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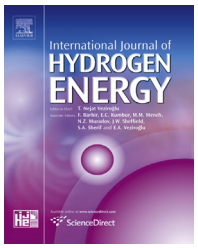

Evidences of oxidative stress during hydrogen photoproduction in sulfur-deprived cultures of *Chlamydomonas reinhardtii*

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ABSTRACT

Biological hydrogen production is being evaluated for use as fuel, since it is a promising substitute for carbonaceous fuels owing to its high conversion efficiency and high specific energy content. The microalga *Chlamydomonas reinhardtii* when grown under sulfur-deprived conditions, switches the metabolism toward the production of hydrogen. A better understandings of physiological and biochemical changes occurring during each phases of the process, represents a prerequisite to enhance the hydrogen output. The aim of this work was to study whether the activation of enzymes of the antioxidant defense system, such as catalase (CAT), ascorbate peroxidase (APOX) and guaiacol peroxidase (GPOX), takes place during the entire process of hydrogen production by *C. reinhardtii* CC 124. Kinase activities present in the crude protein extract and the mitotic specific ones associated with CKS1 protein were assayed to determine how the conditions leading to hydrogen production affected the activities of mitotic and growth associated kinases. We present evidences that oxidative stress enzymes are active during the entire hydrogen production process, besides their activities are higher in the anoxic phase. Stress condition during hydrogen photoproduction provoked at least partial cell cycle arrest leading to block of mitosis and cell division. These findings are in line with the known down-regulation or block of cell cycle related processes in stressed or starved cells.

Introduction

The need for new energy sources as a result of the higher consumption of conventional fuels, caused by world population increase, has fostered to explore a sustainable way to produce energy with zero CO_2 emission as photobiological H_2. Biohydrogen is the ideal energy carrier producing only water as combustion product and no other toxic or polluting

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by-products; therefore it doesn't contribute to air pollution or global warming.

Microalgae are among the groups of organisms able to produce clean, carbon free energy as hydrogen from natural sources, such as sun and water, through the expression of hydrogenase enzymes. Hydrogen can be not only used to generate electricity but it can also be by itself transported, distributed and regenerated, and used as an on-site form of energy.

Chlamydomonas reinhardtii has the metabolic capabilities and the flexibility that allows adaptations to a rapidly changing environment; under specific conditions various strains and mutants produce varying amount of biohydrogen. Studies have been made about the roles of PSII and starch accumulation in hydrogen photoproduction in entrapping algal cells in thin alginate film [1]. Kosourov et al. [2] demonstrated that a truncated antenna mutant of *C. reinhardtii* shown an improvement of photosynthetic productivity in mass culture as well as an increase of the efficiency of biohydrogen photoproduction with respect to the parental strain. The optimized conditions for biohydrogen production are represented by a two-step production strategy developed by Melis [3]. In this set-up cells are first grown under aerobic and sulfur replete conditions, then transferred to anaerobic sulfur-deprived conditions where hydrogenase is induced and biohydrogen release occurs. Phosphorus deprived condition can also induce biohydrogen production as it inhibits the oxygen evolving activity of algae, but this inhibition is slower. It is worth to mention that physiological response of algal cultures to both sulfur and phosphorus depletion are significant similar [4]. In order to improve hydrogen production, the physiological and biochemical changes during each phase of this process need to be better understood.

Oxidative stress is a complex chemical and physiological phenomenon that is related to both biotic and abiotic stresses in living organisms, and develops as a result of overproduction and accumulation of reactive oxygen species (ROS). ROS are substances containing one or more activated atoms of oxygen; they are not always radicals, as is the case for H_2O_2. Free radicals are chemical species that exist independently and contain unpaired electron(s). Both ROS and free radicals promote oxidative stress through oxidation of cell compounds. Oxidative stress is the physiological state when loss of electrons (oxidation) exceeds the gain of electrons (reduction) leading to chemical (oxidative) damage of cell compounds [5]. Oxidative stress is therefore associated with severe and long-term redox (reduction/oxidation) imbalance due to lack of electrons. ROS have important roles as signaling intermediaries in a large number of cellular processes, especially in relation to interactions with the environment. Exposure of cells to ROS may result not only in cell death by excessive oxidation of biomolecules but also in the activation of cellular stress signaling pathways [6]. Anti-oxidation systems are widely distributed among all organisms in order to cope with molecular oxidation and its consequences; the energy invested in these systems helps to prevent the deleterious effect, and it is in fact a defense mechanism against cell death. A complex network of low molecular weight antioxidants, ROS scavenging enzymes, and enzymes that maintain antioxidant pools are required to control the levels of ROS in all subcellular compartments, suppressing the accumulation of these reactive and harmful intermediates under normal growth conditions. Oxidative stress causes a very wide spectrum of genetic, metabolic, and cellular responses. Most oxidative stress conditions that cells might actually encounter will modulate gene expression, may stimulate cell growth, or may cause a protective temporary growth arrest at the point of division, as a transient adaptive response [7].

Oxidative stress inducing agents can cause damage to proteins, lipids, carbohydrates, and nucleic acids, inhibition of enzymes, and chlorophyll degradation [8]. In DNA, ROS can react directly with deoxyribose as well as with the purines and pyrimidines in the DNA, leading to the formation of single strand breaks [7]. Proteins exposed to ROS undergo typical modifications, including specific amino acid alterations, polypeptide fragmentation, aggregation, denaturation and susceptibility to proteolysis [9,10]. Similarly, cellular enzymes and structural proteins, membranes, simple and complex sugars, DNA and RNA are all susceptible to oxidative damage [7]. High levels of ROS production are frequently associated with lipid peroxidation and initiation of radical chain reactions that may lead to membrane damage, changing and disrupting lipid structure as well as membrane organization and integrity [8,11]. A set of antioxidant metabolites and enzymes detoxify ROS and lipid peroxides in the various cell compartments such as chloroplasts, cytosol, mitochondria, and peroxisomes. A sequence of detoxification steps mediated by different enzymes such as SOD (superoxide dismutase), CAT (catalase), APOX (ascorbate peroxidase), GR (glutathione reductase) and GPOX (guaiacol peroxidase) is required to avoid the conversion of one reactive species into a different, more harmful one. Therefore, the knowledge about how enzymes work under oxidative stress during hydrogen production could give insights into a potential tool to employ, or a strategy to develop in the future, related to the increase of production rates.

The aim of this work was to study reaction of the antioxidant defense system during the entire process of biohydrogen production by *C. reinhardtii* CC 124. To this end, the activity and temporal occurrence of some antioxidant enzymes such as CAT, APOX, and GPOX were studied during the sulfur starvation phases. The tested hypotheses were: i) the antioxidant enzymes' activity isn't detectable during biohydrogen production phase as it is in a reductive environment; ii) antioxidant enzymes are active during all phases of biohydrogen production process, and their activity is higher in the phase where anoxic conditions occurs. As complementary analyses, kinase activity in the crude protein extract and the mitotic specific ones associated with CKS1 protein were assayed, in order to determine how the conditions leading to hydrogen production affected the activities of mitosis- and growth-associated kinases related to cell cycle progression and cell cycle block.

Materials and methods

Strain and culture conditions

C. reinhardtii strain CC 124 from the culture collection of the Institute for Ecosystems Study – CNR, Florence, (Italy) was

used. A two-stage protocol, with aerobic phase followed by anaerobic phase, was used for biohydrogen production [3,12]. Cells were grown photomixotrophically in glass columns (5 cm i.d.) of ~400 ml volume in Tris-acetate-phosphate (TAP) medium (pH 7.2 ± 0.2) under continuous illumination of 70 μmol photons m^{-2} s^{-1} supplied from both sides, immersed in a water bath at 28.0 ± 0.5 °C and sparged with a mixture of air–CO_2 (v/v, 97/3). The starting cell concentration was 310 ± 0.2 mg/l. Cultures in logarithmic phase were used to inoculate flat photobioreactors (PBR) (1 l) [13].

For biohydrogen production experiments, algal cells were transferred into a sulfur-deprived medium (TAP-S) before the induction of the anaerobic phase. The cells were harvested by centrifugation, washed 3 times with TAP-S medium, according to Melis et al. protocol [3], then subsequently re-suspended in TAP-S and transferred into a sealed glass flat PBR with mixing operated by a multiple-impeller stirring device [14]. Cultures were illuminated from both sides with 70 μmol photons m^{-2} s^{-1}. The incident light on the surface of the PBR was measured with a quantum radio photometer (Li 250 A, LI-COR). Initial chlorophyll content was 12 ± 2 mg/l. Chlorophyll concentration was determined spectrophotometrically according to Lichtenthaler [15]. The F_v/F_m (variable-to-maximum fluorescence) which indicates the maximum quantum yield of Photosystem II, was measured in dark adapted cells (15 min) using a portable pulse-amplitude-modulation fluorometer, PAM-2100 (H. Waltz, Effeltrich, Germany) operated by means of PamWin (version 2.00f) PC software [16]. For online chlorophyll fluorescence measurements, the fiber-optics of the PAM-2100 was placed directly on the surface of the PBR, at an angle of 90° with respect to incident light. Minimum fluorescence, F_0, was measured by modulated light (<0.5 μmol photons m^{-2} s^{-1}) from an LED (peak wavelength at 655 nm, 600 Hz). A single high-intensity flash (6000 μmol photons m^{-2} s^{-1}, 0.8 s in duration) provided by a miniature 8 V/20 W halogen lamp (Bellphot Osram, model SL, Walz) was used to raise the fluorescence yield to the maximum, F'_m; flashes were administered at 1 min intervals until the culture reached anaerobiosis and thereafter at 5 min intervals. The effective quantum yield of PSII was calculated as follows: $(F'_m - F_s)/F'_m$) were F'_m and F_s were the maximum and the steady-state fluorescence under light exposure, respectively. The fluorescence nomenclature follows Van Kooten and Snel [17]. Fluorescence data collected online by PAM-2100 were exported into a spreadsheet program (Excel; Microsoft Corp., Redmond, WA, USA). Dry weight (DW) was determined according to Lee et al. [18]. Carbohydrate content of the cells was measured using the phenol-sulfuric acid method [19] using D+ glucose as a standard (three replicates). These measurements were done at the beginning and at the end of the sulfur starvation phase. Oxygen concentration, pH and redox potential values were recorded continuously during sulfur starvation phase by means of electrodes inserted hermetically into the PBR.

Hydrogen gas measurements

H_2 production by the cultures was measured according to the system designed by Kosourov et al. [20], which consisted of five elements: (1) a PBR equipped with four probes for continuous monitoring of culture parameters, for example, culture temperature, pH, redox potential (a platinum/gold electrode), dissolved O_2 concentration; (2) a gas-to-liquid conversion bottle; (3) a liquid accumulating bottle; (4) a digital balance; (5) a lap-top computer for continuous data recording. The digital balance, monitoring the changes in the weight of the liquid accumulating bottle (Acculab, ALC models, Sartorius Group, Goettingen, Germany), was connected to the lap-top computer via a RS232 to RS485/422 converter (Intelligent DA&C module ND 6520 USAM Chemitec, Florence, Italy). A software for automatic culture control and data acquisition was developed (Chemitec, Florence, Italy). Biogas was analyzed with a gas chromatograph (Clarus 500, Perkin Elmer) using a packed column (model Carbosieve S-II Spherical Carbon, Supelco) and nitrogen as a carrier gas [13].

Biochemical measurements

Enzyme extraction

Aliquots of 40 ml of culture from the PBR were collected with a syringe and centrifuged at 3000 *g* for 5 min. Pellets were re-suspended in 3 ml of 25 mM sodium phosphate buffer (pH 7.3) at 4 °C with 0.1% w/v Triton X-100. The enzyme extraction was performed by sonication in cold buffer using two cycles of one minute each. At the end of this procedure, the enzyme extract was separated from debris by centrifugation [21]. Determinations were performed in duplicate.

Enzyme determination. Catalase (EC 1.11.1.6) activity was determined spectrophotometrically by measuring the consumption of H_2O_2 at 240 nm (ε: 0.036 mM^{-1} cm^{-1}) in a reaction medium containing 50 mM sodium phosphate buffer (pH 7), 33 mM of H_2O_2 and enzymatic extract [22].

Ascorbate peroxidase (EC 1.11.1.11) activity was determined by oxidation of ascorbate, following the decrease in absorbance at 290 nm (ε: 2.8 mM^{-1} cm^{-1}) in a reaction medium containing 50 mM sodium phosphate (pH 7), 10 mM H_2O_2, 5 mM ascorbate and enzymatic extract [23].

Guaiacol peroxidase (EC 1.11.1.7) activity was determined by measuring the increase in absorption at 470 nm due to the formation of tetraguaiacol (ε: 26.6 mM^{-1} cm^{-1}) in a reaction mixture containing 50 mM potassium phosphate buffer (pH 7), 10 mM guaiacol, 10 mM H_2O_2, and enzymatic extract [24].

CAT, APOX and GPOX activities were expressed as enzyme units (U) mg^{-1} protein min^{-1}, where 1 enzyme unit is defined as a change of 0.01 absorbance min^{-1} caused by the extract sample at 25 °C.

CAT and APOX kinetics were determined at the beginning of the sulfur starvation (T_0) and at 24, 48, 120 and 144 h, while GPOX activity was determined at the beginning (T_0) and at the end (T = 144 h).

Protein determination. Protein determination was performed according to Lowry method [25] using bovine serum albumin as standard.

Kinase assay

H1 histone kinase activity was assayed by the following modification of the method described by Moreno et al. [26] at

the beginning of the sulfur starvation condition (T_0) and at the end (144 h).

Preparation of protein lysate. Pellets containing approximately 2×10^6 cells were harvested washed with SCE buffer (100 mM sodium citrate, 2.7 mM EDTA-Na_2, pH 7 (citric acid)), fast frozen in liquid nitrogen and stored at −70 °C. Protein extracts were prepared according to Bisova et al. [27], the protein concentration was set to 0.5 mg/ml.

Kinase activity in crude extracts and affinity purification of CDKs on CrCKS1 beads. To purify mitotic CDKs, 30 μl of protein lysate containing 15 μg of protein were diluted ten times with RIPA (radioimmunoprecipitation assay) buffer (50 mM HEPES(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), pH 7.5, 150 mM NaCl, 5 mM EDTA (ethylenediaminetetraacetic acid), 5 mM EGTA(ethylene glycol tetraacetic acid), 0.1% w/v SDS (sodium dodecyl sulfate), 1% w/v NP-40 (nonyl phenoxypolyethoxylethanol) containing 1× protease inhibitor cocktail (Sigma P9599, www.sigma-aldrich.com), 1 mM Na_3VO_4, 1 mM benzaminidine, 10 mM NaF and incubated for 2 h at 4 °C with 20 μl of 50% CrCKS1 beads slurry. Unbound proteins were washed out by four consecutive washes with RIPA buffer and two washes with kinase buffer (20 mM HEPES (pH 7.5), 15 mM $MgCl_2$, 5 mM EGTA, 1 mM DTT) [28,29].

The kinase activity was measured according to Bisova et al. [27]. To assay kinase activity in crude extracts, 7 μl of protein lysate containing 3.5 μg of protein were assayed under the same conditions as for CrCKS1 purified kinases. Phosphorylated histone bands were visualized by autoradiography. Kinase activities present in crude extract and those purified by affinity chromatography on CrCKS1 beads represent growth associated kinase activities and mitotic kinase activities respectively [30,27] due to the fact that CrCKS1 protein (homolog of suc1 protein in fission yeast) binds specifically mitotic CDK/cyclin complexes [31,32].

Statistical analysis

One-way ANOVA was used to assess the differences in enzymatic activity in conjunction with Tuckey's multiple range tests and Dunnett's test ($p < 0.05$). All statistical analyses were performed using Statistica v. 8 (StatSoft).

Results

H_2 production and changes in the physical–chemical parameters

As already reported, the entire hydrogen production process can be split into five consecutive phases [20] (Fig. 1): I) an O_2 producing phase, characterized by accumulation of O_2 in the culture; (II) an O_2 consumption phase, during which photosynthesis cannot keep up with respiration; (III) a lag phase, during which neither O_2 nor H_2 is evolved by the cells; (IV) a H_2 production phase, during which H_2 is released; and (V) a termination phase during which the accumulation of H_2 ceases. In our experiments, at the end of the oxygen consumption phase, i.e., in coincidence of the onset of anaerobiosis, a drop in the redox potential was observed (down to −110 mV), reflecting the instauration of a reductive environment inside the culture. Afterwards, the redox potential continued to decline steadily until hour 120, when it reached stability at 500 mV. The hydrogen production started after a lag phase of 24 h, and coincided with a fast drop in the effective quantum yield of PSII ($\Delta F/F'_m$) from 0.7 to about 0.2. The total amount of hydrogen produced by the culture was 110 ml in 48 h, at a mean rate of 2.3 ml L^{-1} h^{-1}. Chlorophyll content of algal cells decreased at the end of biohydrogen production phase, in respect to its initial value, due to pigment degradation caused by the lack of sulfur in TAP-S medium which provoked a functional loss of PSII reaction centers. Carbohydrates storage in the algal cells at 18 h of sulfur starvation increased by 30% of the initial value, reaching 359.4 mg/l at the end of oxygen production phase.

Antioxidant enzymes activity

Enzymatic activity of CAT, APOX and GPOX during the sulfur starvation phase and when hydrogenase activity was induced, resulting in biohydrogen production by the algal cells, are shown in Fig. 2. CAT activity was significantly higher than APOX at all measured times intervals (Fig. 2). CAT activity was

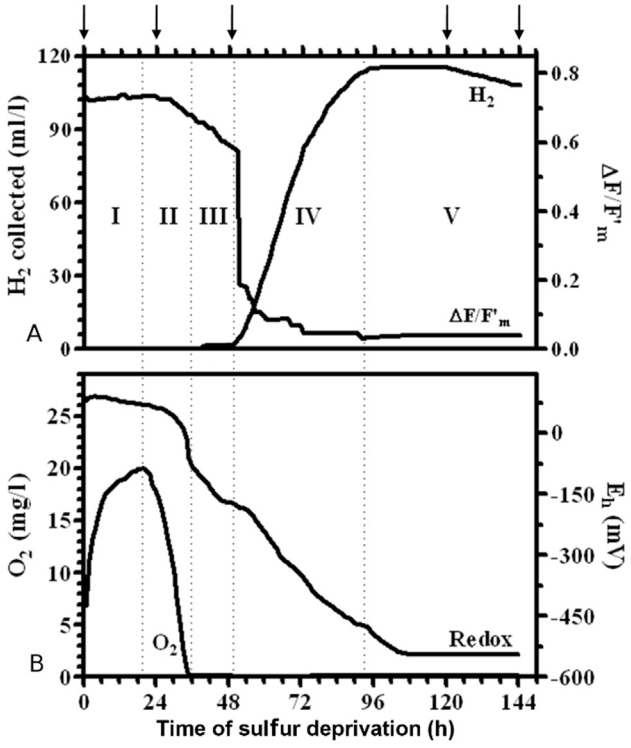

Fig. 1 – (A) Time courses of the output of H_2 gas, the effective quantum yield of photosystem II ($\Delta F/F'_m$), (B) redox potential (E_h), and dissolved oxygen concentration (O_2). Five transition phases as I–V are separated by vertical lines. The experiments were carried out with sulfur-deprived *C. reinhardtii* CC 124 cultures exposed to a photon flux density of 70 μmol m^{-2} s^{-1} supplied on both sides of the reactor. Chlorophyll concentration was 12 mg L^{-1}. Arrows indicate sampling points for enzyme analysis.

significantly lower ($p < 0.05$ ANOVA–Dunnett) at 24 h respect to T_0, (begin of phase II, 24 h of Fig. 1), a decrease in enzymatic kinetic was observed. However, APOX activity at 24 h did not differ significantly from T_0 (Fig. 2).

At the beginning of phase IV, (48 h of Fig. 1) both enzymes' activity showed a significant decrease compared to T_0 ($p < 0.05$ ANOVA–Dunnett) (Fig. 2).

At the peak of hydrogen production during the anaerobic phase, activity of both enzymes was significantly higher thanatT_0: CAT activity 45% higher and APOX increased by 168% (Fig. 2).

On the whole, during sulfur starvation period, enzymatic activity of APOX at 48 and 144 h were significantly lower compared to T_0 and 24, whereas CAT activity at 24, 48 and 144 h were significant lower than values recorded at T_0. Both APOX and CAT activity from 48 h did not differ from that of 144 h (Fig. 2).

GPOX activity at the end of the hydrogen production (144 h) was significantly lower than the activity recorded at T_0. This fact was also observed in CAT and APOX, so the three enzymes studied showed lower activity at the end of the whole period than at the beginning.

Kinase assays

Kinase activity assays were performed to determine how the conditions leading to hydrogen production affected the activities of mitotic and growth associated kinases. In this study, kinase activity in the crude protein extract and the mitotic specific activity associated with CKS1 protein were assayed. The kinase activity in crude protein extract reflects the growth-associated CDK-like kinases, e. g. kinase activity at the commitment point, as well as mitotic CDK-like kinase activity [30,31]. In contrast, affinity binding to CKS1 protein enables specific purification of mitotic CDK/cyclin complex [31,32]. The same amount of protein in a constant volume was used to assay growth-associated and mitotic kinase activities both at the beginning of the starvation period (T_0) and at the end of the experiment (T144 h). For crude extract related kinase assay (growth-associated kinases), 3.5 μg of total protein extract was used, while 15 μg of total protein was used for affinity purification with CKS1 beads (mitotic kinase).

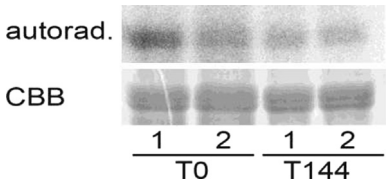

Fig. 3 – Kinase activity in hydrogen producing cells of *C. reinhardtii* CC 124 at T_0 and T144 h. Only bands corresponding to 21 kDa band of lysine rich histone H1 used as a substrate is shown. Upper panel correspond to the autoradiography (autorad.) while the lower one shows the same bands stained by Commassie-briliant blue (CBB) as a loading control. 1 corresponds to activity in crude protein extracts; 2 corresponds to activity purified by CKS1 affinity chromatography, for details on differences between the two activities see Material and methods.

The kinase assay results therefore reflected the changes in kinase activity per amount of protein in the sample. In both types of extracts, the CDK-like kinase activity was lower compared to kinase activity present in normally dividing cells. The kinase activity in the T_0 samples was consistently higher than in the T144 h ones. The crude extract kinase activity at T_0 was also higher than the CKS bound one (Fig. 3). The fact that mitotic kinases in both types of samples were negligible indicates that the cells were not undergoing mitosis as would be expected for starved cells probably due to cell cycle block caused by the starvation treatment [33,34].

Discussion

In this study, we present evidence that oxidative stress enzymes were active during the entire hydrogen production process, i.e., both during the aerobic and anaerobic phases.

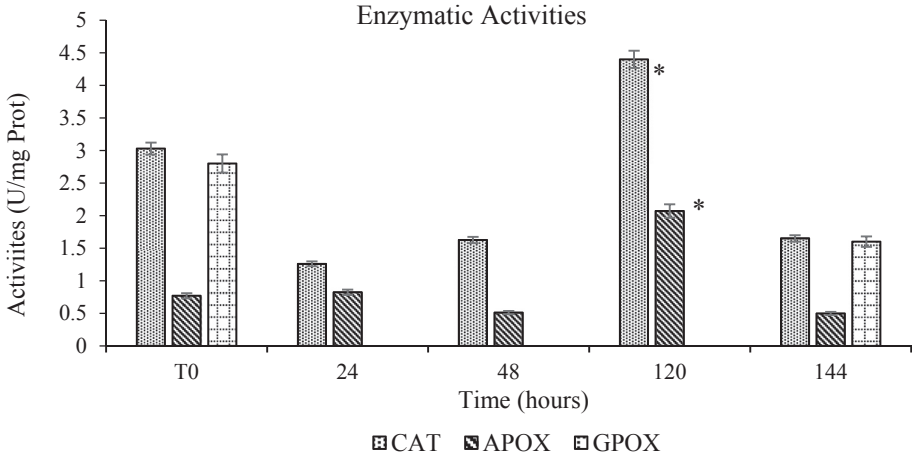

Fig. 2 – Enzymatic activities of CAT, APOX and GPOX measured at time intervals in *C. reinhardtii* CC 124 cells during the sulfur-deprivation process. * Significantly higher from T_0 at $P < 0.05$ (One-way ANOVA–Dunnett test).

However, their activities were higher during the anoxic phase. In contrast, the activities of the cell cycle regulating CDK-like kinases were very low both in the case of growth-associated kinases and the mitotic specific ones. This indicated a stop or block of cell cycle progression at the end of the experiment, which is in line with a down-regulation or even a halting of cell cycle related processes in stressed or starved cells [35–37]. The activities of growth-associated kinases were higher than those of mitotic specific ones at the beginning of the experiment. This might indicate that at least some of the cells were actively growing at this time although they clearly were not undergoing mitosis due to negligible mitotic kinase activity.

We found that CAT activity was high during the whole experiment. There are some reports about CAT increase in response to anoxia in genomic studies of *Chlamydomonas* performed in microarray systems, which have improved our understanding about anaerobic metabolism in this organism, including the metabolic fluxes in anoxic algal physiology [38]. High-density, oligonucleotide-based microarrays have provided unique insights into the genome-wide responses to anoxic acclimation. The levels of more than 500 transcripts were observed to be significantly up-regulated as a consequence of anoxia. Several of these transcripts were associated with fermentative metabolism. Moreover, several genes encoding homologues of proteins involved in transcriptional/translational regulation, posttranslational modification, and stress responses also were up-regulated as the cells were entering anoxia. Insights from the microarray data are being integrated into metabolomics and proteomic data sets, and will soon be integrated into larger systems in order to understand and describe the plasticity of whole cell metabolism. Surprisingly, microarray data revealed an increased expression of genes related to oxidative stress, particularly catalase. This was not unexpected given that genes encoding oxygen sensitive proteins in other organisms are present in genomic regions that also encode high-affinity oxidases. Since acclimation of cells to anoxia requires a substantial energy investment for the biosynthesis of oxygen labile proteins, it is conceivable that these organisms have also evolved mechanisms to protect oxygen sensitive enzymes from reactive species formed by exposure to low levels of oxygen [38]. CAT acts directly and efficiently as scavenger of H_2O_2 after the action of SOD, localized in peroxisomes. This would be the first signal of oxidative stress generated by all ROS species as a consequence of anoxia.

APOX is an enzyme localized in chloroplasts, and its action is mainly directed to generation of ROS as a result of oxidative reactions occurring within the photosystems. Therefore, its activation would be related to ROS generated from the decline of PSII activity and modification of proteins of the light harvesting complex occurring during hydrogen production.

On the other hand, Sakr et al. [39] found a relationship between protein regulators, oxidative stress and hydrogen production in prokaryotic cyanobacterium *Synechocystis* PCC6803. They reported that AbrB2 (gene encoding a protein regulator of hydrogen production operon) down-regulates hydrogen production and other stress defense genes in the absence of stress. In contrast, in the presence of oxidative stress, AbrB2 is oxidized (cystein glutathionylated) and it is no longer able to repress hydrogen production leading to electrons evacuation to other antioxidant processes. After recovery from oxidative stress, AbrB2 activity is restored and starts to repress hydrogen production and antioxidant process, which are no longer required to protect from the energy of the photosynthetic electrons.

Hence, the maintenance of oxidative stress conditions would favor hydrogen production as AbrB2 would be oxidized by glutathionylation, unable to repress the hydrogen production operon.

Another interesting study reported the role of kinase in response to oxidative stress. Li et al. [40] found that DNA-PKcs (DNA-dependent protein kinase catalytic subunit) is a critical regulator of the oxidative stress response and contributes to the maintenance of redox homeostasis, and that DNA-PKcs is required for cellular resistance to oxidation.

Enhancing cell resistance to stress conditions (for example increasing the activity of oxidative stress-related enzymes) could improve the efficiency of the hydrogen production process, making it more stable and for a longer period. With an increased enzyme activity, less energy would be invested into reactions and molecular biosynthesis required to cope with anoxic stress (in the conditions required for H_2 production). This increased resistance could be achieved by selecting or engineering strains with increased oxidative stress protection. Such strains, in combination with biotechnological advances (such as high performance photobioreactors), or practical strategies (as the removal of hydrogen gas from de culture medium, reducing the partial pressure in the photobioreactor headspace) [41] will improve the feasibility of biohydrogen production processes.

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