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Integrated biological hydrogen production

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Abstract

Biological systems offer a variety of ways by which to generate renewable energy. Among them, unicellular green algae have the ability to capture the visible portion of sunlight and store the energy as hydrogen (H_2). They hold promise in generating a renewable fuel from nature's most plentiful resources, sunlight and water. Anoxygenic photosynthetic bacteria have the ability of capturing the near infrared emission of sunlight to produce hydrogen while consuming small organic acids. Dark anaerobic fermentative bacteria consume carbohydrates, thus generating H_2 and small organic acids. Whereas efforts are under way to develop each of these individual systems, little effort has been undertaken to combine and integrate these various processes for increased efficiency and greater yields. This work addresses the development of an integrated biological hydrogen production process based on unicellular green algae, which are driven by the visible portion of the solar spectrum, coupled with purple photosynthetic bacteria, which are driven by the near infrared portion of the spectrum. Specific methods have been tested for the cocultivation and production of H_2 by the two different biological systems. Thus, a two-dimensional integration of photobiological H_2 production has been achieved, resulting in better solar irradiance utilization (visible and infrared) and integration of nutrient utilization for the cost-effective production of substantial amounts of hydrogen gas. Approaches are discussed for the cocultivation and coproduction of hydrogen in green algae and purple photosynthetic bacteria entailing broad utilization of the solar spectrum. The possibility to improve efficiency even further is discussed, with dark anaerobic fermentations of the photosynthetic biomass, enhancing the H_2 production process and providing a recursive link in the system to regenerate some of the original nutrients.

Keywords: *Chlamydomonas reinhardtii*; Green algae; *Rhodospirillum rubrum*; Photosynthetic bacteria; *Clostridium sp.*; Fermentative bacteria; Photobiology; Hydrogen production

1. Introduction

1.1. Aims

The goal of this project is to develop the foundation for a sustainable and commercially viable integrated biological hydrogen production process. This would be achieved through the coalition of three distinct biological processes. Photosynthetic H_2 production by green algae, utilizing the visible region of the solar spectrum, is coupled to H_2 production by anoxygenic photosynthetic bacteria, utilizing the near infrared region of sunlight. Biomass accumulation in the course of photosynthesis by the two organisms is subsequently utilized in dark anaerobic fermentations for the further production of H_2. Small organic acids accumulate as a by-product of the dark anaerobic fermentation; these can serve as substrate to support further hydrogen production by green algae and photosynthetic bacteria.

The foundation of such an integrated biological H_2 production is the oxygenic photosynthesis of unicellular green algae (e.g. *Chlamydomonas reinhardtii*), a

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process that utilizes the energy of sunlight to convert water, carbon dioxide and other inorganic nutrients, into the basic building blocks of life. In Stage 1 of this integrated biological H_2 production, green algal photosynthesis generates biomass and, via the [Fe]-hydrogenase enzyme, quantities of H_2 gas. This stage employs recently developed technologies in green algal H_2 production, in which photosynthetic activity is geared towards both hydrogen and biomass accumulation. In Stage 2, the green algal process is coupled to that of anoxygenic photosynthetic bacteria (e.g. *Rhodospirillum rubrum*) that utilize complementary wavelengths of sunlight to produce ATP, required for the evolution of hydrogen via the nitrogenase enzyme. In Stage 3, fermentative bacteria (e.g. *Clostridium pasteurianum*) catabolize the green algal/photosynthetic bacterial biomass and further enhance the yield of biological hydrogen production. This system holds the possibility for increased efficiency by utilizing the end products of the fermentative H_2 production—small organic acids—as a recursive supply of nutrients for further photosynthetic growth.

Fundamentally, this research provides a "systems biology" opportunity to study the integration of metabolism of a consortium of organisms, while further elucidating the diverse metabolic pathways leading to H_2 production. Advancements in the molecular genetics and biochemistry of these processes are directing the isolation of green algal and photosynthetic bacterial strains that are optimized for ease of cultivation and high yield of H_2 production. Species cocultivation (e.g. *Chlamydomonas reinhardtii* and *R. rubrum*) has been established for enhancement in light utilization and biomass accumulation. Practically, information gained from this functionally integrated research effort would be useful in efforts to further optimize the three-organism system and enhance product accumulation.

1.2. Rationale

The establishment of a sustainable and efficient process of biological hydrogen production would present tremendous environmental and social benefits, both locally and globally. Hydrogen is recognized as an ideal energy carrier that does not contribute to pollution of the environment nor to global climate change. Hydrogen and electricity could team to provide attractive options in future transportation and power generation. Interconversion between these two forms of energy suggests on-site utilization of hydrogen to generate electricity, with the electrical power grid serving in energy transportation, distribution, utilization and hydrogen regeneration, as needed. A challenging problem in establishing hydrogen as a source of energy for the future is the cost-effective, renewable, and environmentally friendly generation of large quantities of hydrogen gas.

Current projections of potential fossil fuel shortfall make it important to develop alternative energy carriers that are renewable and environmentally friendly. The advent of abundant energy through renewable biohydrogen will bring about technological innovations in many fields, including the transportation industry, power generation, and other as yet unforeseen applications. Photobiological H_2 production, in particular, will increase the value of world agriculture, as scaled-up application of the method would additionally produce substantial amounts of biomass, and contain several high value bioproducts. This process would especially provide a boost to the economy of underdeveloped and developing nations, as commercial exploitation of green algae and bacteria would be a low-tech business. Such H_2 production would positively impact global warming, environmental pollution, and the question of energy supply and demand.

2. Current state of the art

Hydrogen metabolism is exhibited primarily by microorganisms, i.e., bacteria and microalgae [1]. Within these groups, it involves many taxonomically diverse species, a variety of enzymes and several metabolic pathways and processes [2–6].

2.1. Hydrogen photoproduction by green algae

Hydrogen production with high specific activity occurs in unicellular green algae (e.g. *Chlamydomonas reinhardtii*) and anaerobic fermentative bacteria (e.g. *Clostridium pasteurianum*). The reaction is catalyzed by [Fe]-hydrogenase enzymes [3,6–9], which mediate the donation of high potential-energy electrons to protons (H^+), according to the reaction:

$$2H^+ + 2FD^- \rightarrow H_2 + 2FD. \quad (1)$$

The enzymatic turnover rate of the [Fe]-hydrogenase is in the 6000–9000 s^{-1} range, consistent with the high specificity of the reaction. In green algae (e.g. *Chlamydomonas reinhardtii*), electrons (e^-) and protons (H^+) are extracted from water (H_2O) through photosynthesis, or from endogenous substrate through chlororespiration [10–13]. The potential energy of these electrons is elevated in the thylakoid membrane of photosynthesis, via the absorption/utilization of visible

sunlight (400–700 nm region of the solar spectrum). Following this light-driven electron transport in the thylakoid membrane, the high potential-energy electrons (e^-) and protons (H^+) are combined to generate molecular H_2. The process of photosynthetic electron transport in green algae can operate with a photon conversion efficiency of 85–90% [14,15] and normally generates biomass from inorganic minerals, CO_2 and H_2O. Given the high solar conversion efficiency and directness of the H_2 production process, green algae are thought to be promising in long-term efforts of photobiological H_2 production. The present work, although based on green algal H_2 production, nevertheless, seeks to enhance potential yields by developing an integrated process that combines and exploits the strengths of green algae, anoxygenic photosynthetic bacteria and dark anaerobic fermentative bacteria to achieve superior yields of H_2 production.

Historically, hydrogen evolution activity in green algae was induced upon a prior anaerobic incubation of the cells in the dark [4,16–19]. This treatment was necessary and sufficient to remove oxygen (O_2) from the medium, as O_2 is a powerful suppressor of the [Fe]-hydrogenase gene expression [9,20,21] and a potent inhibitor of the [Fe]-hydrogenase enzymatic activity [22]. Following such a dark anaerobic induction, and in the course of a subsequent illumination by which to drive green algal photosynthesis, the activity of the [Fe]-hydrogenase is manifested, but it is only transient in nature [23]. It lasts from several seconds to a few minutes only. This is because photosynthetic O_2 quickly accumulates upon illumination and effectively interferes with all aspects of the cellular H_2 metabolism.

At present, the simultaneous production of O_2 and H_2 by the photosynthetic apparatus of green algae offers a number of challenges, mainly due to the great sensitivity of the [Fe]-hydrogenase to O_2, which is evolved upon illumination of the cells [22,24]. An additional problem, assuming that the mutual incompatibility of O_2 and H_2 coproduction is overcome, entails the separation of the two gases, a costly and technologically challenging feat. Nevertheless, a direct photosynthetic O_2 and H_2 coproduction promises the highest yields and efficiencies, approaching 13–15% (light energy to H_2), as H_2 is produced soon after the primary energy conversion event in photosynthesis, i.e., the conversion of sunlight energy into chemical energy. In support of the feasibility of this approach, it has been shown that O_2 and H_2 coproduction by green algae can be prolonged under conditions designed to actively remove O_2 from the reaction mixture [15,17,25]. Moreover, genetic engineering of the green algae can be applied to enhance the O_2 tolerance of the [Fe]-hydrogenase, so as to permit O_2 and H_2 coproduction [26].

A recently developed protocol for the temporal separation of normal photosynthesis (O_2 evolution) from H_2 production in green algae permitted a sustained photobiological hydrogen gas production. The basis of this method was the specific but reversible slow-down of oxygen evolution in the green alga *Chlamydomonas reinhardtii* [27] without affecting the rate of mitochondrial respiration [28]. This was achieved upon withholding sulfur nutrients from the algal growth medium. Sulfur is a component of two essential amino acids, cysteine and methionine, and, consequently, of proteins. In the absence of externally provided sulfur nutrients, cells could not accumulate protein and, therefore, could not grow [28,29]. Specifically, green algae could not perform high rates of de novo protein biosynthesis, required for the frequent replacement of the photosystem-II (PSII) D1/32 kD reaction center protein [30]. Inhibition of the frequent replacement of the D1 protein forced a lowering in the rate of photosynthesis by the cells, to the point where the rate of photosynthetic oxygen evolution in the chloroplast became less than the rate of respiratory oxygen consumption in the mitochondria. In sealed cultures, attenuation of photosynthesis to a level below that of respiration (Fig. 1A) resulted in internal respiratory consumption of O_2 by the cells (Fig. 2), causing anaerobiosis in the growth medium. It was shown that expression of the [Fe]-hydrogenase is elicited in the light under these conditions, automatically leading to H_2-photoproduction by the algae [22,28]. For the first time in the 60-plus year history of this field, it was possible to photoproduce and accumulate bulk amounts of H_2 gas, emanating as bubbles from the green alga culture [10], a sustainable process that could be employed continuously for a few days (Fig. 1B). Thus, progress was achieved by circumventing the sensitivity of the [Fe]-hydrogenase to O_2 through a lowering of the activity of oxygenic photosynthesis and the ensuing consumption of photosynthetic O_2 by respiration [28]. The absence of sulfur nutrients from the growth medium of the algae acted as a metabolic switch, which selectively and reversibly lowered the photosynthesis/respiration (P/R) ratio. Thus, in the presence of sulfur nutrients ($P/R = 4:1$), green algae perform normal photosynthesis (H_2O oxidation, O_2 evolution and biomass accumulation). In the absence of sulfur and absence of O_2 ($P/R < 1$), photosynthesis in *Chlamydomonas reinhardtii* slips into the H_2 production mode. Reversible application of the switch (presence/absence of S) permitted the algae to alternate between O_2 production and H_2 production

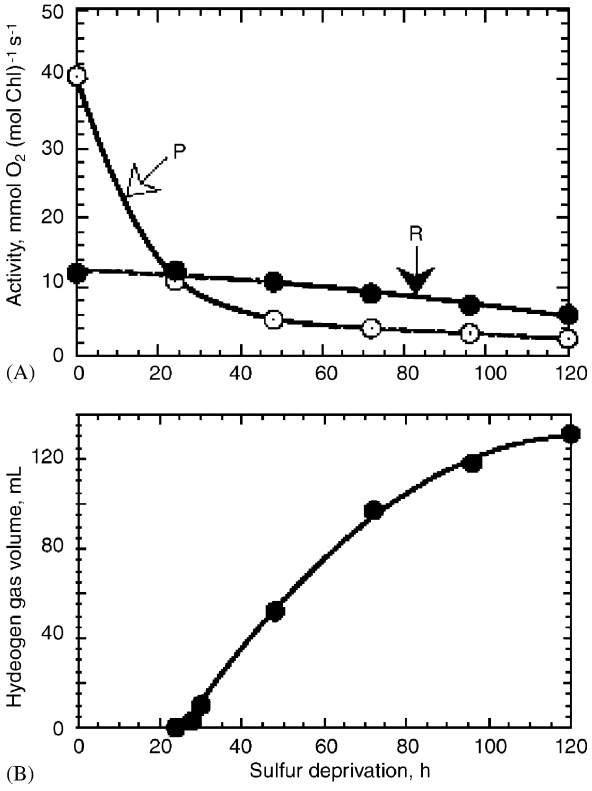

Fig. 1. Photosynthesis, respiration, and H_2 production as a function of time in sulfur nutrient deprivation in the green alga *Chlamydomonas reinhardtii.* (A) Absolute activity of oxygenic photosynthesis (*P*, open circles) and respiration (*R*, solid circles) in *Chlamydomonas reinhardtii* suspended in media lacking a source of sulfur. Note that within 24 h of S-deprivation, the photosynthesis/respiration ratio is lowered to less than 'unity' (P/R ratio < 1). In consequence, sealed cultures of S-deprived *Chlamydomonas reinhardtii* quickly consume all dissolved oxygen and become anaerobic [22], even though they are maintained under continuous illumination. For each time point, the rate of cellular respiration (R) was recorded in the dark, followed by measurement of the light-saturated rate of photosynthesis (P). (B) H_2 gas production and accumulation by *Chlamydomonas reinhardtii* cells suspended in media lacking sulfur nutrients. Gases were collected in an inverted burette and measured from the volume of water displacement. Adapted from [32].

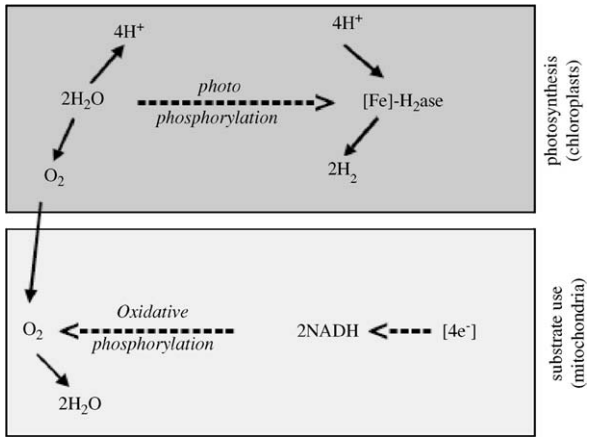

Fig. 2. Coordination of photosynthetic and respiratory electron transport and coupled phosphorylation during H_2 production in $P/R < 1$ green algae. Photosynthetic electron transport from H_2O to H_2 drives *photo-phosphorylation*, i.e., the synthesis of ATP (adenosine triphosphate) in the chloroplast. Mitochondrial electron transport to O_2 ($4e^-$ derived from the breakdown of endogenous glucose) drives ATP synthesis via *oxidative phosphorylation*. Release of molecular H_2 by the chloroplast and consumption of photosynthetically generated O_2 by the mitochondria helps to sustain these linked processes.

[22], thus bypassing the incompatibility and mutually exclusive nature of the O_2 and H_2 producing reactions.

Analysis of this novel process revealed the occurrence of hitherto unknown morphological, physiological and metabolic processes in the green alga *Chlamydomonas reinhardtii,* dramatically altering cell shape, gas exchange, and starch and protein flux in the cell [29,31]. This method served as an important tool by which to probe and improve green alga hydrogen production [10,32]. It also served as the foundation for the development of an integrated biological hydrogen production process that utilizes green algae, anoxygenic photosynthetic bacteria, and dark anaerobic fermentative bacteria for commercially viable H_2 production.

2.2. Hydrogen production by anoxygenic photosynthetic bacteria

Anoxygenic photosynthetic bacteria (e.g. *R. rubrum*) are photoheterotrophs that can grow anaerobically and produce hydrogen in the light [33–36] by means of the enzyme nitrogenase [37–39]. This process depends on utilization of sunlight and of small organic acids [40,41]. These photosynthetic bacteria are nitrogen-fixing, utilizing the enzyme nitrogenase to catalyze the reduction of molecular nitrogen (N_2) to ammonia (NH_3). However, the simultaneous evolution of H_2 by this enzyme is inherent to the process [42], according to the equation:

$$N_2 + 8e^- + 8H^+ + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16Pi. \quad (2)$$

In the absence of N_2 gas, the enzyme may simply reduce protons (H^+) to generate H_2 [43]. Near infrared sunlight (700–950 nm) can be specifically absorbed by the photosynthetic apparatus of these bacteria [44]. It plays a critical role in driving this reaction, as photosynthesis in these microorganisms generates the ATP, needed for the catalysis of the H_2 production reaction

(Eq. (2)). Anoxygenic photosynthetic bacteria, utilizing infrared radiation and small organic acids, can achieve high yields of H_2 production [45]. However, solar conversion efficiencies are low due to the high energetic demand of 4 ATP/H_2 (Eq. (2)), and due to the very low intensity for the saturation of their photosynthesis, which prevents efficient utilization of bright solar irradiance [40,41]. Accordingly, the enzymatic turnover rate of the nitrogenase is thought to be in the range of 6–7 s^{-1} [46], consistent with the low specificity of the reaction.

The nitrogenase enzyme is also O_2-sensitive, as is the [Fe]-hydrogenase of the green algae, and requires anaerobiosis for its expression and function. Moreover, anoxygenic photosynthetic bacteria do not have the ability to oxidize H_2O in order to extract electrons and protons. They depend on small organic acids for growth and H_2 production, and cannot evolve O_2 [47]. However, they can specifically utilize the near infrared (700–950 nm) region of the spectrum [44] to drive photosynthetic electron transport for the generation of chemical energy in the form of ATP. The latter is critical for the function of the nitrogenase in the H_2 production process by these organisms (Eq. (2)). Thus, utilization of the infrared region of the spectrum by anoxygenic photosynthetic bacteria offers a supplementary avenue of solar energy conversion to H_2. This is important, as the near infrared (700–950 nm) region of the solar spectrum would increase the energy content of photosynthetically active radiation (PAR) by about 50%, or nearly double the photons of the solar irradiance converted (Fig. 3). Currently, H_2 production research with photosynthetic bacteria is primarily performed in pure cultures, which are provided with small organic acids as the initial carbon source [36,48–51]. Rates of H_2-production in these systems are typically 40–60 mL H_2 per liter culture per hour, although occasionally higher rates have been reported [41]. The near infrared absorbance of sunlight by photosynthetic bacteria (700–950 nm), such as *R. rubrum*, complements that of green algae (400–700 nm), such as *Chlamydomonas reinhardtii*, raising the prospect of cocultivation of the two organisms for substantially enhanced rates and yields of photobiological H_2 production.

2.3. Hydrogen production by anaerobic fermentative bacteria

Dark anaerobic fermentative bacteria (e.g. *Clostridium pasteurianum*) are not equipped to utilize the energy of the sun for H_2 production. Instead, they rely solely on the catabolism of organic matter, which must be added to the growth medium. Hydrogen is one of the end products of their anaerobic metabolism. An example of the biochemistry that underlines this process is shown in Eq. (3) below

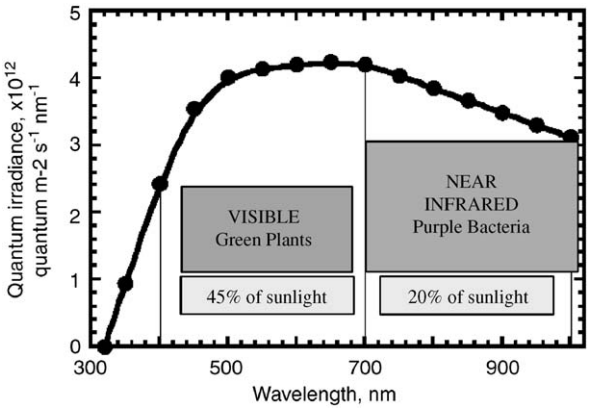

Fig. 3. Solar quantum irradiance distribution as a function of wavelength in the UV-A, visible, and near infrared regions of the spectrum. Adapted from [78]. The visible portion of the solar spectrum (400–700 nm) contains ~ 45% of the total solar energy, whereas the near infrared region (700–1000 nm) contains an additional 20% of the total solar energy incident to the surface of the earth.

$$\text{Glucose} + 2H_2O \rightarrow 4H_2 + 2CO_2 + 2 \text{ acetates}. \quad (3)$$

Anaerobic fermentative bacteria (e.g. *Clostridium pasteurianum*) produce H_2 from carbohydrate and other organic molecules at rates ranging between 25–55 mL H_2 per liter culture per hour [35,36,52,53]. Higher rates of fermentative H_2 production have been reported with a consortium of mesophilic or hyperthermophilic bacteria in optimized bioreactors [54,55]. Depending on the bacterial species and organic nutrients employed, such fermentations result in the generation of an abundance of small organic acids, such as malate, lactate, propionate, butyrate, and/or acetate [41,56–62]. The further conversion of these small organic acids to H_2 is not an energetically favorable reaction and cannot be supported by the fermentative metabolism of the anaerobic bacteria. Hence, small organic acids accumulate in the growth medium. As such, they cause inhibition in the rate of growth and limit the yield of H_2 production by the anaerobes. In consequence, the duration of the H_2 production reaction (Eq. (3)) could be relatively short, and yields can be limited because of the accumulating small organic acids. However, the latter can be removed and utilized in support of growth and H_2 production by green algae and anoxygenic photosynthetic bacteria. An additional concern with these methods is the origin of

the feedstock: while many methods focus on utilizing waste materials, little research has been performed on the direct fermentation of photosynthetic biomass such as microalgae [63].

3. Concepts and feasibility of integrated hydrogen production

The metabolic and H_2 production properties of the organisms described above suggest the design of an integrated system in which oxygenic and anoxygenic photosynthesis are employed in tandem to harvest the visible and near infrared energy of the sun and to convert it into H_2 energy. Hydrogen can be collected using a light–dark cycle [64], while biomass extracted from this integrated process can be converted, through the use of industrial enzymes, into cellulolytic material composed of hydrolysates of polyglucose and protein, which can feed directly into dark anaerobic bacterial fermentations. Alternatively, cellulosome-containing fermentative bacteria can be used [65] and research has shown the dark anaerobic hydrogen production of intact algal biomass [66]. Such non-photosynthetic anaerobic bacterial fermentations would generate H_2 and a variety of small organic acids. The latter can feed back into the anoxygenic photosynthetic bacterial H_2 production reactions according to the schematic in Fig. 4. Such an integrated system would constitute a high-yield, sustainable, and commercially viable H_2 production process.

3.1. Chlamydomonas reinhardtii/cocultivation with Rhodospirillum rubrum

Wild type strains of the green alga *Chlamydomonas reinhardtii* and the anoxygenic photosynthetic bacterium *R. rubrum* were precultured in TAP and Ormerod media, respectively [41,67,68]. A cocultivation medium was devised (Table 1) to contain all the nutrients of both media in a single volume, and precultures in early logarithmic growth phase were inoculated into sealed bottles. Growth under these cocultivation conditions was measured at different light intensities. We found that, for a given cocultivation medium, the relative growth of the two microorganisms could be regulated by the level of irradiance. Fig. 5 shows two such cocultivation cultures in which either *R. rubrum* (Fig. 5 left-side bottle) or *Chlamydomonas reinhardtii* (Fig. 5 right-side bottle) are dominant. Cells were pelleted upon centrifugation in a hematocrit tube for biomass quantitation purposes (Fig. 6). In such "packed cell volume" measurements, the heavier green algae pelleted first, followed by the photosynthetically competent (purple) *R. rubrum*, followed by aerobically repressed (non-photosynthetic) yellowish-white *R. rubrum.*

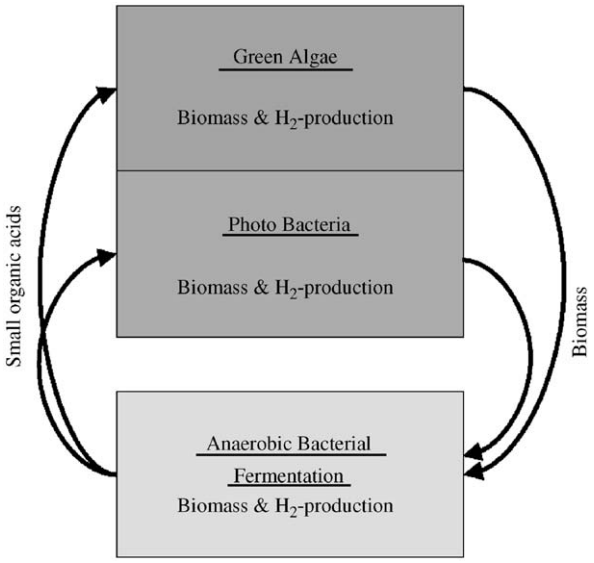

Fig. 4. Functional integration of a three-organism system for H_2 production. Green algae and photosynthetic bacteria are cocultivated in the same photoreactor, thereby minimizing facility costs. Anaerobic bacterial fermentations take place in traditional fermentors, where land surface area is not a requirement. Integration of the three processes ensures cycling of products and promises high yields of H_2 production. Green algal/photosynthetic bacterial system inputs are H_2O, CO_2 and small organic acids, outputs are biomass, sugars and H_2. Anaerobic bacterial fermentation system inputs are biomass and sugars, outputs are biomass, small organic acids, H_2 and CO_2.

Table 1
Growth medium for functional integration of *Rhodospirillum rubrum* and *Chlamydomonas reinhardtii* (COCULT medium)

Nutrient	Concentration
Tris-Hcl, pH 7.0	20 mM
K_2HPO_4	10 mM
$MgSO_4$	2.0 mM
NH_4Cl	7 mM
Acetate	16 mM
Na-Succinate	16 mM
Na-Glutamate	3 mM
$CaCl_2$	680 μM
Na-EDTA	135 μM
Fe-Citrate	138 μM
$ZnSO_4$	76 μM
H_3BO_3	234 μM
$MnCl_2$	36 μM
$CuCl_2$	7 μM
Na_2MoO_4	12 μM
$CoCl_2$	7 μM
Biotin	0.06 μM

Fig. 5. Integrated two-organism cocultivation for hydrogen production. *R. rubrum* and *Chlamydomonas reinhardtii* were grown into the same growth medium under cocultivation conditions to yield a *R. rubrum* to *Chlamydomonas reinhardtii* ratio of 10:1 (left-side bottle) or 1:10 (right-side bottle).

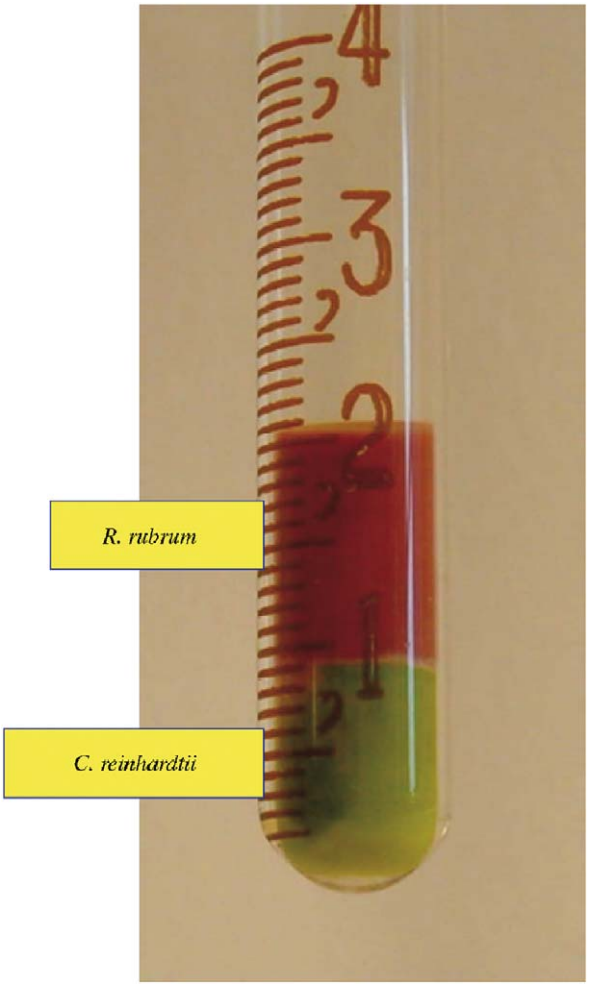

Fig. 6. Hematocrit measurement of *R. rubrum* and *Chlamydomonas reinhardtii* packed cell volume. The heavier green algae pelleted first, followed by the photosynthetically competent (purple) *R. rubrum*. Whenever present, aerobically repressed yellowish-white *R. rubrum* pelleted on top of the purple sample (not shown).

A systematic analysis of *R. rubrum*/*Chlamydomonas reinhardtii* growth under cocultivation conditions was undertaken at three different limiting intensities of growth irradiance, i.e., 30, 90, and 150 μmol photons $m^{-2}\,s^{-1}$ (Fig. 7). These light intensities were below, at about the level of, and slightly above the light compensation point for wild type *Chlamydomonas reinhardtii*, respectively. (The light compensation point is defined as the intensity at which the rate of oxygen evolution by photosynthesis is equal to the rate of oxygen consumption by respiration in *Chlamydomonas reinhardtii*.) At 30 μmol photons $m^{-2}\,s^{-1}$, *Chlamydomonas reinhardtii* and *R. rubrum* grew under anaerobic conditions in coculture, with the *R. rubrum* accumulating biomass at a faster rate relative to that of the *Chlamydomonas reinhardtii* (Fig. 7, 30 μmol photons $m^{-2}\,s^{-1}$).

At 90 μmol photons $m^{-2}\,s^{-1}$, *Chlamydomonas reinhardtii* and *R. rubrum* grew under microaerobic conditions in coculture, again with the *R. rubrum* accumulating biomass at a faster rate relative to that of *Chlamydomonas reinhardtii* (Fig. 7, 90 μmol photons $m^{-2}\,s^{-1}$). At this light intensity, and probably due to the microaerobic conditions prevailing, a substantial fraction of the *R. rubrum* cells (about 20–25%) failed to acquire the chromatophores associated with the development of the photosynthetic apparatus. This fraction of *R. rubrum* appeared as colorless (or yellowish) biomass in the coculture and was as such distinguished in the packed cell volume measurements.

At 150 μmol photons $m^{-2}\,s^{-1}$, *Chlamydomonas reinhardtii* and *R. rubrum* grew under aerobic conditions in the coculture, with the *Chlamydomonas reinhardtii* now accumulating biomass at a faster rate relative to that of *R. rubrum* (Fig. 7, 150 μmol photons $m^{-2}\,s^{-1}$). At this light intensity, the rate of oxygen evolution by photosynthesis is faster than the combined respiratory activities of both organisms, resulting in the presence of

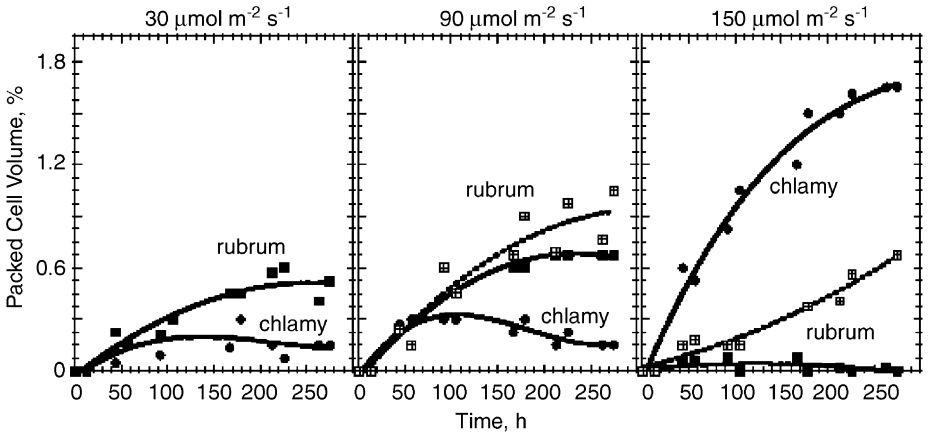

Fig. 7. Packed cell volume of *Rhodospirillum rubrum* (rubrum) and *Chlamydomonas reinhardtii* (chlamy) accumulation as a function of time in coculture under three different growth irradiance intensities. Solid circles: *Chlamydomonas reinhardtii* (chlamy), solid squares: purple *R. rubrum* (rubrum), revealing presence of the photosynthetic apparatus. Open squares: yellowish-white *R. rubrum* (rubrum), indicating aerobic repression and, therefore, absence of the photosynthetic apparatus.

substantial amounts of oxygen in the growth medium. Under these conditions, *Chlamydomonas reinhardtii* biomass accumulated faster than that of *R. rubrum*. Interestingly, and probably due to the aerobic environment in this coculture, nearly 100% of the *R. rubrum* cells failed to acquire the chromatophores associated with the development of the photosynthetic apparatus. In consequence, the entire *R. rubrum* biomass appeared as colorless (or yellowish) in the coculture.

The above-described preliminary experiments revealed that it is possible to cocultivate *Chlamydomonas reinhardtii* and *R. rubrum* in a suitable growth medium. The two cell types coexisted and produced biomass in a facultative process in which *R. rubrum* could potentially benefit from small organic acids exuded by the *Chlamydomonas reinhardtii* cells during such an anaerobic photofermentation.

Any plan to establish a green alga/photosynthetic bacterium (*Chlamydomonas reinhardtii*/*R. rubrum*) integrated system would normally falter due to the great capacity of wild type green algae to produce O_2 photosynthetically under bright sunlight conditions (photosynthesis/respiration $P/R = 4:1$ ratio). Oxygen is a powerful suppressor of both the [Fe]-hydrogenase and nitrogenase gene expression, and an inhibitor of the function of the respective enzymes [69,70]. In this respect, efforts are under way to generate algal photosynthesis/respiration mutants in which the P/R ratio is closer to 1:1 rather than the 4:1 ratio in the wild type. The recent application of antisense technology with the *SulP* gene [71–73] offers a promising avenue of research in lowering the P/R ratio of the cells. Specifically, isolation of green algal strains with a lower photosynthesis/respiration ratio would permit, for the first time, unrestricted cocultivation of a green alga with a photosynthetic bacterium, under anaerobic conditions, for a codepended H_2 production system with superior rates and yields. An integrated green alga/photosynthetic bacterium system is promising in this respect as it offers the prospect of continuity and enhanced yields.

Under optimal conditions of illumination and nutrient supply, and on the basis of current technology, individual *Chlamydomonas reinhardtii* and *R. rubrum* cultures can each produce a maximum of about 50 ml H_2 per liter culture per hour. One advantage of the cocultivation approach is that it alleviates the need for two separate photoreactors for the two organisms (economic considerations), while permitting each to perform efficiently in photosynthesis and H_2 production. This would constitute a photosynthetically efficient and cost-effective H_2 production system in which the duration and yield of the integrated process far exceeds that of the individual components.

3.2. Attenuation of the photosynthesis/respiration (P/R) ratio in green algae

Critical for the efficient cocultivation of green algae and photosynthetic bacteria is the requirement of limiting the process of O_2 evolution by the former. This requirement entails a lowering of the capacity of photosynthesis in green algae to a level that is near to or lower than that of respiration. This objective has been attempted experimentally in the laboratory. Through DNA insertional mutagenesis and screening

in *Chlamydomonas reinhardtii*, a novel chloroplast envelope-localized sulfate permease and the genes that code for it have been identified [71–73]. Evidence at the molecular genetic, protein, and regulatory levels suggested this to be an ABC-type chloroplast envelope-localized sulfate transporter in the model unicellular green alga *Chlamydomonas reinhardtii*. From the four nuclear genes encoding this sulfate permease holocomplex, two are coding for chloroplast envelope-targeted transmembrane proteins (SulP and SulP2), a chloroplast stroma-targeted ATP-binding protein (Sabc), and a substrate (sulfate)-binding protein (Sbp) that is localized on the cytosolic side of the chloroplast envelope. The sulfate permease holocomplex is postulated to consist of a SulP–SulP2 chloroplast envelope transmembrane heterodimer, flanked by the Sabc and the Sbp proteins on the stromal side and the cytosolic side of the inner envelope, respectively. The mature SulP and SulP2 proteins contain seven transmembrane domains and one or two large hydrophilic loops, which are oriented toward the cytosol [74].

As discussed earlier in this paper, sulfate availability to the chloroplast regulates the rate of oxygenic photosynthesis. In principle then, application of antisense technology in *Chlamydomonas reinhardtii* to down-regulate *SulP* expression may lead to the generation of a transformant with a lower capacity of photosynthesis that is equal to or less than that of cellular respiration. Such antisense transformants grow well in the presence of organic carbon (acetate or TAP media). Sealed cultures of such strains become anaerobic in the light, as the capacity for respiration is equal to or greater than the capacity of photosynthesis. In sealed cultures, such strains express the "[Fe]-hydrogenase pathway" and produce H_2 upon illumination, even when sulfate nutrients are abundant in the growth medium [Melis, submitted]. The engineering of a variety of such *Chlamydomonas reinhardtii* strains would permit a continuous H_2 production process in the light, as it would alleviate the need to perform nutrient replacement (S-deprivation) or nutrient titration (S-titration) in order to induce the H_2 production activity of the green algae.

The antisense experimentation with the *SulP* gene successfully provided a genetic approach by which to alter the relationship between photosynthesis and respiration in green algae and thus to probe the function of the [Fe]-hydrogenase pathway. Specifically, a green alga transformant (*antisulP29*) has been isolated in which the P/R ratio (=2 : 1) is substantially lower than that of the wild type (=4 : 1). The *antisulP29* strain showed induction of H_2 photoproduction in the presence of sulfur nutrients [73, Melis, submitted].

3.3. Integrating dark anaerobic fermentative bacteria into photosynthetic H_2 production

A number of dark anaerobic fermentative bacteria can produce H_2 upon fermentation of a variety of organic substrates. For example, *Enterobacter aerogenes* and *Clostridium beijerinckii* can produce hydrogen from glucose and starch [57–60,75]. *Lactobacillus amylovorus*, *Vibrio fluvialis*, and *Clostridium butyricum* are known to convert cellulolytic materials into H_2 [63,66]. During such anaerobic fermentation and H_2 production, small organic acids (glycolate, acetate, lactate, malate etc.) accumulate in the growth medium as inevitable metabolic end-products [76,77]. Accumulation of small organic acids stops H_2 production, as it causes inhibition in the rate of metabolic fermentation and growth by acidifying the medium.

Accumulated biomass from an integrated green alga/photosynthetic bacterium system (*Chlamydomonas reinhardtii*/*R. rubrum*) can be utilized as a fermentation substrate for H_2 production by dark anaerobic fermentative bacteria. A variety of *Clostridium* species can serve in such anaerobic fermentation of cell wall and other metabolites that will be generated from a *Chlamydomonas reinhardtii*/*R. rubrum* system. Soluble and insoluble extracts from the *Chlamydomonas reinhardtii*/*R. rubrum* biomass can be tested separately for yields of H_2 production by these *Clostridium* species. Insoluble biomass can be used directly or upon prior incubation and digestion with appropriate cellulolytic enzymes in vitro. The benefit to be derived from the small organic acids, accumulated as a by-product of the dark anaerobic fermentation, is that they can be recycled (Fig. 4) and utilized as a source of organic carbon, suitable to sustain growth and H_2 production by the integrated green alga/photobacterium system.

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