Introducing pyruvate oxidase into the chloroplast of Chlamydomonas reinhardtii increases oxygen consumption and promotes hydrogen production

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Abstract
In an anaerobic environment, the unicellular green algae Chlamydomonas reinhardtii can produce hydrogen (H₂) using hydrogenase. The activity of hydrogenase is inhibited at the presence of molecular oxygen, forming a major barrier for large scale production of hydrogen in autotrophic organisms. In this study, we engineered a novel pathway to consume oxygen and correspondingly promote hydrogen production in Chlamydomonas reinhardtii. The pyruvate oxidase from Escherichia coli and catalase from Synechococcus elongatus PCC 7942 were cloned and integrated into the chloroplast of Chlamydomonas reinhardtii. These two foreign genes are driven by a HSP70A/RBCS2 promoter, a heat shock inducing promoter. After continuous heat shock treatments, the foreign genes showed high expression levels, while the growth rate of transgenic algal cells was slightly inhibited compared to the wild type. Under low light, transgenic algal cells consumed more oxygen than wild type. This resulted in lower oxygen content in sealed culture conditions, especially under low light condition, and dramatically increased hydrogen production. These results demonstrate that pyruvate oxidase expressed in Chlamydomonas reinhardtii increases oxygen consumption and has potential for improving photosynthetic hydrogen production in Chlamydomonas reinhardtii.

1. Introduction

Hydrogen is an ideal, clean, and potentially sustainable energy source. It is currently produced primarily from fossil fuel (oil, coal, natural gas) [1], but can also be produced by photosynthetic microorganisms [2–5]. Different biological pathways of hydrogen production have been reported in different biological organisms [4]. Generally, biological hydrogen generation can be classified into four categories: (1) biophotolysis of water using algae and cyanobacteria [6–8],
(2) photodecomposition of organic compounds by photosynthetic bacteria [9–11], (3) fermentative hydrogen production from organic compounds [12–14] and (4) hybrid systems using photosynthetic organisms and fermentative bacteria [15]. Though much research has been done on the process of hydrogen production, so far, large scale biological production has not yet been accomplished mainly due to the low production efficiency [3].

One of the primary organisms for studying hydrogen production is Chlamydomonas reinhardtii, a green alga capable of producing hydrogen under stress conditions. A sustained hydrogen production for about 50–100 h has been reported in Chlamydomonas reinhardtii under anaerobic and sulfur deprived conditions [16]. C. reinhardtii uses solar energy to split water (H₂O) into protons (H⁺), electrons (e⁻), and oxygen (O₂) [6]. The [Fe–Fe] hydrogenase [17] then catalyzes the synthesis of hydrogen from H⁺ and e⁻. This reaction is inhibited in the presence of oxygen, which diffuses into the enzyme’s catalytic center and irreversibly binds to it, halting the catalytic capacity [18]. Furthermore, oxygen was found to inhibit the mRNA synthesis of hydrogenase as well [19]. One of the major research focuses on hydrogen production is identifying ways to overcome the inhibition of oxygen on hydrogen production, through either engineering the oxygen sensitivity of the enzyme or decreasing the oxygen levels in the cell [20].

Depletion of sulfur in the medium of C. reinhardtii is shown to affect photosystem II (PSII) and consequently dramatically decreases O₂ production [18], resulting in a significant increase in the hydrogenase activity and an extended H₂ production for over 4–5 days. This sulfur-deprivation process consists of two phases: in the initial phase O₂ evolution gradually declines and there is over-accumulation of carbohydrates in the form of starch, followed by a H₂-production phase driven by residual photosynthetic water oxidation and the anaerobic degradation of starch [18]. The observed decrease in the water oxidation activity of PSII is correlated with a decrease in the turnover of the D1 protein, an essential component of PSII that is inactivated in the light and re-synthesized at very high rates in the dark subsequently [21,22]. At the absence of sulfur, the D1 turnover is impaired due to the lack of sulfurylated residues and PSII activity is gradually lost [23]. Though this method can be used to produce hydrogen, the productivity was much lower than the predicted capacity due to the depletion of electron sources from PSII as a result of the decreased activity [16,24].

Removing sulfur from the medium is a potential useful way to decrease oxygen concentration in the cells, which is required for hydrogen production [18]. There are additional methods to decrease the oxygen concentration inside C. reinhardtii, either in the mitochondria or the chloroplast. In the mitochondria, oxygen is reduced by the complex IV, i.e. cytochrome oxidase, which is at the end of oxidative electron transport chain, or by alternative oxidase (AOX), that bypasses the proton-pumping complexes III and IV [25]. Plastid terminal oxidase (PTOX) is the third way to consume oxygen [26]. Under normal conditions these reactions can not consume all the oxygen generated by photosynthesis, therefore identifying new metabolic engineering options to further decrease the cellular oxygen level and enhance biohydrogen production is a major research focus [27,28].

In this study, we introduced a new way to decrease cellular oxygen levels and increase the hydrogen productivity. This way utilizes pyruvate oxidase (POX), an enzyme that catalyzes the decarboxylation of pyruvate to acetyl-phosphate and CO₂ [29].

\[
\text{Pyruvate} + \text{Orthophosphate} + \text{O}_2 \leftrightarrow \text{Acetyl – phosphate} + \text{H}_2\text{O}_2 + \text{CO}_2
\]

This enzyme has been characterized in E. coli [30–33]. Though previously this enzyme has been considered non-essential in the early stationary phase of E. coli [34], more recent studies suggest that pyruvate oxidase might play an important role in the aerobic growth of E. coli, possibly related to preserving the pool of free CoA [35]. Here we hypothesize that the expression of E. coli POX in the chloroplast of C. reinhardtii can increases the oxygen consumption. Given that H₂O₂, a product of POX, can potentially damage the cell [36], we introduced enzyme catalase (CAT) to reduce H₂O₂.

2. Material and methods

2.1. POX and CAT cloning and vector construction

The full-length fragments of pyruvate oxidase (POX) from Escherichia coli (Genbank accession No. M28208) and catalase (CAT) from Synechococcus elongatus PCC 7942 (Genbank accession No. D61378) were cloned by PCR. Primers are shown in Table 1. The forward primers were inserted with restriction enzyme sites and ribosomal binding sequence (AGGGAGGG) in front of ATG. The coding sequence fragments were confirmed by sequencing. The POX fragment was inserted into a modified pBluescript SK vector containing HSF70A/RBSC2 promoters (heat shock inducible promoter) [37] using restriction endonucleases XhoI and ClaI. Subsequently the CAT fragment was inserted using restriction endonucleases SpeI and NotI. The fragment, containing the HSF70A/RBSC2 promoter, POX and CAT, was inserted into the chloroplast transformation vector ppcg40 [38] using

<table>
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<th>Length of fragments</th>
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<tr>
<td>CAT</td>
<td>446 bp</td>
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<td>atpB</td>
<td>596 bp</td>
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restriction endonucleases SacI and NotI to create pcg40-HPC. The complete construct can be seen in Fig. 1.

2.2. Chloroplast transformation and transformant screening

Particle bombardment transformation was conducted on C. reinhardtii strain cc503 using a helium-driven particle gun (Bio-Rad™ Model PDS1000/He Biolistic particle delivery system) as described by Purton [39]. Before transformation, 10 μg of plasmid pcg40-HPC was linearized by the restriction enzyme EcoRI. After transformation, the algal cells were grown on normal TAP (Tris-Acetate-Phosphate) plates in the dark for 18 h at 25 °C, and washed with fresh TAP liquid medium once. Afterwards, these cells were cultured on TAP plates containing 100 μg/ml spectinomycin under continuous illumination at 25 °C for 1–2 weeks to screen transformants. After selection, transformants were subcultured on TAP plates containing 100 μg/ml spectinomycin for at least six times to reach homoplasmicity. The transgenic strain was named ccHPC.

2.3. PCR analysis

DNA extraction from C. reinhardtii was performed according to Ramesh [40]. Total RNA was extracted from 1.5 ml of C. reinhardtii culture using EZ Spin Column total RNA Purification Kit (Shanghai Sangon Ltd., China) and was subsequently treated with RNase-free DNase I (Fermentas Canada Inc.) for the removal of DNA from the total RNA. The first strand cDNAs were synthesized by RevertAid™ First Strand cDNA Synthesis Kit (Fermentas Canada Inc.). PCR was carried out using the primers listed in Table 1. The PCR program was: 30 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C for 30 cycles.

2.4. Algal culture and growth measurement

C. reinhardtii strain cc503 and transgenic strain ccHPC were grown phototrophically on TAP agar plates or in TAP liquid medium under continuous illumination (about 75 μmol photons m⁻² s⁻¹) at 25°C. To induce expression of POX and CAT, heat shock treatments were performed at 40 °C for 1 h every 6 h. Cell density was checked by measuring absorbance at 750 nm.

2.5. Measurement of photosynthetic oxygen evolution and respiration rates

Photosynthetic oxygen evolution and respiration rates were measured at 25 °C with a Clark-type oxygen electrode (Hansatech, UK). Specifically, 2 ml algal cells were taken after growing in the dark for 24 h and heat-shocked once every 6 h. The cells were placed in an O₂-electrode chamber and equilibrated with NaHCO₃ which has a final concentration of 10 mM.

2.6. Measurement of oxygen evolution and hydrogen production

30 ml algal cells in the middle of the exponential growth phase were transferred to cylindrical glass bottles. The glass bottles were sealed with a rubber gas-tight septum and incubated in the dark for 24 h to create an anaerobic liquid medium. The volume of the top air space of the bottle is 30 ml. From the second day, the sealed bottles were placed under continuous illumination of 30 μmol photons m⁻² s⁻¹ (LL, low light) or 100 μmol photons m⁻² s⁻¹ (HL, high light). Evolved gas was collected from the top air space of the bottle using a gas-tight lockable syringe and injected into a gas chromatograph (Agilent™ 7890) with a thermal conductivity detector to measure gas composition.

2.7. Statistical analysis

Two-way ANOVA was used to analyze the effects of time and strain type on the growth rates, oxygen evolution and hydrogen production rates.

3. Results

3.1. Identification of transformant and foreign gene expression

Fig. 2A shows the timescale of the heat shock treatments and moments of algal sample taking. We took samples at three time points corresponding to the end of a dark growth period, after one-hour heat shock treatment and at the end of 6 h light treatment. No fragments of either POX or CAT were detected in the wild-type cc503 (Fig. 2B, lane 1). However specific POX and CAT fragments were found in the chloroplastic DNA of the transgenic algae ccHPC (Fig. 2B, lane 2). RT-PCR products were amplified from Dnase-treated RNA using specific primers for POX and CAT to ensure no DNA contamination. The mRNA expressions of foreign enzymes were kept at high levels after different heat shock treatments (Fig. 2B). The selected house-keeping gene atpB gene showed consistent high expression levels (Fig. 2B). These results indicated that the POX and CAT genes were integrated into chloroplastic DNA of the transgenic algae and transcribed with high efficiency after continuous heat shock treatments.

3.2. Growth rate of the transgenic algae

We examined the growth rate to analyze whether the expression of two foreign genes affected the growth of algal cells. The

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**Fig. 1** — A Diagram of the pcg40-HPC construct used in the chloroplast transformation of **Chlamydomonas reinhardtii**.

**cpDNA**: fragment of chloroplast DNA; **aadA**: Aminoglycoside 3'-Adenytransferase, a spectinomycin-resistance gene; **HSP70A/RBCS2**: heat-shock promoter; **ecoPOX**: pyruvate oxidase from *E. coli*; **7942CAT**: catalase from *Synechococcus elongatus* PCC 7942.
absorbance at 750 nm was used to estimate cell density, the change of which was correlated with growth rate. The growth rate of transgenic algae ccHPC was slightly slower compared to wild type cc503 (Fig. 3). No statistically significant difference in chlorophyll content was identified between transgenic strain ccHPC and wild type strain cc503 (data not shown).

3.3. Reduced oxygen evolution of the transgenic algae

To examine whether the POX expression in the Chlamydomonas chloroplast affects oxygen concentration inside transgenic algae cells, oxygen evolution rates of algal cells were measured by a Clark-type oxygen electrode. The results showed that oxygen evolution rates of transgenic algae were significantly decreased at different light levels (Fig. 4). At 30 μmol photons m^{-2} s^{-1} illumination, the O2 consumption rate of ccHPC was about 25 μmol O2 mg\textsuperscript{-1} Chl h\textsuperscript{-1}, while the wild type had a rate of about 11 μmol O2 mg\textsuperscript{-1} Chl h\textsuperscript{-1}. At 100 μmol photons m^{-2} s^{-1} illumination, the difference in O2 evolution rates is much smaller than that at 30 μmol photons m^{-2} s^{-1} illumination.

3.4. Hydrogen production in the transgenic algae

After algae cells were incubated in the dark for 24 h, the content of dissolved oxygen in the algal cultures gradually decreased. The oxygen content of the top air space of transgenic algal cultures was lower than that of wild type algal cultures, both under low light (30 μmol photons m^{-2} s^{-1}, Fig. 5A) and high light (100 μmol photons m^{-2} s^{-1}, Fig. 5A). The hydrogen production of transgenic algae was higher than that of wild type algae under low light (30 μmol photons m^{-2} s^{-1}, Fig. 5C). After 48 h, the hydrogen production in the sealed culture of the transgenic algae was about three times higher than that of the wild type. Hydrogen production had a low efficiency under high light (100 μmol photons m^{-2} s^{-1}, Fig. 5D), either in transgenic algae or in the wild type. No statistical difference was detected between the hydrogen production rate under high light between the wild type and the transgenic algae cells.

4. Discussion

The hydrogenase of C. reinhardtii is irreversibly inactivated in the presence of oxygen [17]. In an anaerobic environment the photosynthetic hydrogen production in C. reinhardtii is
Sulfur deprivation inhibits the photosynthetic oxygen evolution and also slightly decreases respiration, and therefore can assist in the formation of an anaerobic state [18]. However, inhibiting photosynthesis also decreased production of the protons and electrons required for hydrogen production. Furthermore, sulfur deprivation also decreases the lifetime of algae [16,24]. To obtain a sustainable hydrogen photoproduction in C. reinhardtii, many researchers have been focusing on creating an oxygen-tolerant hydrogenase [20]. In this study we designed a novel method to decrease the oxygen concentration inside the cell. Specifically, we introduced a fused pyruvate oxidase and catalase gene cassette into the chloroplast of C. reinhardtii. Under low light conditions, transgenic algal cells evolved less oxygen than wild type C. reinhardtii cc503 (Fig. 4) and correspondingly the hydrogen production rate dramatically increased (Fig. 5).

In this study, the foreign genes in transgenic algae were driven by a heat shock inducible promoter. Under normal temperature (25 °C), the foreign genes will not be expressed. After continuous heat shock treatments, POX and CAT were expressed at high levels (Fig. 2). We also analyzed the pyruvate concentration, and did not find a significant difference between transgenic and wild type C. reinhardtii cc503 (Fig. 4) and correspondingly the hydrogen production rate dramatically increased (Fig. 5).

In this study, the foreign genes in transgenic algae were driven by a heat shock inducible promoter. Under normal temperature (25 °C), the foreign genes will not be expressed. After continuous heat shock treatments, POX and CAT were expressed at high levels (Fig. 2). We also analyzed the pyruvate concentration, and did not find a significant difference between transgenic and wild type C. reinhardtii cc503 and ccHPC is 0.167 ± 0.026 fmol/cell and 0.167 ± 0.010 fmol/cell, respectively. This might be because there are many reactions that pyruvate participates in and its concentration might be well regulated. For example, in C. reinhardtii, pyruvate can be oxidized by pyruvate formatelyase (PFL) and pyruvate-ferredoxin oxidoreductase (PFR), and also be generated by pyruvate kinase (PYK) and malic enzyme (ME) [44]. Furthermore it is also oxidized to acetyl-CoA and CO₂ by the pyruvate dehydrogenase complex (PDC), a cluster of enzymes located in the mitochondria of eukaryotic cells and in the cytosol of prokaryotes. While PDC and PFL are regarded as major enzymes catalyzing pyruvate oxidation to acetyl-CoA under aerobic and anaerobic conditions in E. coli [45,46], pyruvate oxidase is usually regarded as a non-essential enzyme [30]. In our study, the transgenic algae which expressed foreign POX have low oxygen evolution (Fig. 4), but did not show a significant inhibition in growth rate (Fig. 3). This shows that POX in C. reinhardtii improves hydrogen production without decreasing growth. On this aspect, it is also worth noting that previous research demonstrated that PoxB (POX in E. coli) is used preferentially at low growth rates [2].

In this study, though the oxygen evolution rate was increased by expressing POX in C. reinhardtii at all light levels, we did not obtain a statistically significant enhanced rate of hydrogen production under high light (100 μmol photons m⁻² s⁻¹, Fig. 5D). This might be because under low light levels, oxygen evolution rate is low; therefore enhancing oxygen consumption rate can greatly influence the oxygen concentration in the algae cell culture. Under high light levels, the rate of oxygen generation is high; therefore much greater enhancement of oxygen consumption rate is required to generate the same degree of decrease in the oxygen content in the algae cell culture as under low light.

Though this new approach holds potential to enhance the hydrogen production, the rate of hydrogen production...
Comparison of the maximal hydrogen production rates using different methods with different algae strains.

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| Expression of lehemogloabin Gene [48] | cc849 | 1.2 | 3 |
|--------------------------------------|------|-----|
| light level (µmol photons m⁻² s⁻¹) | 100 | 100 |
| cell density (mg chl/L)    | 7.5 | 7.5 |
| cell density (cells/L)     | 4 × 10⁻⁶ | 4 × 10⁻⁶ |
| Sulfur concentration (µM)  | 100 | 0 |

achieved is still much lower compared to that obtained in -S treatment (Table 2). This might be partially due to different chlamydomonas strains and different culture medium used. However, compared to the wild-type, the transgenic strain increased the hydrogen production rate by about 2 times, suggesting that this new methodology still holds potential as another additional approach to increase hydrogen production. There are a few strategies to further improve the hydrogen production efficiency of the transgenic POX strain. The first approach is to use a constitutive promoter to drive POX expression. While the heat shock inducing promoter can induce foreign enzymes to express at a high level 2 h after heat shock and keep the activity high for 6 h [37], continuous heat shock causes damage to the cell. A constitutive promoter would drive foreign genes to express at high level without adding external stress. The second approach is to express a codon-optimized fragment of POX. A proof of concept of using such an optimized codon to improve enzyme expression has been shown in a study, where dramatically increased GFP expression level, i.e. with 80-fold increase in C. reinhardtii, has been achieved by optimizing its codon bias [47]. Both approaches can potentially increase the hydrogen production. Thirdly, new approaches to combine the advantage of both the current transgenic approach with previous engineering or cultivating methods might be an area to concentrate future research on, given that these different approaches have different advantage and disadvantages.

5. Conclusion

In this work, we designed a novel approach to decrease the oxygen levels in C. reinhardtii and increase hydrogen production without decreasing any essential ingredients of the culture medium. This approach holds potential to become a new approach towards sustainable large scale production of hydrogen using C. reinhardtii. The advantage of this engineering approach is that the modified cells have a higher oxygen consumption rate without disrupting the photosynthetic rate. Results from this work suggest that this new approach can decrease the cellular oxygen concentration and correspondingly increase the hydrogen production. Much more work is still needed to further optimize this approach to enhance the hydrogen production efficiency.

Acknowledgment

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References


Table 2 – Comparison of the maximal hydrogen production rates using different methods with different algae strains.

Maximal hydrogen production (ml hydrogen/L culture)


