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COMPARISON OF HYDROGEN PRODUCTION IN MICROALGAE UNDER AUTOTROPHIC AND MIXOTROPHIC MEDIA

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Abstract

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Hydrogen is an alternative source of energy of considerable interest, because it is environmentally friendly. Biological hydrogen production processes involving green microalgae are of significant interest. However, until present only few microalgae genera have been studied and almost all of those studies have focused only on cultivation using mixotrophic or heterotrophic media, which are expensive, and can be easily contaminated. This study aimed to compare the potential of biohydrogen production from novel green microalgae under autotrophic and mixotrophic media. A total of ninety strains of six orders of green microalgae were investigated for their capabilities of hydrogen production. The results showed that eleven novel hydrogen-producing microalgae genera were found. The hydrogen production in each order was influenced by the medium. Moreover, several strains presented notable levels of autotrophic hydrogen production and performed at over twice of the mixotrophic medium. These results should be supportive information for the selection and cultivation of hydrogen-producing microalgae in further studies.

Keywords: Chlorophyta, hydrogen, photolysis, renewable energy, sulphur-deprivation.

INTRODUCTION

Hydrogen is considered the cleanest form of renewable fuel. Its combustion results only in water being emitted into the atmosphere. Moreover, hydrogen gas provides a high heating value and lower energy consumption (Brentner et al., 2010; Lam & Lee, 2013). Hydrogen can be produced through several processes including the electrolysis of water, the thermocatalytic reformation of hydrogen-rich organic compounds, and various biological processes. Nevertheless, biohydrogen production from photosynthetic microorganisms has the capability of reducing costs and environmental influences, because these microorganisms use only sunlight as an energy source to split water in the production of H_2 and O_2 (Eroglu & Melis, 2011). Biohydrogen production can be established by several processes, i.e. the biophotolysis of water using algae and cyanobacteria, the photodecomposition of organic compounds by photosynthetic bacteria, fermentative hydrogen production from organic compounds, and the hybrid systems that use photosynthetic and fermentative bacteria (Das & Veziroğlu, 2001). Primarily, *Scenedesmus obliquus* (Turpin) Kützing, unicellular green microalga, has been reported for the capabilities of hydrogen production under anaerobic conditions (Gaffron, 1939). Nowadays, several strains of green algae have been identified for high biohydrogen production, especially *Chlamydomonas reinhardtii* Dangeard (Melis et al., 2000; Kosourov & Seibert, 2009; Faraloni et al., 2011). The biohydrogen production achieved with

microalgae is affected by several cultivation factors such as light intensity, carbon sources, nutrients, pH, temperature and atmosphere gas composition (Pholchan et al., 2017). Furthermore, the hydrogenase enzyme is an important factor in generating hydrogen through photosynthesis (Lam & Lee, 2013). Nevertheless, oxygen gas is an inhibitor of the hydrogenase enzyme activity and also a suppressor to hydrogenase gene expression. Thus, the process of supplementation with inert argon gas to eliminate O_2 until 0.1% or lower (Ghirardi et al., 2009; Melis et al., 2007) combined with cultivation under sulphur deficiency to activate PSII-dependent O_2-activity due to the inhibition of D1 protein synthesize that it is known for (Wykoff et al. 1998), are necessary to decrease oxygen during photosynthesis. However, the hydrogen production potential may be diverse and may depend on the relevant taxonomic groups.

In Thailand, biohydrogen involving various green microalgae such as *Carteria* sp. AARL G045, *Chlamydomonas* sp., *Chlorella lewinii* Bock et al., *Chlorella sorokiniana* Shihira & Krauss, *Chlorella* sp., *Coelastrella* sp., *Micractinium* sp., *Monoraphidium* sp., *Pediastrum duplex*, *Scenedesmus* sp. AARL G022, *Scenedesmus* sp. KMITL-01, *Scenedesmus* sp., *Tetraspora* sp. CU2551, and some green algae obtained from rice paddy fields (Maneeruttanarungroj et al., 2010; Rattana et al., 2010; Pongpadung et al., 2015; Rameshprabu et al., 2015; Pholchan et al., 2017) is of significant interest. Most of these processes have only focused on hydrogen production under mixotrophic or heterotrophic conditions. However, the organic carbon sources supplied under these conditions are costly and can be easily contaminated. Thailand has a high diversity of green microalgae. Approximately one hundred strains belonging to several orders of the green microalgae were isolated and maintained in the culture collection of the Applied Algal Research Laboratory, Chiang Mai University, but many strains have yet to be investigated for their capabilities of biohydrogen production. In addition, previous studies have reported that a certain microalgal strain could produce hydrogen using autotrophic medium (Song et al., 2011; Hwang et al., 2014; Pholchan et al., 2017), which could address the problems associated with mixotrophic or heterotrophic cultures. Thus, this study aims to compare the potential of biohydrogen production from various strains of green microalgae using autotrophic and mixotrophic media. This information will assist both the culture-screening and medium-selection processes for hydrogen-producing microalgae in further studies.

MATERIALS AND METHODS

Microalgae samples

A total of 90 green microalgae were obtained from the Applied Algal Research Laboratory of the Department of Biology of the Faculty of Science of the Chiang Mai University, Thailand. All microalgae were grown autotrophically under Jaworski's medium (JM) (Stein, 1973) and mixotrophically using Tris Acetate Phosphate Medium (TAP) (Harris, 1989) at 25°C under the illumination of a white fluorescent lamp (30.8 µmol m^{-2} s^{-1}), under continuous shaking. Microalgal growth was measured in terms of optical density at 665 nm by a spectrophotometer (GENESYS™ 20 Visible Spectrophotometer) every second day until the optical density reached 1.0 or the stationary phase.

Screening for biohydrogen production

The cells were transferred to fresh sulphur-deprived JM (JM-S) medium or sulphur-deprived TAP (TAP-S) medium. They were grown at 25°C under illumination of a white fluorescent lamp (30.8 µmol m^{-2} s^{-1}), under continuous shaking for 24 hours. Cells were harvested by centrifugation at 4500 rpm, washed three times with fresh JM-S medium or TAP-S medium and re-suspended in the same medium. Microalgae were transferred to 60 ml sealed sterile vial serum bottles with 50 ml of sulphate-deprived medium (Fig. 1). Oxygen was eliminated by flushing the bottles with argon gas. The vials were incubated under a light intensity of 54 µmol m^{-2} s^{-1} at 25°C by manual shaking being applied twice a day.

Fig. 1. Microalgae cultivation in 60 ml sealed sterile vial serum bottle for hydrogen production

Measurement of biohydrogen production

Hydrogen production in the headspace of the vials 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. Hydrogen production was calculated in terms of the amount of hydrogen evolved per chlorophyll content per time (μmol H_2 mg Chl a^{-1} h^{-1}) (Maneeruttanarungroj et al., 2010; Rameshprabu et al., 2015). The percentage difference between H_2 production using JM-S and TAP-S was calculated through the use of the following equation:

(H_2 production of JM-S – H_2 production of TAP-S)/ H_2 production of JM-S * 100 (1)

The percentage difference value ≥50 indicated over double the level of hydrogen production of JM-S when compared to that of TAP-S, while the value ≤ –100 indicated over double the level of hydrogen production of TAP-S when compared to JM-S.

Measurement of chlorophyll content

Chlorophyll in the cell cultures was extracted using 90% methanol according to Becker (1994) and Chlorophyll *a* was calculated using the following equation:

$$\text{Chlorophyll } a \text{ (mg/L)} = \frac{(11.6(A_{665}-A_{750})-1.31(A_{645}-A_{750})-0.14(A_{630}-A_{750})}{\text{Sample (L)} \times 1/\text{path of cuvette}} \times \text{methanol (mL)}$$

Analysis of similarity (ANOSIM)

Significant differences in the overall hydrogen production of both JM-S and TAP-S within the order were analysed using the analysis of similarity (ANOSIM) in the R package vegan (Dixon, 2003).

RESULTS AND DISCUSSIONS

A total of ninety strains of green microalgae obtained from the Applied Algal Research Laboratory, classified into six orders, i.e. Chaetophorales (1 strain), Chlorellales (7 strains), Sphaeropleales (63 strains), Ulotrichales (1 strain), Volvocales (6 strains) and Zygnematales (12 strains), were investigated for their hydrogen production capacities in autotrophic and mixotrophic media. The results showed that all of the examined microalgae could produce biohydrogen at levels of 0.12–0.71 μmol H_2 mg Chl a^{-1} h^{-1} (Table 1). The biohydrogen production achieved using microalgae is affected by several cultivation factors such as culture media, the nutrients and the carbon sources along with the relevant culture conditions including light intensity, pH, temperature and atmospheric gas composition. The previous study has reported that the different levels of hydrogen production are dependent on the relevant strains and trophic conditions (Yu & Takahashi, 2007). Our results revealed that 57% of the microalgae investigated in this study favoured autotrophic medium in generating biohydrogen (Table 2). Of these, seven strains represented double the level of biohydrogen production using JM-S medium compared to those using TAP-S medium (% difference ≥50 in Table 1), such as *Acutodesmus dimorphus* AARL G021, *A. obliquus* AARL G090, *Coelastrum indicum* AARL G043, *Desmodesmus armata* var. *bicaudatus* AARL G019, *D. armata* var. *bicaudatus* AARL G025, *D. maximus* AARL G026 and *Pandorina morum* AARL G010. Although only a few isolates revealed the ability to achieve double the level of biohydrogen production using TAP-S medium compared to those using JM-S medium (% difference ≤ –100 in Table 1), including *Selenastrum bibraianum* AARL G052, *Chlamydomonas* sp. AARL G031, *Pandorina* cf. *charkowiensis* AARL G005 and *Staurastrum* sp. AARL G057, the highest level of biohydrogen production was achieved using the mixotrophic medium (TAP-S) as 0.71 μmol H_2 mg Chl a^{-1} h^{-1}.

The results of this study are in agreement with those of other studies. TAP medium could promote hydrogen production among various microalgae such as *Chlamydomonas reinhardtii*, *Chlorella sorokiniana* Ce, *Carteria* AARL G045 and *Tetraspora* sp. CU2551 (Melis et al., 2000; Chader et al., 2009; Maneeruttanarungroj et al., 2010; Nakkhunthod et al. 2015).

Even though hydrogen production achieved through the use of microalgae has been reported since 1939 (Gaffron, 1939), up to now only some genera have been studied. In this study, several gen-

Table 1. Comparison of hydrogen production from 90 strains of green microalgae in JM-S and TAP-S media

Green microalgae	H_2 production (μmol H_2 mg Chl a^{-1} h^{-1})		Difference, %*
	JM-S	TAP-S	
Order Chaetophorales			
Stigeoclonium sp. AARL G030	0.32	0.27	15.63
Order Chlorellales			
Actinastrum gracillimum AARL G033	0.39	0.28	28.21
Chlorella sp. AARL G014	**0.46****	0.49	–6.52
Chlorella sp. AARL G017	0.31	0.36	–16.13
Chlorella sp. AARL G049	0.18	0.19	–5.56
Dictyosphaerium cf. *ehrenbergianum* AARL G004	0.28	0.21	25.00
Dictyosphaerium sp. AARL G008	0.38	0.34	10.53
Micractinium sp. AARL G009	0.22	0.26	–18.18
Order Sphaeropleales			
Acutodesmus acuminatus AARL G092	0.34	0.18	47.06
Acutodesmus acuminatus AARL G107	0.32	0.29	9.38
Acutodesmus dimorphus AARL G021	0.34	0.08	**76.47**
Acutodesmus dimorphus AARL G091	0.31	0.30	3.23
Acutodesmus obliquus AARL G022	0.27	0.21	22.22
Acutodesmus obliquus AARL G084	0.30	0.31	–3.33
Acutodesmus obliquus AARL G090	0.35	0.16	**54.29**
Acutodesmus obliquus AARL G093	0.34	0.19	44.12
Acutodesmus obliquus AARL G100	0.38	0.30	21.05
Acutodesmus obliquus AARL G104	0.30	0.28	6.67
Acutodesmus obliquus AARL G105	0.34	0.29	14.71
Acutodesmus obliquus AARL G106	0.27	0.25	7.41
Acutodesmus obliquus AARL G108	0.18	0.16	11.11
Coelastrum indicum AARL G043	0.31	0.15	**51.61**
Coelastrum microporum AARL G007	0.26	0.19	26.92
Desmodesmus armatus var. *bicaudatus* AARL G019	0.30	0.15	**50.00**
Desmodesmus armatus var. *bicaudatus* AARL G025	0.26	0.10	**61.54**
Desmodesmus armatus AARL G083	0.26	0.27	–3.85
Desmodesmus armatus AARL G109	0.33	0.27	18.18
Desmodesmus communis AARL G072	0.24	0.27	–12.50
Desmodesmus communis AARL G075	0.28	0.31	–10.71
Desmodesmus communis AARL G076	0.23	0.27	–17.39
Desmodesmus communis AARL G077	0.27	0.29	–7.41
Desmodesmus communis AARL G086	0.22	0.26	–18.18
Desmodesmus communis AARL G088	0.27	0.28	–3.70
Desmodesmus communis AARL G099	0.22	0.29	–31.82
Desmodesmus communis AARL G115	0.12	0.21	–75.00
Desmodesmus denticulatus AARL G024	0.39	0.29	25.64
Desmodesmus denticulatus AARL G050	0.29	0.35	–20.69
Desmodesmus denticulatus AARL G081	0.34	0.24	29.41
Desmodesmus hystrix AARL G080	0.30	0.24	20.00
Desmodesmus maximus AARL G026	0.24	0.11	**54.17**
Desmodesmus maximus AARL G051	0.21	0.20	4.76
Desmodesmus maximus AARL G071	0.23	0.29	26.09
Desmodesmus maximus AARL G098	0.28	0.24	14.29
Desmodesmus opoliensis AARL G089	0.29	0.24	17.24
Desmodesmus opoliensis AARL G094	0.34	0.25	26.47
Desmodesmus opoliensis AARL G095	0.27	0.25	7.41

Green microalgae	H_2 production (μmol H_2 mg Chl a^{-1} h^{-1})		Difference, %*
	JM-S	TAP-S	
Desmodesmus opoliensis AARL G096	0.24	0.25	–4.17
Desmodesmus opoliensis AARL G101	0.21	0.28	–33.33
Desmodesmus opoliensis AARL G102	0.30	0.34	–13.33
Desmodesmus perforatus AARL G027	0.33	0.21	36.36
Desmodesmus perforatus AARL G112	0.32	0.30	6.25
Desmodesmus sp. AARL G078	0.29	0.29	0.00
Desmodesmus sp. AARL G110	0.33	0.27	18.18
Dimorphococcus lunatus AARL G048	0.27	0.34	–25.93
Monoraphidium cf. *obtusum* AARL G016	0.35	0.24	31.43
Pectinodesmus pectinatus AARL G097	0.34	0.32	5.88
Pediastrum boryanum AARL G062	0.20	0.25	–25.00
Pediastrum boryanum var. *boryanum* AARL G117	0.33	0.32	3.03
Pediastrum duplex var. *clathratum* AARL G125	0.33	0.27	18.18
Pediastrum duplex var. *duplex* AARL G060	0.28	0.18	35.71
Pediastrum duplex var. *rugulosum* AARL G123	0.30	0.31	–3.33
Pediastrum duplex var. *subgranulatum* AARL G124	0.32	0.35	-9.37
Pediastrum simplex var. *simplex* AARL G061	0.34	0.30	11.76
Pediastrum simplex var. *sturmii* AARL G127	0.20	0.26	–30.00
Pediastrum simplex var. *sturmii* AARL G128	0.33	0.34	–3.03
Pediastrum tetras AARL G063	0.37	0.27	27.03
Scenedesmus acunae AARL G087	0.29	0.32	–10.34
Scenedesmus obtusus AARL G020	0.38	0.33	13.16
Selenastrum bibraianum AARL G052	0.28	**0.71****	**–153.57**
Selenastrum bibraianum AARL G114	0.34	0.25	26.47
Verrucodesmus verrucosus AARL G079	0.28	0.23	17.86
Order Ulotrichales			
Ulothrix cf. *tenerrima* AARL G029	0.26	0.31	–19.23
Order Volvocales			
Carteria cf. *micronucleolata* AARL G044	0.35	0.34	2.86
Carteria cf. *micronucleolata* AARL G045	0.36	0.34	5.56
Carteria cf. *micronucleolata* AARL G046	0.23	0.12	47.83
Chlamydomonas sp. AARL G031	0.20	0.42	**–110.00**
Pandorina cf. *charkowiensis* AARL G005	0.26	0.54	**–107.69**
Pandorina morum AARL G010	0.38	0.19	**50.00**
Order Zygnematales			
Closterium ehrenbergii AARL G056	0.17	0.16	5.88
Closterium moniliferum AARL G041	0.38	0.28	26.32
Closterium moniliferum AARL G055	0.22	0.22	0.00
Cosmarium lundellii AARL G053	0.24	0.30	–25.00
Cosmarium lundellii AARL G054	0.16	0.21	–31.25
Cosmarium sp. AARL G113	0.15	0.24	–60.00
Euastrum denticulatum AARL G001	0.21	0.23	–9.52
Gonatozygon aculeatum AARL G047	0.16	0.28	–75.00
Staurastrum muticum AARL G116	0.38	0.31	18.42
Staurastrum tetracerum AARL G011	0.37	0.34	8.11
Staurastrum sp. AARL G057	0.17	0.39	**–129.41**
Staurodesmus cuspidatus AARL G059	0.25	0.41	–64.00

*Difference, % ≥ 50 indicates over double hydrogen production of JM-S compared to TAP-S (underline), ≤ –100 indicates over double hydrogen production of TAP-S compared to JM-S (double underline), ** imply to the highest hydrogen production in each medium.

Table 2. Comparison of hydrogen production in autotrophic and mixotrophic media of the various orders of green microalgae

Order	Number of strains				Percentage	
	JM-S higher	TAP-S higher	Equal	Sum	JM-S higher	TAP-S higher
Chaetophorales	1	0	0	1	100	0
Chlorellales	3	4	0	7	43	57
Sphaeropleales	39	23	1	63	62	37
Ulotrichales	0	1	0	1	0	100
Volvocales	4	2	0	6	67	33
Zygnematales	4	7	1	12	33	58
Total	51	37	2	90	57	41

era of microalgae were reported for the first time in terms of their capabilities of hydrogen production, including *Actinastrum*, *Closterium*, *Cosmarium*, *Dictyosphaerium*, *Dimorphococcus*, *Euastrum*, *Pandorina*, *Selenastrum*, *Staurastrum*, *Stigeoclonium* and *Ulothrix*. Surprisingly, many of these displayed potent capabilities of biohydrogen production, for instance *Selenastrum bibraianum* AARL G052, *Pandorina* cf. *charkowiensis* AARL G005 and *Staurastrum* sp. AARL G057 (Fig. 2). Compared to the degree of hydrogen productivity reported in other studies, the hydrogen productivity levels of microalgae presented in this study were determined to be un-equivalent. The other studies have determined that the genus *Chlorella* and the genus *Chlamydomonas* are of significant interest for their high biohydrogen productivity under conditions of nutrient deficiency. Notably, *Chlorella sorokiniana* KU204 could produce up to 1.3 mL of H_2 L^{-1} h^{-1} under N-limitation conditions and when using TAP-S medium (Pongpadung et al., 2015), *Chlamydomonas reinhardtii* presented 4.3 mL of H_2 L^{-1} h^{-1} when TAP-S medium was used (Laurinavichene et al., 2006), while *C. reinhardtii* cc124 showed up to 12.5 mmol of H_2 mg Chl a^{-1} h^{-1} under the same conditions (Kosourov & Seibert, 2009). However, the levels of hydrogen production in this study have not been optimized yet. The optimal culture conditions such as medium composition and nutrient stress and environmental factors should be considered in order to fulfil the obligations of biohydrogen production for the potent strains being described in this research.

The overall hydrogen production potential, when using both autotrophic and mixotrophic media of the chlorophycean distinguished by the orders, was evaluated by the analysis of similarities (ANOSIM). The results demonstrated that significantly different levels of hydrogen production within each order were found (p = 0.001) (Fig. 3). The microalgae in the order Chlorellales and the order Volvocales tended to expose high H_2 production levels, which differed from those of the order Sphaeropleales and the order Zygnematales. However, there was some overlap ($0.25 < R < 0.5$). This result corresponds with the findings of the previous studies, which reported that *Chlamydomonas* belonging to the order Volvocales, and *Chlorella* belonging to the order Chlorellales are

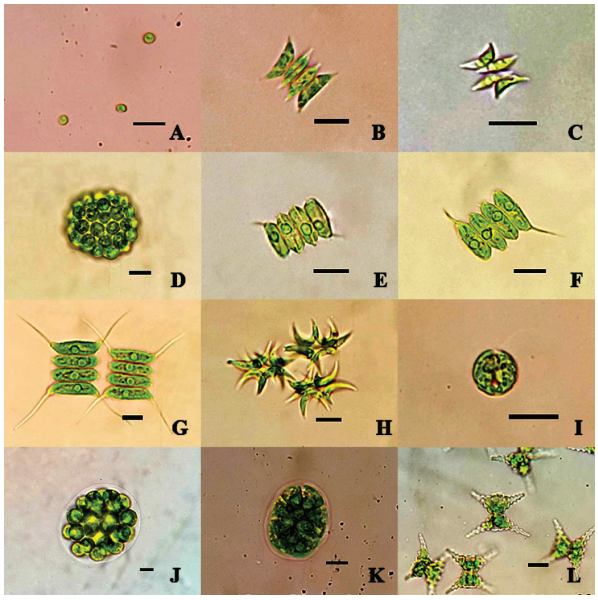

Fig. 2. Green microalgae – a potential biohydrogen producers: (A) *Chlorella* sp. AARL G014, (B) *Acutodesmus dimorphus* AARL G021, (C) *A. obliquus* AARL G090, (D) *Coelastrum indicum* AARL G043, (E) *Desmodesmus armatus* var. *bicaudatus* AARL G019, (F) *D. armatus* var. *bicaudatus* AARL G025, (G) *D. maximus* AARL G026, (H) *Selenastrum bibraianum* AARL G052, (I) *Chlamydomonas* sp. AARL G031, (J) *Pandorina* cf. *charkowiensis* AARL G005, (K) *P. morum* AARL G010, (L) *Staurastrum* sp. AARL G057 (scale bar = 10 μm)

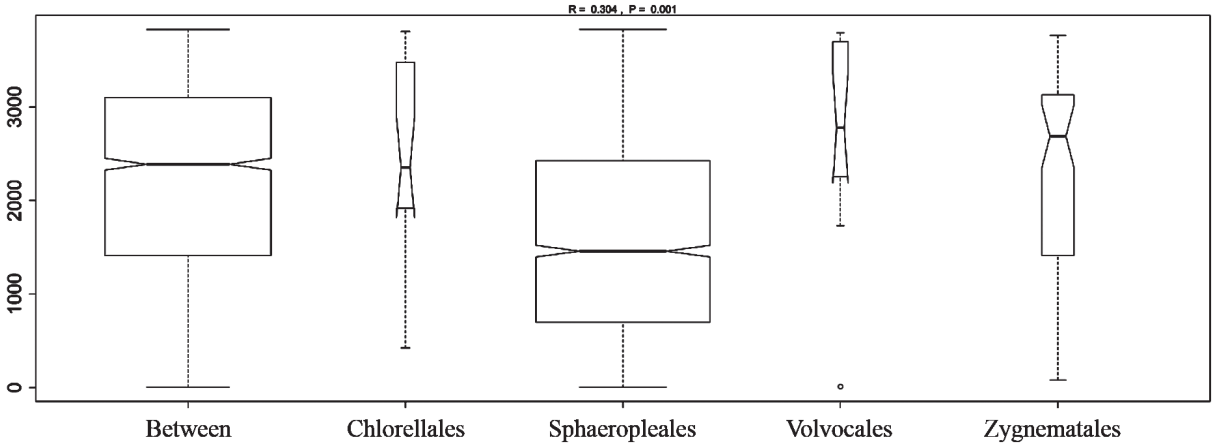

Fig. 3. Comparison of biohydrogen production between four orders of green microalgae: Chlorellales, Sphaeropleales, Volvocales, and Zygnematales, using the analysis of similarities (ANOSIM)

the most promising strains of microalgae for hydrogen production. However, the other species obtained from different chlorophycean orders presented in this study are also note-worthy in terms of their abilities of being cultivated in JM-S medium.

To summarize, the hydrogen production in ninety strains of green microalgae was investigated by using autotrophic and mixotrophic media. Microalgae were found to be able to produce biohydrogen under light conditions of sulphur deficiency in Jaworski's medium (JM-S) and under conditions of sulphur deficiency in Tris Acetate Phosphate Medium (TAP-S). Of these, eleven novel hydrogen producing microalgal genera were reported. The promising microalgal orders to be noticed were Chlorellales and Volvocales. However, it is also important to note that a suitable medium should be considered for each microalgal order or genus. Thus, these data should be considered advantageous for the discovery and cultivation of hydrogen-producing microalgae in further studies.

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AUTOTROFINĖMIS IR MIKSOTROFINĖMIS SĄLYGOMIS AUGINAMŲ MIKRODUMBLIŲ VANDENILIO GAMYBOS PALYGINIMAS

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Santrauka

Vandenilis kaip energijos nešėjas yra vienas perspektyviausių ekologiškų ateities energijos šaltinių. Pastaruoju metu ypač išaugo susidomėjimas biologine vandenilio gamyba pasitelkiant žaliadumblius, kadangi tik keletas šios dumblių grupės rūšių yra ištirta. Taip pat, mikrodumbliai buvo auginami miksotrofinėmis ar heterotrofinėmis sąlygomis, kas lemia didelius gavybos kaštus ir dažną kultūrų užsikrėtimą. Atliktais tyrimais buvo siekiama ištirti vandenilio gamybą auginant dar netirtas žaliadumblių rūšis autotrofinėmis ir miksotrofinėmis mitybos sąlygomis. Iš viso buvo analizuojama devyniasdešimt žaliadumblių rūšių, priklausančių šešioms eilėms. Tyrimų rezultatai atskleidė vienuolika naujų vandenilio gavybai perspektyvių žaliadumblių rūšių. Skirtingoms eilėms priklausančių žaliadumblių vandenilio gamyba skyrėsi priklausomai nuo auginimo terpės. Keletas kamienų išsiskyrė didesnėmis vandenilio gamybos savybėmis auginant juos autotrofinėje terpėje, tačiau produkavo dvigubai didesnius vandenilio kiekius jas auginant miksotrofinėmis sąlygomis. Tyrimo rezultatai yra svarbūs pasirenkant perspektyvias vandenilio gamybai mikrodumblių rūšis bei jų tolesniam auginimui.