RSC Advances

ROYAL SOCIETY OF CHEMISTRY

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Journal Name

ARTICLE

Variations of cellular components on *Thermosynechococcus sp.* CL-1 under electrochemical treatment

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

www.rsc.org/

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To investigate the potential for biofuel production by using cyanobacterium photosynthesis integrated with electrochemical treatment, several protein expressions and cellular components were experimentally analyzed. *Thermosynechococcus sp.* CL-1 (TCL-1) was chosen because it shows a significant photocurrent response at 0.4 V without the addition of artificial mediators in an H-type two-compartment electrolysis cell. The crude lipid content in the TCL-1 cells in either the anodic membrane or the suspended medium at 0.4 V was higher than that of the control experiments. In addition, the cells attached to the anodic membrane showed a considerably higher crude lipid content and lower carbohydrate content compared with the suspended cells. The experimental results show that electrochemical treatment redistributed the cellular components and proteomics of TCL-1 in the suspended medium and anodic membrane without increasing the total production.

Introduction

The combustion of fossil fuel increases CO_2 concentration in the atmosphere and results in severe global warming. Sunlight is not only the main contributor to global warming but also a potential source for new energy technologies. The U.S. Department of Energy indicated that 1-h of sunlight can provide energy for 1-y. Hence the development and application of solar cells constitute one of the dominant energy strategies in most countries. Because photosynthesis can fix CO_2 in the Calvin cycle through power supply (adenosine triphosphate, ATP and NADH) from sunlight, the production of electric power or biofuel precursors with CO_2 fixation by photosynthetic organisms can be developed for eliminating energy risk and global warming. Furthermore, photosynthetic microorganisms are more suitable candidates for the aforementioned application than higher plants deduced for higher photosynthetic efficiency.[1]

Microbial fuel cells (MFCs) are devices that generate the electric energy by using microorganisms as biocatalysts. In recent years, MFCs have been used for generating energy generation with organic compounds in wastewater as the energy source. Because sunlight is one of the energy sources, instead of heterotrophic bacteria, MFCs comprising photosynthetic microorganisms (PMFCs) must be developed for capturing energy from sunlight. External electron mediators are used increasingly less because they are highly toxic and expensive.[2,3]

A previous study provided information on a PMFC with mediator-free by using a pure culture, the attached cyanobacteria, Spirulina platensis, as the anode.[4] Electric power in the dark (1.64 mW m^{-2}) is substantially higher than that in light (0.132 mW m^{-2}), thus revealing that heterotrophic metabolism is dominant in the electrical production and that photosynthesis provides only the accumulation of glycogen. Recently, Pisciotta demonstrated that the direct light-dependent electrogenic activity exits in mesophilic cyanobacteria.[5] Through a series of experiments related to inhibitors on photosystem (PS) I and PS II, they also indicated that plastoqunione (PQ) is the major mediator.

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Furthermore, we observed the current production directly from a thermophilic cyanobacterium, *Thermosynechococcus sp.* CL-1 (TCL-1), by photosynthesis without the addition of artificial mediators.[6] Current production is the "stress" process of the electrons outflow from the inner of the cells. Based on photosynthetic energy conversion, the cellular components could be changed under stress.[7] The cyanobacteria have adapted to various extreme environmental conditions (e.g., high pH, high salinity etc.), therefore, they are found worldwide. A previous study showed that the cultivation of *Synechocystis sp. strain* PCC 6803 at a high temperature (40°C) increased the production of highly saturated monoglucosyldiacylglycerol (MGlcDG) in a thylakoid membrane.[8] Consequently, using environmental stress as a trigger to facilitate biosynthesizing a specific cellular component is reasonable in biofuel research.

In addition, a proteomics survey is a well-known tool for determining cyanobacterial stress physiology.[9-11] Ehling-Schulz and Scherer detected 430 spots by using two-dimensional electrophoresis analysis on *Nostoc commune*, and at least 30% of all spots were significantly affected by ultraviolet (UV)-B.[9] However, no data are available to indicate the possible roles of any of these proteins. Huang screened the proteomics of salt-stress-induced changes in the plasma membranes of *Synechocystis sp.* strain PCC 6803. The results indicated that the highest enhancement in proteins during salt stress was related to the protection of PS II under iron deficiency and in thylakoid membrane formation.[10]

Because studies on using electrochemical treatment as a stress to produce biofuels are rare, investigating the variations in cellular components and proteomics in TCL-1 under electrochemical treatment is worthwhile.

Materials and Methods

Microbial species

The *Thermosynechococcus sp.* CL-1 (TCL-1) strain was isolated from Chin-Lun hot spring (pH 9.3, 62°C) in Taiwan, as described previously [6]. A modified Fitzgerald medium was used as the growth culture, consisting of (in mg L^{-1}) 496 $NaNO_3$, 39 K_2HPO_4, 75 $MgSO_4 \bullet 7H_2O$, 27 $CaCl_2$, 58 Na_2SiO_3, 6 $FeC_6H_5O_7$, 6 citric acid, 1 ethylenediaminetetraacetic, and a 1 mL L^{-1} Caffron solution in distilled water.[12] The medium was used for pre-culture and additional tests under various conditions.

Electrochemical treatment preparation

An H-type two-compartment electrolysis cell was used in this study (Fig. 1). The 2.5 L anode chamber was combined with a 2 L working volume of a culture medium containing 47 mM DIC at an initial pH of 9.5, which was prepared using a mixture of $NaHCO_3$ and Na_2CO_3, a piece of carbon felt, a man-made reference cathode, and a Pt wire connected to a potentiostat. Carbon fibre (B0050, Toray, Japan) was fixed inside the anode chamber with a given area elastic accessory and connected to the potentiostat with a Pt wire for electron transportation. Pre-treated procedures on the carbon fibre are (1) washing with a 0.1 N HCl solution, (2) washing whin a 0.1 N NaOH solution overnight for cleaning, and (3) drying at 60°C overnight, thus avoiding impurities. In addition, a man-made reference electrode composed of a KCl solution was connected to the potentiostat with a Pt wire for voltage reference. Furthermore, the cathode chamber containing 20 ml of a medium was connected to the potentiostat with a Pt wire for electron transportation. The anode and cathode chambers were separated by a cellophane membrane, thus allowing ion transport for balance.

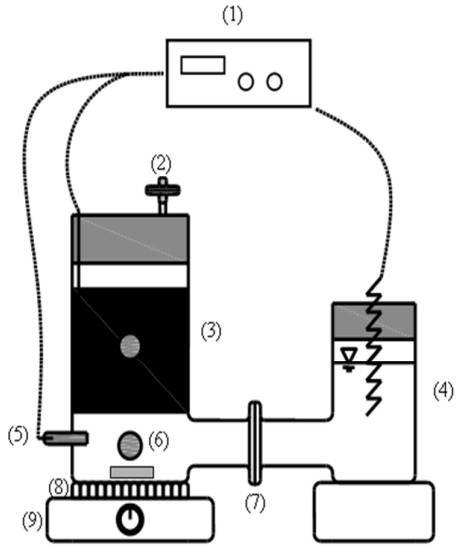

Fig. 1. Schematic of an H-type two-compartments electrolysis cell. (1) Potentiostat; (2) 0.45 µm felt for gas exchanged; (3) Carbon filter as working electrode; (4) Pt wire as counter electrode; (5) Reference electrode; (6) Sampling hole; (7) Cellophane membrane for ion balance; (8) Florescence light; (9) Magnetic stirrer

Experimental procedure

The turbulence of the cultivation solution in the anodic chamber was controlled by a magnetic stirrer at a constant speed to enhance the mixing of the reactor content, thus avoiding biomass settling. In addition, the cultivation temperature was controlled within an illuminated incubator (FH-130w, Taiwan) at 50ºC. Light intensity, measured at the nearest distance from the fluorescent lamps in the centre of the anodic chamber by using a Lux meter (TM 50000, TOMEI), was 20 kLx.

After fabricating the H-type two-compartment electrolysis cell, the steady state of blank current response, approximately 5 µA, was obtained after 1 hr at a given voltage of 0.4 V by using a potentiostat/galvanostat (HA-151A, Japan). Next, 5 ml of a medium containing TCL-1

(initial $OD_{680\ nm}$ = 0.1) was injected into the anode chamber for seeding and the half-hour data of current response were then recorded until the end of each run. The $OD_{680\ nm}$ was measured using an UV-visible spectrophotometer (DU730, Beckman Coulter, USA) approximately twice a day, and the pH was measured at the same frequency. Finally, the cells were harvested in their log growth phases and stored at -70°C.

Cellular compositions and fatty acid methyl esters (FAME) measurement

An analytical method for carbohydrate detection was performed, followed by a colorimetric measurement at 485 nm.[13] The crude protein content was determined by multiplying the nitrogen content by 6.25, and the nitrogen content was determined using an element analyser. Crude lipids were extracted according to the method by Holm-Hansen.[14] Briefly, total lipids were extracted from 10 mg of the TCL-1 cell pellet by sonication. Phase separation was performed using methanol-chloroform-water (10:10:9, v/v/v). The chloroform phase was evaporated to dryness under a gentle stream of nitrogen, dried under vacuum, and then weighed. Transesterification was catalysed by 0.5 N NaOH/methanol at 80°C for 13 minutes and then 14 %wt/vol BF_3/methanol for 10 minutes. In addition, 1% methyl n-pentadecanoate in hexane was added as the external standard. The fatty acid methyl ester composition was determined using gas chromatography involving a flame ionization detector.

Proteomic survey

Regarding proteomic survey, reagents, including 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), dithiothreitol (DTT), an IPG buffer (pH 4-7), an Immobioline DryStrip pH 4-7, thiourea, urea, and bromophenol blue (GE Healthcare, Piscataway, NJ), were used. To eliminate the interference of Rubisco, the harvested TCL-1 cells used as 2-DE samples were prepared using the procedure described by Krishnan, with some modifications.[15] In Brief, 40 mg of TCL-1 cells was placed in a clean Eppendorf tube and 1 ml of 50 mM Tris-HCl, pH 6.8 containing 1% protease inhibitors and 20 mM DTT wa added. The cell wall was broken down using a sonicater (XL-2000, Misonix, USA) impulsively at 10 W for 30 times at 4°C. The solution was centrifuged at 17,000 × g for 10 min and the supernatant was collected. A 10 mM of $CaCl2.2H_2O$ and sodium phytate as a final concentration were added to the supernatant and this solution was incubated at 42°C for 10 min to remove the interfence of Rubisco.

After the proteins were isolated from the calcium/phytate fraction, the supernatant was suspended in 750 ml of an extraction buffer at pH 7.5 containing 0.7 M sucrose and 20 mM DTT. After incubation on ice for 10 min, the same volumes of saturated phenol were added and this solution was vigorously shaken for 30 mins at room temperature. This solution was centrifuged at 17,000 × g for 10 mins and the supernatant was collected. To this supernatant, 3 volumes of freshly prepared MeOH with 0.1 M ammonium acetate (pre-cooled to -20°C) were added. A precipitate was formed overnight, and this was centrifuged at 17,000 × g for 20 mins and the supernatant was discarded. The protein pellet was resuspended using 100 % methanol containing 0.1 M ammonium acetate followed by centrifugation at 17,000 × g for 10 min. The supernatant was discarded and the residue (insoluble proteins) was washed with 80 % of acetone for three times. Between each step, the solution of insoluble protein was incubated at -20°C for 30 min before centrifugation at 17,000 × g for 10 min at 4°C. In the final step, the protein pellet was dried and then solubilized in an adequate volume of a rehydration buffer containing 7 M urea, 2 M thiourea and 4 % CHAPS. Protein quantifications were performed as described by Bradford.[16]

80 µg of TCL-1 proteins was dissolved in a pH gradient strip containing 1% (v/v) IPG buffer (pH 4-7), 20 mM DTT, and 0.1 % (v/v) bromophenol blue. Isoelectric focusing was performed at 15°C under running conditions of 250 V for 250 voltage-hours (Vh), 300 V for 1 Vh, 3500 V for 2900 Vh and 3500 V for 12600 Vh until 15600 Vh was reached. Two-dimensional electrophoresis was performed on 12 % SDS-PAGE gel with SE-600 Ruby (GE Healthcare, Piscataway, NJ). Protein detection was performed using silver staining, as describer previously.[17] Gel images were scanned by an EPSON EXPRESSION 1000XL scanner (Epson, Japan) at a resolution of 300 dpi. The gel to gel matching and identification of differences in spots was conducted using the analysis software Image-Master 2D Platinum (version 6.0, GE Healthcare). After the analysis of the difference in the spots between those of the control and those receiving electrochemical treatment, the differentially expressed spots were incised and stored at -20°C. The protein identification was authorized by the Proteomics Research Core Laboratory (College of Medicine, National Cheng Kung University, Taiwan).

Results and Discussion

Prior to experimental data were further treated, a statistical model of analysis of variance (ANOVA) is used to analyse the differences among group means and their associated procedures based on the 95% confidence interval. The growth curves of *Thermosynechococcus* sp. CL-1 (TCL-1) with and without electrochemical treatment are similar (Fig.2). However, the harvested mass in these two cases, including suspended and attached cells, are different (Table 1). The harvested biomass of Control (S+M) (456 mg) is considerably higher than that of +0.4V (S+M) (356 mg). The condition of +0.4 V tends to exclude the cells from the membrane (carbon felt) and results in less harvested mass compared with Control-M. Presumably, the outflow of electrons is faster from the membrane than from the medium because of the mass transfer effect. The variations in cells regarding growth and cellular components in the

medium should be lower than those in the membrane. Regarding the suspended TCL-1, the crude lipid and carbohydrate contents after electrochemical treatment (+0.4V-S) (27.9% and 42.3%, respectively) are slightly higher than those for Control-S (26.8% and 38.2%, respectively) (Table 1). Conversely, comparing crude protein content revealed an inverse result (30.6% and 40.9%, respectively). The FAMEs probably changed under various stresses to adapt to the new conditions. For example, Balogi et al. (2005) indicated that *Synechocystis* sp. PCC 6803 at a high temperature (40°C) increases the production of highly saturated monoglucosyldiacylglycerol (MGlcDG) in thylakoid membrane. However, the FAME components in these two cases (treatment and control) are similar and palmitic acid (C16:0) is the major component (ca. 62% of total FAME) (Table 2). These results show that the effect of electrochemical treatment on the suspended cells in the anode chamber is probably too low to change the FAME composition. If the lipid productivity is high, palmitic acid (C16:0) is the suitable material to produce biodiesel. Conversely, this treatment on proteomics screening reveals significant results. As shown in Table 3, two proteins, photosystem (PS) II manganese-stabilizing polypeptide and the elongation factor Tu, which are related to functions in PS II and cytoplasm, were induced. Additionally, four proteins were reduced after the same treatment. The probable functions related to all identified proteins are listed in Table 3. However, investigating the relationship between cellular components and protein expression requires additional data.

Attached cells in the membrane with and without electrochemical treatment

As mentioned, the harvested biomass of the membrane after electrochemical treatment (+0.4V-M) (63 mg) was less than that of Control-M (148 mg). However, the crude lipid content in the TCL-1 cells with +0.4V-M (61.1%) was 14.7% higher than that in Control-M (52.1%) and the crude protein content was lower than that in Control-M (Table 1). The anodic membrane should be the major location for electrons outflow. Therefore, the attached cells in the anodic membrane probably live under stress. Two proteins, glutamate-1-semialdehyde aminotransferase and phycobilisome rod linker polypeptide, in the anodic membrane were reduced after electrochemical treatment. Glutamate-1-semialdehyde aminotransferase is related to the chlorophyll biosynthetic process. Phycobilisome rod linker polypeptide is directly related to photosynthesis in phycobilisome and the thylakoid membrane. Consequently, electrochemical treatment should affect photosynthesis because of electron outflow from the inner of the cells.

Comparison of Suspended Cells and attached cells in the membrane without treatment

As shown in Table 1, the crude lipid content in Control-M (52.1%) was substantially higher than that in Control-S (26.8%) even without electrochemical treatment and also revealed increment by 94.4% based on Control-S. TCL-1 in Control-M attached on the membrane was probably a stress related to cell movement. In addition, TCL-1 in Control-M received lower light illumination than that in Control-S because the anodic membrane was far away from the centre of the reactor. This resulted in a higher respiration activity and decrement by 53.4% of carbohydrate content in Control-M than that in Control-S (glycogen consumption). As shown in Table 2, C16:1 and C18:0 were reduced, and C15:1 was induced as TCL-1 received lower light intensity and a probable stress related to cell movement. As shown in Table 3, all induced and reduced proteins were heat shock proteins under a probable stress and/or lower light condition.

Comparison of suspended cells and attached cells in the membrane under treatment

The crude lipid content in the TCL-1 cells with +0.4V-M was 37% higher than in +0.4V-S and revealed the increment by 127.9% based on Control-S. Inversely, the decrease in carbohydrate and crude proteins in +0.4V-M was considerable compared with that in +0.4V-S. These results show more differences compared with the case without electrochemical treatment. Therefore, the effect of electron outflow on +0.4V-M is more significant than on +0.4V-S because of the mass transfer effect. As shown in Table 3, two proteins, the iron binding protein component of ATP-binding cassette (ABC) iron transporter and the elongation factor Tu, were induced. The elongation factor Tu consists of three-domain guanosine triphosphate (GTP)-ases with an essential function in the elongation phase of messenger ribonucleic acid translation. It promotes the GTP-dependent binding of aminoacyl-transfer RNA to the A-site of ribosomes during protein biosynthesis.[18,19] Consequently, an increase in the elongation factor Tu in this study represents the regulation of genes expression or the occurrence of biosynthesis of some protein. The iron binding protein component of ABC iron transporter, is related to ion transport, iron ion homeostasis, and metal ion binding near the thylakoid membrane.

Cellular components production with or without the electrochemical treatment

After the results of the suspended cells and cells attached to the membrane for +0.4 V and the control were summed, the average contents of crude lipids, carbohydrates, and crude proteins between these two cases were similar. The harvested biomass after the 0.4 V electrochemical treatments was slightly lower than that of the control. This probably resulted from the running off of electrons or an unknown negative effect of repulsion on TCL-1 under electrochemical treatment. Consequently, electrochemical treatment redistributed the cellular components proportion of TCL-1 in the suspended medium and the anodic membrane without increasing the total production.

Conclusion

The present study reveals the high crude lipids content on TCL-1 in the membrane of anode even without electrochemical treatment and different FAME components as compared to the suspended one. Results indicated that +0.4V-S, control-M, and +0.4V-M showed the increments by 4.1%, 94.4%, and 127.9% on crude lipid content comparing to the control-s experiment. However, the same series of experiment reveal the decrement by 31.8%, and 59.5% on biomass for control-M, and +0.4V-M respectively. Electrochemical treatment redistributes cellular components proportion of TCL-1 in the suspended medium and anodic membrane without increasing the total production. The proteomic analyses revealed several proteins can be induced or reduced after attaching on the anodic membrane or electrochemical treatment. However, the relationship between the proteins related to the specific function and cellular components variation are still further recognized.

Acknowledgements

We gratefully acknowledge the Sustainable Environment Research Center (SERC), National Cheng Kung University, Taiwan for their kindly support.

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Table 1. Content and harvested mass of crude lipids, carbohydrates, and crude proteins in *Thermosynechococcus sp.* CL-1 (TCL-1) with or without the electronchemical treatment (N≧4).

Item	Content (%)			Harvested mass (mg)			
	CL	CH	CP	BM	CL	CH	CP
Control-S	26.8±0.1	38.2±1.7	40.9±6.2	221.5±79.8	59.3±21.4	84.6±30.5	90.6±32.7
+0.4V-S	27.9±2.3 (4.1%)	42.3±4.5 (10.7%)	30.6±4.2 (-25.2%)	245.0±67.7 (10.6%)	68.4±18.9 (15.3%)	103.6±28.6 (22.5%)	74.9±20.7 (-17.3%)
Control-M	52.1±5.7 (94.4%)	17.8±4.8 (-53.4%)	24.7±11.3 (-39.6%)	151.0±27.9 (-31.8%)	78.6±14.5 (32.5%)	26.9±5.0 (-68.2%)	37.3±6.9 (-58.8%)
+0.4V-M	61.1±7.3 (127.9%)	11.6±4.2 (-69.6%)	17.2±3.9 (-57.9%)	89.8±24 (-59.5%)	54.9±14.7 (-7.4%)	10.4±2.8 (-87.7%)	15.5±4.1 (-82.9%)
Control(S+M)	37.4±3.4	29.6±2.8	34.1±2.2	372.5±62.2	137.9±14.5	111.5±27.0	127.9±28.0
+0.4V(S+M)	37.1±2.8 (-0.8%)	33.8±2.6 (14.2%)	26.9±1.1 (-21.1%)	334.8±65.3 (10.1%)	123.3±20.5 (-10.6%)	114.0±28.0 (2.2%)	90.3±19.9 (29.4%)

CL: crude lipid, CH: carbonhydrate, CP: crude protein, BM: biomass

"Control-S" indicates suspended cells without electrochemical treatment; "Control M" indicates cells attached to the anode membrane without electrochemical treatment; "+0.4 V-S" indicates suspended cells with the 0.4 V treatment; and "0.4 V-M" indicates cells attached to the anode membrane with the 0.4 V treatment.

*Biomass yield is estimated according to $OD_{680nm} \times 200$ (mg L^{-1}).

§Crude protein content is estimated according to N content × 6.25

Lipid, carbohydrate, and protein yield are estimated according to biomass multiplied by cellular component content

Table 2. Fatty acid methyl ester (FAME) composition in *Thermosynechococcus sp.* CL-1 (TCL-1) with or without the electron-chemical treatment.

Composition	C15:1	C16:0	C16:1	C18:0	C18:1n9c
Control-S	8.1	61.5	7.9	9.2	13.3
+0.4V-S	7.8	62.0	7.8	9.7	12.6
Control-M	22.1	59.8	-	5.9	12.2

"-" means "not detectable"

The case of +0.4V-M did not provide sufficient mass for FAME analysis

Table 3. Expressed proteins identification from various source of treated *Thermosynechococcus sp.* CL-1 (TCL-1)

Item	Expression ratio	Protein description	Component	Probable function(s)
+0.4V-S/ Control-S				
	4.06	photosystem II manganese-stabilizing polypeptide	photosystem II	calcium ion binding
	-3.5	hypothetical protein tlr2403 (Molybdopterin biosynthesis protein)	-	nucleotide binding, catalytic activity
	-2.6	phosphoglycerate kinase	cytoplasm	ATP binding, kinase activity, phosphoglycerate kinase activity
	-4.3	glutamate--ammonia ligase	cytoplasm	glutamate-ammonia ligase activity
	2.4	elongation factor Tu	cytoplasm	GTP binding, GTPase activity,translation elongation factor activity
	-5.8	transketolase(involve glycolysis)	-	transketolase activity
+0.4V-M/ Control-M				
	-7.4	Glutamate-1-semialdehyde aminotransferase	cytoplasm	isomerase activity,transaminase activity,glutamate-1-semialdehyde 2,1-aminomutase activity,pyridoxal phosphate binding
	-12.3	phycobilisome rod linker polypeptide	thylakoid membrane	photosynthesis
Control-M/ Control-S				
	6.2	heat shock protein	cytoplasm	adenyl-nucleotide exchange factor activity,adenyl-nucleotide exchange factor activity,chaperone binding,protein homodimerization activity
	-3.4	heat shock protein	cytoplasm	
	240.7	heat shock protein	cytoplasm	
+0.4V-M/ +0.4V-S				
	2.8	iron binding protein component of ABC iron transporter	thylakoid membrane	metal ion binding
	2.4	elongation factor Tu	cytoplasm	GTP binding, GTPase activity,translation elongation factor activity