mL L^{-1} h^{-1}	S-free L1medium with acetic acid (L1+HAc-S), temperature at 26 °C, darkness	4
*Tetraselmis striata*Butcher	0.34 mL L^{-1} h^{-1}	S-free L1medium with acetic acid (L1+HAc-S), temperature at 26 °C, darkness	4
Dunaliella apiculata	0.018 mL L^{-1} h^{-1}	S-free L1medium with acetic acid (L1+HAc-S), temperature at 26 °C, darkness	4
Pyramimonas sp.	0.062 mL L^{-1} h^{-1}	S-free L1medium with acetic acid (L1+HAc-S), temperature at 26 °C, darkness	4
Chlorella sp.	20 mL L^{-1}	P-free TAP medium, 0.8 mg L^{-1} of chl content, CO_2, light intensity of 45 µE m^{-2} s^{-1} and temperature at 25 °C	12

5. Conclusion

For screening of high H_2 producing green algae isolated from the Gulf of Thailand and the Andaman Sea, the unicellular green alga *Chlorella* sp. LSD-W2 showed the highest H_2 production rate under both light and dark anaerobic conditions. It can be rapidly grown in TAP medium. The highest photobiological hydrogen production rate was obtained when incubated cells in the N-deprived TAP medium. This rate was approximately 20-fold higher than that of cells incubated in a normal TAP medium. From the result, the green alga *Chlorella* sp. LSD-W2 is suggested to be one of the potential H_2 producers. The other environmental factors on H_2 production by this strain need to further study.

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7. References

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