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Biohydrogen production from engineered microalgae *Chlamydomonas reinhardtii*

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Abstract. The green microalgae *Chlamydomonas reinhardtti* is well-known specie in the terms of H_2 production by photo fermentation and has been studying for a long time. Although the H_2 production yield is promising; there are some bottlenecks to enhance the yield and efficiency to focus on a well-designed, sustainable production and also scaling up for further studies. D1 protein of photosystem II (PSII) plays an important role in photosystem damage repair and related to H_2 production. Because *Chlamydomonas* is the model algae and the genetic basis is well-studied; metabolic engineering tools are intended to use for enhanced production. Mutations are focused on D1 protein which aims long-lasting hydrogen production by blocking the PSII repair system thus O_2 sensitive hydrogenases catalysis hydrogen production for a longer period of time under anaerobic and sulfur deprived conditions. *Chlamydomonas* CC124 as control strain and D1 mutant strains (D240, D239-40 and D240-41) are cultured photomixotrophically at 80 μmol photons m^{-2} s^{-1}, by two sides. Cells are grown in TAP medium as aerobic stage for culture growth; in logarithmic phase cells are transferred from aerobic to an anaerobic and sulfur deprived TAP – S medium and 12 mg/L initial chlorophyll content for H_2 production which is monitored by the water columns and later detected by Gas Chromatography. Total produced hydrogen was 82 ±10, 180 ±20, 196 ±20, 290 ±30 mL for CC124, D240, D239-40, D240-41, respectively. H_2 production rates for mutant strains was 1.3 ±0.5 mL/L.h meanwhile CC124 showed 2-3 fold lower rate as 0.57 ±0.2 mL/L.h. Hydrogen production period was 5 ±2 days for CC124 and mutants showed a longer production time for 9 ±2 days. It is seen from the results that H_2 productions for mutant strains have a significant effect in terms of productivity, yield and production time.

Keywords: microalgae; biohydrogen; chlamydomonas reinhardtii

1. Introduction

It is a well-known situation that finding new sources for fuel consumption is an emerging area and researchers are trying to optimize new production strategies for future's world in the terms of energy related fields. The supply of energy in today's world is mostly driven from fossil fuels which also cause problems like environmental pollution, ecological imbalance and global warming (Oncel and Vardar-Sukan 2009 and Oncel 2013). The regulations and new legislations try to encourage the investments about alternative sources of energy for a cleaner world one of which is called bioenergy (Hallenbeck and Benemann 2002). Bioenergy is the new concept based on biological sources for renewable, sustainable and environmental friendly energy production

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(Laurinavichene *et al*. 2006).

Biohydrogen, a promising kind of an alternative source, is referred as an energy carrier gas and has more gravimetric energy potential than conventional fossil fuels (Oncel and Sabankay 2012). Besides; the combustion products are water and oxygen which has no harm in the environmental aspect (Das and Veziroglu 2008).

Biohydrogen can be produced via direct biophotolysis, indirect biophotolysis, photo fermentation and dark fermentation (Levin *et al*. 2004). Among these; direct biophotolysis of hydrogen by microalgae is gaining interest more than 60 years (Gaffron and Rubin 1942). Microalgae are unicellular photosynthetic microorganisms which converts inorganic carbon compounds to organic substances (Ma *et al*. 2010). The microalgal species like *Anabena sp.*, *Chlorella vulgaris*, *Chlorella pyrenoidosa* and *Chlamydomonas reinhartti* are hydrogen producing species (Oncel and Sukan 2011); but *C. reinhartti* catches more interest due to high amount of hydrogen production capacity (Scoma *et al*. 2012 and Gianelli and Torzillo 2012) and short culture growth period. *C. reinhardtti* is also used as a model organism of eukaryotic microalgae studies and its genetic basis is well defined and metabolism is also highlighted for further studies (Specht *et al*. 2010).

The photosynthesis in microalgae is similar to the plants; inorganic carbon compounds and light energy are converted into organic compounds. Photosynthesis has two main stages called light reactions and dark reactions. At the first stage; the ATP is generated to be used for dark reactions. At the second stage; final organic compounds are produced. Photosystem II (PSII) and Photosystem I (PSI) are the protein complexes which are embedded in the thylakoid membranes of chloroplast and they are responsible from photosynthesis (Melis *et al*. 2000).

On the other hand microalgal biohydrogen production is a series of redox reactions in the thylakoid membranes which starts with water splitting and electron transfer from PSII and finalize with the hydrogen gas production. Light driven activation of Photosystem II led water to release electrons to protein ferrodoxin in Photosystem I (Melis and Happe 2001) which requires illuminating culturing conditions. [Fe]-hydrogenase enzyme is activated and electrons are transferred to H^+ ions (Kosourov *et al*. 2002 and Hoshino *et al*. 2012) and hydrogen ions are reduced to H_2 gas by a reversible mechanism in hydrogenase enzyme (Scoma *et al*. 2012) under anaerobic conditions. The key point for anaerobic culturing for biohydrogen production is because of high sensitivity of [Fe]-hydrogenase enzyme to trace amount of O_2 existance (Ghirardi *et al*. 2010).

Even though *C. reinhardtti* is a good candidate is developed by the two stage culture strategy, based on sulfur deprivation, defined by Melis *et al*. (2000). Sulfur is one of the building blocks of amino acids in the PSII reaction center protein D1 (Kosourov *et al*. 2005). D1 protein repairs photo damage in PSII and has role in the generation of oxygen (Edelman *et al*. 1984) which inhibits hydrogenase enzymes. When sulfur is deprived in culture media; D1 protein synthesis thus PSII activation decreases below photosynthesis limits resulting in the suppression of oxygen generation (Zhang *et al*. 2002).

According to two stage culture strategy; microalgae first grow under aerobic culture later transferred into anaerobic sulfur deprived conditions where enzymatic reactions took place for hydrogen production (Melis 2002 and Tsygankov *et al*. 2006). The cells use the oxygen left in the sealed production chamber and after a lag phase of 24-48 hours start to produce hydrogen which peaks at the following 2-3 days and then decrease. This is because the oxygen driven from splitting of water molecules accumulates in the chamber, inhibits the activity of hydrogenases and results as decrease in the hydrogen production (Grihardi *et al*. 2000 and Melis *et al*. 2000).

The main problem of the hydrogen production from green algae is not the capacity but the sustainability. In the assistance of enhanced recombinant gene technology tools, the mutant strains can be obtained for higher volumetric hydrogen production. Photosytem II has D1-D2 heterodimer protein complexes in reaction center (Giardi *et al*. 2013). It is known that this proteins have active gene clusters which is responsible from electron transport and quinone binding to PSII and results in oxygen evaluation. Responsible genes for biohydrogen production, called as hydrogenases, are even sensitive to trace amount of oxygen existence. In that case; besides environmental condition altering such as using sulfur deprived anaerobic cultures another production strategy developed for sustainable biohydrogen evaluations. Deletion mutations are directed to the amino acid residues 234 and 242 effects functional properties of PSII (Kettunen *et al*. 1996). In that manner, the mutant strains has amino acid deletions between residues of 234 and 242. D240 strain has one deletion mutation of amino acid residue 240 meanwhile D23940 and D24041 has the double deletions at the amino acid residues of 239 and 240; 240 and 241; respectively (Faraloni and Torzillo 2010). The genetic basis of the process has evaluated and by genetic manipulation techniques on D1 protein shows long term H_2 production by mutant microalgae species (Scoma *et al*. 2012).

The aim of this study is to evaluate the hydrogen production of 3 mutant strains (D240, D23940, and D24041) of *C. reinhardtti* and CC124 as control 80 + 80 μmol photons m^{-2} s^{-1} by two sides.

2. Materials & methods

All the strains were grown photomixotrophically in 500 ml capacity bubble columns (5 cm internal diameter) in Tris-Acetate-Phosphate (TAP) medium at about pH 7.2 and 27 ± 0,5 ℃. The cultures were continuously illuminated with cool white fluorescent light (~ 40 μE m^{-2} s^{-1}) and sparged with sterile air-CO_2 gas mixture (volume fraction was ~2%). *Chlamydomonas reinhardtii* cells are grown aerobically 1 L Roux type flat glass photobioreactor (PBR) under constant illumination of 80 + 80 μmol photons m^{-2} s^{-1} by two sides and the initial chlorophyll content for culture growth was 4 ± 1 mg/L.

Cells are then transferred to anaerobic phase after chlorophyll concentration is reached to 26 ± 4 mgL^{-1}. Cells in TAP medium, were harvested by centrifugation (3500 g for 3 min),washed 5 times with sulfur deprived TAP medium (TAP-S) and then, resuspended in the TAP-S medium and adjusted to a final concentration of about 12 mg Chlorophyll L^{-1}. TAP-S suspended cells were placed into the same type of flat PBRs and sealed and connected to calibrated water columns to monitor the volumetric hydrogen production (Oncel and Vardar-Sukan 2009, Oncel and Sukan 2011). The hydrogen production experiments were carried out under the same illumination conditions. pH, cell number, dry weight and chlorophyll content and starch are measured at both aerobic and anaerobic phases. The hydrogen gas is measured at calibrated water columns and analysed at Gas Chromatography (GC) (Oncel and Sabankay 2012).

3. Results and discussion

Microalgae have gained attention in terms of biohydrogen production (Giannelli and Torzillo 2012). Among these, *Chlamydomonas reinhardtti* is the model organism because of high biohydrogen production capacity. The problem about producing biohydrogen from Chlamydomonas reinhardtii is not the capacity but the sustainability because the hydrogenase enzymes are sensitive

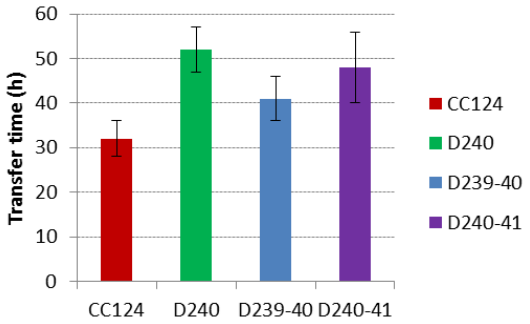

Fig 1 Transfer time regards to chlorophyll (a + b) amount of 26 ±4 mg L^{-1} for hydrogen production phase

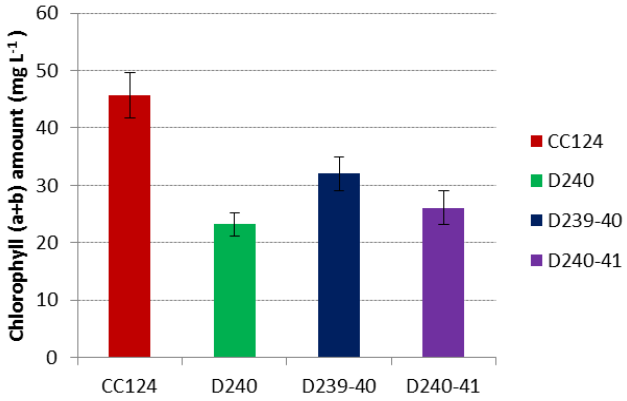

Fig 2 Chlorophyll (a + b) amount reached in aerobic phase by each culture at the end of 72 hours

the trace amount of O_2.

Chlamydomonas cells are cultivated under continuous illumination of 80 μmol photons m^{-2} s^{-1} which is the average light supply for microalgae cultivation with initial chlorophyll concentration of 4 ±1 mg/L.

The initial chlorophyll concentration is an important parameter for cell growth because light cultures are affected negatively and results in longer lag phase (Oncel and Sabankay 2012). In this study; the initial chlorophyll content was 4 ±1 mgL^{-1}. As it is depicted in the Fig. 1, CC124 control strain has a shorter transfer time, 32 ±4 hours meanwhile mutant strains D240, D23940, D24041 has longer 52 ±5, 41 ±5, 48 ±8 hours; respectively. The reason behind this is the D1 protein mutations affect the photosystem mechanism and the photosynthesis rate is slower than the control strains. When the *Chlamydomonas* cells are cultivated under aerobic conditions for 72 hours; CC124 strain has almost 2-fold chlorophyll amount around 45 ±4 mg/L meanwhile mutant strains D240, D23940, D24041 have 23 ±2, 32 ±3, 26 ±3, respectively. This results support the mutation mechanism in the cells in terms of decreasing the photosynthetic activity and oxygen evaluation (Melis and Happe 2001).

The importance of the chlorophyll amount during aerobic growth phase is related to the aging of the cells reproducing in the culture (Fig. 2). It is suggested to transfer cells to hydrogen

production phase at mid-logarithmic growth phase which has chlorophyll amount around 26 ± 4 mg/L (Oncel and Vardar-Sukan 2011).

Regarding to cell numbers and chlorophyll amounts of all the strains in Table 1; the cell number and chlorophyll amount does not have a linear correlation. D240, D23940, D24041 strains have mutation in D1 protein which is responsible for photosynthesis. In that case, when mutations are directed to D1 protein to lower photosynthetic activity the chlorophyll amount and cell numbers do not have linear correlations among each other.

The anaerobic phase is the critical state of biohydrogen production where hydrogenase enzymes are activated and H^+ ions, final electron acceptor in the direct biophotolysis, are converted into H_2 gas form by [Fe]-Hydrogenases (Happe *et al*. 2002). The key production strategy for hydrogen generation is anaerobic cultures and sulphur deprived culture media developed by Melis *et al*. in 2000. Sulfur is one of the elements in the D1 protein amino acids like methionine and cysteine (Melis 2002 and Tsygankov *et al*. 2006). These amino acids are synthased by the sulphate uptake and assimilation. D1 protein in PSII repairs photosystem damage and enhance oxygen evaluation (Forestier *et al*. 2003).

Anaerobic phase starts after harvesting and washing cells with TAP-S medium and sealing the 1 L roux PBR. The initial chlorophyll amount for anaerobic phase is adjusted around 12 mg/L to prevent stress conditions originating from illumination and anaerobiosis. The culture starts to use the residues of oxygen. When the oxygen is consumed a rapid decrease occurs in the fluorescent measurement of PSII activity is the sign of deactivation (Antal *et al*. 2003, Torzillo *et al*. 2009 and Oncel and Subankay 2012).

The lag phase in the cultures shows the duration between starting of the anaerobic phase and initial hydrogen production. The results show that D23940 has a shorter lag phase but in general they almost have the same duration to consume residual oxygen. Cultures for hydrogen production are experimented for 10 days which refers to 240 hours. After 48 hours for CC124 the hydrogen

Table 1 Aerobic phase

Strain	Cell number ($\times 10^5$ mL^{-1})	Dry weight ($mg\ L^{-1}$)	Starch ($mg\ L^{-1}$)	Chlorophyll (a + b) ($mg\ L^{-1}$)	Total carotenoids ($mg\ L^{-1}$)
CC124	2,5	175	8,92	3,47	0,87
D240	2,6	250	13,85	4,14	1,10
D23940	8,5	825	18,05	3,64	1,46
D24041	4,4	625	12,69	3	0,84

Table 2 Hydrogen production stage

Strain	Lag phase (h)	Production time (h)	Total production ($mL\ L^{-1}$)	Time for maximum production (h)
CC124	10 ± 2	144 ± 36	82 ± 10	24
D240	10 ± 2	190 ± 24	180 ± 20	72
D23940	8 ± 1	216 ± 40	196 ± 20	72
D24041	12 ± 3	216 ± 24	290 ± 30	72

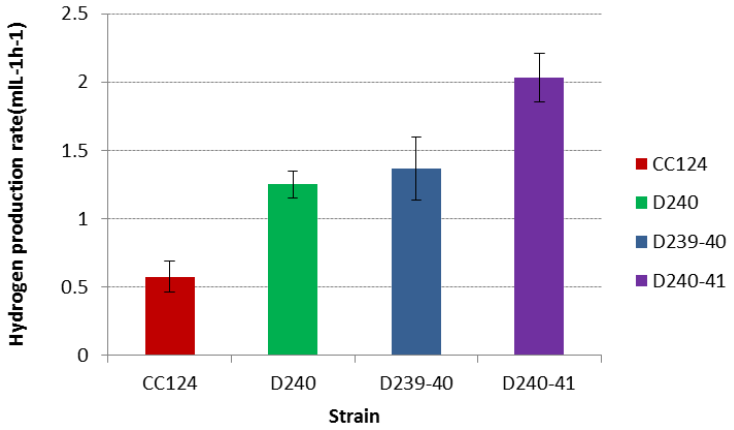

Fig. 3 Hydrogen production rate

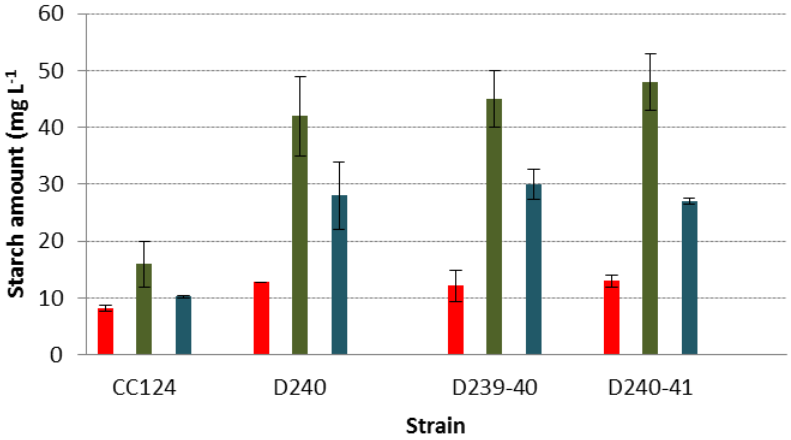

Fig. 4 Change in the amount of starch during hydrogen production phase regards to experiment start x, hydrogen start x and experiment end x

production is decreased and stopped at 144 h. But the mutant strains had longer production duration accompanied with higher volumes of hydrogen, 2-4 folds compared to control strain CC124. The other positive effect of mutation is observed from maximum hydrogen production time which is 24 hours for CC124 and 72 hours for mutant strains. This data show that, the mutations directed to the D1 protein and hydrogenase enzyme has a research worthy issue both in terms of sustainable and higher volumes of hydrogen gas production which is also shown in Table 2. Among this strain, D24041 has the highest volume followed by D23940 and 240 strains.

The hydrogen production rates of mutant strains are also higher than control strain CC124. D24041 has almost $2mlL^{-1}h^{-1}$ of production which is 2 fold for other mutants and 4 fold of CC124 presented in Fig. 3.

Starch deposits are the main energy sources for cells under anaerobic conditions. The photosynthesis mechanism is blocked and cells started to use starch deposits as energy supply and to be able to survive (Kima *et al*. 2006). At the initial stage of anaerobic phase, cells use the

oxygen left in the sealed chamber. Cultures were approximately 12 mg/L chlorophyll content and the initial starch amount of the cells is similar. When H_2 production starts, the starch amount has a peak and the levels of starch decrease at the end of production (Faraloni and Torzillo 2010). The anaerobic environment is a stress for the cells which results in the decrease of the cell number, some of the cells deteriorate. After fully anaerobic conditions, cells use the starch and the starch amount is correlated with the hydrogen production. We can assume the starch amount as the potential liability of the cells and thus D24041 strain showed a higher amount of starch when compared to other strains shown in Fig 4. The control strain has 3-4 fold lesser content of starch at the initial H_2 production.

4. Conclusions

In conclusion, the mutations directed for sustainable biohydrogen production has a positive effect as it is shown in the present study. CC124 is the traditional strain and model for hydrogen producing *C. reinhardtti*. Even the two step sulphur deprived production strategy is well defined and used more than 10 years, the lack of sustainable production makes it necessary to find alternative production strategies.

The biohydrogen derived from microalgae is referred as future's reliable source of energy, but still there is not enough study to optimize production conditions and scale up for commercializing (Scoma *et al*. 2012, Oncel and Sabankay 2012, and Oncel and Kose 2014). The recombinant technology has promising tools for higher hydrogen production because the metabolism and pathways are well described.

This study shows the effects of mutant strains in terms of hydrogen production capacity compared with CC124 control which is also shown in previous studies (Torzillo *et al*. 2009 and Faraloni and Torzillo 2010). It is observed that the PSII activity, starch accumulation, hydrogen production volume and duration is enhanced by the deletion of aminoacid residues at 240, 239 and 240, 240 and 241 for mutant strains D240, D23940 and D24041; respectively. All the mutant strains have total biohydrogen production volume however the strain D24041 has the best results of 2-3 folds for hydrogen production volume and almost 2 folds of production period compared with others. To be able to develop an optimized process for biohydrogen; D24041 strain is a suitable candidate to determine further studies and process design for sustained biohydrogen production from microalgae.

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