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High rates of photobiological hydrogen production by a cyanobacterium under aerobic condition

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Of all the emerging renewable and green energy sources, biohydrogen stands out as a lucrative choice. Hydrogen can be produced by certain groups of microorganisms that possess the nitrogenase and / or bidirectional hydrogenase enzymes [1,2,3] . These enzymes are highly sensitive to oxygen and require special protective mechanisms in order to function in an environment that is predominantly aerobic. Although the potential of this biological process in contributing towards clean energy was realized decades ago, its optimization for large scale production continues to be a challenging endeavor. The poor yield of hydrogen in the strains studied till date, owed largely to the low specific activities of the oxygen sensitive enzymes, has been a major factor impeding progress in this direction. Here, we report about a unicellular, diazotrophic cyanobacterial strain with an exceptional capacity to generate unprecedented amounts of hydrogen under aerobic conditions. Wild type *Cyanothece* 51142 produces hydrogen utilizing solar energy and either high levels of carbon dioxide or glycerol as carbon sources. Our study indicates that hydrogen production in this strain is mediated by an efficient nitrogenase system which can be effectively manipulated to convert solar energy into hydrogen at rates which are an order of magnitude higher compared to any known wild type hydrogen producing strain.

Microbial H_2 production relies on either photosynthetic or fermentative processes. Although the fermentative pathway leads to high yields, economic feasibility studies suggest that a direct and efficient conversion of solar energy to H_2 is necessary for the process to be commercially viable [4,5]. Several photosynthetic microalgal and bacterial species are being studied as prospective model organisms for photobiological H_2 production. Outstanding among them are *Rhodopseudomonas palustris*, a purple photosynthetic , nitrogen fixing bacterium, *Chlamydomonas reinhardtii*, a green alga and *Anabaena* and *Synechocystis,* members of the cyanobacterial group (Table 1A,B). High specific rates of nitrogenase mediated H_2 production have been reported for *R. palustris.* However, like all purple bacteria, *R. palustris* performs

anoxygenic photosynthesis, thereby requiring an anaerobic environment and external sources of energy for H_2 production[6,7,8]. *Anabaena,* a filamentous diazotrophic cyanobacterial strain produces H_2 only in specialized cells called heterocysts which maintain a microaerobic environment to protect the oxygen sensitive nitrogenase enzyme. The low frequency of heterocysts in a filament (one in ten cells differentiate into heterocysts[9]) results in low yields of net H_2 production. In contrast, *C. reinhardtii* and *Synechocystis* sp. PCC 6803 produce H_2 via the bidirectional hydrogenase enzyme and sustainable rates of H_2 production can be achieved in both these organisms only under strictly anaerobic conditions (Table 1 A, B). Although these strains have long been used as model organisms for studying biohydrogen production, the importance of selecting novel and native strains with diverse energy conversion systems that may have evolved as a consequence of ecological pressure has often been emphasized[10, 4].

In this work we describe a cyanobacterial strain with the potential to produce extraordinarily high amounts of H_2 under aerobic conditions. *Cyanothece* 51142 thrives in marine environments limited in dissolved inorganic nitrate salts and has been recognized for its role in maintaining the marine nitrogen cycle [11,12]. This robust cyanobacterium can derive all its nutritional requirements from sunlight, atmospheric carbon dioxide and nitrogen gases (Fig. 1). During the day it photosynthesizes and fixes carbon which gets stored as large reserves of glycogen [13]. At the onset of the dark period high rates of respiration rapidly create an anoxic intracellular environment. This facilitates oxygen sensitive processes such as nitrogen fixation and H_2 production to occur at night at the expense of the accumulated glycogen. The orchestrated diurnal cycling patterns of these central metabolic processes are also evident at the level of gene transcription[14]. These unique attributes of *Cyanothece* 51142, which make it an ideal organism for H_2 production, are possibly the remnants of the metabolic and regulatory processes that aided in the acclimatization of the ancient cyanobacterial strain during its switch from an anaerobic to an aerobic environment. Retention of ancient metabolic traits that originated in the Achaean oceans has been reported in other cyanobacterial strains[15].

Cyanothece 51142 exhibits high specific rates of H_2 production (> 150 µmoles of H_2.mg $Chl^{-1}.hr^{-1}$) under aerobic conditions (Figure 2a). This is in striking contrast to other known unicellular microbial strains which entail a complete anaerobic environment for H_2 metabolism (Table 1). Genome analysis of this strain revealed the presence of both the nitrogenase (NIF) and bidirectional hydrogenase (HOX) enzyme systems[12]. Analysis at the transcription level revealed that *hox* genes are expressed under nitrogen fixing as well as nitrogen sufficient conditions while *nif* is expressed only under nitrogen fixing conditions (Fig 2b, 2c). However H_2 production

can be detected only under diazotrophic conditions and addition of combined nitrogen to the growth media of a culture fixing molecular nitrogen results in immediate reduction in the rates of nitrogen fixation and simultaneous cessation of hydrogen production (Figure 2d). These results suggest that the high rates of H_2 production observed in *Cyanothece* 51142 are largely mediated by the nitrogenase enzyme system.

The rates of hydrogen production in *Cyanothece* 51142 can be greatly enhanced when cells are grown with high concentrations of CO_2 or glycerol. When grown under 8-15% CO_2–enriched air *Cyanothece* 51142 cells accumulate twice the amount of glycogen as compared to cells grown under ambient CO_2 levels and consequently generate higher amounts of hydrogen (Fig. 2a, 3a). Under mixotrophic conditions with glycerol as the carbon source, *Cyanothece* 51142 cells exhibit rapid doubling time, greatly enhanced glycogen accumulation and significantly higher rates of hydrogen production (Fig 2a). A batch culture of *Cyanothece* cells supplemented with 50 mM glycerol can produce upto 850 ml H_2 / L culture over a period of two days. Both CO_2 and glycerol are abundantly available as industrial waste products making biohydrogen production by *Cyanothece* 51142 a commercially attractive option.

Nitrogen fixation is an energy intensive process, requiring 16 molecules of ATP for every molecule of nitrogen fixed and hydrogen produced. However, the process is of paramount importance to diazotrophic species inhabiting ecological niches with very low levels of inorganic nitrates. Consequently, photoautotrophic unicellular strains like *Cyanothece* 51142 are expected to have evolved effective strategies for harvesting and storing solar energy which could be utilized at night when the energy demands are high. Our results indicate that the large glycogen reserves that build up during the processes of photosynthesis and carbon fixation are mobilized as a source of ATP and reductants for the nitrogenase enzyme to function at night (Fig. 3a). In contrast, in the presence of an external carbon source like glycerol, the expression of glycogen phosphorylase (*glgP*), a gene involved in glycogen degradation is down regulated at the end of the incubation phase and the glycogen reserves of the cells still remain high (Fig. 2b). This might indicate a switch from the initial use of intracellular glycogen reserves to the direct use of glycerol as a source of energy and reductants for the nitrogenase enzyme later during the incubation phase.

An important feature of the nitrogenase enzyme system is the potential to channelize all available electrons toward H_2 production in the absence of molecular nitrogen [16,17]. The nitrogenase reaction is also resistant to feedback inhibition from the accumulated H_2[18]. When

Cyanothece 51142 cells are incubated under an argon atmosphere (absence of molecular nitrogen) a 2-3 folds increase in yield is achieved (Fig. 3b, Table 1a). Production rates of up to 470 µmoles of H_2.mg chl^{-1}.hr^{-1} can be achieved from cells grown in glycerol supplemented media under argon environment (Fig. 3b, Table 1b). These rates are several folds higher compared to any other wild type H_2 producing strain known till date (Table 1b). Previous studies revealed that an uptake hydrogenase present in most diazotrophic strains reutilizes part of the hydrogen formed, thus lowering the net yield of nitrogenase mediated H_2 production in several wild type strains. It has been possible to enhance H_2 production by creating uptake hydrogenase mutants[19,20]. The genome sequence of *Cyanothece* 51142 also shows the presence of an uptake hydrogenase. The transcripts for this gene (*hupS*) appear to be down regulated at the end of the light incubation phase of H_2 production (Fig 2b). The *hupS* transcripts are present under both nitrogen sufficient and deficient conditions indicating that its expression is independent of *nif*. Generating *hup*$^-$ mutants in *Cyanothece* 51142 will presumably lead to further enhancement in the rates of H_2 production.

Although decades of research have unveiled various principles underlying biological H_2 production, achieving any significant increase in yield has been a major challenge. Genetic modifications of H_2 yielding pathways have resulted in several folds improvement in the production rates compared to the wild type strains[20,21,22, 8]. However, since the overall H_2 production rates in these wild type strains are considerably low, a twenty fold increase in yield in the mutants is still not sufficient to meet the commercial requirements. Therefore, identification of a cyanobacterial strain exhibiting high rates of H_2 production under ambient aerobic conditions is a remarkable feat in the field of biofuel research. Prior studies have demonstrated the robustness of the cyanobacterial system for H_2 production over a prolonged period of time[23], corroborating the possibility of using high H_2 yielding cyanobacterial strains for large scale production. A systems level understanding of this biological phenomenon in *Cyanothece* 51142 will reveal previously unknown cellular factors and regulatory mechanisms that influence the process so that they can be favorably altered to produce hydrogen for the energy sector.

Methods

Growth Conditions

For H_2 measurement *Cyanothece* 51142 cells were grown in shaking flasks in ASP2 medium [24] without supplemented $NaNO_3$ at 30℃ under 12-h light-dark cycles and 100 µmol photon.m^{-2}.s^{-1} of white light. Cultures were inoculated with 1/4 V of cultures grown in ASP2 medium without $NaNO_3$ under continuous light (50 µmol photon.m^{-2}.s^{-1} white light) which in turn were inoculated with 1/10 V of cultures grown in ASP2 with $NaNO_3$ under similar conditions. For mixotrophic growth, cultures were supplemented with 50 mM glycerol. For growth under high CO_2 conditions, the cultures were aerated with 8% CO_2-enriched air at a flow rate of 100mL/min.

Hydrogen and nitrogenase activity measurement

Cells were transferred at the end of the light cycle (D0) to air tight glass vials and incubated at a light intensity of 100 µmol photon.m^{-2}.s^{-1} for 12 hours. For anaerobic incubation, the glass vials were flushed with argon for 15-30 mins. H_2 accumulated in the headspace of sealed culture vials was withdrawn with an air-tight syringe and quantified on an Agilent 6890N Gas Chromatograph with a molseive 5A 60/80 column (inner dimensions 6'×1/8") and Thermal Conductivity Detector. Injection, oven, and detector temperatures were 100℃, 50℃, and 100℃, respectively. Argon was the carrier gas, and was supplied at a flow rate of 65 ml/min.

The acetylene reduction assay [25] was used to determine nitrogenase activity of the hydrogen producing cultures. Cells were incubated in sealed glass vials in light at 30˚C under a 5% acetylene atmosphere with or without flushing with argon. Gas samples were withdrawn, and ethylene production was measured on an Agilent 6890 N gas chromatograph equipped with a flame ionization detector using argon as the carrier gas (flow rate of 65 ml/min). The temperature of the injector, detector, and oven were 150˚C, 200˚C, and 100˚C, respectively.

Determination of the glycogen content

The cellular glycogen content was measured using a glucose hexokinase assay (Sigma) with glycogen from bovine liver Type IX (Sigma) as standard. The cell pellets were washed twice with 100% ethanol to extract the chlorophyll prior further treatments. In order to remove free glucose, 40% KOH was added and the samples were incubated for 1h at 95℃. Glycogen was precipitated over night at -20℃ with 2 volumes of 100% ethanol. The samples were centrifuged for 1h at 4℃ and 2N HCl was added before incubation at 95℃ for 30 min. The same volume of

2N NaOH and 1/2V of 1M phosphate buffer, pH 7 were added prior dilution with 1V ddH_2O. For the enzyme assay, 75 μL of sample solution was used and mixed with 200 μL of enzyme solution in a microtiter plate (Costar, UV light proof). After 15 min of incubation at ambient temperature the amount of NADPH was measured at 340 nm in a μQuant plate reader (Bio-Tek Instruments).

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR analyses was performed on RNA samples isolated from cultures grown under nitrogen fixing (without combined nitrogen) with and without supplemented glycerol, from cultures grown under aeration with 8% CO_2 enriched air and under non-nitrogen fixing conditions (with combined nitrogen in the form of $NaNO_3$). For the time course experiment samples were harvested every 4 hrs for 24 hrs, starting with one hour into the dark period (D1). In total, 6 samples were collected. For RT-PCR analysis under hydrogen producing conditions, culture samples were taken at the end of the light period at time point D0 and from the assay bottles at the end of the incubation at time point D12. The RNA was isolated and quantified essentially as described in Stöckel et al., 2008. 700ng of DNAse (Promega) treated total RNA samples were used for reverse transcription with the Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and random primers (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The absence of DNA contaminations has been tested for each RNA sample (Figure 2, 3). PCR was carried out at 94°C for 4 min, and 25 cycles of 94°C 30 sec, 58°C 20 sec, 72°C 20 sec with a final extension time of 4 min at 72°C. For the genes *nifH*, *hupS*, *glgP*, *glgA*, and *coxA* 25 cycles and for the gene *hoxH* 26 cycles were used. The following primers were used: *nifH* F: ACC ATT GCT GCG TTA GCT GAA AC, R: TAA TAC CAC GAC CCG CAC ATC CA; *coxA* F: TGA TAT GGC CTT TCC CAC CCT CA, R: AGA GAA CTA AAG CGG CAG CGA GA; *hupS* F: ATA GCT GGT TTC GTT GTC GCT GT, R: CGA AGT CTT GGG TGG TTG CTT TG; *hoxH* F: TGG AGA AGA CGG ACT TTG GGA AC, R: AAA GAA GAG GTC GCT ACA CCA CC; *glgP* F: TCG GCT GAA TTC CTT ATG GGT CG, R: CAG GAA TTT CCA CTT GCC AAC CG; *16S rRNA* F: AGA GGA TGA GCA GCC ACA CT, R: TAA TTC CGG ATA ACG CTT GC.

Figure legends:

Figure 1. A schematic diagram showing biohydrogen production in *Cyanothece* 51142 utilizing solar energy and atmospheric CO_2. CO_2 is fixed during the day to synthesize glycogen which serves as an energy reserve for H_2 production at night. The figure shows an integrated approach of H_2 production utilizing flue gas from coal fired power plants or glycerol waste from biodiesel plants.

Figure 2. *Cyanothece* 51142 exhibits high specific rates of nitrogenase-mediated H_2 production.

a. Specific rates of H_2 production in *Cyanothece* 51142 under aerobic, photoautotrophic conditions utilizing ambient (control) or high concentrations of CO_2 and under mixotrophic growth conditions with glycerol. The cultures were incubated under aerobic conditions for H_2 production. Each column represents an average of measurements from at least three biological replicates. Error bars indicate standard deviation from the average.

b. RT-PCR analysis of the enzyme systems involved in H_2 metabolism in *Cyanothece* 51142. Strong *nifH* expression can be observed from the beginning of the dark phase (D0) in the control (grown in ambient CO_2) as well as in cultures supplemented with high concentrations of CO_2 and glycerol. *nifH* transcripts are also present in the cells under light incubation during H_2 production (D12). *glgP*, a gene involved in glycogen degradation is down regulated in cultures supplemented with glycerol indicating that glycerol is used directly as a source of glycogen. Interestingly, *hoxH* expression is also greatly down regulated in the presence of glycerol. Expression of *hupS* is lower under light incubation.

c. Comparison of expression of genes involved in H_2 metabolism under nitrogen sufficient and nitrogen fixing conditions. *nifH* transcripts can only be observed under nitrogen fixing condition while *hoxH* and *hupS* are present under both.

d. Addition of inorganic nitrate salts to the culture media of a nitrogen fixing culture results in cessation of H_2 production and greatly reduced nitrogen fixation. Each column represents an average of measurements from at least three biological replicates. Error bars indicate standard deviation from the average.

Figure 3. Effect of external carbon sources and argon on H_2 metabolism in *Cyanothece* 51142.

a. Estimation of glycogen utilization for H_2 production. D0 and D12 indicate beginning and end of a 12 hour light incubation period for H_2 production. The difference in the glycogen level between the two time points corresponds to the amount of glycogen utilized for H_2 production. Note that in the culture supplemented with glycerol, there is no significant difference in the glycogen level after the 12 hour incubation period indicating utilization of glycerol and not glycogen for H_2 production. Each column represents an average of measurements from at least three biological replicates. Error bars indicate standard deviation.

b. Higher specific rates of hydrogen production are achieved in the absence of molecular nitrogen in the head space of the incubation bottles.

Each column represents an average of measurements from at least three biological replicates. Error bars indicate standard deviation from the average.

Table 1A - Specific rates of H_2 production by *Cyanothece* 51142 and other model photoautotrophic wild type strains.

Strain	Enzyme	Specific rates of H_2 production (aerobic incubation)	Specific rates of H_2 production (anaerobic incubation)	
		(µmoles/mg chl.h)	(µmoles/mg chl.h)	(µmoles/mg prot.hr^{-1})
Cyanothece 51142	Nitrogenase	152	373	3.5
Anabaena 29413	Nitrogenase	-	45.17 [20]	
Synechocystis 6803	Hydrogenase	-	1.2 [21]	
C. reinhardtii	Hydrogenase	-	*6.6 [26 27]	
R. palustris	Nitrogenase			*0.92 [8,18]

Table 1B – Highest rates of H_2 production by some high hydrogen yielding strains.

Strain	H_2 production		Growth and incubation conditions
	(µmoles/mg prot.h)	(µmoles/mg chl.h)	
Cyanothece 51142	3.5	467	Wild type, 8% CO_2 or 50% glycerol, argon incubation
Anabaena 29413 (PK84)	-	167.6	*hup*-[1] mutant, argon incubation[20]
Synechocystis 6803 (M55)	-	6	*ndhB*-[1] mutant, glucose, glucose oxidase, sulfur deprivation, argon incubation[21]
C. reinhardtii	-	16.26*	WT, photomixotrophic growth, anaerobic incubation[26 27]
R. palustris	3.6	-	Mutations in *hup* and *nifA*, organic carbon sources, anaerobic growth and incubation[18]

*The rates were calculated using information from both references 26 and 27

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