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Factors influencing algal photobiohydrogen production in algal-bacterial co-cultures

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A B S T R A C T

Algal-bacterial co-cultures represent an alternative way for algal biohydrogen generation. Efficient algal hydrogen production requires anaerobiosis and electrons accessible for the algal FeFe-hydrogenases. A number of factors strongly influence the development of this optimal environment. Various algal strains were tested for hydrogen evolution with a selected bacterial partner, a fully hydrogenase deficient *Escherichia coli*. During the hunt for the most efficient algae strains, gas-to-liquid phase ratio, algal optical density and algal cell size were identified as crucial factors influencing algal hydrogen evolution rate, accumulated algal hydrogen yield, carbon dioxide and oxygen levels as well as acetic acid consumption in illuminated algal-bacterial cultures. The highest accumulated hydrogen yields were observed for the different algal partners under similar experimental setup. The combination of a gas-to-liquid phase ratio of 1/1 with an algae cell density of $3.96 * 10^8$ algae cell ml^{-1} (OD_{750}: 1) resulted in the highest accumulated algal hydrogen yields under continuous illumination of ~ 50 µmol $m^{-2} s^{-1}$ light at 25 °C irrespective of the applied algae strain. Accumulated hydrogen yield was also strongly influenced by the algal cell size, smaller cell size correlated with higher hydrogen evolution rate. The highest accumulated algal hydrogen yield (88.98 ± 2.19 ml H_2 l^{-1} d^{-1}) was obtained with *Chlorella* sp. MACC 360 -*E. coli* Δ*hypF* co-culture.

1. Introduction

Green algae are able to evolve hydrogen by FeFe-hydrogenases in both fermentative and photochemical ways [1]. Since oxygen is a strong inhibitor of the algal FeFe-hydrogenase activity and expression, anaerobic environment is a prerequisite of algal hydrogen evolution [2]. Plastidic ferredoxin receiving the electrons from the photosystem I (PSI) serves as direct electron donor of the FeFe-hydrogenase. Three different electron pathways were described for algal hydrogen evolution. Two of them are connected to the photosynthetic electron transport, while the third one represents the fermentative degradation of the endogenous stored compounds. However, the photosynthetic electron transport dependent pathways are strikingly different. The electrons are originated from water splitting in the photosystem II (*PSII*) dependent pathway, while electrons are provided by starch degradation in the *PSII* independent pathway and these electrons are transferred to the plastoquinone (PQ) pool in the electron transport chain *via* the NAD(P)H:plastoquinone oxidoreductase [3]. The electrons migrate from the electron transport chain to ferredoxin in both cases and reduced ferredoxin donates electrons to the FeFe-hydrogenases [4,5].

The FeFe-hydrogenases are extremely sensitive to oxygen [2]. Anaerobiosis is fundamental for their efficient operation, but O_2 is generated during photosynthetic activity and inhibits hydrogen evolution [6]. Various approaches have been utilized to overcome this bar-

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rier [7]. During dark adaptation the sealed algal cultures consume the oxygen and establish anaerobic environment by the algal respiration [1,8]. Under illumination the initial rate of hydrogen evolution is high, but the evolving oxygen instantly inhibits the FeFe-hydrogenases. Many attempts have been made to elaborate long-term continuous H_2 photoproduction [7]. It can be achieved during illumination, when the algae cultures are constantly purged with inert gas to maintain a stable anaerobic environment by rapidly removing the photosynthetically evolved oxygen [9]. Establishment of anaerobic environment is possible through nutrient deprivations by depleting either sulfur, phosphorus, nitrogen or magnesium from the medium [7]. The oxygen production rate of *PSII* is diminished in response to nutrient deprivation, however it takes time, anaerobic environment is achieved in 1 to 8 days depending on the applied nutrient deprivation approach [10–13]. The oxygen content of the closed cultures is consumed by the algal respiration, which can be enhanced by the addition of acetic acid [14]. Establishment of anaerobic environment is possible without nutrient deprivation by using low light intensity and adding acetic acid. Under 50 μmol photon $m^{-2} s^{-1}$, the oxygen evolved by the low activity of *PSII* is efficiently respired by the algae which allow hydrogen production [15]. By the addition of bacterial partners to the algal culture in TAP (Tris-Acetate-Phosphate) medium the rate of the net oxygen consumption can be further enhanced in the sealed algal-bacterial cultures. This decreases the time necessary for the establishment of the anaerobic environment in the liquid phase to a few hours (compared to 1 to 8 days). The elevated total respiration rate enables the use of higher illumination resulting in higher hydrogen production. A number of studies focused on the determination of the optimal light intensity for the most efficient hydrogen production [16,17]. Besides changing the incident illumination power, the consumable light yield can be influenced by the density of the cells in the liquid cultures [18]. To obtain the maximal hydrogen evolution rate of an axenic algal or a mixed algal-bacterial culture the optimal cell density values must be determined experimentally.

The hydrogen production capacities of the axenic algal or mixed alga-bacterial cultures are also strongly influenced by the concentration of the H_2. The FeFe-hydrogenases have hydrogen evolving and consuming functions as well [19]. The partial pressure of the ambient hydrogen influences the establishment of the equilibrium between the concentration of H_2 and the level of the reduced ferredoxin (Fd). This phenomenon has been investigated in anaerobic bacteria during dark fermentation [20]. The equilibrium levels show significant differences between the different bacterial (and possibly algal) strains as well. In the case of *Chlamydomonas reinhardtii* the midpoint redox potential of the major photosynthetic electron transport Fd (encoded by *petF*) is around −0.4 V [21]. When the hydrogen redox potential at pH 7 is more negative than this value the hydrogenase starts consuming hydrogen, while hydrogen production is initiated at less negative redox potential [22]. This phenomenon is of high importance when the goal is to achieve the highest possible accumulative hydrogen level. There are two possible solutions to eliminate the inhibitory effect of the accumulated hydrogen; the appropriate setup of the liquid *versus* gas volume ratio, as well as regular purging of the headspace gas to control the concentration of the accumulated hydrogen [15,22]. Both methods have been applied with promising results for pure algae cultures incubated in sulfur-depleted TAP medium.

The application of the bacterial partners allows significantly earlier algal hydrogen production compared to the nutrient deprived axenic algal cultures. Furthermore, carefully selected bacteria enhance algal biomass production leading to even higher hydrogen production rate [23–25]. The aim of this study was the further improvement of the hydrogen production efficiency. To achieve maximal hydrogen accumulation and hydrogen photoproduction rate the most appropriate algae strains were selected, the algal culture density was optimized and the gas-to-liquid ratio was fine-tuned in a strain specific way. The effect of H_2 partial pressure on algal H_2 production was investigated in detail using three selected algae strains (Table 1).

Table 1
List of algal and bacterial strains.

Strain	Relevant genotype or phenotype	Reference or source
Chlamydomonas reinhardtii cc124	Wild type	[26]
Chlorella sp. MACC 360	Wild type	[27]
Chlorella sp. MACC 411	Wild type	This work
Chlamydomonas sp. MACC 530	Wild type	This work
Chlamydomonas sp. MACC 549	Wild type	[28]
Chlamydomonas sp. MACC 772	Wild type	This work
Chlamydomonas sp. MACC 775	Wild type	This work
Chlorella sp. PAG	Wild type	This work
Escherichia coli JW5433	BW25113, Δ*hypF*:*kan*	[29]

2. Materials and methods

2.1. Cultivation of axenic and mixed algae cultures

Chlamydomonas sp. MACC 549, *Chlorella* sp. MACC 360 and all the MACC algae strains were selected from the Mosonmagyaróvár Algal Culture Collection (MACC) (Table 1). *Chlamydomonas reinhardtii* cc124 and *Chlorella* sp. PAG were received from the Institute of Plant Biology, Biological Research Centre of the Hungarian Academy of Sciences. *E. coli* JW5433 (Δ*hypF*) strain was selected from Institute of Biochemistry, Biological Research Centre of the Hungarian Academy of Sciences. Pure algae strains were grown and maintained on TP (Tris-Phosphate medium) medium supplemented with rifampicin (50 μg ml^{-1}). The TP medium is a modified TAP (Tris-Acetate-Phosphate medium) medium where acetic acid is replaced with HCl to obtain pH 7 [30]. The algae strains were constantly incubated on TP plates under 50 μmol $m^{-2} s^{-1}$ light intensity at 25 °C. Algae used for hydrogen-evolution experiments were directly inoculated from TP plates into liquid TAP medium. Algae cultures in TAP medium were incubated in closed Erlenmeyer flasks, under 50 μmol $m^{-2} s^{-1}$ light intensity at 25 °C shaken at 180 rpm. Algae stock solutions were generated using fresh cultures by centrifugation and re-suspending the cells in fresh TAP medium. *Escherichia coli* JW5433 (Δ*hypF*) was pre-grown for experiments on LB (Luria-Bertani medium) plates at 30 °C in the dark [31]. *Escherichia coli* JW5433 (Δ*hypF*) was harvested from LB plates and suspended into TAP media for the preparation of concentrated bacterial stock solution. Algal-bacterial liquid cultures were established by mixing the axenic algal and pure bacterial stock solutions in 40 ml serum bottles. Six different liquid final volumes were set in the bottles: 10, 15, 20, 25, 30 and 35 ml. First the proper amount of algae was measured into the 40 ml bottles from the algae stock solutions. Second, the proper yield of *E. coli* Δ*hypF* was measured into the 40 ml bottles from the bacterial stock solutions. Finally, TAP medium was added to the dense algal-bacterial co-cultures to obtain the required culture volumes and algal and bacterial optical densities. The final optical densities of the algae cells were set to 0.7, 1, 2, 3, 4 and 5 (OD_{750}) in each volume in the *Chlorella* sp. MACC 360 – *E. coli* Δ*hypF* co-cultures. The final optical densities of the algae cells were set to 0.7, 1, 2, 3 and 4 (OD_{750}) in each volume in the *Chlamydomonas* sp. MACC 549 – *E. coli* Δ*hypF* co-cultures. The final optical densities of the algae cells were set to 0.7, 1 and 2 (OD_{750}) in each volume in the *Chlamydomonas reinhardtii* cc124 – *E. coli* Δ*hypF*. The final optical density of *E. coli* Δ*hypF* was set to 0.5 (OD_{600}). This way the mixed cultures had differential algal cell numbers for the different algae strains (Table 2) while the bacterial cell number was fixed at $8.31 \pm 0.95 * 10^9$ cells ml^{-1}. Mixed cultures were incubated under 50 μmol $m^{-2} s^{-1}$ light intensity at 25 °C shaken at 180 rpm. All

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