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Original scientific paper

Wolffia globosa as a biocatalyst in plant-based biofuel cells

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Abstract

The rootless duckweed Wolffia globosa, not explored toward electrogenicity till now, is investigated as a putative biocatalyst in plant-based biofuel cells (P-BFC) for the electrical current generation and its basic metabolic changes during the polarization are depicted. After a short adaptation period, the open-circuit voltage of P-BFC, utilizing W. globosa as an anodic biocatalyst, reaches values of 630 mV. At a connected external resistor of 1 kΩ in the electric circuit, stable current densities of 170±10 mA m^{-2} are achieved. The electrical outputs depend on the anodic potential, reaching negative values of ca. -200 mV (vs. SHE). W. globosa produces an electrochemically active compound, acting as an electron shuttle. The polarization intensifies the W. globosa metabolism, expressed in a double increased glucose and starch content along with 1.82 times higher specific amylase activity of 70.0±2.8 U g^{-1} wet biomass in the organelle-enriched fractions of the explored as biocatalysts plants compared to the control. The results reveal that Wolffia globosa can be utilized as a biocatalyst in P-BFC for simultaneous electricity generation and increased carbohydrate and protein content.

Keywords

Duckweed; polarization; metabolic activity; electricity generation

Introduction

Decarbonization is one of the main goals of the EU energy system, needing new technologies. As primary producers, plants are those that convert CO_2 into organic matter. Recently, a new technology named plant-based biofuel cell (P-BFC), mimicking the processes imposed in nature, has been proposed as an alternative approach for intensified CO_2 capturing and utilization. The principles of P-BFC, based on the fact that specific bacteria in the rhizodeposits of plants can transfer electrons to the anode of the fuel cell located in the soil, were proved in 2008 [1,2]. It has been verified that by

P-BFC, solar energy and CO_2 can be harvested for plant biomass production with a simultaneous electric current generation [3]. *Oryza sativa*, *Spartina anglica, Arundinella anomala,* and *Glyceria maxima* have been proven as suitable biocatalysts in P-BFCs [4]. As far as these plants cooperate with the soil microbes, the systems are called plant microbial fuel cells (P-MFC). The P-BFC technology was further developed by using aquatic plants as biocatalysts, which can directly communicate with the anode of the bioelectrochemical system (BES) [5,6]. In a previous study [5], we reported that higher aquatic plant *Lemna minuta* duckweeds can be used as biocatalysts in direct photosynthetic plant fuel cells (DPPFC) without the participation of electrogenic bacteria. The influence of abiotic factors such as temperature, humidity, and light intensity, as well as the day/night alternation, on the electrical outputs obtained by the BES, was established. It has been demonstrated that *Lemna minuta* duckweed, autotrophically grown in plant fuel cell under natural sunlight illumination, can generate high power density (380±19 mW m^{-2}), corresponding to 120±6 GJ ha^{-1} year, which remains the highest achieved value reported in the literature [7,8]. Under artificial light sources, P-BFC generated an order of magnitude lower current indicating the significance of the light-dependent processes implemented in the chloroplasts of these anodic biocatalysts. Indeed, tracing the changes in the current during the day and night, higher electrical outputs were achieved during daylight hours. The energy generated by duckweed-based P-BFC so far is summarized and presented in Table 1.

***Table 1**. Summary of the generated current and achieved power by P-BFC carried out with different biocatalysts belonging to the Lemnaceae family upon the anode under different grown conditions*

Duckweed	Light source	Energy by PBFC	$E^{`0}$ / mV*	Proposed plant components participating in EET	Ref.
Lemna minuta	Natural sunlight >10 k Lx	Predicted and achieved: 1.6 A m^{-2} ≅ 400 mW m^{-2} (120 GJ ha-1 $year^{-1}$)	−280	dependence on the day/night cycles	[5]
Lemna minuta	Mixed lab light 1.5 k Lx	Measured: 160 mA m^{-2} predicted by polarization curve: 250 mA m^{-2}, 50 mW m^{-2}			[5]
Lemna valdiviana	Mixed lab light 1.5 k Lx	226 mA m^{-2}, predicted by polarization curve: 1.2 A m^{-2} and 140 mW m^{-2}	−266	Light-dependent processes of chloroplasts, respiration	[6]
Lemna minor	60 k Lx coherent light source, irradiation by optical fibers, with polarized monochromatic light: **red** (λ = 650 nm, 0.059 W m^{-2}); **violet** (λ = 450 nm, 0.732 W m^{-2}); visible non-polarized (1.5 W m^{-2})	17.5 mA m^{-2} 13.0 mA m^{-2} 15.5 mA m^{-2}		cytochrome b6f , Red light-absorbing photosystems PS I and PS II	[9]
Wolffia globosa	Mixed lab light 1.5 k Lx	170±10 mA m^{-2}, predicted 1 A m^{-2}, 90 mW m^{-2}	−260	Confirms the above	This study

*determined by CV, 10 mV s^{-1}

Besides *Lemna minuta* [5], only two more duckweed species *Lemna valdiviana* [6] and *Lemna minor* [9] have been investigated for exoelectrogenic properties so far. Although under artificial illumination these systems generate lower electrical current, the exploration of model aquatic plants in the laboratory conditions contributes to the improvement of the technology, especially when clarifying the mechanisms of extracellularly transferred electrons. The common feature of the plants belonging to the *Lemnaceae* family is that they all have roots, which are in contact with the P-BFC anode and therefore are supposed to contribute to the electrical current generated by a direct

extracellular electron transfer (EET). Depending on the illumination intensity and the period of cultivation, the response of the duckweeds to the fuel cell polarization has been also related to the membrane potential of the fronds/roots as well as to the secretion of plant endogenous mediators. The direct EET mechanism was further verified when *Lemna minor* duckweeds have been grown in a P-BFC under total light source control conditions [9]. The plants were grown in fuel cells which were wrapped with black non-transparent folio with a black light-tight lid. Polarized monochromatic light and, as a control light from the visible spectrum was diffused over the plants through optical fibers and the electrical current was recorded over time. Of special interest were the P-BFCs which were irradiated with polarized monochromatic light with the precise wavelength of 650 nm (red light) and 450 nm (blue-violet light) [9]. The higher current values during the photoperiods and the analyses of the charge transfer by the electrochemical impedance spectroscopy suggested that a direct, photo-induced charge transfer between the duckweed and the fuel cell anode took place with the participation of the light-absorbing photosystems. Although the plant' chlorophyll *"a"* absorbs at a maximum of about 450 nm wavelength, the red light is more effective because both photosystems (PS I and PS II) absorb light of wavelengths in the red specter. When PS II absorbs light, electrons in the chlorophyll reaction center are excited to a higher energy level. Photo-excited electrons travel through the cytochrome b6f complex to photosystem I via an electron transport chain set in the thylakoid membrane [9].

In the present study, the rootless duckweed *Wolffia globosa*, the smallest flowering plant world-wide, with free-floating fronds, is investigated for the first time as a biocatalyst in P-BFC regarding its capability of generating electricity. The influence of the polarization conditions on the plant's metabolism was examined in the organelle fractions of *W. globosa* obtained from duckweeds which are grown in P-BFC and compared to those of normally grown plants (control).

Experimental

Experimental setup

The fuel cell construction consists of a transparent glassy body where the cathode and the anode have been placed (Figure 1). A rectangular piece of carbon felt with dimensions 3.5×4.5 cm upon cork pontoons, served as an anode.

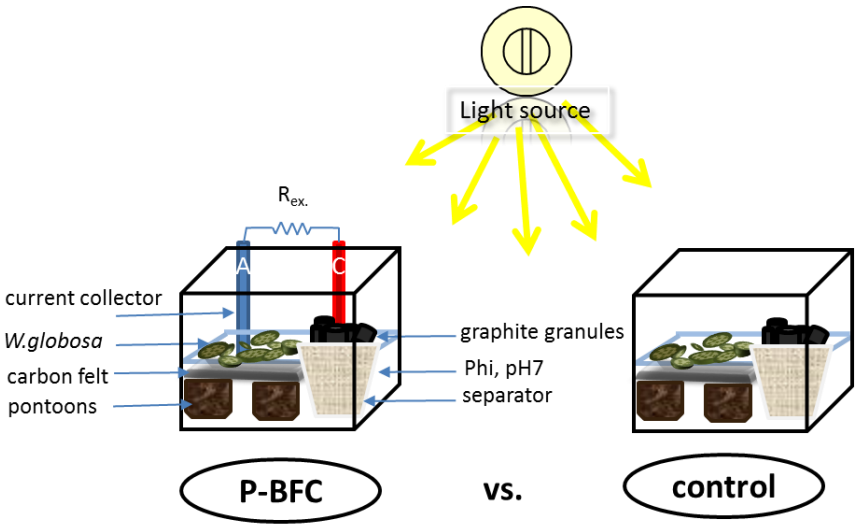

Figure 1. *Scheme of the experimental setup. The duckweed was grown under polarization conditions of the biofuel cell and compared with plants grown as a control without connection in an electrical circuit*

1 g *Wolffia globosa* has been grown in the fuel cell in 40 ml 67 mM phosphate buffer, pH 7.0, for enhanced conductivity, at open-air conditions. The losses from the evaporation and water uptake by the plants were daily compensated. Mixed light (artificial and natural sunlight) was used as a light source to irradiate the plants. Graphite granules (4.3 g total weight), placed into a 100 µm Christean handy water filter as a separator, were used as an air cathode. Identical P-BFCs have been developed and different experiments were carried out in triplicate – the first experimental setup consisted of P-BFCs operating across a 1 kΩ load resistor (a closed electrical circuit), the second - as control samples (open circuit conditions).

Electrochemical measurements

The voltage across the resistor was measured daily. The resistor was switched off and after 10 minutes of stabilization the open-circuit voltage (OCV), and the anodic potential were measured against Ag/AgCl (3 M KCl) reference electrode. The P-BFC was also characterized by polarization curves obtained by varying the external resistance from 100 kΩ to 10 Ω by using a resistance decade box. The load resistances were changed through 2 units at each decade. The cell voltage, *U*, was recorded 5 min after switching a given resistance by using a digital multimeter DMM2700 (Keithley Instruments Inc., US). The current was calculated according to the equation I / mA = U / R, where U/mV is the measured cell voltage, R / Ω is the external resistance, and the current density according to j / mA m^{-2} = I / S, where S / m^2 is the geometric area of the anode. The power density was calculated as P /mW m^{-2} = j U / 1000.

The electrochemical activity of the exploited anolyte was explored by cyclic voltammetry (CV). The anolyte was filtered through a 0.2 µm microporous sterile filter. The CV was carried out in a three-electrode arrangement, consisting of a Pt-wire as a working electrode, a platinized titanium mesh as a counter electrode, and Ag/AgCl as a reference electrode, with a scan rate of 10 mV/s.

Biochemical and enzymatic analyses

Fractionation

1 g of the used as a biocatalyst or control grown duckweeds was mechanically disintegrated for 10 min in 2 ml 67 mM phosphate buffer, pH 7.0. After centrifugation at 12,000 *g* for 10 minutes, the organelles (chloroplasts and mitochondria) have been harvested in the pellet fractions, re-suspended in 1 ml 67 mM phosphate buffer, pH 7.0, and used for biochemical and enzymatic analyses. These parameters have been also examined in the supernatant fractions and the anolyte/water samples. The electrolyte, respectively the buffer solution, is filtered through a sterile filter with a pore size of 0.25 µm by a syringe.

Proteins and sugars quantification

The intracellular protein concentration was analyzed by the modified Bradford method (Merck) and estimated in mg ml^{-1} by using a BSA-calibration curve. The determination of reducing sugars (glucose equivalents) was carried out by using the DNS method [10]. The iodine method of Halick and Keneaster was used for the determination of starch [11]. The method was modified as described in [5]. Both glucose and starch concentrations in the samples were determined in mg ml^{-1} by using calibration curves.

Specific amylase activity

The amylase activity was assayed by quantifying the reducing sugars (glucose equivalents) liberated from soluble starch using the method described by Bernfeld [12]. One unit of enzyme

activity (U) is defined as the amount of enzyme required to release 1 µmol of glucose from soluble starch per minute. The determined glucose amount in the pellet fraction was subtracted from that estimated after the enzyme assay. The results are calculated and presented as a specific amylase activity (U g^{-1} wet biomass).

Quantification of inorganic phosphates

The molybdenum blue method [13] was used for the quantitative determination of inorganic phosphates. The concentration is estimated as mM by using a calibration curve after subtracting the amount of the inorganic phosphate in the buffer.

Specific phytase activity

Phytase, myo-inositol hexakisphosphate phosphohydrolase (EC. 3.1.3.8), catalyzes the hydrolysis of phytic acid to inositol polyphosphates and free orthophosphoric acid. The reaction (400 µl volume) was carried out by using 0.2 mM Na-phytate in 0.1 M acetate buffer, pH 5.5, at 37 °C for 60 min. The reaction was stopped by adding two parts of freshly prepared acetone: 5 N Sulfuric acid: 10 mM ammonium molybdate in proportion 2:1:1. After mixing, 40 µl of 1.0 M citric acid was added to each tube. The orthophosphates released from phytate were determined by Engelen's method [14]. The phytase activity is defined as 1 µmol inorganic orthophosphate released from the phytate per minute and presented as a specific phytase activity (U g^{-1} wet biomass).

Each biochemical or enzymatic parameter has been analyzed in triplicate and the results are presented as a mean value ± a standard deviation.

Results and discussion

Electrical and electrochemical properties of W. globosa-based P-BFC.

At the beginning of the experiment, the open-circuit voltage (OCV) of *W.* globosa-based P-BFC was ca. 330 mV but after about six days of polarization the OCV increased and reached steady-state values of 615±15 mV over the rest of the period (Figure 2a). As far as the cathodic potential was relatively constant (ca. +250 mV *vs.* Ag/AgCl) over time, the decreasing values of the anodic potential up to –400 mV *vs*. Ag/AgCl confirmed the role of this parameter and the contribution of the biological component to the current generation processes (Figure 2b).

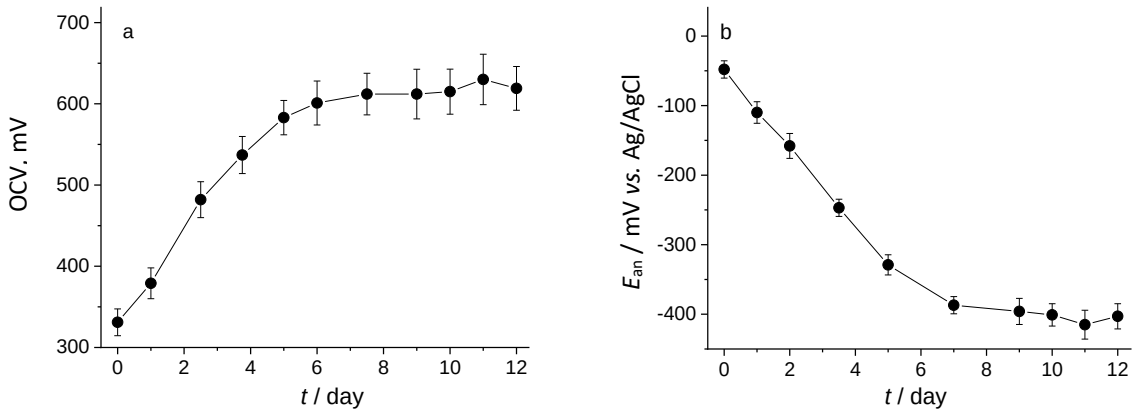

Figure 2. *Changes of (a) the open-circuit voltage, (b) the anodic potential of W. globosa - P-BFC over time*

After stabilization of the OCV, the P-BFC was analyzed by polarization and power curves. The results, shown in Figure 3a, reveal that the developed bioelectrochemical system can generate a current reaching density of 1 A m^{-2} and a maximal power density of ca. 90 mW m^{-2}. The current

density was close to that previously established for *Lemna valdiviana* - based P-BFC [6]. The generated over time current by *W. globosa* - P-BFC at a continuously switched external resistor in the circuit increased much faster than the OCV. After two days of operation, steady-state current values in the range of 170±10 mA m^{-2} were achieved (Figure 3b). Although the achieved current was lower than that predicted by the polarization and power curves, the estimated current densities were comparable with the current densities generated by the duckweed *L. minuta*, which has been grown in P-BFC under artificial illumination [5].

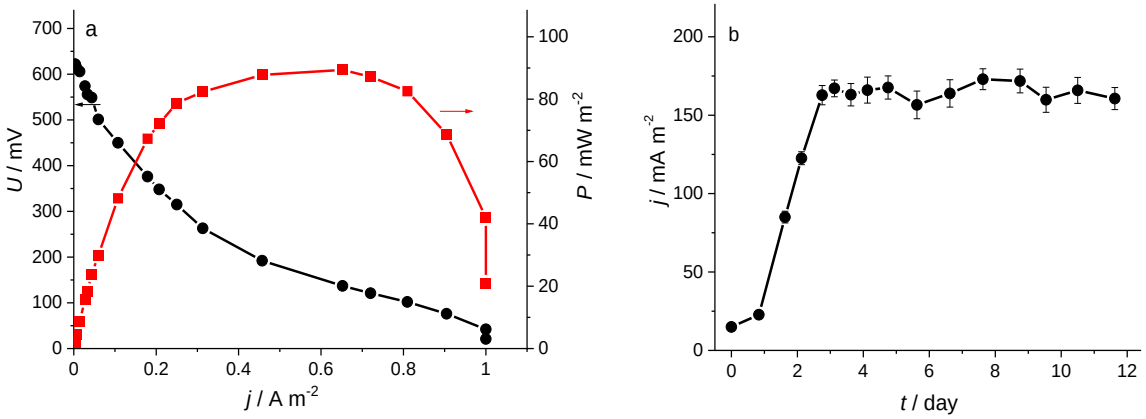

***Figure 3**. (a) Polarization (*U *as a function of* j*) and power (*P *as a function of* j*) curves of* W. globosa *- P-BFC on the 6th day of operation, (b) current density recorded during P-BFC operation across an external load of 1 kΩ*

Till now, the technology of the P-BFC includes the utilization of duckweed species, spread onto the carbon felt surface, and being in permanent contact with the anode by their roots while swimming on the water surface. One of the main differences between the duckweeds of the genus *Lemna* and that of *Wolffia* is that the latter are rootless. To check the possible mechanism, by which this aquatic plant transfers electrons extracellularly to the anode, additional cyclic voltammetry (CV) of the spent anolyte was carried out. The CV results (Figure 4) suggest that the grown in the biofuel cell *W. globosa* secretes an electrochemically active compound in the anolyte, which could contribute to the generated current acting as an endogenous electron transfer mediator (EnM), shuttling electrons between the plants and the anode. This mediated electron transfer mechanism is in good agreement with previously proven EnMs for both aquatic plants [5,6] and yeast cells [15,16]. However, so far only a few EnMs have been identified from bacteria, which is the reason why they are collectively called "endogenous mediators" [17]. While *Shewanella oneidensis* secretes flavin molecules, *Pseudomonas aeruginosa* self-produces nitrogen-containing heterocyclic phenazine metabolites such as pyocyanin [18]. Phenazines are derived from the shikimic acid pathway that is highly conserved in most organisms including plants [19]. Chorismic acid serves as the phenazine branch point once the phenazine biosynthetic genes are expressed. The polarization conditions and the results obtained suggest that secondary metabolites are included in the duckweed response to polarization. The duckweed is capable of expressing phenolic compounds and flavonoids to maintain the redox state of the plant. Comparing the CV of *Wolffia globosa* with the CVs of other duckweed species (Table 1), it is seen that the formal potential $E^{`0}$ values of −260 mV *vs.* Ag/AgCl (−55 mV *vs.* SHE) are close to that of *L. valdiviana* and slightly shifted from that of *L. minuta*. The potential of the oxidation peak reaction was recorded at −114 mV *vs.* Ag/AgCl, corresponding to *ca.* 90 mV *vs.* SHE, while that of the reduction peak reaction was −404 *vs.* Ag/AgCl (*ca.* -200 mV *vs.* SHE). Although the nature of the EnM in duckweeds needs further clarification, the established redox potentials overlap that of the 2-cyanophenazine [20] suggesting that it could be

a phenazine derivate. The duckweeds grown normally as a control do not express redox activity (Figure 4-inset), indicating the redox substance is produced only under polarization conditions.

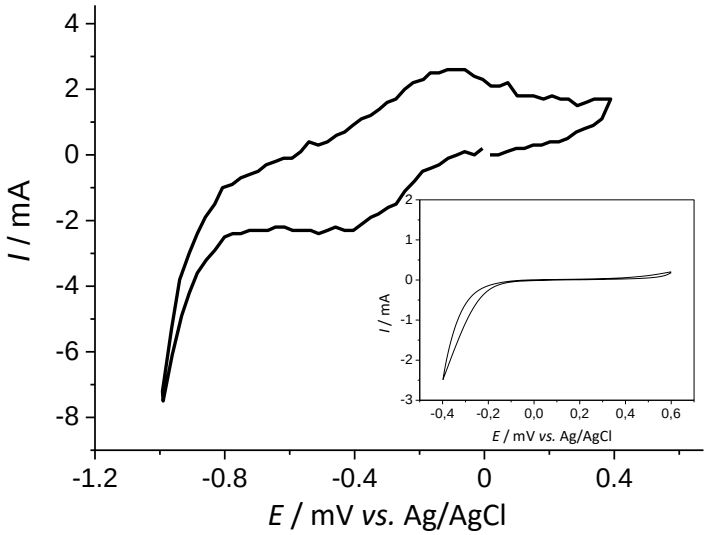

Figure 4. *Cyclic voltammogram of the* W. globosa *- P-BFC –anolyte (phosphate buffer, pH 7.0) on the 12th day of operation. Inset - negative control toward polarization. Scan rate: 10 mV s^{-1}, the second scan*

In conclusion, *W. globosa*-based fuel cell gives high and relatively stable electrical outputs over time, which reveals that the smallest flowering plants *W. globosa* are promising biocatalysts for further development of the P-BFC biotechnology. Although rootless, they are secreting redox-active compounds (EnM), which shuttle electrons to the anode.

Influence of the polarization on the W. globosa biochemical parameters

The extracellular electron transfer from living organisms to the anode is associated with metabolic changes caused by the applied polarization [6,15]. To examine how *W. globosa* responds to the polarization, the explored in biofuel cells duckweeds and those grown as a control have been collected and partially fractionated for receiving an organelle-enriched fraction. The basic biochemical parameters such as the intracellular content of proteins, glucose, starch, and inorganic phosphate have been studied. The enzymes catalyzing the degradation of the reserve carbohydrates and phosphates (in form of phytates) - the amylase and phytase, respectively, have been analyzed as well. The results from these analyses are summarized in Table 2. Besides, these parameters have been also inspected in the supernatants obtained by centrifugation as well as in samples taken from the liquid media (anolyte and water) and presented as Suppl. Table S-1.

Table 2. *The basic biochemical parameters and the specific activities of the enzymes responsible for the degradation of carbohydrates and phosphorus reserves in the enriched with chloroplasts and mitochondria fractions obtained from* W. globosa *grown under normal (control) and polarization conditions (P-BFC)*

Organelles enriched fraction	Content, mg g^{-1} wet biomass			Activity, U g^{-1} wet biomass		$c_{PO_4^{3-}}$ / mM
	Protein	Glucose	Starch	Amylase	Phytase	
W. globosa - P-BFC	495.0±10.3	15.5±3.1	125.1±8.7	70.0±2.8	4.3±0.2	160.8±30
W. globosa - control	392.5±20.2	7.8±2.0	73.5±3.5	38.5±1.9	2.8±0.1	22.4±3.3

About a 25 % increase in the total protein content of the organelle-enriched fraction has been registered under polarization conditions, while the protein content of the supernatant was twice lower than that of the control, indicating that the polarization influences processes implemented in the chloroplasts and mitochondria of the duckweeds. Indeed, a much more sensitive change is

established for the carbohydrate content – the twice higher quantity of reducing sugars and 1.7 times higher reserve carbohydrates in the form of starch of the same fraction. Having in mind that the synthesis of glucose in plants takes place in the Calvin cycle in the chloroplasts, it can be assumed that polarization affects the photosynthetic processes of the duckweeds. At the same time, peroxisomal photorespiratory enzymes have been reported to protect against stress [21]. For example serine: glyoxylate aminotransferase can contribute to the polarization stress response [21,22]. Negligible amounts of starch and glucose were registered in the anolyte/water (Suppl. Table S 1), indicating that the plants are intact, the cellular membranes are impermeable, and do not allow the loss of valuable nutrients. At the same time, the specific amylase activity was 1,82 times higher than that of the control, indicating that not only more starch is produced under polarization, but also more reserve carbohydrates were hydrolyzed to cover the plant's energy demands. The results show that both catabolic and anabolic processes are enhanced in *W. globosa* grown as a biocatalyst under polarization. The higher content of inorganic phosphates as well as enhanced phytase activity supports the hypothesis about intensified metabolic processes. From the initial 67 mM phosphates, in which the control plants grew, one-third was established in the water (Suppl. Table S-1), showing that ca. 40 mM phosphates were sufficient to supply the plants with the required amount of phosphorus. 20 mM phosphates were determined in the organelle fraction of the control, while these values were extremely higher in the respective fraction of the plants grown under polarization. Having in mind that the duckweeds contain inositol phosphates (phytates) as a reserve form for storing phosphorus [15,23] and to check whether the change in the number of inorganic phosphates in the duckweeds grown during polarization is associated with additional degradation of inositol phosphates, the phytase activity of the samples was also determined. The results show a 53 % increase in the specific phytase activity, suggesting, on the one hand, an explanation for the higher phosphate content, and on the other hand, that the phytate is synthesized and hydrolyzed simultaneously to a greater extent under polarization. The results for phytate degradation suggest that duckweeds can be used not only as food/feed resources and pharmaceuticals but as well as for phytoremediation [24].

Conclusions

Wolffia globose duckweed can be grown in biofuel cells and is capable of generating an electrical current whose value is comparable with the current achieved by duckweed species belonging to the *Lemnaceae* family. The electrons are transferred to the anode by secretion of an electrochemically active substance acting as an endogenous mediator of electron transfer.

In addition, intensified metabolism in aquatic plants used as biocatalysts in Plant-BFC is observed at polarization, expressed in increased photosynthetic properties, phosphorylation, and dephosphorylation processes. The increased content of proteins in *W. globosa* grown in the fuel cell specifies the benefit of the technology for increasing the plant biomass at polarization. Furthermore, the double increased glucose and starch amounts propose the significance of the used P-BFC as a cheap way to obtain valuable raw materials that can be used in food and bioethanol production, while utilizing atmospheric CO_2 and generating electrical current. The results also suggest the application of the plant-BFC as a beneficial approach for increasing yield in duckweed farming.

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