Volume 1 | Number 1 | Jan 2013 | Pages 1–100

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1 **Bacterial symbionts enhance photo-fermentative hydrogen evolution of**

2 *Chlamydomonas* **algae**

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1 The green algae *Chlamydomonas* sp. strain 549 and *Chlamydomonas reinhardtii* cc124 were
2 investigated for their hydrogen-evolution capability in mixed algal-bacterial cultures. Stable
3 bacterial contaminations were identified during *Chlamydomonas* sp. strain 549 cultivation.
4 The bacterial symbionts belonged to various genera, mostly *Brevundimonas*, *Rhodococcus*,
5 and *Leifstonia*, each of which enhanced algal hydrogen production. This phenomenon was not
6 limited to the natural associations. Increased algal hydrogen evolution was achieved by simple
7 artificial algae-bacteria communities, as well. Algal-bacterial co-cultures were designed and
8 tested in hydrogen-evolution experiments. The highest hydrogen yields were obtained when
9 hydrogenase-deficient *Escherichia coli* was applied as a symbiotic bacterium
10 (*Chlamydomonas* sp. strain 549 generated 1196.06 ± 4.42 μL H_2 L^{-1}, while *C. reinhardtii*
11 cc124 produced 5800.54 ± 65.73 μL H_2 L^{-1}). The results showed that oxygen elimination is
12 the most crucial factor for algal hydrogen production and that efficient bacterial respiration is
13 essential for the activation of algal Fe-hydrogenase. The algae-based hydrogen-evolution
14 method described represents a novel combination of fermentative and photolytic hydrogen-
15 generation processes. Active photosynthesis was maintained during the entire hydrogen
16 evolution process, which contributes to the sustainability of hydrogen production.

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19 Keywords: green algae, symbiotic interaction, biohydrogen, hydrogenase, bacterial
20 contamination
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1 Introduction

2 Increasing attention has been paid to the diverse exploitability of various algae and algae
3 products, and several further fields for the use of algal biomass are expected to open in the
4 near future. Algae are considered as healthy food and food supplements and sold all over the
5 world[1]. Algal biomass is commonly used in agriculture as animal feed and soil fertilizer [2, 3].
6 Algae extracts are used to develop various natural and green pharmaceutical products[4]. Algae-
7 bacterial associations are beneficial in certain industrial applications. The bacteria coexisting
8 with algae in wastewater improve biodegradation efficiency of complex substrates. Algae
9 represent an extremely diverse group of living organisms in terms of lifestyles, morphology
10 and adaptation capability. The metabolic versatility of certain unicellular algae makes them
11 attractive eukaryotic model organisms in several fields of basic and applied science including
12 the research for clean, green energy carriers. Green algae are capable of absorbing sunlight
13 and converting solar energy into hydrogen gas. This process takes advantage of the
14 photosynthetic system of the algae which links water splitting to H_2 production[5, 6]
15 Biologically generated H_2 has therefore the potential to be an alternative fuel of the future and
16 shows great promise for generating large scale sustainable energy.

17 Hydrogen is considered as a potential non-carbon based alternative energy carrier, or
18 as a future fuel in the field of transportation[7]. Hydrogen has a high energy content per mass
19 (120 MJ/kg) compared to petroleum (automotive gasoline 46.4 MJ/kg, automotive diesel 45.6
20 MJ/kg)[8]. Hydrogen is considered environmentally friendly energy carrier, since its
21 combustion produces water as the sole product and no CO_2 emission is linked to its use.
22 Hydrogen can be generated from clean and green sources[9]. However, at present only a very
23 low percent of hydrogen is produced from renewable sources.

24 Biological H_2 production can be categorized into three major groups: direct
25 biophotolysis, photofermentation (indirect photolysis), and dark fermentation. Direct
26 biophotolysis is considered the most desirable and eco-friendly route for generating H_2, certain
27 photosynthesizing microalgae are capable of channeling the protons and electrons (generated
28 in the water-splitting step of photosynthesis) to their Fe-hydrogenases instead of utilizing
29 them to create photosynthates by fixing and converting CO_2 into organic carbon sources.
30 *Chlamydomonas reinhardtii* is the best studied unicellular green microalgae capable of
31 biohydrogen production. Two major methods are known for sustained *Chlamydomonas*-based
32 hydrogen evolution. Hydrogen production using sulfur-deprived photoheterotrophic cultures
33 of *C. reinhardtii* is the most widespread approach[10]. Sulfur deprivation leads to complete
34 anoxia via inactivation of the photosynthetic system. Thus, the extremely oxygen-sensitive
35 algal Fe-hydrogenase will become functional[11]. Illumination is essential in this approach, as
36 the photosynthetic ferredoxin (an electron donor) links the Fe-hydrogenase to the electron
37 transport chain in the chloroplast. The major drawback of sulfur deprivation method is the
38 need for the separation of biomass generating and H_2 producing phases including the change
39 of cultivation media. Another algal hydrogen evolution approach is possible through dark
40 fermentation[12]. In dark, anaerobic conditions, algae can catabolize endogenous carbohydrates
41 or secondary metabolites, generating organic acids, ethanol, carbon dioxide (CO_2), and
42 hydrogen gas (H_2).

1   The vast majority of algal hydrogen evolution studies were conducted using pure algae
2 cultures, although algae almost exclusively live in complex ecosystems in nature where they
3 interact with multiple micro- and macroorganisms. Consequently, algae cultivation in pure
4 cultures requires special attention and particularly expensive resources. Thus, natural algae
5 consortia are expected to receive more attention in algae research and algae-based industries,
6 as well. Knowledge of the exploitability of algal-bacterial consortia in biohydrogen
7 production is limited. Most of the published studies describe consortial systems, where
8 ultimately bacterial strains produce hydrogen, and algae strains are used as an organic carbon
9 source utilized by these bacteria. Mixed cultures of *C. reinhardtii* and *Dunaliella tertiolecta*
10 were used as starch source for a hydrogen production system. Starch of algae source was
11 degraded in this system to lactic acid by *Lactobacillus amylovorus,* which served as substrate
12 for the photosynthetic bacterium *Rhodobium marinum* A-501 to produce hydrogen[13].
13 Similarly, green alga *Chlorella sorokiniana* was grown as substrate in air–CO_2 gas mixture
14 for dark fermentation-based hydrogen production achieved by *Enterobacter cloacae* IIT-BT
15 08 [14]. Much less data are available and algal hydrogen production in co-cultures. Biohydrogen
16 production capacity and growth rate of *C. reinhardtii* cc849 or the transgenic strain Iba co-
17 cultured with *Bradyrhizobium japonicum* have been investigated[15]. The sulfur-deprivation
18 method together with *B. japonicum* inoculation resulted in enhanced rates of the oxygen
19 consumption in the cultures, increased growth rate of algae, and significantly improved
20 hydrogen evolution rates. In our described approach the bacterial partner enhances the algal
21 hydrogen production by effective respiration which leads to rapid anaerobization. The goal of
22 our study was to elucidate the nature and dynamics of algal hydrogen evolution observed in
23 various algal-bacterial interactions in order to exclude the disadvantages of sulfur deprivation
24 and open the door to a widespread, straightforward algae cultivation for hydrogen production
25 purpose.

26 **Materials and methods**

Strain	Relevant genotype or phenotype	Reference or source
Rhodococcus sp.	Wild type	This work
Leifstonia sp.	Wild type	This work
Brevundimonas sp.	Wild type	This work
Chlamydomonas reinhardtii cc124	Wild type	[16]
Chlamydomonas sp. 549	Wild type	This work
Escherichia coli MC4100	Δ*hypF*	[17]
Escherichia coli K-12	Wild type	[18]
Ralstonia eutropha HF441	SH⁻, MBH⁻, Δ*hypF1F2*	[19]

Ralstonia eutropha H16	Wild type	[20]

1 **Table 1. List of algae and bacterial strains**

2 **Chemicals**

3 **TAP medium[21]** (for 1 L medium)

4 **1. TAP salts** (dissolved in 1 liter distilled water): 25 mL
5 NH_4Cl: 15.0 g
6 $MgSO_4 . 7H_2O$: 4.0 g
7 $CaCl_2 . 2H_2O$: 2.0 g

8 **2. Phosphate solution** (dissolved in 100 mL distilled water): 0.375 mL
9 K_2HPO_4: 28.8 g
10 KH_2PO_4: 14.4 g

11 **3. Hutner's trace elements[22]:** 1.0 mL

12 EDTA disodium salt (dissolved in 250 mL distilled water): 50 g
13 $ZnSO_4 . 7 H_2O$ (dissolved in 100 mL distilled water): 22 g
14 H_3BO_3 (dissolved in 200 mL distilled water): 11.4 g
15 $MnCl_2 . 4 H_2O$ (dissolved in 50 mL distilled water): 5.06 g
16 $CoCl_2. 6 H_2O$ (dissolved in 50 mL distilled water): 1.61 g
17 $CuSO_4 . 5 H_2O$ (dissolved in 50 mL distilled water): 1.57 g
18 $(NH_4)_6Mo_7O_{24}. 4 H_2O$ (dissolved in 50 mL distilled water): 1.10 g
19 $FeSO_4. 7 H_2O$ (dissolved in 50 mL distilled water): 4.99 g

20 **4. Tris:** 2.42 g

21 **5. Glacial acetic acid**: 1.0 mL

22 **6. HCl** (for Tris minimal medium)

23 **7. Agar** (for solid medium): 15g L^{-1}

24 **LB (Luria Broth) media[23]** (for 1 L media):

25 Tryptone: 10 g
26 Yeast Extract: 5 g
27 NaCl: 10 g

28 **DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea**) (for 10 mL stock solution): 0.025 g

29 **Cultivation of pure and mixed cultures**

30 Algae strains were selected from the Mosonmagyaróvár Algal Culture Collection (MACC).
31 MACC was established by the Institute of Plant Biology, University of West-Hungary in
32 1992. The MACC is the 3rd largest soil algae culture collection in Europe containing 588 -
33 mainly Brazilian - soil and 382 freshwater microalgae, including 70 *Chlamydomonas* strains.

1   *Chlamydomonas* sp. strain 549 and *C. reinhardtii* strain cc124 were grown on TAP (TRIS-
2   acetate-phosphate medium) and TP (TRIS-acetate-phosphate medium) medium supplemented
3   with rifampicin. The TP medium is a modified TAP medium where acetic acid is replaced
4   with HCl[21]. The TAP and TP plates were constantly incubated under 50 μmol m^{-2} s^{-1} light
5   intensity at 25°C. Algae used for hydrogen-evolution experiments were harvested as fresh
6   cultures grown on agarized TP plates complemented with rifampicin and transferred into
7   liquid TAP medium. Sulfur-deprivation was achieved by suspending and pelleting the algae
8   cultures three times in TAP-S (TRIS-acetate-phosphate medium without sulfur) media before
9   inoculation. The algae stock solutions were equally distributed into 40-mL Hypo-Vial bottles,
10 resulting in 35 mL final volume and a final optical density (OD_{750}) of 0.7. Bacterial colonies
11 with clearly different phenotypes were isolated from the algal-bacterial co-cultures. *E. coli*
12 and *R. eutropha* strains were selected form Institute of Biochemistry, Biological Research
13 Centre of the Hungarian Academy of Sciences and Biotechnology Department, University of
14 Szeged, respectively. Isolated bacteria, *E. coli* and *R. eutropha* were grown and maintained on
15 LB (Luria-Bertani medium) plates at 30°C in the dark. *Rhodococcus* sp. generated single
16 colonies in two days, while *Brevundimonas* and *Leifstonia* strains were incubated for three
17 days. Bacteria used for algal co-cultivation were harvested from agarized LB plates and
18 transferred into liquid TAP medium. These bacterial stock solutions were equally distributed
19 into 40-mL Hypo-Vial bottles, resulting in 35 mL final volume and a final optical density
20 (OD_{600}) of 0.5. Algal-bacterial liquid cultures were established by mixing the pure algae and
21 bacterial stock solutions into 40-mL Hypo-Vial bottles, resulting in 35 mL final volumes and
22 the equivalent final optical densities as the previous ones. Bottles were sealed with butyl
23 rubber stoppers and aluminum caps. All experiments were performed using three parallel
24 samples.

25 **Identification of natural bacterial contaminants**

26 Bacterial genomic DNA isolations from pure cultures were performed using traditional
27 genomic DNA isolation methods[24]. Total purified DNA was used for 16S rDNA-based PCR
28 identification. Universal 16S rDNA oligonucleotides were used for the amplification (f27 5'-
29 AGAGTTTGATCCTGGCTCAG-3' and r1492 5'-ACGGCTACCTTGTTACGACTT-3' for
30 16S rDNA). The PCR products were isolated from agarose gel, and capillary Sanger
31 sequencing of the isolated PCR products was used to determine the exact sequence of the
32 amplified   16S   rDNA   fragments.   Homology   searches   with   BLAST
33 (http://blast.ncbi.nlm.nih.gov/) were used to identify the origin of the 16S rRNA sequences.

34 **Determination of algal and bacterial cell numbers**

35 Optical density of algae cultures in TAP media was determined. The cell number was counted
36 in a Burker chamber. Optical densities of the mixed algal-bacterial and pure algae cultures in
37 TAP media were measured spectrophotometrically at 750 nm, while pure bacterial cultures
38 were measured at 600 nm. The colony forming cunits (CFU) were counted on LB plates using
39 serial dilutions. All experiments were repeated three times.

40 **Gas chromatography analyses**

1   The hydrogen and oxygen levels in the headspace of Hypo-Vial bottles were measured with
2   gas chromatography. An Agilent 7890A gas chromatograph equipped with a thermal
3   conductivity detector and an Agilent HP-Molsieve column (length 30 m, diameter 0.320 mm,
4   film 12.0 μm) was used in splitless mode. Linde HQ argon 5.0 gas was used as carrier and
5   reference gas. The temperatures of the injector, TCD detector and column were kept at 150
6   ºC, 160 ºC and 60 ºC, respectively. The column pressure was 47,618 psi. The flow rate of the
7   column was 12 ml/min. Samples of 50 μL volumes were analyzed. Three biological replicates
8   were used for the measurements.

9   Hydrogen calibration curve was used to determine accurate gas volumes. Serial
10   dilutions of pure hydrogen gas were prepared in 25 mL gas-tight vials, identical volumes
11   were injected into the gas cromatograph, data from three replicates were used to draw the H_2
12   calibration curve. The following formula was used for the conversion of simple GC unit:
13   $x=y/239.13$ (x: volume of pure hydrogen gas in μl, y: measured GC unit). The measured
14   yields were normalized for the production of 1 L algae culture: $x=y/239.13*2857$.

15   Oxygen yields were correlated to ambient oxygen concentration. The measured
16   oxygen yields were divided with the average of the ambient oxygen yields in GC unit and
17   multiplied with the ambient oxygen content: $x=y/438.57*21$ (x: oxygen content in percentage,
18   y: measured GC unit).

19   **Determination of dissolved oxygen concentration**

20   Oxygen concentration values in the liquid phase of the cultures were monitored continuously
21   at 25°C by fiber-optic oxygen meter (Precision Sensing GmbH, Fibox 3 Minisensor Oxygen
22   Meter, Germany). Calibration was performed without temperature sensor using a
23   conventional two-point calibration in oxygen-free water (1g Na_2SO_3 in 100mL water) and air-
24   saturated water.

25   **Inhibition of photosynthesis**

26   The herbicide DCMU[25] (3-[3,4-dichlorophenyl]-1,1-dimethylurea) (200 μM final
27   concentration) was used to block electron transfer in the photosynthetic electron transport
28   chain at Q_B-binding site[26-28].

29   **Chlorophyll fluorescence measurements**

30   Detection of chlorophyll (Chl) fluorescence changes provides a sensitive tool for monitoring
31   photosystem II (PSII) activity[29]. Illumination of dark-adapted photosynthetic samples with
32   continuous actinic light results in an increase of fluorescence from the low F_o (or O-origin
33   measured at 10 μs) level to its maximal F_m (or P-peak at ~200 ms) yield following
34   characteristic triphasic kinetics with intermediate steps denoted by J (at ~3 ms) and I (at ~20
35   ms)[30, 31]. Light-dependent changes in Chl fluorescence yield are determined by the redox state
36   of Q_A, the primary quinone electron acceptor of PSII, but they also reflect the reduction of the
37   photosynthetic electron transport chain[32]. The OJ phase is the photochemical part of the OJIP
38   (Chl a fluorescence induction transient) transient, and its relative amplitude reflects the redox
39   state of the PQ pool, such that the higher the PQH_2/PQ ratio in the dark, the higher the J-
40   level[33].

Flash-induced fluorescence increase and subsequent decay as well as fast fluorescence induction upon continuous illumination, so-called OJIP-transients, were measured after 3 min of dark adaptation by a double-modulation FL3000 fluorometer (Photon System Instruments, Czech Republic). Flash-induced fluorescence changes were detected by logarithmic timing of weak pulses in the time range of 150 μs–100 s after applying a single-turnover, saturating flash with a length of 30 μs[34]. Fast fluorescence increase was induced by 1000 μmol m^{-2} s^{-1}-intensity actinic light, and OJIP transients were detected in a time range of 10 μs–2 s. Mixed samples were kept throughout the entire experiment and during measurements in the same airtight, closed cuvettes.

Flash-induced fluorescence relaxation curves were analyzed as described by[35]. Multicomponent deconvolution of the monotonically decreasing curves was performed using a fitting function with two exponential components (fast and middle phase) and one hyperbolic component (slow phase):

$$F_{v,corr} = A_1 exp(-t/T_1) + A_2 exp(-t/T_2) + A_3/(1 + t/T_3) + A_0$$

where $F_v = F(t) - F_o$, $F(t)$ is the fluorescence yield at time t, F_o is the basic fluorescence level before the flash, A_1–A_3 are the amplitudes, and T_1–T_3 are the time constants. The non-decaying fluorescence component in the time span of the measurement is described by a constant A_0 amplitude. $F_{v,corr}$ is the variable fluorescence yield corrected for nonlinearity, taking into consideration the nonlinear correlation between fluorescence yield and the redox state of Q_A using the Joliot model[36] with a value of 0.5 for the energy-transfer parameters between PSII units.

Maximal PSII quantum yield, $F_v/F_m = (F_m - F_o)/F_m$, was determined using a Dual-PAM-100 (Walz, Germany) fluorometer[37, 38]. The minimal and maximal Chl fluorescence yields, F_o and F_m, respectively, were detected after 10 min of dark adaptation. The maximal yield was achieved by illuminating the cultures in the sealed Hypo-Vial bottles for 600 ms with a long saturating light pulse of 10000 μmol m^{-2} s^{-1} intensity.

Acetic acid analytical measurements

Acetic acid levels were analyzed by HPLC. The instrument consisted of a Gynkotek M 480 pump, a TOSOH 6040 UV detector (210 nm), a Rheodyne 8125 injector with a 20-mL loop, and an Aminex HPX-87H column (300 x 7.8 mm; 5 mm) (BioRad Co., USA). The mobile phase was 0.005 M H_2SO_4 applied at a 600-μL min^{-1} flow rate and 60°C column temperature. The injected analyte volume was 20 μL. Quantitative analysis for acetic acid (Sigma-Aldrich Co., USA) performed via five-point calibration. Three replicates were used for each measurement.

Results

Natural bacterial contaminations of *Chlamydomonas* sp.

Chlamydomonas sp. strain 549 was contaminated in the culture collection by various bacterial species (bacterial contamination of green algae is a common phenomenon). Bacterial colonies

1 of various color were observed around the young green algae plaques on TAP plates.
2 Similarly, an opalescent film layer was observed at the surface of the bottles when
3 *Chlamydomonas* sp. 549 was cultured in liquid TAP medium. Contaminated bacterial
4 colonies were isolated and identified using 16S rDNA amplification followed by capillary
5 sequencing. Most of the contaminating bacteria belonged to three bacterial genera:
6 *Brevundimonas*, *Rhodococcus*, and *Leifstonia*. Various antibiotics were tested for the bacteria
7 and for the algae. Rifampicin proved to be selective and effective against all contaminating
8 bacterial strains and was, therefore, suitable for maintaining pure algae cultures. Effects of
9 natural bacterial contaminants on algae biomass yield were assessed. The data showed that the
10 bacterial partners generally promoted algal growth and resulted in significantly elevated algae
11 biomass (Fig. 1. Panel A). Microscopy investigations supported this observation, indicating
12 that the biomass increase was the result of the higher density of algae cells in the algal-
13 bacterial association cultures compared to the pure algae samples. In order to evaluate the
14 contribution of the different bacteria to the increase in biomass, biomass of pure algae cultures
15 was compared to algae harboring *Rhodococcus* sp., *Brevundimonas* sp., and *Leifstonia* sp.
16 (Fig. 1. Panel B). The average biomass yield of the pure algae cultures was set at 100%. In co-
17 cultures with *Rhodococcus* and *Brevundimonas* bacteria, the algae biomass increased by 46%
18 and 26%, respectively, while algae biomass decreased by 27% when *Leifstonia* sp. was the
19 contaminating bacterium. Based on these data, a *Rhodococcus* strain as a natural bacterial
20 symbiont was selected for further investigations of the co-cultivation effects on algal
21 hydrogen-evolution capabilities.

22 **Growth characteristics of single strains versus designed co-cultures**

23 Selected bacterial partners including *Ralstonia eutropha*, *E. coli*, and one of the natural
24 partners, *Rhodococcus* sp., were tested for co-culturing with *Chlamydomonas* sp. strain 549.
25 All experiments were performed in both TAP and TP growth media. Two growth phases
26 could be distinguished for the *Chlamydomonas* sp. 549-*Rhodococcus* sp. mixed cultures in
27 TAP regardless of the bacterial partner. An intensive growth phase of four days was followed
28 by a stationary phase during the next 3–4 weeks. Interestingly, the algae cell number of the
29 mixed cultures significantly exceeded the cell number of the parallel pure algae cultures,
30 while the cell numbers of both culture types remained constant in the stationary phase.
31 However, no variation in the algae cell number was observed between the pure and mixed
32 cultures in TP medium. In addition to counting algae cells, colony forming units (CFU) for
33 the bacterial partners were determined. A continuous and drastic decrease was observed in
34 bacterial CFU in most cultures. The only exception was the algal-bacterial mixed culture
35 propagated in TAP medium under illumination, where bacterial CFU slightly increased the
36 first day, although this initial bacterial propagation was followed by a decrease in CFU (Fig.
37 1. Panel C). The optical density of the mixed cultures increased continuously to a maximum
38 level of OD_{750} 2.0 in the first four days, which was similar to the optical density of the pure
39 algae cultures under illumination. The optical density of the pure bacterial cultures slightly
40 decreased throughout the experiment, indicating the continuous decay of the bacterial
41 population in the TAP medium. As expected, in dark conditions the optical densities of the

1   pure and mixed cultures decreased slowly but continuously during the 28 days of the
2   experiment (Fig. 1. Panel D).

3   The pH values of the mixed cultures and the pure algae cultures increased from an
4   initial 7.3 to a final value of 8.7 in the first phase of the experiments under illumination.
5   However, the pH of the bacterial cultures remained constant (7.3). In contrast to the
6   illuminated samples, no significant changes in the culture pH were detected in dark
7   conditions. The initial pH value of 7.3 remained stable in all types of cultures, including the
8   mixed ones.

9   In order to explain the observed pH and algae cell number data, acetate concentrations
10   were continuously measured both in the pure *Chlamydomonas* sp. 549 culture and in the
11   mixed algal-bacterial cultures under illumination (Fig. 1. Panel E). Significant differences
12   were observed from the beginning of the experiment. The acetic acid consumption rate was
13   much higher in the mixed cultures than in the pure algae cultures. These results are in good
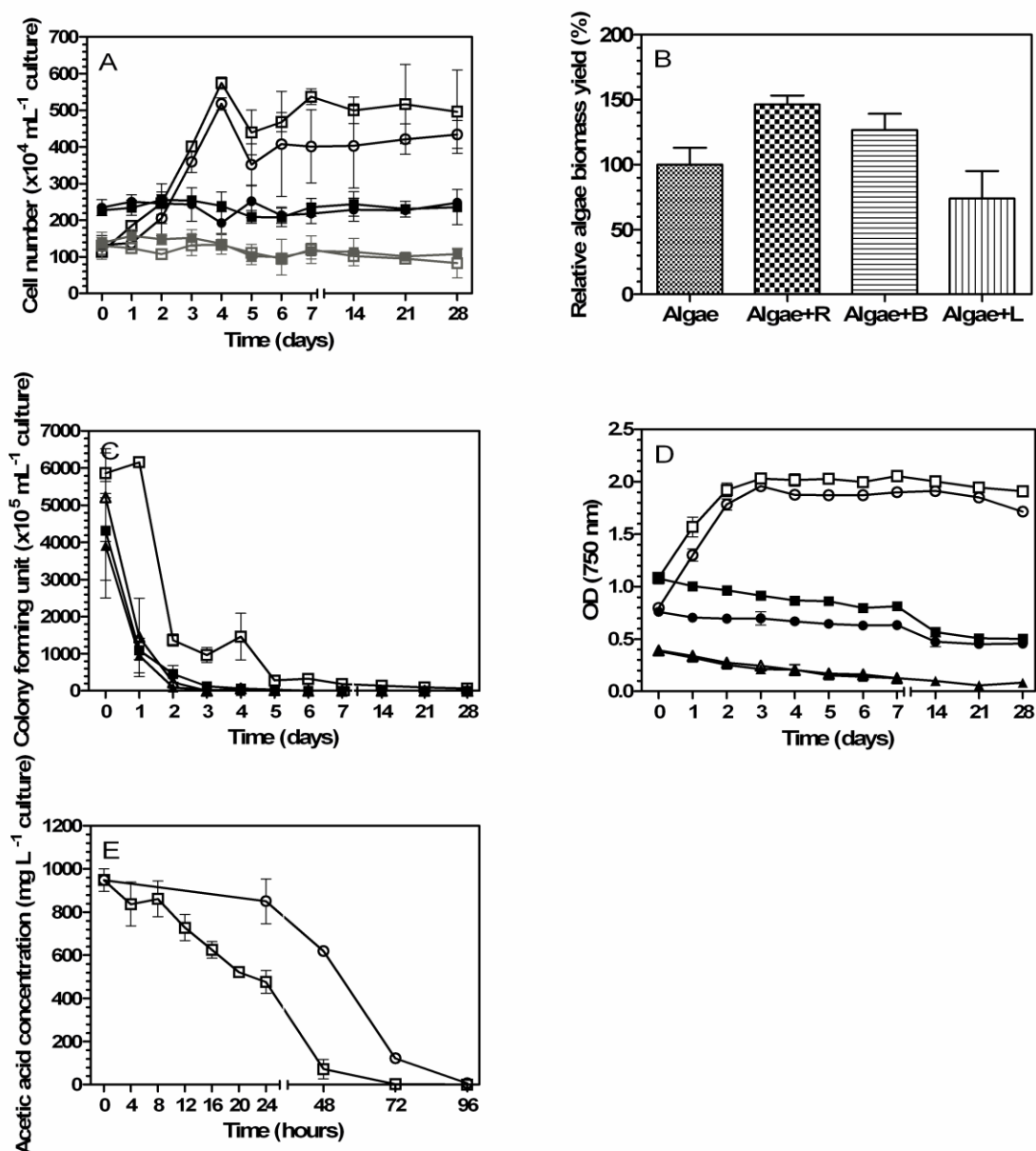14   agreement with the intensive growth of the algae cell number in algal-bacterial cultures.

1
2 **Figure 1. Growth characteristics of single strains versus designed co-cultures. Panel A**:
3 Algae cell number determination using a Burker counting chamber. *Chlamydomonas* sp. 549
4 (circles) and mixed *Chlamydomonas* sp. 549-*Rhodococcus* sp. cultures (squares) were
5 incubated in TAP (black) and TP (grey) medium, in light (open marks) and dark (closed
6 marks) conditions. **Panel B**: Relative algae biomass yields in the pure algae (Algae) and the
7 mixed algal-bacterial cultures: *Rhodococcus* sp. (Algae + R), *Brevundimonas* sp. (Algae + B),
8 *and Leifstonia* sp. (Algae + L). **Panel C**: Colony forming unit (CFU) determination of
9 *Rhodococcus* sp. during the co-cultivation experiment. Pure *Rhodococcus* sp. cultures
10 (triangles) were compared to the mixed *Rhodococcus* sp.-*Chlamydomonas* sp. (squares) co-
11 cultures propagated in TAP medium under illumination (open marks) and in darkness (closed
12 marks). **Panel D**: Optical density (OD) measurements of pure *Chlamydomonas* sp. (circles),
13 *Rhodococcus* sp. (triangles), and mixed *Chlamydomonas* sp. 549-*Rhodococcus* sp. cultures
14 (squares) incubated in darkness (closed marks) and under illumination (open marks). **Panel E**:

1 HPLC measurements of acetic acid concentration in *Chlamydomonas* sp. 549 (open circles)
2 and *Chlamydomonas* sp. 549-*Rhodococcus* sp. (open squares) cultures propagated in TAP
3 medium under illumination.

4 **Improvement of the efficiency of hydrogen evolution**

5 The hydrogen-producing system described comprises two major microbial elements: a
6 photosynthetic unicellular green alga (*Chlamydomonas* sp. 549) in combination with a
7 selected heterotrophic bacterium (*Rhodococcus* sp., *R. eutropha*, or *E. coli*). In principle, both
8 components are capable of hydrogen production under appropriate conditions. All tested
9 microorganisms possess hydrogenase enzymes; the bacteria applied all harbor at least one
10 NiFe hydrogenase. However, it was shown that in TAP medium, only the algal partner could
11 generate hydrogen. To improve the co-cultivation system efficiency, experiments were
12 performed using bacterial partners lacking active hydrogenase enzymes. All parameters were
13 identical with the original experimental setup except that the *hypF* mutant derivatives of *R.*
14 *eutropha* and *E. coli* strains were used. The lack of the pleiotropic HypF protein causes a
15 block in the biosynthesis of NiFe hydrogenases, resulting in inactive hydrogenase enzymes.
16 Interestingly, significant differences were observed between the hydrogen production rate of
17 the mixed cultures with the hydrogenase mutants and wild-type bacterial partners. The
18 combinations harboring mutant bacterial strains developed higher amounts of hydrogen due to
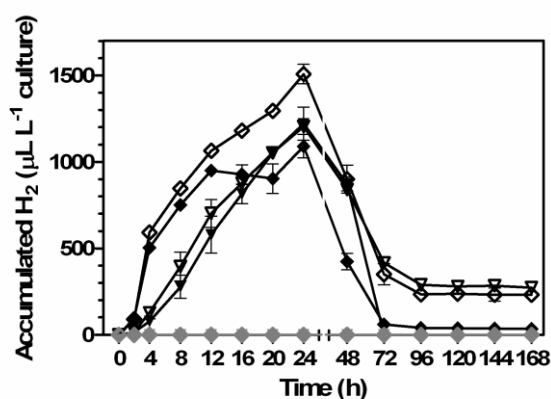19 the inhibition of bacterial hydrogen uptake (Fig. 2.).

20
21 **Figure 2. Improvement of the efficiency of hydrogen evolution.** Amount of H_2
22 accumulated in the headspace of the sealed bottles produced by mixed *Chlamydomonas* sp.
23 549 and by artificially added *Ralstonia eutropha* (reverse triangles) or *Escherichia coli*
24 (diamonds) cultures.. Both wild-type (closed marks) and hydrogenase-deficient (open marks)
25 versions of each bacterial partner were tested. The H_2 yields of mixed algal-bacterial (black
26 marks, both open and closed) and pure bacterial (grey marks, both open and closed) cultures
27 were compared. Grey marks are in the line with x axis, because pure bacterial cultures didn't
28 produce any hydrogen.

29 **Characteristics of algal hydrogen evolution under illumination and in darkness**

1   Hydrogen-production capabilities of *Chlamydomonas* sp. 549 were assessed. The *C.*
2   *reinhardtii* cc124 strain was used as benchmark strain, as it has been rigorously investigated
3   for sulfur-deprived hydrogen evolution. Hydrogen production was detected only in the algal-
4   bacterial cultures under illumination using TAP medium (the total amount of accumulated H_2
5   was 1196.06 ± 4.42 μL L^{-1} [strain 549] and 5800.54 ± 65.73 μL L^{-1} [strain cc124] produced in
6   one day) (Fig. 3. Panel A). Neither the pure algae nor the pure bacterial cultures incubated in
7   TAP media generated any hydrogen. Hydrogen production in the mixed cultures was
8   maintained for 16−24 h following a 2−4 h initial lag phase. The average rate of hydrogen
9   production was 49.83 ± 0.18 μL L^{-1} h^{-1} (strain 549) and 241.68 ± 2.73 μL L^{-1} h^{-1} (strain
10   cc124). Hydrogen production ceased after 24 hours, and in the case of *Chlamydomonas* sp.
11   549-*E. coli hypF* mixed cultures the level of the accumulated hydrogen slowly started
12   decreasing. Interestingly, the hydrogen production showed late fluctuations in the *C.*
13   *reinhardtii* cc124-*E-coli hypF* mixed cultures, after a 24 h long consumption period (between
14   24h and 48 h) the hydrogen level stabilized, even a second hydrogen production period was
15   observed until the 6th day of the experiment. The two sulfur-deprived, algal-bacterial co-
16   cultures evolved different total amounts of hydrogen. Strain 549 produced 2637.49 ± 555.42
17   μL L^{-1} hydrogen in 3 days, while strain cc124 evolved 47241.3 ± 4660.69 μL L^{-1} hydrogen in
18   7 days (Fig. 3. Panel B). There were also noticeable differences in hydrogen production
19   between the two pure sulfur-deprived algal strains. The hydrogen evolution of
20   *Chlamydomonas* sp. 549 was barely measurable (193.5 ± 66.81 μL L^{-1} produced in 2 days)
21   compared to that of strain cc124 (25028.1 ± 3943.47 produced in 7 days). The reason for this
22   remarkable difference is the highly different rate of the D1 protein degradation in these two
23   algae strains[39] (Fig. 3. Panel F).
24   Strikingly different rates of hydrogen generation were observed in the sulfur-deprived
25   pure *C. reinhardtii* cc124 and mixed *C. reinhardtii* cc124 algal-bacterial cultures. The total
26   amount of accumulated H_2 was 25028.1 ± 3943.47 in the case of pure *C. reinhardtii* cc124
27   algal culture, while almost double the amount of hydrogen (47241.3 ± 4660.69 μL L^{-1}) was
28   detected in the headspace of the *C. reinhardtii* cc124 algal-bacterial cultures. The length of
29   the hydrogen evolution period also showed remarkable differences between cultures
30   incubated in the sulfur-deprived (7 days) and complete media (3 days).
31   Hydrogen evolution in the mixed cultures initiated once oxygen concentration in the
32   headspace decreased to 4−5% from the initial atmospheric level (21%) (Fig. 3. Panel C). This
33   oxygen level remained constant until the end of the second day (48 h) in the culture of
34   *Chlamydomonas* sp. 549. After this period, the oxygen level rapidly increased and reached a
35   relatively constant level of 58−60%. The oxygen level in the headspace of *C. reinhardtii*
36   cc124 mixed cultures remained at a low level (2%) during the experiment. The headspace
37   oxygen concentrations of the pure algae cultures were a constant 18−20% during the first 48
38   hours, but then, similar to the mixed algal-bacterial cultures, they quickly increased and
39   reached the same concentration as the mixed cultures (58−60%) in the case of strain 549. The
40   oxygen level of the pure bacterial cultures was maintained at a constant low value of 4−6%,
41   which was similar to the mixed cultures.
42   Dissolved oxygen concentrations were also measured in both the mixed and pure
43   cultures (using *Chlamydomonas* sp. 549 as the algal partner) using a fiber-optic oxygen meter

1   (Fig. 3. Panel E). These measurements were in good agreement with the gas chromatographic
2   headspace investigations. The pure bacterial and mixed algal-bacterial cultures became
3   completely anaerobic in 3–3.5 h (dissolved oxygen concentration decreased from 257 μmol L^{-1}
4   to 1–2 μmol L^{-1}). The pure bacterial cultures maintained this fully anaerobic environment
5   during the entire seven-day monitoring period. Similar to that observed in the headspace
6   measurements, the mixed algal-bacterial cultures reinitiated oxygen generation after
7   approximately 48–60 h, reaching an oxygen level six times greater than the level at the
8   beginning of the experiment (257 μmol L^{-1} vs. 1600 μmol L^{-1}). An increasing oxygen level
9   was detected in the liquid phase of the pure algae cultures in the first few hours after
10   inoculation (650 ± 100 μmol L^{-1}), which was maintained during the next 48–72 h. After 48 h,
11   the oxygen evolution rate increased rapidly and reached 1600 μmol L^{-1} in the case of the
12   mixed cultures (Fig. 3. Panel E). The relative oxygen levels in the headspace of the pure
13   *Chlamydomonas* sp. 549 and *C. reinhardtii* cc124 sulfur-deprived algae cultures decreased
14   from 21% to 15–16% during the first day (Fig. 3. Panel D). However, starting at the second
15   day, the oxygen levels distinctly changed in the pure algae cultures. The oxygen content
16   increased to 45% in the headspace of *Chlamydomonas* sp. 549 but decreased to 1–2% in the
17   case of *C. reinhardtii* cc124 (Fig. 3. Panel D). The mixed sulfur-deprived cultures (with both
18   algal partners) reached a low (4–5%) oxygen concentration in the headspace during the first
19   four hours of co-cultivation. This low rate of oxygen remained constant in the mixed *C.*
20   *reinhardtii* cc124 cultures, while in the mixed *Chlamydomonas* sp. 549 cultures the oxygen
21   level rapidly increased to 20% on the fourth day of the experiment. This phenomenon was
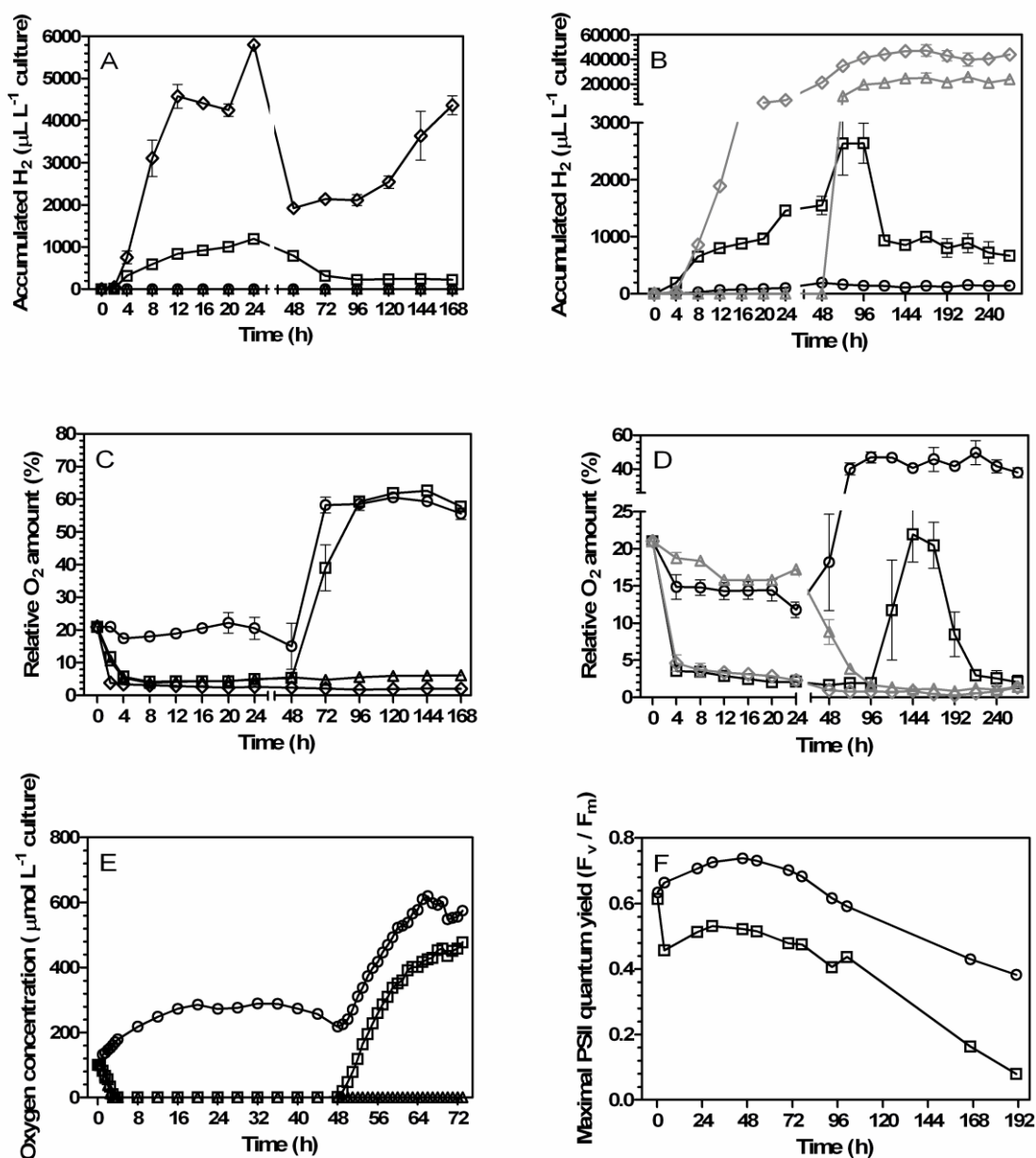22   temporary, as the oxygen level returned to 3% by the seventh day and remained stable.

1
2 **Figure 3. Algal hydrogen-evolution characteristics under illumination. Panel A**: Amount
3 of H_2 gas accumulated in the headspace of the sealed bottles using TAP growth medium under
4 illumination. Pure *Chlamydomonas* sp. 549 (open circles), mixed *Chlamydomonas* sp. 549-
5 *Escherichia coli hypF* (open squares), *C. reinhardtii* cc124-*Escherichia coli hypF* (open
6 diamonds), and pure *Escherichia coli hypF* (open triangles) were compared for their H_2-
7 evolution capabilities. Open circles and open triangles are in the line with the x axis, while the
8 hydrogen production of the pure *Chlamydomonas* sp. 549 and *Escherichia coli hypF* was
9 undetectable. **Panel B**: Amount of H_2 accumulated in the headspace of the sealed bottles
10 using TAP-S growth medium under illumination. Pure *Chlamydomonas* sp. 549 (open black
11 circles), pure *C. reinhardtii* cc124 (open grey triangles), mixed *Chlamydomonas* sp. 549-
12 *Escherichia coli hypF* (open black squares), and mixed *C. reinhardtii* cc124-*E. coli hypF*
13 (open grey diamonds) were compared for their H_2-evolution capabilities. **Panel C**: Relative

1   oxygen (O_2) level in the headspace of the sealed bottles incubated in TAP medium under
2   illumination. Pure *Chlamydomonas* sp. 549 (open circles), mixed *Chlamydomonas* sp. 549-
3   *Escherichia coli hypF* (open squares), *C. reinhardtii* cc124-*Escherichia coli hypF* (open
4   diamonds), and pure *Escherichia coli hypF* (open triangles) were compared for their
5   respiratory activities. **Panel D**: Relative O_2 level in the headspace of the sealed bottles
6   incubated in TAP-S medium under illumination. Pure *Chlamydomonas* sp. 549 (open black
7   circles), pure *C. reinhardtii* cc124 (open grey triangles), mixed *Chlamydomonas* sp. 549-*E.*
8   *coli hypF* (open black squares), and mixed *C. reinhardtii* cc124-*E. coli hypF* (open grey
9   diamonds) were compared. **Panel E**: Oxygen concentrations in the liquid phase of the
10   samples incubated in TAP medium under illumination. Pure *Chlamydomonas* sp. 549 (open
11   circles), mixed *Chlamydomonas* sp. 549-*Escherichia coli hypF* (open squares), and pure
12   *Escherichia coli hypF* (open triangles) were compared for their respiratory activities. **Panel F**:
13   Maximal PSII quantum yield of *Chlamydomonas* sp. 549 in pure algae (open circles) and co-
14   cultures (open squares) incubated in TAP-S media under illumination.

15   In the absence of light, hydrogen evolution was detected in both pure algae and algal-
16   bacterial mixed cultures. Similar to the illuminated mixed cultures, multiple phases of
17   hydrogen evolution could be observed. Hydrogen production started after a short lag phase
18   (2–4 h) following inoculation. Hydrogen production was maintained for three days in both
19   cases. Significant differences were not detected between the total hydrogen production of the
20   pure algae and the mixed cultures (5621 ± 645 µL L^{-1}). In the dark, the rates of hydrogen
21   generation by either the algae alone or by the algal-bacterial mixed cultures were
22   approximately 60.5 ± 2.5 µL L^{-1} h^{-1}. As expected, hydrogen evolution was not detected in the
23   pure bacterial cultures (Fig. 4. Panel A).
24   In dark, the headspace of the liquid cultures retained the same oxygen values
25   compared to the illuminated cultures. The algal-bacterial mixed and the pure bacterial cultures
26   reached the minimum levels of the oxygen 4 h after inoculation (3–5%), while the pure algae
27   cultures reached the same level in 8 h (Fig. 4. Panel B). This microaerobic state was
28   maintained throughout the experiment. In the liquid phase of the cultures, similar oxygen
29   values were measured as with the illuminated samples (1–2 µmol L^{-1}). The pure bacterial and
30   algal-bacterial cultures approached a continuous anaerobic state in 1 h, while the pure algae
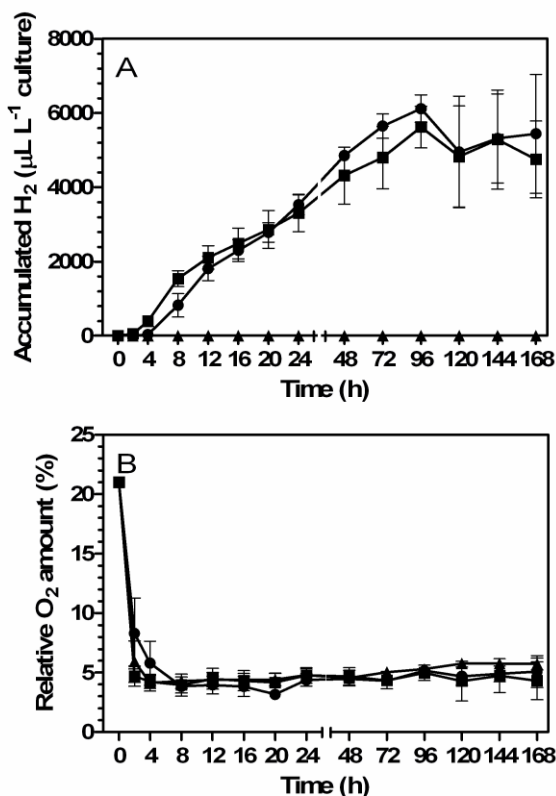31   reached this level in 5 h.

1

2   **Figure 4. Algal hydrogen-evolution characteristics in darkness. Panel A**: Amount of H_2
3   accumulated in the headspace of the sealed bottles incubated in TAP medium in darkness.
4   Pure *Chlamydomonas* sp. 549 (closed circles), mixed *Chlamydomonas* sp. 549-*Rhodococcus*
5   sp. (closed squares), and pure *Rhodococcus* sp. (closed triangles) were compared for their H_2-
6   evolution capabilities. **Panel B**: Relative O_2 levels in the headspace of the sealed bottles
7   incubated in TAP medium in darkness. Pure *Chlamydomonas* sp. 549 (closed circles), mixed
8   *Chlamydomonas* sp. 549-*Rhodococcus* sp. (closed squares), and pure *Rhodococcus* sp. (closed
9   triangles) were compared for their respiratory activities.

10   **Dual mechanism of hydrogen evolution**

11   To determine the mechanism of hydrogen production in the algae, the photosynthetic system
12   was inhibited with DCMU. The DCMU molecule blocks the PQ binding site of PS II,
13   inhibiting electron flow from PSII to PQ. Application of this inhibitor caused a reduction of
14   hydrogen evolution in algae after one day (*Chlamydomnas* sp. 549-*E. coli hypF* mixed culture
15   completed with 2% ethanol 3194.84 ± 159.23 μL L^{-1}, DCMU treated *Chlamydomonas* sp.
16   549-*E. coli hypF* mixed culture 778.34 ± 115.71), indicating that a significant part of the total
17   evolved hydrogen is of a photolytic source. A large portion of the hydrogen is derived from
18   fermentative pathways based on the data of the pH and acetic acid measurements (Fig. 1.
19   Panel E) as well as hydrogen evolution characteristics under illumination and in darkness.

20   **Fluorescence investigations on the algal photosynthetic system**

1 We investigated the state of the algal photosynthetic system during our experiments.
2 Characteristic changes in PSII activity were observed in the case of algal-bacterial mixed
3 cultures using Chl fluorescence measurements. The OJIP transients of algal cells measured
4 after 24 h of starting the mixed cultures showed a considerable increase in J-level (Fig. 5.
5 Panel A, closed circles) compared to control cells without bacteria (Fig. 5. Panel A, open
6 triangles), indicating that the PQ-pool was significantly reduced under these conditions. After
7 bubbling the culture with air for 10 min, the J-level returned (Fig. 5. Panel A, closed squares),
8 and the OJIP-transient level was very similar compared to controls. The calculated F_v/F_m
9 values in in mixed cultures that received air bubbles and controls were very similar (0.62 and
10 0.66, respectively), indicating that the maximal quantum efficiency of PSII did not change
11 significantly due to presence of bacteria.

12 Flash-induced fluorescence decay curves reflect the reoxidation processes of Q_A^- and
13 can be explained by the widely used two-electron gate model[35]. The fast phase (few hundred
14 microseconds) decay component reflects Q_A^- reoxidation via forward electron transport in
15 centers that contain bound PQ at the Q_B site before the flash. The middle phase (few
16 milliseconds) arises from Q_A^- reoxidation in centers that had an empty Q_B site at the time of
17 the flash and must bind a PQ molecule from the PQ pool. The slow phase (few seconds)
18 reflects Q_A^- reoxidation via a back reaction with the S_2 state of the water-oxidizing complex.
19 The flash-induced fluorescence decay of algae was significantly slower in the mixed cultures
20 (Fig. 5. Panel B, closed circles) compared to the controls (Fig. 5. Panel B, open triangles)
21 after 24 h of growth. The fast phase amplitude completely disappeared, while the slow phase
22 amplitude increased under anaerobic conditions due to the presence of bacteria (Table 2). This
23 indicates that in the absence of oxygen in the dark, Q_B binding pockets do not contain
24 oxidized or semi-reduced PQ molecules capable of accepting electrons from Q_A^-. The PQ
25 molecules can bind to the Q_B site and reoxidize Q_A^- in the middle decaying phase only after
26 oxidation of PQH_2 molecules by PSI through the $cytb_6/f$ complex. This resulted in slower
27 linear electron transport between PSII and PSI under anaerobic conditions. This retardation of
28 electron transport was completely reversible by bubbling the culture with air for 10 min, as
29 suggested by the flash-induced fluorescence decay curve (Fig. 4B, closed squares) being
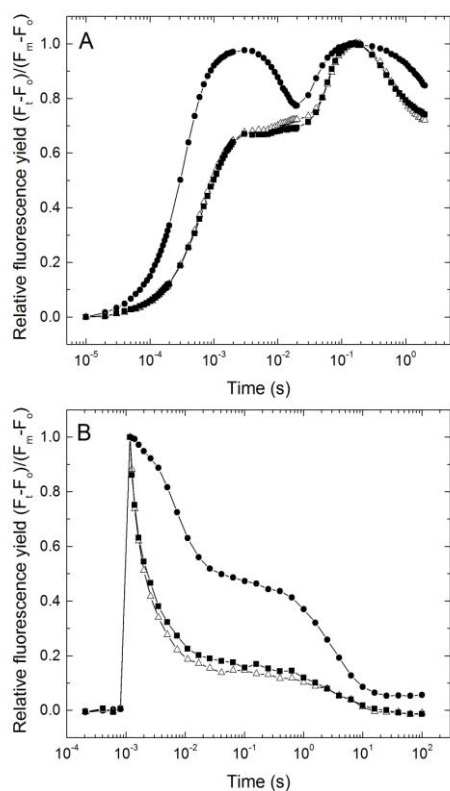30 almost identical to the control one.

1

Figure 5. Fluorescence investigations on the algal photosynthetic system. The effect of
bacteria-induced anaerobic conditions on fast chlorophyll fluorescence changes in
Chlamydomonas sp. 549 medium cells. The OJIP transients (**Panel A**) and flash-induced
fluorescence relaxation curves (**Panel B**) were produced from mixed cultures containing algal
and bacterial cells (closed symbols) and also from those containing only algal cells (open
symbols). Measurements were performed 24 h after starting the cultures without further
treatment (circles, triangles) or after bubbling with air for 10 min (squares). The curves were
double-normalized between F_o and F_m.

	Fast phase	Middle phase	Slow phase	
	T_1 (µs) / A_1 (%)	T_2 (ms) / A_2 (%)	T_3 (s) / A_3 (%)	A_0 (%)
Pure	585 ± 98 / 38.9 ± 2.8	5.0 ± 0.7 / 34.6 ± 3.1	3.5 ± 0.5 / 26.5 ± 0.7	0
Mixed	– / 0	8.9 ± 0.7 / 33.6 ± 0.8	3.1 ± 0.2 / 61.4 ± 0.8	5.0 ± 0.8
Mixed, 10 min air	511 ± 92 / 35.2 ± 2.4	5.4 ± 0.6 / 34.8 ± 2.5	2.9 ± 0.3 / 30.0 ± 0.7	0

**Table 2. Decay kinetics of flash-induced variable fluorescence in algal-bacterial mixed
cultures and in pure algal cultures after 24 h of growth.** Multicomponent deconvolution of
the fluorescence relaxation curves presented in Figure 4B. The kinetics were analyzed in
terms of two exponential (fast and middle phase), one hyperbolic (slow phase), and a non-
decaying component as described in the materials and methods. Values after slashes are

1   relative amplitudes as a percentage of total variable yield. Standard errors of the calculated
2   parameters are also indicated.

3   **Discussion**

4   Effects of bacterial co-cultivation on the hydrogen evolution properties of selected
5   *Chlamydomonas* sp. 549 algae were investigated. Both the liquid and gaseous phase
6   environments of the algal-bacterial consortia were controlled and monitored. Characteristic
7   fluctuations were detected in the headspace gas compositions and in respiratory activities of
8   the analyzed communities.
9   The green alga *Chlamydomonas* sp. 549 was shown to readily associate with various
10 bacterial species during cultivation. A number of natural bacterial partners were separated and
11 identified, most belonging to *Rhodococcus*, *Brevundimonas*, and *Leifstonia* genera. Each
12 combination was characterized for growth and biomass-yield properties. *Rhodococcus*
13 partners were shown to be the most abundant and most rapidly growing bacterial participants
14 in the associations. Therefore, this association produced the most hydrogen among the natural
15 combinations. Aside from natural partners, various further bacterial strains were able to
16 efficiently reduce the oxygen level and further enhance hydrogen production. Use of the *E.*
17 *coli* pleiotropic hydrogenase mutant strain *hypF* resulted in the generation of the largest
18 hydrogen amount[17]. The hydrogen evolution characteristics of *Chlamydomonas* sp. 549 were
19 compared to those of the widely used green alga *C. reinhardtii* cc124. Comparisons were
20 made with pure algae and mixed algal-bacterial cultures using either full medium (TAP) or
21 the sulfur-deprived method, which is the benchmark method for algae-based hydrogen
22 evolution. Striking differences were observed between the various algae strains in terms of
23 oxygen consumption intensity, starting time, and duration of hydrogen evolution. The
24 complete anaerobiosis in sulfur-deprived pure *C. reinhardtii* cc124 cultures was established in
25 96 h under illumination, while the algal-bacterial cultures─either with or without sulfur
26 deprivation─generated fully anaerobic environments in 2–4 h as measured in the headspace
27 of the bottles. We showed that pure, sulfur-deprived *Chlamydomonas* sp. 549 algae cultures
28 were unable to entirely consume the oxygen due to slower degradation of the D1 protein in
29 this algae strain[39] (Fig. 3. Panel F). Thus, the presence of oxygen prevented hydrogen
30 production (Fig. 3. Panels B and D). These data also reflect the highly different hydrogen-
31 production capabilities of various *Chlamydomonas* algae. Similar to oxygen consumption,
32 hydrogen evolution started after 48 h in pure, sulfur-deprived *C. reinhardtii* cc124 cultures,
33 which corroborates with the results of other experiments using the sulfur-deprivation method
34 (Table 3.). In the algal-bacterial cultures hydrogen evolution was detected much earlier - 2–4
35 h after preparation of co-cultures – compared to the sulfur deprived *C. reinhardtii* cc124
36 cultures (Table 3.). This rapid hydrogen evolution can be beneficial in the future industrial
37 application of algal hydrogen evolution. In the cases of the sulfur-deprived mixed cultures,
38 hydrogen evolution continued for three (*Chlamydomonas* sp. 549) and seven days (*C.*
39 *reinhardtii* cc124). Thus, the combination of the two methods (sulfur deprivation and algae-
40 bacteria co-cultivation) resulted in the highest efficiency of hydrogen evolution. However,
41 significant differences were observed in hydrogen-production capabilities of the sulfur-
42 deprived algal-bacterial co-cultures in terms of duration of hydrogen production and hydrogen

1 yield. The mixed culture of *C. reinhardtii* cc124 and *E. coli hypF* produced 47241.3 ±
2 4660.69 μL L^{-1} hydrogen in seven days, while the consortium of *Chlamydomonas* sp. 549.
3 and *E. coli hypF* evolved 2637.49 ± 555.42 μL L^{-1} hydrogen in three days. It is important to
4 emphasize that hydrogen-evolution performance of the sulfur-deprived mixed cultures was
5 significantly higher than that of sulfur-deprived pure algae cultures under the same conditions
6 (47241.3 ± 4660.69 μL L^{-1} versus 25028.1 ± 3943.47 μL L^{-1} hydrogen produced in seven
7 days in the *C. reinhardtii* cc124-*E. coli hypF* system). After the hydrogen-evolution period, a
8 significant part of the accumulated hydrogen was consumed in the batch system from the
9 *Chlamydomonas* sp. 549-*E. coli hypF* co-cultures. This uptake process is also closely linked
10 to acetic acid metabolism. Once acetic acid is consumed, the intensive algae respiration
11 ceases, oxygen level increases, and algal hydrogenases stop functioning. Apparently,
12 *Chlamydomonas* sp. 549 has a significantly higher uptake activity than *C. reinhardtii* cc124,
13 attributed again to variability of algal hydrogenases.
14

Total hydrogen production	Starting time of hydrogen production	Length of measurements	Experimental setup	References
1.2 mL L^{-1}	2-4 h	24 h	Growth medium: TAP, algae strain: *Chlamydomonas* sp. 549, bacterial partner: *E. coli hypF*, volume: 35 mL, pH = 7.2, 25 °C, non-synchronized growth, light intensity: 50 μmol m^{-2} s^{-1}	This work
2.6 mL L^{-1}	2-4 h	72 h	Growth medium: TAP, algae strain: *Chlamydomonas* sp. 549, bacterial partner: *E. coli hypF*, volume: 35 mL, pH = 7.2, 25 °C, non-synchronized growth, light intensity: 50 μmol m^{-2} s^{-1}	This work
5.8 mL L^{-1}	2-4 h	24 h	Growth medium: TAP, algae strain: *Chlamydomonas reinhardtii* cc124, bacterial partner: *E. coli hypF*, volume: 35 mL, pH = 7.2, 25 °C, non-synchronized growth, light intensity: 50 μmol m^{-2} s^{-1}	This work
47 mL L^{-1}	2-4 h	168 h	Growth medium: TAP-S, algae strain: *Chlamydomonas*	This work

			reinhardtii cc124, bacterial partner: *E. coli hypF*, volume: 35 mL, pH = 7.2, 25 °C, non-synchronized growth, light intensity: 50 μmol m^{-2} s^{-1}	
85 mL L^{-1}	46 h	140 h	Growth medium: TAP-S, algae strain: *Chlamydomonas reinhardtii* cc124, volume: 1.2 L, pH = 7.2, 28 °C, 4 h synchronous growth, light intensity: 300 μmol m^{-2} s^{-1}	40
71.6 mL	32 h	140 h	Growth medium: TAP-S, algae strain: *Chlamydomonas reinhardtii* cc124, volume: 1.2 L, pH = 7.2, temperature: 28 °C, , light intensity: 300 μmol m^{-2} s^{-1}	40
56.4 mL L^{-1}	~ 90 h	~ 140 h	Growth medium: TAP-S, algae strain: *Chlamydomonas reinhardtii* cc124, volume 550 mL, pH = 7.4, addition of CO_2, temperature: 28 °C, light intensity: 110 μmol m^{-2} s^{-1}	41
570.5 mL L^{-1}	~ 24 h	~ 160 h	Growth medium : TAP-S, algae strain: *Chlamydomonas reinhardtii* L159I-N230Y, volume 800 mL, pH = 7.2, addition of CO_2, temperature: 28 °C, light intensity: 140 μmol m^{-2} s^{-1}	39
262.0 mL L^{-1}	~ 24 h	~ 80 h	Growth medium: TAP-S , algae strain: *Chlamydomonas reinhardtii* cc124 volume 800 mL, pH = 7.2, addition of CO_2, temperature: 28 °C, light intensity: 140 μmol m^{-2} s^{-1}	39
16.68 mL L^{-1}	~ 24 – 48	312 h	Growth medium: TAP-S, algae strain: *Chlamydomonas*	42

| | | h | | | *reinhardtii* Cr849 + *Pseudomonas* sp. A8, volume 40 mL, pH = 7.0, temperature: 25 °C, light intensity: 60 μE m^{-2} s^{-1} | |

Table 3. Comparison of various algal-bacterial and algal hydrogen producing approaches

Fluorescence investigations were used to examine the state of the photosynthetic system in algae during the experiments under illumination. A highly reduced PQ pool was observed in the mixed cultures. Features of the OJIP curve measured in samples kept under anaerobic conditions were consistent with data in the literature[43]. Short air-bubbling restored the usual OJIP and flash-induced fluorescence decay curves seen in pure cultures. Thus, algal PSII remained intact and fully active with respect to hydrogen production by algal-bacterial cultures in contrast to the decreased PSII activity observed in sulfur-deprived ones[10].

In darkness, the bacterial co-cultivation only had a minor effect on algal (*Chlamydomonas* sp. 549) hydrogen production. Similar total hydrogen amounts were generated by the algae in the mixed and pure algae cultures. Also, only slightly faster oxygen consumptions were measured in mixed cultures compared to pure algae cultures.

Our data showed that the presence of actively respiring bacterial communities has a major impact on algal hydrogen evolution exclusively under illumination. Thus, active algal photosynthesis may play an important role in the enhanced algal hydrogen productivity. We hypothesize that certain photosynthates are used by the bacteria; the algal products might be essential for the active respiration of the bacterial partner, leading to complete anaerobiosis. Acetate was also shown to have a central role in the hydrogen-evolution process observed in the algal-bacterial co-cultures. Algal hydrogen generation ceased as acetic acid concentration decreased to a particular level in the case of *Chlamydomonas* sp. 549. Thus, the efficiency of the algal hydrogen production in the co-cultivation system is highly dependent upon the concurrent presence of the metabolically active bacteria and on the availability of acetic acid for algal cells. The major role of the bacterial partner is the efficient respiration (i.e., consumption of oxygen), which makes it possible for the algae to use acetic acid for hydrogen evolution. The highly different rates of acetic acid metabolism of the mixed algal-bacterial and pure algae cultures might seem contradictory with the observed similar pH profiles of the cultures. It is presumed that the higher ratio of dissolved CO_2 in the mixed cultures balances the pH level, although this issue must be resolved in future analyses. Additional questions regarding bacterial-algal interactions (e.g., the role of the partners in acetic acid metabolism) exist. Community-level transcriptome analyses are planned to provide answers for these questions.

Conclusion

The interaction of *Chlamydomonas* sp. 549 and *C. reinhardtii* cc124 with actively respiring bacterial partners represents an alternative, novel approach for algae-based biohydrogen evolution. The creation of a continuous, sustainable algae hydrogen-evolving system is

1   possible by combining photosynthesis-driven algae biomass generation and concomitant
2   hydrogen evolution (a combination of photolytic and fermentative hydrogen production)
3   under illumination with fermentation-based hydrogen production in darkness using a single
4   algae-bacterium consortium.

Acknowledgements

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References

14   1.   O. Pulz and W. Gross, *Applied microbiology and biotechnology*, 2004, 65, 635-648.
15   2.   P. Spolaore, C. Joannis-Cassan, E. Duran and A. Isambert, *Journal of bioscience and*
16   *bioengineering*, 2006, 101, 87-96.
17   3.   U. Dahl, C. R. Lind, E. Gorokhova, B. Eklund and M. Breitholtz, *Ecotoxicology and*
18   *environmental safety*, 2009, 72, 351-357.
19   4.   E. E. Luiten, I. Akkerman, A. Koulman, P. Kamermans, H. Reith, M. J. Barbosa, D. Sipkema and
20   R. H. Wijffels, *Biomolecular Engineering*, 2003, 20, 429-439.
21   5.   M. L. Ghirardi, A. Dubini, J. Yu and P.-C. Maness, *Chemical Society Reviews*, 2009, 38, 52-61.
22   6.   A. Dubini and M. L. Ghirardi, *Photosynthesis research*, 2014, 1-13.
23   7.   M. Momirlan and T. N. Veziroglu, *International Journal of Hydrogen Energy*, 2005, 30, 795-
24   802.
25   8.   M. Ni, M. K. Leung, K. Sumathy and D. Y. Leung, *International Journal of Hydrogen Energy*,
26   2006, 31, 1401-1412.
27   9.   S. Manish and R. Banerjee, *International Journal of Hydrogen Energy*, 2008, 33, 279-286.
28   10.   A. Melis, L. Zhang, M. Forestier, M. L. Ghirardi and M. Seibert, *Plant Physiology*, 2000, 122,
29   127-136.
30   11.   M. L. Ghirardi, L. Zhang, J. W. Lee, T. Flynn, M. Seibert, E. Greenbaum and A. Melis, *Trends in*
31   *biotechnology*, 2000, 18, 506-511.
32   12.   A. R. Grossman, C. Catalanotti, W. Yang, A. Dubini, L. Magneschi, V. Subramanian, M. C.
33   Posewitz and M. Seibert, *New Phytologist*, 2011, 190, 279-288.
34   13.   H. Kawaguchi, K. Hashimoto, K. Hirata and K. Miyamoto, *Journal of bioscience and*
35   *bioengineering*, 2001, 91, 277-282.
36   14.   K. Kumar, S. Roy and D. Das, *Bioresource technology*, 2013, 145, 116-122.
37   15.   S. Wu, X. Li, J. Yu and Q. Wang, *Bioresource technology*, 2012, 123, 184-188.
38   16.   S. N. Kosourov, M. L. Ghirardi and M. Seibert, *International journal of hydrogen energy*, 2011,
39   36, 2044-2048.
40   17.   T. Maier, U. Binder and A. Böck, *Archives of microbiology*, 1996, 165, 333-341.
41   18.   S. S. Cohen, *The Journal of biological chemistry*, 1949, 177, 607-619.
42   19.   I. Wolf, T. Buhrke, J. Dernedde, A. Pohlmann and B. Friedrich, *Archives of microbiology*, 1998,
43   170, 451-459.
44   20.   A. Holzenburg, F. Mayer, G. Harauz, M. Van Heel, R. Tokuoka, T. Ishida, K. Harata, G. Pal and
45   W. Saenger, *Nature*, 1987, 325, 730-732.

1 21. D. S. Gorman and R. Levine, *Proceedings of the National Academy of Sciences of the United*
2   *States of America*, 1965, 54, 1665.
3 22. S. Hutner, L. Provasoli, A. Schatz and C. Haskins, *Proceedings of the American Philosophical*
4   *Society*, 1950, 152-170.
5 23. J. Sambrook and D. Russell, Cold Spring Harbor Laboratory Press Cold Spring Harbor, New
6   York, 2001.
7 24. M. R. Green and J. Sambrook, *Molecular cloning: a laboratory manual*, Cold Spring Harbor
8   Laboratory Press Cold Spring Harbor, New York, 2012.
9 25. J. G. Metz, H. B. Pakrasi, M. Seibert and C. J. Arntzer, *FEBS letters*, 1986, 205, 269-274.
10 26. B. R. Velthuys, *FEBS Letters*, 1981, 126, 277-281.
11 27. C. Wraight, *Israel Journal of Chemistry*, 1981, 21, 348-354.
12 28. A. Volgusheva, S. Styring and F. Mamedov, *Proceedings of the National Academy of Sciences*,
13   2013, 110, 7223-7228.
14 29. G. C. Papageorgiou, *Chlorophyll a fluorescence: a signature of photosynthesis*, Springer, 2004.
15 30. R. J. Strasserf and A. Srivastava, *Photochemistry and photobiology*, 1995, 61, 32-42.
16 31. G. C. Papageorgiou, M. Tsimilli-Michael and K. Stamatakis, *Photosynthesis Research*, 2007,
17   94, 275-290.
18 32. G. Schansker, S. Z. Tóth and R. J. Strasser, *Biochimica et Biophysica Acta (BBA)-Bioenergetics*,
19   2005, 1706, 250-261.
20 33. M. Tsimilli-Michael, K. Stamatakis and G. C. Papageorgiou, *Photosynthesis research*, 2009, 99,
21   243-255.
22 34. M. Trtílek, D. M. Kramer, M. Koblížek and L. Nedbal, *Journal of luminescence*, 1997, 72, 597-
23   599.
24 35. I. Vass, D. Kirilovsky and A.-L. Etienne, *Biochemistry*, 1999, 38, 12786-12794.
25 36. A. Joliot and P. Joilot, *Comptes Rendus Hebdomadaires des Seances de l Academie des*
26   *Sciences* 1964, 258, 4622-4625.
27 37. U. Schreiber, U. Schliwa and W. Bilger, *Photosynthesis research*, 1986, 10, 51-62.
28 38. F. Perreault, N. Ait Ali, C. Saison, R. Popovic and P. Juneau, *Journal of Photochemistry and*
29   *Photobiology B: Biology*, 2009, 96, 24-29.
30 39. A. Scoma, D. Krawietz, C. Faraloni, L. Giannelli, T. Happe and G. Torzillo, *Journal of*
31   *biotechnology*, 2012, 157, 613-619.
32 40. A. Tsygankov, S. Kosourov, M. Seibert and M. L. Ghirardi, *International Journal of Hydrogen*
33   *Energy*, 2002, 27, 1239-1244.
34 41. A. A. Tsygankov, S. N. Kosourov, I. V. Tolstygina, M. L. Ghirardi and M. Seibert, *International*
35   *Journal of Hydrogen Energy*, 2006, 31, 1574-1584.
36 42. X. Li, S. Huang, J. Yu, Q. Wang and S. Wu, *International Journal of Hydrogen Energy*, 2013, 38,
37   10779-10787.
38 43. S. Z. Tóth, G. Schansker and R. J. Strasser, *Photosynthesis research*, 2007, 93, 193-203.

39
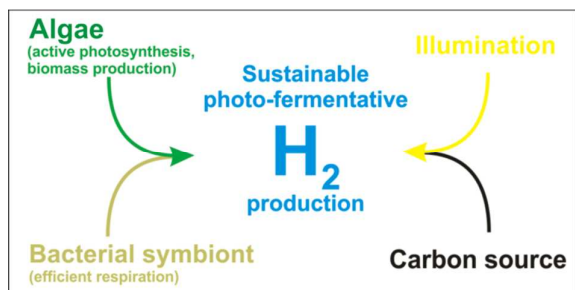
The association of photosynthesizing *Chlamydomonas* algae and actively respiring bacterial partners represents an alternative, efficient and sustainable approach for biohydrogen generation.