sciendo

10.2478/botlit-2019-0016

BOTANICA
ISSN 2538-8657

2019, 25(2): 145–155

PHOTOAUTOTROPHIC PRODUCTION OF HYDROGEN IN *CARTERIA CRUCIFERA* AARL G045 CO-CULTURED WITH BACTERIAL FLORA

Kritsana Duangjan[1], Watsamon Nakkhunthod[2], Chayakorn Pumas[1, 2*]

[1]Chiang Mai University, Science and Technology Research Institute, 239 Huay Kaew Road, T. Suthep, Muang District, Chiang Mai THA-50200, Thailand
[2]Chiang Mai University, Faculty of Science, Department of Biology, 239 Huay Kaew Road, T. Suthep, Muang District, Chiang Mai THA-50200, Thailand
*Corresponding author. E-mail: chayakorn.pumas@gmail.com

Abstract

Duangjan K., Nakkhunthod W., Pumas C., 2019: Photoautotrophic production of hydrogen in *Carteria crucifera* AARL G045 co-cultured with bacterial flora. – Botanica, 25(2): 145–155.

This study reports on high potential of photoautotrophic biohydrogen production in co-culture of the novel hydrogen producing microalga *Carteria crucifera* AARL G045 with its bacterial flora. Hydrogen production from immobilised and free cells were compared under photoautotrophic and photofermentation conditions. Two bacterial floras of *Carteria crucifera* AARL G045 cultures were isolated and identified by 16S rDNA sequence analysis as *Agrobacterium tumefaciens* G045-01 and *Aeromicrobium kwangyangensis* G045-02. As a result, co-culture of *Carteria crucifera* AARL G045 and *A. kwangyangensis* G045-02 generated higher level of hydrogen gas than that of the mono algal culture by about 1.32 times. In addition, co-culture of both free cells of the microalga and the bacterium produced higher hydrogen than both co-immobilisation in calcium alginate by about 2.04 times.

Keywords: biohydrogen, co-cultivation, free cells, immobilised cells, microalgae.

INTRODUCTION

Several microorganisms can produce photo catalytic hydrogen such as algae, cyanobacteria and some photosynthetic bacteria (Das & Veziroğlu, 2001). Since microorganisms use only sunlight as an energy source to split water in the production of H_2 and O_2, so they have the ability to reduce cost and the environmental impact on hydrogen production (Eroglu & Melis, 2011). Green microalga *Scenedesmus obliquus* (Turpin) Kützing has been firstly reported for the abilities of hydrogen production beneath anaerobic conditions (Gaffron, 1939). Several strains of green microalgae have been recognized for their hydrogen production. Most of the studies focus on hydrogen production in *Chlamydomonas reinhardtii* Dangeard under mixotrophic or heterotrophic conditions (Melis et al., 2000; Kosurov & Seibert, 2009; Faraloni et al., 2011). However, the commercial disadvantages of those trophic conditions are higher cost from organic carbon source and obligate axenic operation. Thus, autotrophic conditions have been considered for hydrogen production of microalgal strains (Hwang et al., 2014; Duangjan et al., 2017).

According to the previous study of Duangjan et al. (2017), several green algae have been described for the capacity of hydrogen production under autotrophic conditions. Among these, *Carteria crucifera* AARL G045 is a promising strain because of its rapid growth rate, greater hydrogen production and easy to be harvested because of large cell size (Duangjan et al., 2017). The genus *Carteria* is a unicellular green microalga, which is classified in the family Chlamydomonadaceae, the same as *Chlamydomonas*, a model microorganism for biohydrogen production. *Carteria* cells are round or al-

most round shape with eye spot. They contain four flagella at the top of the cell and 2–4 vacuoles near the flagella (Fig. 1). This genus is widely distributed in freshwater and terrestrial habitats. Furthermore, previous study has recognized the capacity of these strains for high lipid accumulation, potent wastewater treatment and promising hydrogen production strain (Janta et al., 2013; Boonkhot et al., 2015; Duangjan et al., 2017).

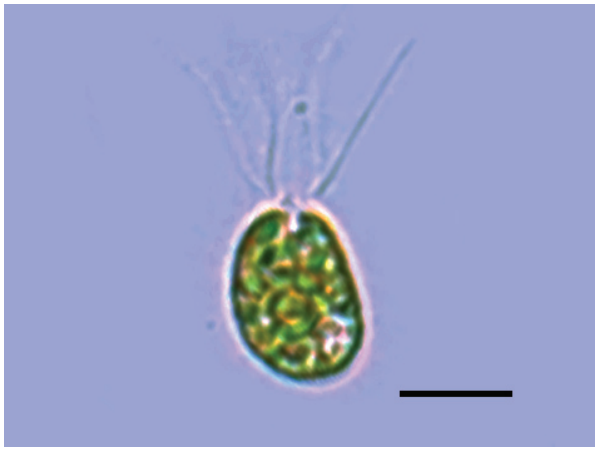

Fig. 1. Morphology of *Carteria crucifera* AARL G045 observed by using the light microscope (scale bar = 10 µm)

Hydrogen production in green microalgae depends on various parameters such as light intensity, carbon sources, nutrients, pH, temperature, atmosphere gas composition as well as activity of hydrogenase enzyme (Lam & Lee, 2013; Pholchan et al., 2017). Nevertheless, hydrogenase is extremely sensitive to oxygen, as a suppressor of hydrogenase gene expression (Srirangan et al., 2011). Thus, certain techniques have been established to reduce or eliminate the effect of oxygen such as replacing oxygen with inert gas, cultivation under two stage photosynthesis by separation of oxygen and hydrogen production process through sulphur deprivation, photo-periods, chemical reducing agents and co-culturing with bacteria (Melis et al., 2000; Saifuddin & Priatharsini, 2016; Xu et al., 2017). Previous studies have revealed that some bacteria have the capacity to enhance hydrogen production in algae-bacterial co-cultivation by consuming of oxygen from algal photosynthesis. The example for algae-bacterial co-cultivation were such as *Azotobacter chroococcum*, *Bacillus subtilis*, *Bradyrhizobium japonicum*, *Escherichia coli*, *Escherichia coli* ΔHypF, *Microbacterium paraoxydans* strain 591, *Pseudomonas* sp. strain D, *Pseudomonas* sp. A8, *Rhizobium* sp., *Stenotrophomonas maltophilia* strain 776 and *Thuomonas intermedia* BCRC 17547 with the genera *Chlamydomonas*, *Chlorella* and *Scenedesmus* (Ergoglu & Melis, 2011; Wu et al., 2012; Li et al., 2013; Wirth et al., 2015; Xu et al., 2016; Lakatos et al., 2017; Ban et al., 2018). Besides, they often form mutualistic relationships and secrete some useful factors for algae growth (Tanabe et al., 2015).

Moreover, immobilisation is another technique that can enhance H_2 yields from microalgae (Saifuddin & Priatharsini, 2016). This technique has more advantages, i.e. immobilised cells occupy less space, require small volume of growth medium, easier to handle and can be reused (Eroglu & Melis, 2011). The enhancement of hydrogen production by immobilised *C. reinhardtii* cells on various polymer matrices have been investigated, such as Al-borosilicate porous glass sheets, thin alginate films, Ca^{2+}-alginate films. These immobilised conditions provide significantly increased duration of the hydrogen production phase; stimulate hypoxic environment and very rapid decline of PSII activity within the alginate cells (Laurinavichene et al., 2006; Kosurov & Seibert, 2009).

In this study, novel potential hydrogen producing green microalga *Carteria crucifera* AARL G045 was introduced. The hydrogen production of this strain under phototrophic and photo-fermentation was evaluated. In addition, the bacterial floras were isolated and co-cultured individually with the microalga. Photoautotrophic hydrogen production in free microalgae or bacteria-cultured cells was compared to those under immobilisation conditions.

MATERIALS AND METHODS

Algae and bacterial strains

Algal strain, *Carteria crucifera* AARL G045, was obtained from Algal Culture Collection of the Applied Algal Research Laboratory, Department of Biology, Faculty of Science, Chiang Mai University, Thailand. Algal cells were maintained under phototrophic conditions containing Jaworski's medium (consisting of $Ca(NO_3)_2.4H_2O$, KH_2PO_4, $MgSO_4.7H_2O$, $NaHCO_3$, EDTA Fe Na, EDTA Na_2, H_3BO_3, $MnCl_2.4H_2O$,

$(NH_4)6Mo_7O_{24}.4H_2O$, Cyanocobalamin, Thiamine HCl, Biotin, $NaNO_3$, $Na_2HPO_4.12H_2O$) (Schlösser, 1982) at 25 ± 2ºC continuous shaking under illumination of a white fluorescent lamp (50 µmol $m^{-2}s^{-1}$).

Bacterial flora strains in the algal culture were isolated according to the following procedure: bacterial flora in the algal culture was obtained by spreading on mixed Luria Bertani (Atlas, 2010) and Jaworski's medium (1:1) agar plate after a serial dilution and incubated at 37ºC in the dark for 24–48 hours; bacterial colonies with clearly different phenotypes were picked and restreaked on fresh agar plate for pure strains (Li et al., 2013). These strains were co-cultured with *Carteria crucifera* AARL G045 to promote hydrogen production.

Identification of bacterial flora in algal culture

The complete genomic DNA of bacterial isolates was manually extracted and purified (Evans et al., 2013). Bacterial 16S rDNA was obtained by PCR amplification using universal primer pair 27F/1492R (27F: 5'-AGAGTTTGATCCTGGCTCAG-3'; 1492R: 5'-TACCTTGTTACGACTT -3') (Bio Basic Canada Inc.). The PCR products were purified using a GF-1 AmbiClean (PCR&Gel), ViVantis (Nucleic Acid Extraction Kit Hand Book) and sequenced on an ABI3730xl DNA analyser. Taxonomic classification of bacterial 16s rDNA gene was analysed using the nucleotide-nucleotide BLAST database. A phylogenetic tree was constructed by MEGA programme using the Neighbor joining method.

Hydrogen production in *Carteria crucifera* AARL G045 under photoautotrophic and photo-fermentation conditions

Green microalga *Carteria crucifera* AARL G045 cells were harvested by centrifugation at 4500 rpm for five minutes and washed three times in sulphur-deprived medium. *Carteria* cells were transferred to a 1 litre experimental bottle containing 800 ml medium consisted with two trials: sulphur-deprived Jaworski's medium (JM-S) and sulphur-deprived Tris Acetate Phosphate medium (TAP-S) (Harris, 1986). Photo-fermentation is the fermentative conversion of organic substrate to biohydrogen production by a diverse group of photosynthesis bacteria. TAP medium contains acetate compound, which represent photo-fermentation mode (Atif et al., 2005). However, Jaworski's medium does not contain organic substrate, microalgae use carbon dioxide as a carbon source, thus, Jaworski's medium is defined as a photoautotrophic condition. Initial optical density of algae cells was defined to OD_{665} of 0.5 (chlorophyll *a* about 442.82 mg L^{-1}). Oxygen gas in the system was eliminated by flushing with argon gas. The experimental bottles were sealed and incubated at 25 ± 2ºC under illumination of a white fluorescent lamp (27 ± 2 µmol m^{-2} s^{-1}) on magnetic stirrer.

Hydrogen production was measured by replacing water in cylinder. Hydrogen concentration in the headspace of cylinder was determined using a gas chromatography device (Agilent HP 6890 gas chromatograph, thermal conductivity detector (TCD), HP-PLOT molecular sieve 5A (30 m)). The oven temperature was maintained at 40ºC. The injector was kept at 180ºC, whereas the detector was kept at 220ºC. Helium gas was used as the carrier gas during hydrogen analysis. Besides, oxygen levels in the culture medium on the first and last days of the experiments were determined using Dissolved Oxygen Meters (HORIBA – LAQUAact OM-71-02). Moreover, algal growth was measured by chlorophyll *a* measurement (Saijo, 1975).

Co-culture of *Carteria crucifera* AARL G045 and its isolated bacterial flora for H_2 production

Bacterial flora cells in agar plate were maintained with Luria Bertani (LB) broth in the dark under continuous shaking at 125 rpm 37ºC for 18–24 hours until exponential bacterial growth. Then, cells were harvested by the centrifugation at 12000 rpm for 10 minutes. Green microalga *Carteria crucifera* AARL G045 in Jaworski's medium was harvested by centrifugation at 4500 rpm for five minutes. Algal cell palates were washed three times in sulphur-deprived Jaworski's medium (JM-S) before inoculation. Both alga and bacterial flora palates were transformed to a 1 litre experimental bottle containing 800 ml of JM-S culture medium. Initial optical density of the alga and bacterial cells were defined to OD_{665} of 0.5 (chlorophyll *a* about 442.82 mg L^{-1}) and OD_{600} of 0.5, respectively (Lakatos et al., 2017). The condition of hydrogen production was the same as in the above methods. Hydrogen production in the headspace of cylinder, and oxygen levels in the culture medium were determined.

Co-culture of *Carteria crucifera* AARL G045 and the selected bacterial flora with immobilised and free cells for H_2 production

The selected bacterial flora and microalga were co-cultured in JM-S culture medium under immobilised and free cell condition. Four treatments, consisting of free cell of alga and free cell of bacterium, immobilised alga and immobilised bacterium, free cell of alga and immobilised bacterium, immobilised alga and free cell of bacterium, were cultivated in a 1 litre experimental bottle containing 800 ml of JM-S culture medium. The condition of hydrogen production was the same as in the above methods (Fig. 2).

For immobilisation, the initial microalgal cell was about 1×10^6 cells mL^{-1}. The cells were centrifuged at 4500 rpm for 10 minutes. Then, microalgal pellets were mixed with 2% sodium alginate before forming in 0.1 M calcium citrate. Finally, the calcium alginate (about 3 mm diameters) was soaked in calcium chloride and kept at 4ºC for two hours (Fig. 3) (De Bashan & Bashan, 2010; Boonkhot et al., 2015).

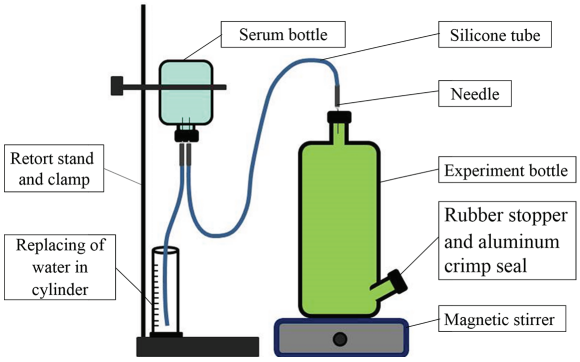

Fig. 2. Diagram of hydrogen production system in *Carteria crucifera* AARL G045 and the selected bacterial flora with immobilised and free cells for H2 production

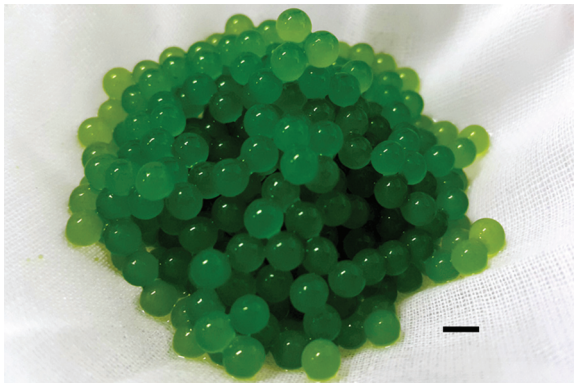

Fig. 3. Immobilised *Carteria crucifera* AARL G045 cells in calcium alginate (scale bar = 3 mm)

Measurements of algal and bacterial growth

On the first and last day of the experiments, bacterial density was determined on LB-JM plates using serial dilutions, while algal growth was determined by chlorophyll *a* content (Saijo, 1975). Briefly, algal cells from co-culture were filtered through GF/C glass microfiber filters; re-suspended in 90% (v/v) methanol and incubated at 70ºC for 20 minutes; the supernatants were collected by centrifugation (MSE Sanyo Harrier 18/80) at 6000 rpm for five minutes. Chlorophyll *a* was calculated according to the following equation (1):

$$\text{Chlorophyll } a \text{ (mg.L}^{-1}) = \frac{[11.6(A665–A750)–1.31(A645–A750)–0.14(A630–A750) \times \text{methanol (mL)}]}{[\text{Sample (L)} \times 1/\text{path of cuvette}]} \quad (1)$$

For immobilised cells, Ca^{2+} alginates were dissolved in 7% sodium bicarbonate.

GenBank accession number

A GenBank accession number for nucleotide sequence of green microalga *Carteria crucifera* AARL G045 is MN223983, and its bacterial flora *Agrobacterium tumefaciens* G045-01 and *Aeromicrobium kwangyangensis* G045-02 are MN220636 and MN220637, respectively.

RESULTS AND DISCUSSION

Identification of *Carteria crucifera* AARL G045 bacterial flora in the algal culture

After serial dilution and purification, two bacterial flora strains were isolated from *Carteria* culture by LB-JM medium agar. Those strains were named G045-01 and G045-02. From phenotype observation, bacterial flora strain G045-01 is a rod gram-negative bacterium, which form small white colony, convex, circular with an entire edge. Second strain, G045-02 is a rod gram-positive bacterium, which form big yellow colony, flat, circular with an entire edge. Two isolated bacterial floras were identified by sequencing of their 16S rDNA gene and phylogenetic tree, respectively. The results exposed that G045-01 showed high similarity up to 99% with that of *Agrobacterium tumefaciens* and G045-02 was closely related to *Aeromicrobium kwangyagenesis* with 99% similarity, based on the GenBank data (Fig. 4). Typically, *Agrobacterium tumefaciens* (*Rhizobium radiobacter*) is a widespread naturally occurring soil bacterium,

gram-negative, aerobic, rod-shaped, motile and often associated with plants, which causes the crown gall disease in various dicotyledonous plants (Guo et al., 2017), whereas *Aeromicrobium* is a gram-positive, aerobic, rod-shaped, non-motile actinomycete strain (Niu et al., 2015).

In routine culture, mutualistic bacterium is usually observed in algal culture and its secretion has a tendency to promote algal growth. Previously, the studies have exposed that several bacteria have the capacity to enhanced hydrogen production in algae-bacterium co-cultivation (Li et al., 2013; Wirth et al., 2015; Xu et al., 2016; Lakatos et al., 2017; Ban et al., 2018). Thus, bacterial flora in *Carteria crucifera* AARL G045 cultivation may assist to enhanced hydrogen production in *Carteria*-bacterium co-cultivation.

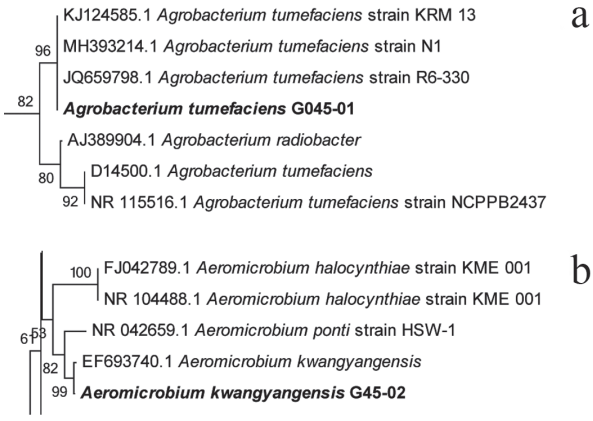

Fig. 4. Phylogenetic analysis of bacteria isolated from *Carteria crucifera* AARL G045 culture base on 16S rDNA sequences. Phylogenetic tree was constructed by MEGA programme using the Neighbor joining method. a – *Agrobacterium tumefaciens* G045-01, b – *Aeromicrobium kwangyangensis* G045-02

Hydrogen production in *Carteria crucifera* AARL G045 under photoautotrophic and photo-fermentation conditions

Biohydrogen gas from microalga in headspace of the experiment bottles was monitored every 24 hours. The gas content in the headspace was hydrogen, nitrogen and carbon dioxide. Hydrogen production under photoautotrophic condition (JM-S) had higher levels than that of photo-fermentation condition (TAP-S) during 72 hours of the cultivation. The highest hydrogen accumulation in JM-S was 4.55 mL L^{-1} culture (203 µmol H_2) (Fig. 5). Since the two-stage bio-photosynthesis was used for temporal separation of oxygen by sulphur-deprivation, lacking sulphur had direct effect on two essential amino acids in D1 protein (methionine and cysteine) synthesis. After that, D1 protein, the major protein in photosystem II reaction centre, was damaged, which decreased oxygen production and the condition become an anaerobic state (Melis et al., 2000; Srirangan et al., 2011). Then, hydrogenase enzyme could function. Some studies have reported that hydrogenase activity may be frequently noticeable after 3–4 hours under anaerobic conditions (Forestier et al., 2003). In this study, higher level of hydrogen production was observed under photoautotrophic conditions (Jaworski's medium). Since each species of microalgae is suitable for different medium and culture conditions, they also have a variety of biochemical mechanisms and different physiological processes. Thus, each species of microalgae may have different photosynthesis and hydrogen production (Chader et al., 2009). As a result, *Carteria crucifera* AARL G045 grew well in Jaworski's medium under photoautotrophic conditions and high level of hydrogen production was revealed as well. The results are supported by several investigations suggesting that photoautotrophic condition may promote hydrogen production in various green microalgae. However, it is also important to note that each microalga has a different suitable medium for hydrogen production (Song et al., 2011; Hwang et al., 2014; Duangjan et al., 2017; Pholchan et al., 2017). Concerning the economic and upscale production, the direct biophotolysis is more advantageous as the media does not require any organic carbon source thereby obtaining a low-cost medium (Duangjan et al., 2017; Nagarajan et al., 2017). Amount of oxygen in culture medium and algal growth was also demonstrated during 72 hours of inoculation. The results showed significantly decreasing level of oxygen in TAP-S medium ($p < 0.05$), whereas significantly increasing level of oxygen in JM-S medium ($p < 0.05$) was observed (Fig. 6). The previous study has demonstrated that acetate substance has a direct effect on photo-system II (PSII). The cultivation of *Chlamydomonas reinhardtii* under mixotrophic conditions in acetate-supplemented media (TAP medium) has revealed that the O_2 level in thylakoids of mixotrophic *C. reinhardtii* is less than that in photo-

autotrophic cultures (Roach et al., 2013). As a result, acetate in TAP-S medium may have the same effect on *Carteria crucifera* AARL G045, which belongs to the same taxonomic order of Chlamydomonadales. Even though a higher level of hydrogen production was observed under photoautotrophic condition (JM-S medium), oxygen gas could also be detected. This result corresponds to the report by Hwang and co-researchers, who have demonstrated that the H_2ase gene (HYDA) of some eukaryotic microalgae could be expressed and detected even at high concentrations of oxygen (≥ 21%, initially atmospheric condition) (Hwang et al., 2014). Additionally, the reduction of oxygen by co-culture with bacteria may enhance hydrogen production. Consequently, Jaworski's medium was designated for hydrogen production by co-culture *Carteria crucifera* AARL G045 with bacterial flora in the next step.

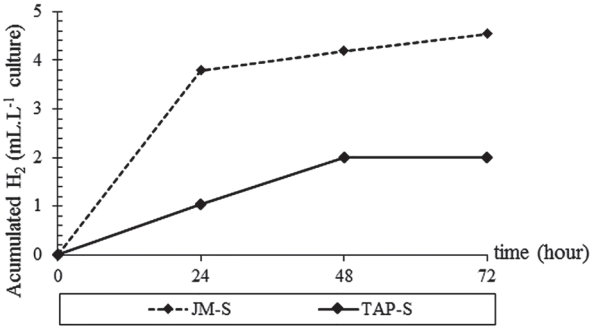

Fig. 5. Accumulated hydrogen production from the cultivation of *Carteria crucifera* AARL G045 in sulphur-deprived Jaworski's medium (JM-S) and sulphur-deprived Tris acetate phosphate medium (TAP-S)

Co-culture of *Carteria crucifera* AARL G045 and isolated bacterial flora for H_2 production

The microalga *Carteria crucifera* AARL G045 was co-cultured with its isolated bacterial flora, *Agrobacterium tumefaciens* G045-01 and *Aeromicrobium kwangyangensis* G045-02 in JM-S culture medium and investigated for potential H_2 production. It was found that hydrogen production start producing at the first 24 hours and the highest accumulated H_2 was recognized as 6.58 mL L^{-1} (293.75 μmol H_2) in the co-culture with *A. kwangyangensis* G045-02, which was higher level of H_2 than that of the single algal culture by about 1.32 times (Fig. 7). Moreover, the results showed significantly decreasing level of oxygen in the co-culture with *A. kwangyangensis* G045-02 ($p < 0.05$), whereas significantly increasing level of oxygen in single microalga ($p < 0.05$) (Fig. 7). *Aeromicrobium* is an aerobic bacterium that is commonly isolated from the environment, including soil and water (Miller et al., 1991; Lee & Lee, 2008). Earlier studies have noted that increased respiration rate or O_2 consumption of co-cultured bacterial strains is the main reason for enhancement of H_2 yield. It is corresponding with our study, in which we found that the lowest of dissolved oxygen was observed in co-culture algae with *A. kwangyangensis* G045-02 (Fig. 7). This result is in accordance with several other studies. According to Li and co-researchers, co-culturing of *C. reinhardtii* strain with *Stenotrophomonas maltophilia* strain 776 and *Pseudomonas* sp. A8 also provides higher hydrogen production by about four times more than single algal culture together with the lowest oxygen content (Li

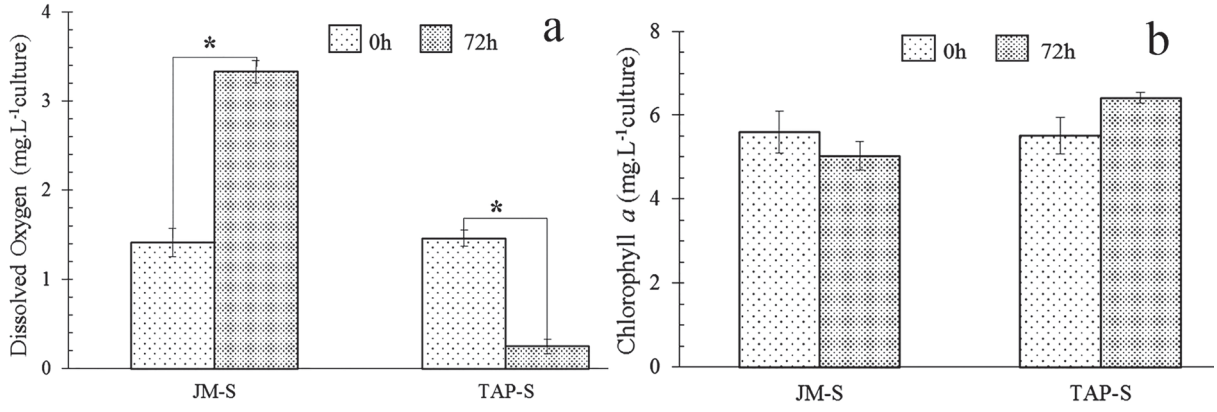

Fig. 6. Measurement of dissolved oxygen and algae growth in cultivation of *Carteria crucifera* AARL G045 for hydrogen production in sulphur-deprived Jaworski's (JM-S) and sulphur-deprived (TAP-S) Tris acetate phosphate medium. **a** – dissolved oxygen in culture medium, **b** – chlorophyll *a*, * – significant difference by the Paired Samples Test

et al., 2013). Moreover, several bacterial strains have been considered to co-culture with algae to enhance H_2 yield, including *Bradyrhizobium japonicum*. This strain is known to improve the H_2 yield by increasing the biomass, respiration rates, hydrogenase activity and starch content of pure algae cultures. Co-culturing with this bacterium provides maximum hydrogen production by about 17-fold of the pure algal culture (Xu et al., 2017). Although algal strain is the most important for hydrogen production, gas-to-liquid phase ratio, algal optical density and algal size are crucial factors that influence hydrogen production (Lakatos et al., 2017). Moreover, optimized condition of microalga and bacterium co-cultivation may enhance more H_2 production as well as immobilisation. An immobilised technique has been reported for greater hydrogen production in microalgae (Saifuddin & Priatharsini, 2016). Thus, in our study, this technique was reported to help produce higher hydrogen gas.

Co-culture of *Carteria crucifera* AARL G045 and the selected bacterial flora with immobilised and free cells for H_2 production

In this study, immobilised and free cell cultivation was used for high levels of H_2 production, and *Aeromicrobium kwangyangensis* G045-02 was selected to co-culture with the microalga. Although other studies have reported that the immobilisation technique has an ability to support high producing H_2 yields, which is easier to handle, such as transferring to new medium, repeated for generated products, and protecting cells from outer environment (Moreno-Garrido, 2008), our result was in contrast. The highest involved H_2 was recognized from both free cells of algae and bacterial flora as 6.01 mL L^{-1} culture (268.30 µmol H_2) (Fig. 8). However, both immobilised algae and bacterial flora cells, free algae cells and immobilised bacterial flora cells, immobilised algae and free bacterial flora cells demonstrated lower involved hydrogen gas as 2.94, 2.10, and 0.66 mL L^{-1} culture, respectively (Fig. 8). The hydrogen gas was produced within first 24 hours in the treatments with inoculated free cell of alga and free cell of bacterium, immobilised alga and immobilised bacterium and free cell of alga and immobilised bacterium. However, H_2 production was detected only after 72 hours in the treatment with immobilised alga and free cell of bacterium.

In this study, initial dissolved oxygen in the culture medium was defined between 1.37–1.47 mL L^{-1}. However, after 72 hours of inoculation, only oxygen content in both free cells of alga and bacterial flora treatment significantly decreased to 0.37 mL L^{-1}($p < 0.05$) (Fig. 8), while the oxygen content of the other treatments significantly increased ($p < 0.05$), especially in the immobilised algal cells. Possibly due to the fundamental changes in the cellular metabolism, the increase of oxygen from the immobilised state has been reported by Hameed & Ebrahim (2007). Since hydrogen production in both alga and bacterial flora in free cells were greatly developed, it may be the synergic effect from bacterial growth and did not appear to be affected. The growths of microalga were not significantly different in most treatments, except in free cell of alga and immobilised bacterium. How-

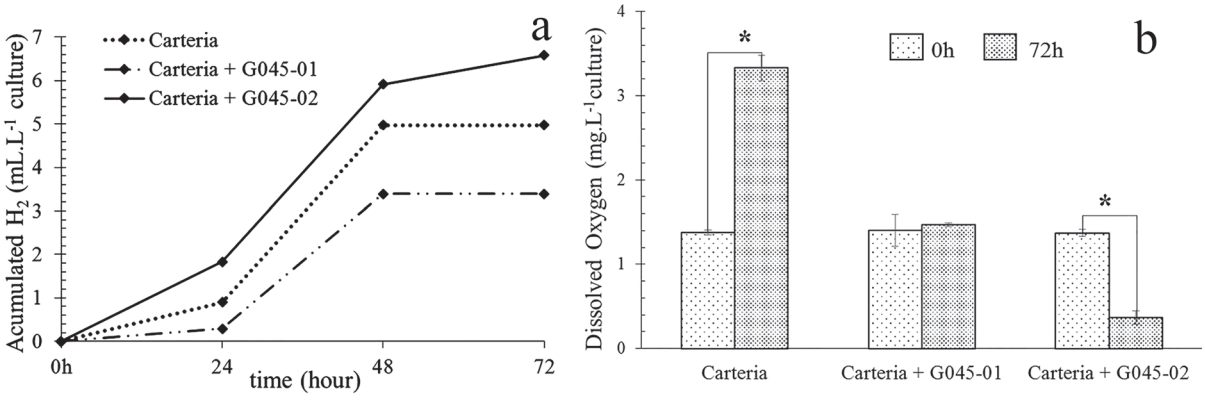

Fig. 7. Gas composition derived from inoculation of *Carteria crucifera* AARL G045 and bacterial flora in sulphur-deprived Jaworski's medium. **a** – accumulated hydrogen production, **b** – dissolved oxygen in culture medium, G045-01 – *Agrobacterium tumefaciens* G045-01, G045-02 – *Aeromicrobium kwangyangensis* G045-02, * – significant difference by the Paired Samples Test

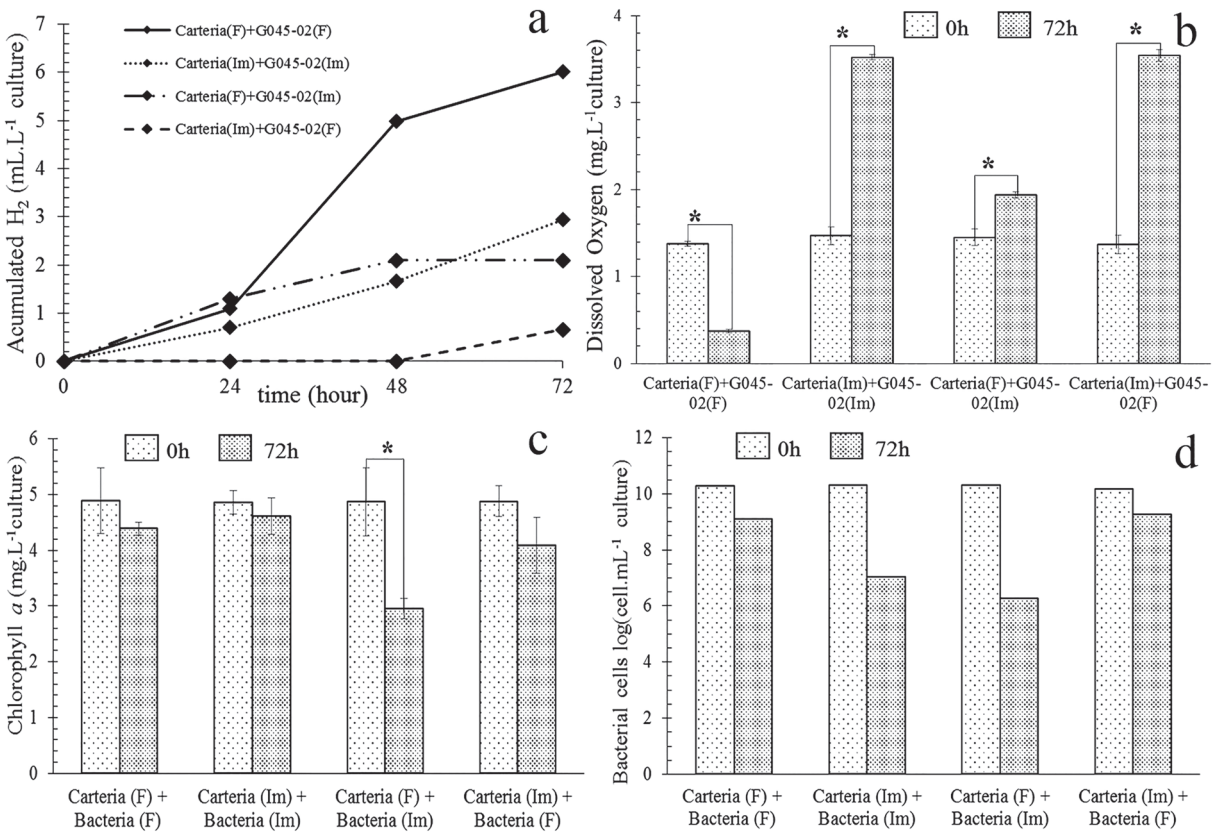

Fig. 8. Gas composition derived from inoculation of *Carteria crucifera* AARL G045 with *Aeromicrobium kwangyangensis* G045-02 in sulphur-deprived Jaworski's medium compared to algal and bacterial growth. **a** – accumulated hydrogen production, **b** – dissolved oxygen in culture medium, **c** – chlorophyll *a*, **d** – bacterial cells, Im – immobilised cell, F – free cell, * – significant difference by the Paired Samples Test

ever, it seems that bacterium *A. kwangyangensis* G045-02 did not prefer the immobilised condition, which was noticeable from decreasing cell number of bacterial cells (Fig. 8). According to the results, appropriate conditions of free microalga-bacterium co-cultivation may be considered to enhance hydrogen production.

CONCLUSIONS

Co-culture of microalga *Carteria crucifera* AARL G045 and its aerobic bacterial flora *Aeromicrobium kwangyangensis* AARL G045-02 generated higher level of hydrogen gas than that of a single algal culture by about 1.32 times. As a result, photoautotrophic condition was considered to be an alternative method to enhance hydrogen production from green microalga. Co-culture of both free cells of the microalga and the bacterium produced higher hydrogen than co-immobilisation. However, optimized condition of *Carteria* and bacterium flora co-cultivation may assist high hydrogen production.

ACKNOWLEDGEMENTS

This study was financially supported by the Biodiversity-Based Economy Development Office (Public organization) and the Post-Doctoral Fellowship 2018, Chiang Mai University, Thailand. We would like to thank Mr Russell Kirk Hollis, a lecturer at Chiang Mai University, the Faculty of Humanities, for linguistic assistance.

REFERENCES

Atif A.A.Y., Fakhru'l-Razi A., Ngan M.A., Morimoto M., Iyuke S.E., Veziroglu N.T., 2005: Fed batch production of hydrogen from palm oil mill

effluent using anaerobic microflora. – International Journal of Hydrogen Energy, 30: 1393–1397.

Atlas R.M., 2010: Handbook of microbiological media. – Florida.

Ban S., Lin W., Wu F., Luo J., 2018: Algal-bacterial cooperation improves algal photolysis-mediated hydrogen production. – Bioresource Technology, 251: 350–357.

Boonkhot P., Pekkoh J., Pumas C., 2015: Growth and biomass production of microalgae immobilized in different matrices. Proceeding of the 7th National Conference on Algae and Plankton (NCAP2015). Bangkok, Thailand, 25–27 March 2015: 14–26. – Bangkok.

Chader S., Hacene H., Agathos S.N., 2009: Study of hydrogen production by three of *Chlorella* isolated from the soil in the Algerian Sahara. – International Journal of Hydrogen Energy, 34(11): 4941–4946.

Das D., Veziroğlu T.N., 2001: Hydrogen production by biological processes: a survey of literature. – International Journal of Hydrogen Energy, 26: 13–28.

De Bashan L.E., Bashan Y., 2010: Immobilized microalgae for removing pollutants: review of practical aspects. – Bioresource Technology, 101: 1611–1627.

Duangjan K., Nakkhunthod W., Pekkoh J., Pumas C., 2017: Comparison of hydrogen production in microalgae under autotrophic and mixotrophic media. – Botanica Lithuanica, 23(2): 169–177.

Eroglu E., Melis A., 2011: Photobiological hydrogen production: Recent advances and state of the art. – Bioresource Technology, 102: 8403–8413.

Evans J.D., Chen Y.P, Cornman R.S., De La Rua P., Foret S., Foster L., Genersch E., Gisder S., Jarosch A., Kucharski R., Lopez D., Lun C.M., Moritz R.F.A., Maleszka R., Muñoz I., Pinto M.A., Schwarz R.S., 2013: Standard methods for molecular research in *Apis mellifera*. – Journal of Apicultural Research, 52(4): 1–54.

Faraloni C., Ena A., Pintucci C., Torzillo G., 2011: Enhanced hydrogen production by means of sulfur-deprived *Chlamydomonas reinhardtii* cultures grown in pretreated olive mill wastewater. – International Journal of Hydrogen Energy, 36: 5920–5931.

Forestier M., King P., Zhang L., Posewitz M., Schwarzer S., Happe T., Ghirardi M.L., Seibert M., 2003: Expression of two [Fe]-hydrogenases in *Chlamydomonas reinhardtii* under anaerobic conditions. – European Journal of Biochemistry, 270: 2750–2758.

Gaffron H., 1939: Reduction of CO_2 with H_2 in green plants. – Nature, 143: 204–205.

Guo M., Huang Z., Jing Yang J., 2017: Is there any crosstalk between the chemotaxis and virulence induction signalling in *Agrobacterium tumefaciens*? – Biotechnology Advances, 35: 505–511.

Hameed M.A., Ebrahim O.H., 2007: Biotechnological potential uses of immobilized algae. – International Journal of Agriculture and Biology, 9(1): 183–192.

Harris E.H., 1986: The *Chlamydomonas* Sourcebook: a Comprehensive Guide to Biology and Laboratory Use. – San Diego.

Hwang J., Kim H., Choi J., Abou-Shanab R.A.I., Dempsey B.A., Regan J.M., Kim J.R., Song H., Nam I., Kim S., Lee W., Park D., Kim Y., Choi J., Ji M., Jung W., Jeon B., 2014: Photoautotrophic hydrogen production by eukaryotic microalgae under aerobic conditions. – Nature communications, 1–6.

Janta K., Pekkoh J., Tongsiri S., Pumas C., Peerapornpisal Y., 2013: Selection of some native microalgal strains for possibility of bio-oil production in Thailand. – Chiang Mai Journal of Science, 40: 593–602.

Kosourov S.N., Seibert M., 2009: Hydrogen photoproduction by nutrient-deprived *Chlamydomonas reinhardtii* cells immobilized within thin alginate films under aerobic and anaerobic conditions. – Biotechnology and Bioengineering, 102(1): 50–58.

Lakatos G., Balogh D., Farkas A., Ördög V., Nagy P.T., Bíró T., Maróti G., 2017: Factors influencing algal photobiohydrogen production in algal-bacterial co-cultures. – Algal Research, 28: 161–171.

Lam M.K., Lee K.T., 2013: Biohydrogen production from algae. – In: Pandey A., Chang J., Hallenbeck P.C., Larroche C. (eds), Biohydrogen: 161–183. – San Diego.

Laurinavichene T.V., Fedorov A.S., Ghirardi M.L., Seibert M., Tsygankov A.A., 2006: Demonstration of sustained hydrogen photoproduction by immobilized, sulfur-deprived *Chlamydomonas*

reinhardtii cells. – International Journal of Hydrogen Energy, 31(5): 659–667.

Lee D.W., Lee S.D., 2008: *Aeromicrobium ponti* sp. nov., isolated from seawater. – International Journal of Systematic and Evolutionary Microbiology, 58: 987–991.

Li X., Huang S., Yu J., Wang O., Wu S., 2013: Improvement of hydrogen production of *Chlamydomonas reinhardtii* by co-culture with isolated bacteria. – International Journal of Hydrogen Energy, 38: 10779–10787.

Melis A., Zhang L., Forestier M., Ghirardi M.L., Seibert M., 2000: Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga *Chlamydomonas reinhardtii*. – Journal of Plant Physiology, 122: 127–135.

Miller E.S., Woese C.R., Brenner S., 1991: Description of the Erythromycin-Producing Bacterium *Arthrobacter* sp. Strain NRRL B-3381 as *Aeromicrobium erythreum* gen. nov., sp. nov. – International Journal of Systematic and Evolutionary Microbiology, 41(3): 363–368.

Moreno-Garrido I., 2008: Microalgae immobilization: Current techniques and uses. – Bioresource Technology, 99: 3949–3964.

Nagarajan D., Lee D., Kondo A., Chang J., 2017: Recent insights into biohydrogen production by microalgae from biophotolysis to dark fermentation. – Bioresource Technology, 227: 373–387.

Niu L., Xiong M., Tang T., Song L., Hu X., Zhao M., Zhang K., 2015: *Aeromicrobium camelliae* sp. nov., isolated from Púer tea. – International Journal of Systematic and Evolutionary Microbiology, 65: 4369–4373.

Pholchan M., Kaewseesuk K., Klayraung S., Sompong U., 2017: Effect of light intensities and atmospheric gas conditions on biohydrogen production of microalgae isolated from fisheries wastewater. – Environment and Natural Resources Journal, 15(2): 21–29.

Roach T., Sedoud A., Krieger-Liszkay A., 2013: Acetate in mixotrophic growth medium affects photosystem II in *Chlamydomonas reinhardtii* and protects against photoinhibition. – Biochimica et Biophysica Acta, 1827: 1183–1190.

Saifuddin N., Priatharsini P., 2016: Developments in bio-hydrogen production from algae: a review. – Research Journal of Applied Sciences, Engineering and Technology, 12(9): 968–982.

Saijo Y.A., 1975: Method for determination of chlorophyll. – Japanese Journal of Limnology, 36(3): 20–43.

Schlösser U.G., 1982: Sammlung von algenkulturen. – Berichte der Deutschen Botanischen Gesellschaft, 95(1): 181–276.

Song W., Rashid N., Choi W., Lee K., 2011: Biohydrogen production by immobilized *Chlorella* sp. using cycles of oxygenic photosynthesis and anaerobiosis. – Bioresource Technology, 102: 8676–8681.

Srirangan K., Pyne M.E., Chou C.P., 2011: Biochemical and genetic engineering strategies to enhance hydrogen production in photosynthetic algae and cyanobacteria. – Bioresource Technology, 102(18): 8589–8604.

Tanabe Y., Okazaki Y., Yoshida M., Matsuura H., Kai A., Shiratori T., Ishida K., Nakano S., Watanabe M.M., 2015: A novel alphaproteobacterial ectosymbiont promotes the growth of the hydrocarbon-rich green alga *Botryococcus braunii*. – Scientific Reports, 5: 10467.

Wirth R., Lakatos G., Maróti G., Bagi Z., Minárovics J., Nagy K., Kondorosi É., Rákhely G., Kovács K., 2015: Exploitation of algal-bacterial associations in a two-stage biohydrogen and biogas generation process. – Biotechnology for Biofuels, 8: 59.

Wu S., Li X., Wang Q., 2012: Increased hydrogen production in co-culture of *Chlamydomonas reinhardtii* and *Bradyrhizobium japonicum*. – Bioresource Technology, 123: 184–188.

Xu L., Li D., Wang Q., Wu S., 2016: Improved hydrogen production and biomass through the cocultivation of *Chlamydomonas reinhardtii* and *Bradyrhizobium japonicum*. – International Journal of Hydrogen Energy, 41: 9276–9283.

Xu L., Cheng X., Wang Q., 2017: Effect of cocultivation of *Chlamydomonas reinhardtii* with *Azotobacter chroococcum* on hydrogen production. – International Journal of Hydrogen Energy, 42: 22713–22719.

KULTIVUOJAMŲ SU BAKTERIJOMIS MIKRODUMBLIŲ *CARTERIA CRUCIFERA* AARL G045 FOTOAUTOTROFINĖ VANDENILIO PRODUKCIJA

Kritsana Duangjan, Watsamon Nakkhunthod, Chayakorn Pumas

Santrauka

Pirmą kartą buvo nustatyta, kad *Carteria crucifera* AARL G045 mikrodumblis, kultivuojamas su bakterijomis, turi didelį potencialą fotoautotrofinėmis sąlygomis gaminti bio-vandenilį. Tyrimai atlikti su imobilizuotomis kalcio alginato granulėmis ir laisvomis mikrodumblių ir bakterijų ląstelėmis, o vandenilio produkcija tirta fotoautotrofinėmis ir anaerobinės foto-fermentacijos sąlygomis. Dvi su *Carteria crucifera* AARL G045 kultūra asocijuotos bakterijos buvo išskirtos ir, atlikus 16S rDNR sekų analizę, identifikuotos kaip *Agrobacterium tumefaciens* G045-01 ir *Aeromicrobium kwangyangensis* G045-02. Rezultatai atskleidė, kad mikrodumblis *Carteria crucifera* AARL G045 ir bakterija *A. kwangyangensis* G045-02 auginami kartu vienoje kultūroje apie 1,32 karto padidino vandenilio dujų gamybą. Taip pat, abiejų neimobilizuotų bakterijų ir mikrodumblio ląstelių kultūros išskiriamo vandenilio kiekį padvigubino, lyginant su ko-kultivuojamomis imobilizuotomis ląstelėmis.