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Biohydrogen production from engineered microalgae *Chlamydomonas reinhardtti*

*Ayse KOSE[1)], Suphi S. ONCEL[2)]

[1), 2)] *Ege University Department of Bioengineering*, *Izmir*, *Turkey*
[1)] aykoseay@gmail.com

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.

1. INTRODUCTION

There is an undeniable reality about human dependence on fossil fuel sources (Parmar 2011). Parallel to the increase in the world's population, the requirement and consumption of fuels are also increased dramatically. 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

[1)] MSc Student
[2)] Assistant Professor, Dr.

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). 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 which is based on biological sources for renewable, sustainable and environmental friendly energy production (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). This feature makes it a research-worthy topic. 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 techniques; 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. 2011). The microalgal species like *Anabena sp*., *Chlorella vulgaris*, *Chlorella pyrenoidosa* and *Chlamydomonas reinhartti* has the hydrogen production potential (Oncel and Sukan 2011); but *C*. *reinhartti* catches more interest due to high amount of hydrogen production capacity (Scoma *et al*. 2012, Gianelli and Torzillo 2012) and short culture growth period. *C*. *reinhardtti* is 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 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. Illuminating and anaerobic conditions are required for hydrogen production. Light driven activation of Photosystem II led water to release electrons to protein ferrodoxin in Photosystem I (Melis and Happe 2001); thus [Fe]-hydrogenase enzyme is activated and electrons are transferred to H^+ ions (Kosourov *et al*. 2002, Hoshino *et al*. 2012) and hydrogen ions are reduced to H_2 gas by a reversible mechanism in hydrogenase enzyme (Scoma *et al*. 2012). The reason for anaerobic culture condition is the high sensitivity of [Fe]-hydrogenase enzyme to trace amount of O_2 existance (Ghirardi *et al*. 1994).

Even though *C*. *reinhardtti* is a good candidate having a potential for high amount of hydrogen production, the process for sustainable hydrogen and volumetric yield is justified by the two stage culture strategy, based on sulfur deprivation, defined by Melis 2000 et al. 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).

Under the light of this key information, microalgae first grow under standard aerobic culture later transferred into anaerobic sulfur deprived conditions where enzymatic reactions took place for hydrogen production (Melis 2002, Tsygankov *et al*. 2006). The cells use the oxygen left, if any, 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, Melis *et al*. 2000).

The main problem of the hydrogen production from green algae is not the capacity but the sustainability. In the aid of enhanced recombinant gene technology tools, the mutant strains can be obtained for higher volumetric hydrogen production. Hydrogenase enzymes have active gene clusters called HydA1 and HydA2 which catalyze the hydrogen production in microalgae. Homologous mutations are directed to HydA1 which aim more tolerant enzymes to the oxygen, longer production period and volume. 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 difference of 3 mutant strains (D240, D23940, D24041) of *C. reinhardtti* and CC124 as control at 80 *μ*mol photons m^{-2} s^{-1}, by two sides.

All the *Chlamydomonas reinhardtii* 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°C. The cultures were continuously illuminated with cool white fluorescent light (~ 40 *μ*E m^{-2} s^{-1}) and sparged with sterile air-CO_2 gas mixture (volume fraction was ~2%). Algal cells in the bubble columns were grown to logarithmic phase and transferred to 1 L Roux type flat glass photobioreactor (PBR) under continuous illumination of 80 + 80 *μ*mol photons m^{-2} s^{-1}, by two sides and the initial chlorophyll content for culture growth was 4 ± 1 mg/L.

After the aerobic cultivation process final chlorophyll concentration of 26 ± 4 mgL^{-1} observed was an indication for the transfer into the TAP-S medium for the hydrogen production. 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, 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).

2. CONCLUSIONS

Microalgae have gained attention in terms of biohydrogen production (Giannelli and Torzillo 2012). Among these, *C. reinhardtti* is the model organism with high hydrogen production. But because the hydrogen production is inhibited in the existence of oxygen, anaerobic and sulfur deprived cultures are used (Melis *et al*. 2000). The problem about the sustainability of hydrogen production is tried to be realized with mutant strains.

80 *µ*mol photons m^{-2} s^{-1} light supply is the average amount for microalgae cultivation which does not inhibit the early stage cultures and shading problem by dense cultures are also avoided (Laurinavichene *et al*. 2004). *Chlamydomonas* cells are cultivated under continuous illumination 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 because photon accumulation and the lag time of the culture growth will be longer.

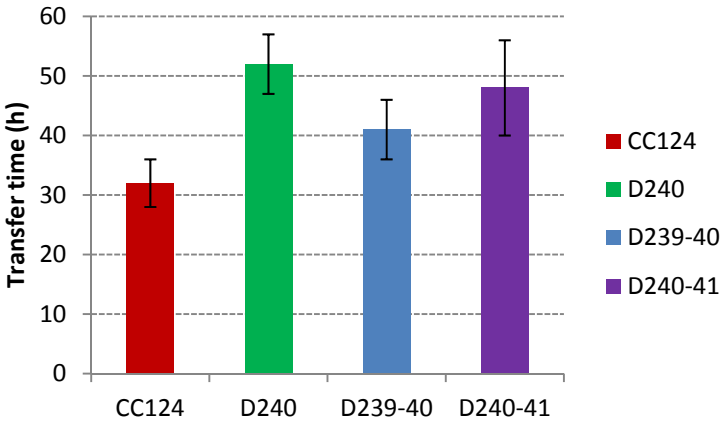

Fig. 1 Transfer time regards to chlorophyll (a + b) amount of 26 ± 4 mg L^{-1} for hydrogen production phase

The same initial chlorophyll amount avoids the differences originating from starting conditions of the culture. At the study done by Oncel and Sabankay (2012) more chlorophyll containing culture reached targeted chlorophyll amount for transfer to anaerobic phase around 2 days. In this study the initial chlorophyll content was in the range of 3-4 mg/L. 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 lowering the photosynthetic activity and oxygen evaluation (Melis and Happe 2001).

The importance of the chlorophyll amount during aerobic growth phase is related to the ages of the cells reproducing in the culture (Fig. 2). It is suggested to transfer cells for hydrogen production phase where the cells are reproducing actively after mid-logarithmic growth phase which has chlorophyll amount around 26 ± 4 mg/L (Oncel and Vardar 2011). The possible methods to obtain the culture age for transfer is to measure either the cell number or chlorophyll amount which chlorophyll amount is preferred in this study.

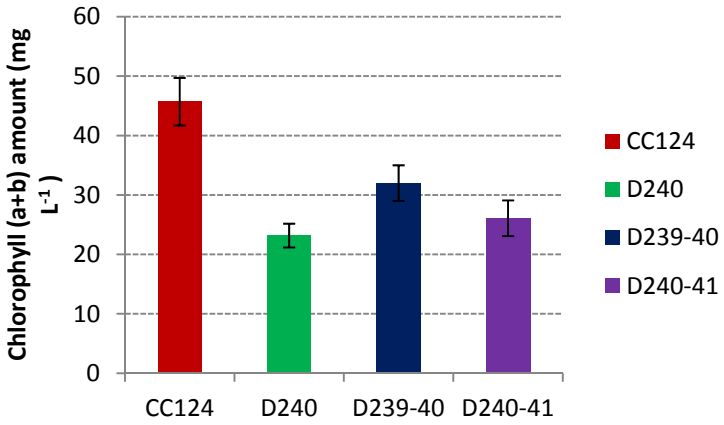

Fig. 2 Chlorophyll (a + b) amount reached in aerobic phase by each culture at the end of 72 hours

As it is shown in Table 1 the cell number and the chlorophyll amount is not linear. CC124 and D23940 have almost the same chlorophyll around 3.5 mg/L but the cell number of D23940 is 8.5 x 10^5 mL^{-1},approximately 4 fold of CC124. This shows that for an efficient culture start, chlorophyll amount is better than cell number because each cell does not contain same amount of chlorophyll which is directly related to photosystem activity of the cells.

Table 1 Aerobic phase

Strain	Cell number (x10^5 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

The anaerobic phase is the critical part of the hydrogen 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*. (2000). Sulfur is one of the elements in the D1 protein amino acids like methionine and cysteine (Melis 2002, 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 fixed around 12 mg/L to prevent light and anaerobic stress. 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, Oncel and Sabankay 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 production volume is decreased and stopped at 144 h. But the mutant strains had longer production duration accompanied with higher volumes of hydrogen, 2-4 folds of 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.

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

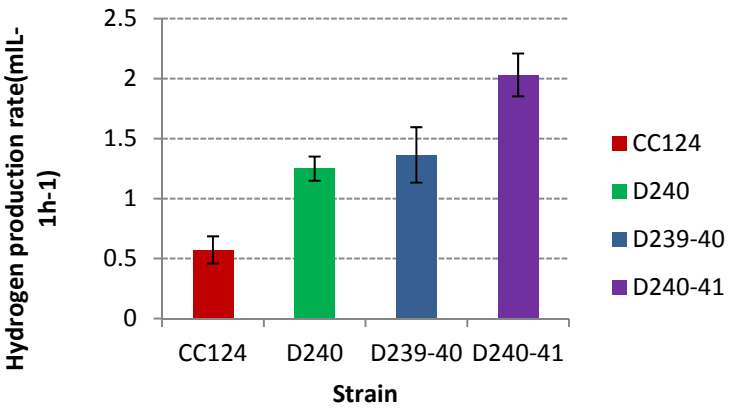

Fig. 3 Hydrogen production rate

The hydrogen production rates of mutant strains are also higher than control strain CC124. D24041 has almost 2 $mlL^{-1}h^{-1}$ 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 H2 production.

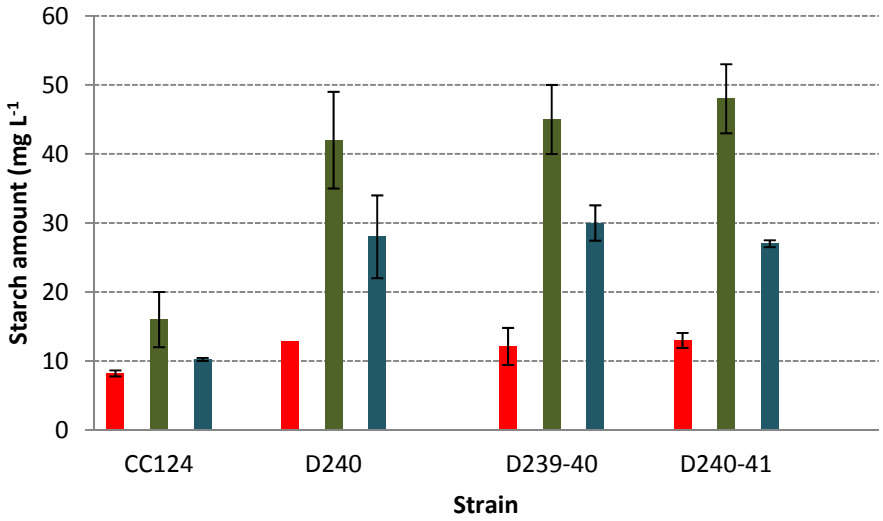

Fig. 4 Change in the amount of starch during hydrogen production phase regards to experiment start x, hydrogen start x and experiment end x

In conclusion, the mutations directed for higher volume of hydrogen 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 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 and scale up (Scoma *et al*. 2012, Oncel and Sabankay 2012). 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, Faraloni and Torzillo 2010). We have observed that the PSII activity, starch accumulation, hydrogen production volume and duration is enhanced by the homologous mutations at the gene clusters HydA1 of [Fe] Hydrgenases. All the mutant strains have increased amount of hydrogen gas production, 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 good candidate to determine further studies and process design.

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REFERENCES

Antal, T.K., Krendelevam, T.E., Laurinavichene, T.V., Makarova, V.V., Ghirardi, M.L., Rubin, A.B., Tsygankov, A.A. and Seibert, M. (2003), "The dependence of algal H2 production on photosystem II and O2 consumption activities in sulfur-deprived *Chlamydomonas reinhardtii* cells", *Biochim*. *Biophys*. *Acta*, **1607**,153-160.

Das, D. and Veziroglu, N. (2008), "Advances in biological hydrogen production in processes", *Int*. *J*. *Hydrogen Energy*, **33**, 604-657.

Edelman, M. and Mattoo, A.K. (2008), "D1-protein dynamics in photosystem II: The lingering enigma", *Photosynth*. *Res*., **98**, 609-620.

Faraloni, C. and Torzillo, G. (2010), "Phenotypic characterization and hydrogen production in Chlamydomonas reinhardtii Q_B binding D1 protein mutants under sulphur starvation: changes in chlorophyll fluorescence and pigment composition", *J*. *Phycol*., **46**, 788-799.

Forestier, M., King, P., Zhang, L., Posewitz, M., Schwarzer, S., Happe, T., Ghirardi, M.L. and Seibert, M. (2003), "Expression of two [Fe]-hydrogenases in Chlamydomonas reinhardtii under anaerobic conditions", *Eur*. *J*. *Biochem*., **270**, 2750-2758.

Gaffron, H. and Rubin, J. (1942), "Fermentative and photochemical production of hydrogen in algae", *J*. *Gen*. *Physiol*., **26**, 219-240.

Ghirardi, M.L., Zhang, L., Lee, J.W., Flynn, T., Seibert, M., Greenbaum, E. and Melis, A. (2000), "Microalgae: a green source of renewable H2", *Trends Biotechnol*., **18**, 506-511.

Giannelli, L. and Torzillo, G. (2012), "Hydrogen production with the microalga Chlamydomonas reinhardtii grown in a compact tubular photobioreactor immersed in a scattering light nanoparticle suspension", *Int*. *J*. *Hydrogen Energy*, **37**, 16951-16961.

Hallenbeck, P.C. and Benemannm J.R. (2002), "Biological hydrogen production;

fundamentals and limiting processes", *Int*. *J*. *Hydrogen Energy*, **27**, 1185-1193.
Happe, T., Hemschemeier, A., Winkler, M. and Kaminski, A. (2002), "Hydrogenases in green algae: do they save the algae's life and solve our energy problems?", *Trends in Plant Sci*., **7**(6).
Hoshino , T., Daniel, J.J. and Joel, L. (2012), "Design of new strategy for green algal photo-hydrogen production: Spectral-selective photosystem I activation and photosystem II deactivation", *Cuello Biores*. *Technol*., **120**, 233-240.
Kima, J.P., Kang, C.D., Park, T.Y., Kim, M.S. and Sim, S.J. (2006), "Enhanced hydrogen production by controlling light intensity in sulphur deprived Chlamydomonas reinhardtii culture", *Int*. *J*. *Hydrogen Energy*, **31**, 1585-1590.
Kosourov, S., Makarova, V., Fedorov, A.S., Tsygankov, A., Seibert, M. and Ghirardi, M.L. (2005), "The effect of sulfur re-addition on H2 photoproduction by sulfur-deprived green algae", *Photosynth Res*., **85**, 295-305.
Kosourov, S., Patrusheva, E., Ghirardi, M.L., Seibert, M. and Tsygankov, A. (2002), "A comparison of hydrogen photoproduction by sulfur-deprived Chlamydomonas reinhardtiiunder different growth condition", *J*. *Biotechnol*., **128**, 776-787.
Laurinavichene, T., Tolstygina, I. and Tsygankov, A. (2004), "The effect of light intensity on hydrogen production by sulfur-deprivedChlamydomonas reinhardtii", *J*. *Biotechnol*., **114**, 143-151.
Laurinavichene, T.V., Fedorov, A.S., Ghirardi, M.L., Seibert, M. and Tsygankov, A.A. (2006), "Demonstration of sustained hydrogen photoproduction by immobilized, sulfurdeprived Chlamydomonas reinhardtii cellsé", *Int*. *J*. *Hydrogen Energy*, **31**, 659-667.
Levin, D.B., Pitt, L. and Love, M. (2004), "Biohydrogen production: prospects and limitations to practical application", *Int*. *J*. *Hydrogen Energy*, **29**, 173-185.
Melis, A. and Happe, T. (2001) "Hydrogen production: green algae as a source of energy", *Plant*. *Physiol*., **127**, 740-748.
Melis, L., Zhang, M., Forestier, M.L. and Ghirardi, M.S. (2000), "Sustained photobiological hydrogen gas production upon reversible inactivation of oxygen evolution in the green alga Chlamydomonas reinhardtii", *Plant Physiol*., **122**, 127-136.
Oncel, S. and Sabankay, M. (2012), "Microalgal biohydrogen production considering light energy and mixing time as the key features for scale up", *Biores*. *Technol*., **121**, 228- 234.
Oncel, S. and Vardar-Sukan, F. (2009), "Photo-bioproduction of hydrogen by Chlamydomonas reinhardtii using a semi-continuous process regime", *Int*. *J*. *Hydrogen Energy*, **34**, 7592-7602.
Oncel, S. and Sukan, F.V. (2011), "Effect of light intensity and the light: dark cycles on the long term hydrogen production ofChlamydomonas reinhardtiiby batch cultures", *Biomass Bioenerg*., **35**, 1066-1074.
Parmar, A., Singh, N.K., Pandey, A., Gnansounou, E. and Madamwar, D. (2011), "Cyanobacteria and microalgae: A positive prospect for biofuel", *Biores*. *Technol*., **102**, 10163-10172.
Scoma, A., Giannelli, L., Faraloni, C. and Torzillo, G. (2012), "Outdoor H2 production in 50-L tubular photobioreactor by means of a sulfur-deprived cultur of the microalgae Chlamydomonas reinhardtii", *J*. *Biotechnol*., **157**- 620-627.
Scoma, A., Krawietz, D., Faraloni, C., Gianelli, L., Happe, T. and Torzillo, G. (2012),

"Sustained H2 production in a Chlamydomonas reinhardtii D1 protein mutant", *J. Biotechnol*., **157**, 613-619.
Specht, E., Miyake-Stoner, S. and Mayfield, S. (2010), "Micro-algae come of age as a platform for recombinant protein production", *Biotechnol. Lett*., **32**, 1373-1383.
Torzillo, G., Scoma, A., Faraloni, C., Ena, A. and Johanningmeier, U. (2009), "Increased hydrogen photoproduction by means of a sulfur deprived Chlamydomonas reinhardtii D1 protein mutant", *Int. J. Hydrogen Energy*, **34**, 4529-4536.
Tsygankov, A.A., Kosourov, S.N., Tolstygina, I.V., Ghirardi, M.L. and Seibert, M. (2006), "Hydrogen production by sulfur-deprived Chlamydomonas reinhardtii under photoautotrophic conditions", *Int. J. Hydrogen Energy*, **31**, 1574-1584.
Zhang, L., Happe, T. and Melis, A. (2002), "Biochemical and morphological characterization of sulfur-deprived and H2-producing Chlamydomonas reinhardtii (green alga)", *Planta*, **214**, 552-561.