OPEN

SUBJECT AREAS:

ENVIRONMENTAL
MICROBIOLOGY

MARINE MICROBIOLOGY

ENVIRONMENTAL
BIOTECHNOLOGY

WATER MICROBIOLOGY

Auto-flotation of heterocyst enables the efficient production of renewable energy in cyanobacteria

Ming Chen^{1,2}, Jihong Li^{1,2}, Lei Zhang^{1,2}, Sandra Chang^{1,2}, Chen Liu¹, Jianlong Wang¹ & Shizhong Li^{1,2}¹Institute of New Energy Technology, Tsinghua University, Tsinghua Garden, Beijing 100084, China, ²Beijing Engineering Research Center for Biofuels, Tsinghua University, Tsinghua Garden, Beijing 100084, China.Received
1 July 2013Accepted
16 January 2014Published
6 February 2014Correspondence and
requests for materials
should be addressed to
S.Z.L. (szli@mail.
tsinghua.edu.cn)

Utilizing cyanobacteria as a bioenergy resource is difficult due to the cost and energy consuming harvests of microalgal biomass. In this study, an auto-floating system was developed by increasing the photobiological H₂ production in the heterocysts of filamentous cyanobacteria. An amount of 1.0 μM of diuron, which inhibited O₂ production in cyanobacteria, resulted in a high rate of H₂ production in heterocysts. The auto-floating process recovered 91.71% ± 1.22 of the accumulated microalgal biomass from the liquid media. Quantification analysis revealed that 0.72–1.10 μmol H₂ per mg dry weight microalgal biomass was necessary to create this auto-floating system. Further bio-conversion by using anaerobic digestion converted the harvested microalgal biomass into biogas. Through this novel coupled system of photobiological H₂ production and anaerobic digestion, a high level of light energy conversion efficiency from solar energy to bioenergy was attained with the values of 3.79% ± 0.76.

The conversion of solar energy into clean chemical energy has attracted much attention in recent years, due to the fossil fuel crisis and the emission of greenhouse gases^{1,2}. Photobiological H₂ production via cyanobacteria is an important strategy for converting solar energy into renewable and sustainable energy^{3–5}; however, a major obstacle in the production of renewable bioenergy from cyanobacteria is its relatively low light energy conversion efficiency (LCE). The theoretical maximal LCE of photosynthesis in cyanobacteria is reported to be approximately 11%⁶. However, the LCE for renewable energy production has always been relatively low because most of the converted solar energy is stored as biomass⁷. For example, the LCE for microalgal H₂ is 0.042% and 0.075% for *Anabaena* sp. strain PCC 7120 (henceforth referred to as *Anabaena* 7120) and *Chlamydomonas reinhardtii* respectively^{8–10}. In these microalgal cells, the electron pathway for hydrogen production is strictly oxygen sensitive, and most of the electrons from water photolysis were utilized to assimilate CO₂ and produce biomass^{6,7}. Converting the light energy stored in microalgal biomass into renewable energy is a promising strategy for a new source of bioenergy.

Unfortunately, biomass harvest is still a technical bottleneck for microalgae-driven solar energy conversion^{11,12}. Many strategies have been attempted for the efficient harvest of the biomass, such as centrifugation, filtration, and flocculation^{11,13}. But each one of these harvesting methods are also energy-intensive and expensive¹⁴. Bio-flocculation has been utilized successfully in recent years to obtain microalgae sedimentation¹⁵, but the recovery of sedimental cells is still a time consuming and costly process. Dissolved air flotation (DAF) is considered to be the primary technology of choice for harvesting of microalgal biomass due to its high efficiency and relatively lower energy consumption compare to centrifugation^{16–18}. However, some of the chemical coagulants used in DAF created additional problems, such as excess cost and pollution to the environment^{15,19}. Furthermore, the operational cost of the DAF unit is still high because of the enormous energy required to achieve the necessary air compression of 390 kPa (56 psig)^{20,21}. Therefore, an auto-floating system without the need of coagulants and compressed air will be a promising strategy for an economical and environmental friendly method to harvest microalgae.

Two key features are essential for an auto-floating system. One required element is the ability to auto-flocculate and sediment, and the other is the ability of the microalgae to produce an adequate amount of gas to enable flotation. The heterocyst-containing N₂-fixing cyanobacterium *Anabaena* 7120 is a photoautotrophic microalga that assimilates CO₂ from the atmosphere in order to grow. This microalga has a marked flocculation activity attributed to its filamentous cellular structure and excretion of mucilage^{22,23}. Using sunlight as its only source of energy, *Anabaena* can generate H₂ gas under anaerobic conditions^{24–26}. Photobiological H₂ production of

Figure 1 | Compared analysis of auto-flocculation process of *Anabaena*, *Chlamydomonas* and *Synechocystis*. Cells in logarithmic phase ($A_{730} = 0.8$ – 1.0) were equally divided into 60 mL glass tubes. Duplicate tubes of each strain were sampled for the analysis. The self-flocculation of the culture was monitored by taking photographs at the indicated time points.

Anabaena 7120 occurs both in vegetative cells and heterocysts. Research has shown that this photobiological activity is mainly catalysed by nitrogenase in the heterocysts^{27–29}.

In this study, auto-flocculation of *Anabaena* 7120 was induced by stimulating photobiological H_2 production through inhibition of photosynthetic O_2 evolution. The relationship between the amount of H_2 production and biomass weight was determined by quantification analysis. Following the auto-floating harvest process, an anaerobic digestion of the separated cellular biomass was performed to convert its stored solar energy to H_2 and CH_4 , contributing to a high total LCE value for clean energy production.

Results

***Anabaena* 7120 rapidly self-flocculated in standing culture.** We investigated the self-flocculation ability of *Anabaena* 7120 and compared it with two other representative photo- H_2 producing microalgae, the green alga *Chlamydomonas reinhardtii* and the cyanobacterium *Synechocystis* sp. PCC 6803. As clearly shown in figure 1, unlike the unicellular microalgae *Chlamydomonas* and *Synechocystis*, the algal biomass of the *Anabaena* culture aggregated and flocculated rapidly. The culture of *Anabaena* 7120 settled completely in just 2 hours.

H_2 production was significantly enhanced in DCMU-treated samples. H_2 gas can be used as a clean and renewable energy, and also act as an effective force to provide buoyancy for flotation of the microalgal biomass. *Anabaena* 7120 can produce H_2 gas under N-starved conditions by using solar light as the energy resource. Previous reports have shown that O_2 is the major inhibitor in the production of photosynthetic H_2 in N_2 -fixing cyanobacteria^{30,31}. Anaerobic conditions induce H_2 production within *Anabaena* 7120 but the amount of H_2 produced under these conditions was quite low. As shown in table 1, the highest rate obtained by our laboratory was $1.26 \mu\text{mol}$ of H_2 per mg of chlorophyll *a* per hour (per mg Chl *a*.h). Moreover, as seen in Supplementary Fig. S1a, O_2 gas (red line) and H_2 gas (blue line) were produced simultaneously in the culture. These results corresponded to previous research results presented in *Anabaena cylindrica*^{24,32}. Although both O_2 and H_2 evolved in *Anabaena* 7120, O_2 is only produced in vegetative cells. The increase of O_2 content (see Supplementary Fig. S1a) revealed the

active photochemical function of photosystem II (PSII). As shown in Supplementary Fig. S1b, O_2 evolution activity of PSII was quite high in the early stage of H_2 production (0–12 h).

To prevent O_2 evolution, diuron (3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCMU), an obligate inhibitor of PSII was added to *Anabaena* 7120 cultures (Fig. 2a). The results showed that the O_2 evolution in the *Anabaena* cells was inhibited by 60% of the original level with the addition of $0.05 \mu\text{M}$ DCMU, and a 100% suppression was achieved when treated with $1.0 \mu\text{M}$ DCMU. To investigate the effects of $1.0 \mu\text{M}$ DCMU on the photochemical properties of PSII, the effective photochemical quantum yield of PSII in dark-adapted samples (Fig. 2b) and the electron transport rate of PSII (ETR II) (Fig. 2c) were also analyzed. These data revealed that the photochemical activity of the PSII reaction center was almost completely suppressed and that electron transfer in PSII was significantly blocked from 26.25 ± 0.65 to $1.25 \pm 0.15 \mu\text{mol electrons m}^{-2}\text{s}^{-1}$. Therefore, a $1.0 \mu\text{M}$ concentration of DCMU efficiently interrupted photosynthetic O_2 evolution in the *Anabaena* 7120 cells.

To determine the photobiological H_2 production in *Anabaena* 7120 treated with $1.0 \mu\text{M}$ DCMU, the treated cells were grown with illumination at $100 \mu\text{E m}^{-2}\text{s}^{-1}$. As shown in figure 3, the H_2 production increased drastically in DCMU-treated cultures and maintained a relative high level for more than 120 hours under continuous illumination. Hydrogen production in untreated and treated cells is presented in table 1. DCMU-treated cultures accumulated H_2 about 200 folds more and around 10 times faster. These results demonstrated that under illumination, *Anabaena* 7120 cells produced a high rate of H_2 in the absence of O_2 .

Table 1 | The rates and accumulation of H_2 production in untreated cells and DCMU treated cells of *Anabaena* 7120

Sample	Accumulated H_2 (μmol) ¹	Maximal rate ($\mu\text{mol/mg Chl. a/h}$)
Untreated cells	0.42 ± 0.03	1.26
DCMU treated cells	77.41 ± 7.36	11.58

¹ The data is the average value of triplicate biological texts of sample tubes containing 40 mL new *Anabaena* 7120 cell cultures ($A_{730} = 0.8$ – 1.0), and all samples were illuminated for 120 hours to produce H_2 .

Microalgal biomass automatically floated with high level of H_2 . From figure 1, *Anabaena* 7120 self-flocculated rapidly in standing culture. The flocculated microalgal biomass cluster treated with DCMU was observed floating within 12 hours (Fig. 4a). The relationship between H_2 accumulation and the amount of floating biomass were investigated (Fig. 4b). Under our experimental conditions, 0.72–1.10 μmol of H_2 per mg of dry weight microalgal biomass was necessary for the biomass to float. Thus, auto-flotation occurred with a relatively high level of H_2 . Additional evidence was provided by the observation that the auto-floating process was disrupted when H_2 production ceased under dark or anaerobic conditions (Fig. 4c). Together, these results demonstrated that auto-flotation of the biomass was a process that depended on a high level of hydrogen. The floating microalgae biomass was harvested at an efficiency of $91.71\% \pm 1.22$.

Heterocyst was required for high level of H_2 production and self-flotation. In untreated *Anabaena* 7120 cells, heterocysts are the major contributors to photobiological H_2 production, however their contribution is unclear in DCMU-treated cultures^{31,33}. Because heterocysts differentiation in *Anabaena* 7120 is dependent on nitrogen starvation, the H_2 production of 1.0 μM DCMU-treated cultures under BG-II (–N) and BG-II (+N) media conditions were evaluated and compared (Fig. 5). Forty mL *Anabaena* 7120 cultures in the logarithmic growth phase were treated with DCMU. During a 120-hour period, H_2 accumulation in DCMU-treated cultures grown in BG-II (–N) containing heterocysts increased significantly from 0.42 μmol to 77 μmol (Fig. 5a). In contrast, DCMU-treated cultures grown in BG-II (+N) without heterocysts accumulated only baseline volume of H_2 gas (0.08 μmol in untreated cells and 0.06 μmol in DCMU-treated cells) shown in figure 5b. To determine whether the

Figure 2 | Photosystem II (PSII) activity assays using O_2 evolution rate measurements and chlorophyll fluorescence analyses. Cells in the logarithmic phase (A_{730} : 0.8–1.0) were treated with DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) for 10 minutes prior to the analysis. (a) Photosynthetic O_2 evolution rates of the *Anabaena* 7120 cells were analysed after treatment with DCMU using various concentrations ranging from 0.005 μM to 5.0 μM . BQ was supplemented as an electron acceptor at a final concentration of 2.0 μM . The O_2 evolution rate of untreated (0 μM DCMU) cells was set as 100%. (b, c) The photochemical activity of PSII was represented as the change in chlorophyll fluorescence between untreated cells and 1.0 μM DCMU-treated cells. All of the tested samples were adjusted to 20 μg of chlorophyll per mL to measure their fluorescence. The images of fluorescence detected a visible decline in the quantum yield of PSII in DCMU-treated cells, which was detected by Imaging-PAM (see the Methods section). During this experiment, the ethanol-treated cells were added as a control because ethanol was used as a solvent for the DCMU treatment experiments. Error bars indicate s.d. values calculated from the average of triplicate experiments.

Figure 3 | Photobiological H_2 production in DCMU-treated cells under anaerobic conditions with continuous illumination. The tested cells were equally divided into 60 mL glass tubes and then sealed with rubber stoppers after being sparged with argon gas. All of the samples were incubated in a 30°C culture box with 100 $\mu E \cdot m^{-2} \cdot s^{-1}$ illumination. The H_2 content in the untreated samples and the DCMU-treated samples was then measured by a GC system at specific time points. Compared to the untreated cells (red symbol and line), H_2 accumulation in the DCMU-treated samples increased quickly and lasted more than 120 hours. The 0 hour, 3 hour, 6 hour, 12 hour and 24 hour time points were recorded as the early phase, and after the 24 hour point, the H_2 accumulation was recorded every 24 hours. Error bars indicate s.d. values calculated from the average of triplicate experiments.

high level of H_2 production and auto-flotation was dependent on heterocyst in BG-II (−N) medium, we tested the H_2 production and flotation feature of a heterocyst differentiation deficient mutant AMC-100 (published as LW1) in which the *nifHDK* promoter was deleted from the chromosome³⁴. AMC-100 was grown in BG-II (+N) medium and then induced for photobiological H_2 production in BG-II (−N) medium with 1.0 μM DCMU. As shown in figure 6, the photobiological H_2 production is quite low in AMC-100 culture and no self-flotation occurred. These results strongly suggested that heterocysts were required for the high rate and sustained photobiological H_2 production in 1.0 μM DCMU-treated cultures. Also shown in Supplementary Fig. S2, other important filamentous cyanobacteria that differentiate into heterocysts also exhibited this auto-floating capacity when treated with DCMU. The flotation behaviours revealed in figure 6 and Supplementary Fig. S2 indicated that heterocyst differentiation is an important component for a microalgal self-flotation harvesting system.

The harvested microalgal biomass was further converted into H_2 and CH_4 by anaerobic digestion. During high levels of photobiological H_2 production, the microalgal biomass was separated from liquid media via the auto-floating process (Fig. 4). The microalgal biomass was anaerobically digested to produce biogas (H_2 and CH_4) by waste anaerobic sludge. As shown, the harvested microalgal biomass further degraded into biogases, yielding 266.3 mL biogas per g of dry weight (Fig. 7). The LCE for direct photobiological H_2 production in *Anabaena* 7120 cells increased to as high as 1.18% with DCMU addition. Furthermore, the conversion of the auto-floating microalgal biomass increased the LCE of the whole system to a maximum value of 4.77% and an average value of 3.79%, shown in table 2.

Discussion

For most species of microalgae, much of the solar energy consumed during photosynthesis is converted and stored as cellular biomass^{6,7}. Thus, only a fraction of the converted solar energy is available for photobiological H_2 production by microalgae. H_2 and CH_4 gas produced in microalgal anaerobic digestion is a type of bioenergy that can be readily used. Sulphur-deprived *Chlamydomonas* and nitrogen-deprived *Cyanothece*, as unicellular microalgae, have shown high levels of photobiological H_2 production under the proper conditions^{35,36}. However, the separation and harvest of the biomass from these species of microalgae is quite difficult and expensive due to the low specific gravity of the individual cells keeping the cells suspended in culture^{16,37}. Furthermore, the LCE for H_2 production in most species of cyanobacteria is relatively low, i.e., below 1%^{8,9}. Compared with unicellular microalgae, flocculation is more likely to occur naturally in *Anabaena* 7120 cultures due to its filamentous cell structure and excretion of mucilage. Figure 1 revealed that a standing culture of *Anabaena* 7120 flocculated and settled automatically in a very short time period (approximately 60 min). This fast self-flocculation activity provides a significant advantage in the creation of an auto-floating system for the efficient and economic harvest of microalgal biomass. According to the requirements of an auto-floating system, as mentioned above, microalgae floated if an adequate amount of H_2 gas was also produced.

In this study, a high level of photobiological H_2 production in heterocysts was stimulated and sustained by the inhibition of O_2 production in the microalgal cells. Previous research had shown that any trace of O_2 ceased the anaerobiosis induced nitrogenase activity³⁸. Even though heterocysts already have low O_2 concentration, the strict anaerobic intracellular condition resulting from DCMU treatment was essential. The same conclusion was drawn in an *Anabaena* strain in which nitrogenase activity within the anaerobic heterocysts was suppressed significantly by exposing to a trace amount of O_2 ³⁸. Argon gas and DCMU induced strict anaerobiosis is the most likely explanation for the high level of photo- H_2 production in *Anabaena* 7120. We also investigated the possible reason for such a stimulated long stage of H_2 production in 1.0 μM DCMU treated cells. As the major carbohydrate storage in N_2 -fixing cyanobacteria, glycogen can provide nitrogenase energy and reducing power for N_2 fixation and H_2 production³⁶. As shown in Supplementary Fig. S3a, the accumulated glycogen was consumed in DCMU treated *Anabaena* 7120 cells during the H_2 production period. In addition, the increase in nitrogenase (*NifH*) expression suggested that more electrons and reductants from glycogen were transferred to nitrogenase for proton reduction and H_2 gas evolving (see Supplementary Fig. S3b).

As a PSII photochemical inhibitor, DCMU addition is an effective strategy to inhibit O_2 evolution and improve the long stage H_2 production in *Anabaena* cultures used in this study. However, DCMU utilization may bring some environmental concerns as well. Metal ion inducible gene expression systems, such as Cu and Ni, could also be chosen as a possible alternative strategy for repression of PSII activity and increase photoautotrophic H_2 production in microalgae³⁹. The protein such as Nac2 which is required for stable expression of the core polypeptide of PSII could be a possible target³⁹. Beside the metal ions, some environmental factors (light intensity, O_2 level and CO_2 concentration) inducible gene systems also had been reported in microalgae^{40–43}, which would eliminate the use of environmentally hazardous inducers.

As shown, *Anabaena* 7120 cells auto-flocculated rapidly and the high level of H_2 gas evolution from the heterocysts induced flotation of the flocculated microalgal biomass. Harvesting efficiency of over 90% was achieved by using this method. This auto-floating system of the filamentous cyanobacterium provided an economical and environmental friendly strategy for microalgal biomass harvests. Moreover, some auto-flocculating microalgae have been utilized successfully to sediment un-flocculating microalgae¹⁵. The former

Figure 4 | Auto-flotation induced in DCMU treated cells. Logarithmic growth cells were used for the assay. (a) The monitoring of microalgal biomass auto-flotation during the photobiological H_2 production of DCMU-treated samples. The 0, 3, 6, 12 and 24 hour time points were recorded as the early phase, and after the 24 hour time point the H_2 accumulation was recorded every 24 hours. (b) The analysis of the quantitative relation between H_2 accumulation and the amount of auto-floated algal biomass. Cells in various concentrations ($A_{730} = 0.6\text{--}1.2$) were sampled for analysis. Auto-flotation occurred between 12–24 h after treated by $1.0 \mu\text{M}$ DCMU in anaerobic conditions in these sealed tubes with various amount of algal biomass. After measurement of H_2 accumulation in tubes, the floated biomass was immediately transferred for dry weight analysis. (c) The analysis of the relationship between H_2 production (c₁) and auto-flotation of biomass (c₂). Each group of cells at the 120 hour time point (DCMU-treated samples under argon with light, under argon in the dark and under air with light, respectively) were recorded and compared. Error bars indicate the s.d. values that were calculated from the average of triplicate experiments.

research revealed that the sedimentation rate of the un-flocculating microalgae was increased considerably when mixed with auto-flocculating microalgae, leading to a higher recovery percentage. Referring to the recent bio-flocculation research of Salim *et al.*¹⁵, such an auto-floating system has the potential to be widely used for harvesting of the un-flocculating microalgae, such as the unicellular microalga *Chlorella*.

The LCE for photosynthesis can reach a theoretical maximum of 11% in cyanobacteria, while the LCE for direct photobiological H_2 production is always less than 0.1% in *Anabaena* 7120. By the auto-floating harvest strategy, the further conversion of microalgal biomass into clean bioenergy can become a promising bioenergy resource with a relatively high LCE. The bioenergy obtained by this auto-floating system can be calculated in two steps: the H_2 gas energy produced during the first stage and the biogas produced in the second stage (Table 2).

During the first stage, photobiological H_2 was produced from the heterocysts. At the second stage, the microalgal biomass was separated from the auto-floating system and further converted by anaerobic digestion into clean energy as H_2 and CH_4 . The auto-floating process combines effective algal photobiological H_2 production and residual microalgal biomass digestion for the production of clean energy. The LCE of this couple system for biofuel production from solar energy was obtained with an average value of 3.79%. Moreover, it avoids costly energy-consuming processes, such as centrifugation and filtration, to harvest the microalgal biomass⁴⁴. The LCE for direct photobiological H_2 production increased to an average value of 3.79%.

Photobiological H_2 production in cyanobacteria can be further increased through genetic engineering or by optimization of growth factors^{8,29,45}. In addition, strategies to improve clean energy production from the accumulated microalgal biomass continued to be

Figure 5 | Morphological and physiological investigation of the relationship between heterocyst development and photobiological H₂ production in *Anabaena* 7120 cells. Cells in the logarithmic growth phase were used for the assay. (a1–a3) A confocal microscopy image of the *Anabaena* 7120 cells grown in BG-II (–N) medium showed normal development of heterocysts. (b1–b3) The confocal microscopy image shows *Anabaena* 7120 cells grown in BG-II (+N) medium without heterocysts. The cells were immobilised with 1% (w/v) agar in BG-II (–N) medium before observation and graphic analysis. A number of heterocysts (white arrows) were observed at the frequencies between 1/10 to 1/20 (heterocyst/vegetative cell). a1, b1-a bright field micrograph of the cells; a2, b2- an auto fluorescence (excited at 488 nm) micrograph of the cells; a3, b3-a combined micrograph of the cells. (a4) Physiological analysis of the photobiological H₂ accumulation in *Anabaena* 7120 cells with normal heterocysts. (b4) Physiological analysis of the photobiological H₂ accumulation in *Anabaena* 7120 cells without heterocysts.

explored⁴⁶. Based on the auto-floating process described here, commercial applications can be developed for the future production of clean energy from microalgae.

Methods

Species and growth conditions. Filamentous cyanobacteria *Anabaena* 7120 (FACHB-418), *Anabaena variabilis* (FACHB-319) and *Anabaena cylindrica* (FACHB-1038) were purchased from the Institute of Hydrobiology, Chinese Academy of Sciences. The *nifHDK* operon promoter mutant AMC-100 was provided by Professor Jame W. Golden. The unicellular cyanobacterium *Synechocystis* 6803 and green alga *Chlamydomonas reinhardtii* CC503 were kind gifts from Professor Quanxi Wang. Microalgal cells were cultured at 30°C in BG-II media (with or without nitrogen)⁴⁷ and buffered with Tris-HCl (5 mM, pH 8.0). The liquid culture was bubbled with 2–3% (v/v) CO₂ in air under continuous illumination by fluorescent lamps (30 μE m^{–2}s^{–1}).

Determination of cell density and chlorophyll *a* content. The cell density was calculated using light absorption at 730 nm (A_{730}) in a UV-Vis mini-1240 spectrophotometer (SHIMADZU, Kyoto, Japan) with a 3 mL cuvette and an optical path of 1 cm. Chlorophyll *a* (Chl *a*) content was measured as previously described⁴⁸. In summary, *Anabaena* 7120 cells (1.5 mL) were collected by centrifugation and washed twice with 1 mL of fresh BG-II medium. The supernatant was discarded after centrifugation; 1.5 mL of methyl alcohol was added to the cell pellet; and the mixture was incubated at 4°C for 6–14 h. The supernatant was then collected by centrifugation and absorbance at 665 nm (A_{665}) was measured. The concentration of Chl *a* (μg mL^{–1}) was calculated as $13.9 \times A_{665}$ ⁴⁹.

Sample preparation for photobiological H₂ production. *Anabaena* 7120 cells were cultured in 0.5 L BG-II (–N) medium for 5 days ($A_{730} = 0.8$ – 1.0) and were transferred to 60 mL glass tubes (leaving 20 mL of head space). DCMU was added to the cultures for a final concentration of 1.0 μM. The tubes were sparged with Ar gas

Figure 6 | Characterization of photo-H₂ production and auto-flotation of wild type *Anabaena* 7120 and the heterocyst deficient mutant AMC-100. The AMC-100 strain was grown in non-diazotrophic BG-II (+N) medium. The logarithmic phase cells were then washed and suspended in BG-II (−N) medium and treated by 1.0 μM DCMU. The accumulated H₂ gas in the sample tubes was detected after 24 h of treatment. Error bars indicate the s.d. values that were calculated from the average of triplicate experiments.

for 2 minutes to eliminate the air in the samples and then sealed with rubber stoppers. The samples were incubated at 30°C under continuous illumination by fluorescent lamps (100 μE m^{−2} s^{−1}) to induce photobiological H₂ production. The incubated samples were then subjected to H₂ and O₂ assays at specific time points.

Biogas production. After the *Anabaena* 7120 microalgal biomass finished producing photobiological H₂, it was collected and transferred to the biogas incubator and monitoring system for sludge-based anaerobically digested biogas production. Substrate fermentation was conducted in batch tests as follows: 100 mL biogas batch fermenters were filled with 5.0 g of anaerobic sludge from a mesophilic anaerobic digester in the Gaobeidian Sewage Treatment Plant in Beijing, China. The substrate was equivalent to 9.0 g of wet microalgal biomass per test. It was loaded and the fermenters were then sealed with rubber septa and incubated in a 37°C water bath for dynamic monitoring of biogas production. The incubation and monitoring process were performed in the Automatic Methane Potential Test System (AMPTS) II

(Bioprocess Control Sweden AB, Lund, Sweden). The seeding sludge contained 10.8% total solids (TS) and 5.57% volatile solids (VS).

Gas analysis. To measure the amount of photobiological H₂ production and O₂ evolution from the samples, 500 μL of gas was withdrawn at predetermined time intervals from the bottles with a gas-tight syringe and injected into a gas chromatograph equipped with a thermal conductivity detector (TCD). The column was a 5 Å molecular sieve column (2 m × 1/8 mm), which could assay H₂, O₂ and N₂ simultaneously. Highly pure argon gas (≥99.999%) was used as the carrier gas at a flow rate of 21 mL per minute, which was based on the effective separation and assay of the gas mixture in our samples.

To measure the amount of dark fermentative biogas production, the volume of evolved gas was monitored every 24 hours by AMPTS II. The measured volume was then converted to a volume of gas at standard temperature and pressure using the Ideal Gas Law. The methane content of the biogas was analysed every day using a SP-6890A gas chromatograph, (Lu Nan Analytic Instruments Co., Ltd., Shandong, China) equipped with a TCD. A 1.83 m column was packed with Porapak Q (50/80 mesh). The operating temperatures of the column, injector port and the detector were 90°C, 40°C and 50°C, respectively. Ar gas was used as the carrier gas at a flow rate of 30 mL per minute. A standard gas mixture which was composed of 15.0% H₂, 60.1% CH₄ and 19.9% CO₂ was used to calibrate the system. The H₂ and CH₄ contents were multiplied by the production of biogas.

Confocal laser scanning microscopic (LSM) analysis. Following the details of methods described in previous studies^{50–52}, the *Anabaena* 7120 filamentous cells grown in BG-II (−N) and BG-II (+N) were sampled for morphological vegetative cell and heterocyst analysis and examined using a Zeiss LSM 710 confocal microscope (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Bright field and auto fluorescence (excited at 488 nm) micrographs were captured to illustrate the visible differences between the microalgal cells with and without heterocysts.

PSII activity analysis. The PSII activity was determined using the benzoquinone (BQ) photosynthetic evolution rate of O₂ and the quantum yield efficiency, which detected the changes in chlorophyll fluorescence. Photosynthetic O₂ evolution was measured at 25°C (Hansatech Instruments, Ltd., Norfolk, England) with a Clark-type oxygen electrode, according to the method described by Mi *et al.* (1995)⁵³. Cells (2 mL) were collected from the sample tube and injected into the reaction chamber, followed by suspension in BQ, to a final concentration of 10 μM. The intensity of the action light (AL) used to measure photosynthetic O₂ evolution was 800 μE m^{−2} s^{−1}. Changes in chlorophyll fluorescence were measured using a pulse amplitude modulated (PAM) chlorophyll fluorometer (Heinz Walz GmbH, Effeltrich, Germany) and an emitter-detector-cuvette assembly (ED-101US; Heinz Walz GmbH) with a unit 101ED, as described elsewhere^{54–56}. To detect a visible change between the PSII activity of the treated and untreated cells, the quantum yield of PSII was detected by a new pulse-amplitude modulated imaging system that measures active fluorescence (Heinz Walz GmbH, Germany), which is described in detail elsewhere^{57,58}.

Calculation of light energy conversion efficiency (LCE). The LCE of the whole conversion process consists of two steps: direct photobiological H₂ production and the anaerobic digestion of microalgal biomass for biogas production. The LCE of step 1 was determined based on the supplied light energy during the H₂-producing phase; the LCE of step 2 was based on the supplied light energy during the cell growth of the biomass accumulation phase. The efficiency of each step was calculated according to the following equation:

Figure 7 | Biogas production from the microalgal biomass substrate after photobiological H₂ production. (a) The detected total biogas volume of the blank sample and the sample with algal biomass as a substrate. (b) The gas composition (H₂ and CH₄) analysis during the anaerobic digestion stage. Error bars indicate the s.d. values that were calculated from the average of triplicate experiments.

Table 2 | The LCE calculation of the two stages and the whole process

Stage	light energy absorption (J)	Energy production (J)	LCE (%)
Algae growth	79619.48	—	—
Photobiological H ₂ production	2948.87	18.36 ± 1.75	0.62 ± 0.06
Anaerobic dark digestion	—	3112.77 ± 631.47	—
Couple process	82568.35	3131.13 ± 631.50	3.79 ± 0.76

Conversion efficiency(%) = (Energy content of H₂ and CH₄) / (Absorption of light energy) × 100 (1)

In full expression the equation is in form:

$$\eta_{\text{H}_2 + \text{CH}_4} (\%) = 100 E_{\text{H}_2 + \text{CH}_4} / Es A$$

$$= 100 (\Delta G_{\text{H}_2}^{\circ} R_{\text{H}_2} + \Delta G_{\text{CH}_4}^{\circ} R_{\text{CH}_4}) / (EsA) \quad (2)$$

where $\Delta G_{\text{H}_2}^{\circ}$ and $\Delta G_{\text{CH}_4}^{\circ}$ are the standard Gibbs energy for the energy storage reaction generating H₂ (237200 J mol⁻¹ at 297 K) and CH₄ (890310 J mol⁻¹ at 297 K) respectively, R_{H_2} and R_{CH_4} are the amounts of accumulated H₂ and CH₄ gas respectively, Es is light energy radiation, and A is the illumination area^{59,60}.

- Jeffries, T. & Lindblad, P. We march backwards into the future. *Curr. Opin. in Biotechnol.* **20**, 255–256 (2009).
- Scholes, G. D., Fleming, G. R., Olaya-Castro, A. & van Grondelle, R. Lessons from nature about solar light harvesting. *Nat. Chem.* **3**, 763–774 (2011).
- Benemann, J. R. Hydrogen production by microalgae. *J. Appl. Phycol.* **12**, 291–300 (2000).
- Ghirardi, M. L., Dubini, A., Yu, J. & Maness, P. C. Photobiological hydrogen-producing systems. *Chem. Soc. Rev.* **38**, 52–61 (2009).
- Prince, R. C. & Kleshgi, H. S. The Photobiological Production of Hydrogen: Potential Efficiency and Effectiveness as a Renewable Fuel. *Crit. Rev. Microbiol.* **31**, 19–31 (2005).
- Ducat, D. C., Way, J. C. & Silver, P. A. Engineering cyanobacteria to generate high-value products. *Trends Biotechnol.* **29**, 95–103 (2011).
- Dismukes, G. C., Carrieri, D., Bennette, N., Ananyev, G. M. & Posewitz, M. C. Aquatic phototrophs: efficient alternatives to land-based crops for biofuels. *Curr. Opin. in Biotechnol.* **19**, 235–240 (2008).
- Lindblad, P. et al. Photoproduction of H₂ by wildtype *Anabaena* PCC 7120 and a hydrogen uptake deficient mutant: from laboratory experiments to outdoor culture. *Int. J. Hydrogen Energy* **27**, 1271–1281 (2002).
- Hallenbeck, P. C. & Benemann, J. R. Biological hydrogen production; fundamentals and limiting processes. *Int. J. Hydrogen Energy* **27**, 1185–1193 (2002).
- Berberoglu, H. & Pilon, L. Maximizing the solar to H₂ energy conversion efficiency of outdoor photobioreactors using mixed cultures. *Int. J. Hydrogen Energy* **35**, 500–510 (2010).
- Grima, E. M., Belarbi, E. H., Acien-Fernández, F. G., Robles-Medina, A. & Chisti, Y. Recovery of microalgal biomass and metabolites: process options and economics. *Biotechnol. Adv.* **20**, 491–515 (2003).
- Uduman, N., Qi, Y., Danquah, M. K., Forde, G. M. & Hoadley, A. Dewatering of microalgal cultures: A major bottleneck to algae-based fuels. *J. Renew. Sust. Energy* **2**, 12701–12715 (2010).
- Golueke, C. G. & Oswald, W. J. Harvesting and Processing Sewage-Grown Planktonic Algae. *J. Water Pollut. Con. F.* **37**, 471–498 (1965).
- Wijffels, R. H. & Barbosa, M. J. An Outlook on Microalgal Biofuels. *Science* **329**, 796–799 (2010).
- Salim, S., Bosma, R., Vermuë, M. & Wijffels, R. H. Harvesting of microalgae by bio-flocculation. *J. Appl. Phycol.* **23**, 849–855 (2011).
- Bare, W. F. R., Jones, N. B. & Middlebrooks, E. J. Algae Removal Using Dissolved Air Flotation. *Water Pollut. Con. F.* **47**, 153–169 (1975).
- Chung, Y., Choi, Y. C., Choi, Y. H. & Kang, H. S. A demonstration scaling-up of the dissolved air flotation. *Water Res.* **34**, 817–824 (2000).
- Teixeira, M. R. & Rosa, M. J. Comparing dissolved air flotation and conventional sedimentation to remove cyanobacterial cells of microcystis aeruginosa: Part I: The key operating conditions. *Sep. Purif. Technol.* **52**, 84–94 (2006).
- Lee, A., Lewis, D. & Ashman, P. Microbial flocculation, a potentially low-cost harvesting technique for marine microalgae for the production of biodiesel. *J. Appl. Phycol.* **21**, 559–567 (2009).
- Wiley, P. E., Brennen, K. J. & Jacobson, A. E. Improved Algal Harvesting Using Suspended Air Flotation. *Water Environ. Res.* **81**, 702–708 (2009).
- Féris, L. A. & Rubio, J. Dissolved air flotation (DAF) performance at low saturation pressures. *Filtr. Sep.* **36**, 61–65 (1999).
- Avnimelech, Y., Troeger, B. W. & Reed, L. W. Mutual Flocculation of Algae and Clay: Evidence and Implications. *Science* **216**, 63–65 (1982).
- Walsby, A. E. Mucilage secretion and the movements of blue-green algae. *Protoplasma* **65**, 223–238 (1968).
- Benemann, J. R. & Weare, N. M. Hydrogen Evolution by Nitrogen-Fixing *Anabaena cylindrica* Cultures. *Science* **184**, 174–175 (1974).
- Tsygankov, A. A., Serebryakova, L. T., Rao, K. K. & Hall, D. O. Acetylene reduction and hydrogen photoproduction by wild-type and mutant strains of *Anabaena* at different CO₂ and O₂ concentrations. *FEMS Microbiol. Lett.* **167**, 13–17 (1998).
- Dutta, D., De, D., Chaudhuri, S. & Bhattacharya, S. Hydrogen production by Cyanobacteria. *Microb. Cell Fact.* **4**, 36 (2005).
- Peterson, R. B. & Burris, R. H. Hydrogen metabolism in isolated heterocysts of *Anabaena* 7120. *Arch. Microbiol.* **116**, 125–132 (1978).
- Houchins, J. P. & Burris, R. H. Occurrence and localization of two distinct hydrogenases in the heterocystous cyanobacterium *Anabaena* sp. strain 7120. *J. Bacteriol.* **146**, 209–214 (1981).
- Masukawa, H., Mochinaru, H. & Sakurai, H. Disruption of the uptake hydrogenase gene, but not of the bidirectional hydrogenase gene, leads to enhanced photobiological hydrogen production by the nitrogen-fixing cyanobacterium *Anabaena* sp. PCC 7120. *Appl. Microbiol. Biotechnol.* **58**, 618–624 (2002).
- Fay, P. Oxygen relations of nitrogen fixation in cyanobacteria. *Microbiol. Rev.* **56**, 340–373 (1992).
- Tamagnini, P. et al. Hydrogenases and Hydrogen Metabolism of Cyanobacteria. *Microbiol. Mol. Biol. Rev.* **66**, 1–20 (2002).
- Weissman, J. C. & Benemann, J. R. Hydrogen production by nitrogen-starved cultures of *Anabaena cylindrica*. *Appl. Environ. Microbiol.* **33**, 123–131 (1977).
- Ghirardi, M. L. et al. Hydrogenases and Hydrogen Photoproduction in Oxygenic Photosynthetic Organisms. *Annu. Rev. Plant Biol.* **58**, 71–91 (2007).
- Golden, J. W., Whorff, L. L. & Wiest, D. R. Independent regulation of *nifHDK* operon transcription and DNA rearrangement during heterocyst differentiation in the cyanobacterium *Anabaena* sp. strain PCC 7120. *J. Bacteriol.* **173**, 7098–7105 (1991).
- Melis, A., Zhang, L., Forestier, M., Ghirardi, M. L. & Seibert, M. Sustained Photobiological Hydrogen Gas Production upon Reversible Inactivation of Oxygen Evolution in the Green Alga *Chlamydomonas reinhardtii*. *Plant Physiol.* **122**, 127–136 (2000).
- Bandyopadhyay, A., Stöckel, J., Min, H., Sherman, L. A. & Pakrasi, H. B. High rates of photobiological H₂ production by a cyanobacterium under aerobic conditions. *Nat. Commun.* **1**, 139 (2010).
- Craggs, R. J., McAuley, P. J. & Smith, V. J. Wastewater nutrient removal by marine microalgae grown on a corrugated raceway. *Water Res.* **31**, 1701–1707 (1997).
- Rippka, R. & Stanier, R. Y. The Effects of anaerobiosis on nitrogenase synthesis and heterocyst development by nostocacean cyanobacteria. *J. Gen. Microbiol.* **105**, 83–94 (1978).
- Surzycki, R., Cournac, L., Peltier, G. & Rochoix, J. D. Potential for hydrogen production with inducible chloroplast gene expression in *Chlamydomonas*. *P. Natl. Acad. Sci.* **104**, 17548–17553 (2007).
- Hihara, Y., Kamei, A., Kanehisa, M., Kaplan, A. & Ikeuchi, M. DNA Microarray Analysis of Cyanobacterial Gene Expression during Acclimation to High Light. *Plant Cell* **13**, 793–806 (2001).
- Murata, N., Takahashi, S., Nishiyama, Y. & Allakhverdiev, S. I. Photoinhibition of photosystem II under environmental stress. *BBA-Bioenergetics* **1767**, 414–421 (2007).
- Sicora, C. I., Ho, F. M., Salminen, T., Styring, S. & Aro, E. M. Transcription of a “silent” cyanobacterial *psbA* gene is induced by microaerobic conditions. *BBA-Bioenergetics* **1787**, 105–112 (2009).
- Hanawa, Y., Watanabe, M., Karatsu, Y., Fukuzawa, H. & Shiraiwa, Y. Induction of a high-CO₂-inducible, periplasmic protein, H43, and its application as a high-CO₂-responsive marker for study of the high-CO₂-sensing mechanism in *Chlamydomonas reinhardtii*. *Plant Cell Physiol.* **48**, 299–309 (2007).
- Greenwell, H. C., Laurens, L. M. L., Shields, R. J., Lovitt, R. W. & Flynn, K. J. Placing microalgae on the biofuels priority list: a review of the technological challenges. *J. R. Soc. Interface* **7**, 703–726 (2010).
- Burrows, E. H., Chaplen, F. W. R. & Ely, R. L. Optimization of media nutrient composition for increased photofermentative hydrogen production by *Synechocystis* sp. PCC 6803. *Int. J. Hydrogen Energy* **33**, 6092–6099 (2008).
- Yen, H. W. & Brune, D. E. Anaerobic co-digestion of algal sludge and waste paper to produce methane. *Bioresour. Technol.* **98**, 130–134 (2007).
- Allen, M. M. Simple conditions for growth of unicellular blue-green algae on plates. *J. Phycol.* **4**, 1–4 (1968).
- Ma, W., Shi, D., Wang, Q., Wei, L. & Chen, H. Exogenous expression of the wheat chloroplast fructose-1,6-bisphosphatase gene enhances photosynthesis in the transgenic cyanobacterium *Anabaena* PCC 7120. *J. Appl. Phycol.* **17**, 273–280 (2005).
- Arnon, D. I. Copper enzymes in isolated chloroplasts polyphenoloxidase in *Beta Vulgaris*. *Plant Physiol.* **24**, 1–15 (1949).
- Wang, L. et al. Treatment with moderate concentrations of NaHSO₃ enhances photobiological H₂ production in the cyanobacterium *Anabaena* sp. strain PCC 7120. *Int. J. Hydrogen Energy* **35**, 12777–12783 (2010).

51. Lehner, J. *et al.* The morphogene AmiC2 is pivotal for multicellular development in the cyanobacterium *Nostoc punctiforme*. *Mol. Microbiol.* **79**, 1655–1669 (2011).
52. Sukenik, A., Kaplan-Levy, R. N., Welch, J. M. & Post, A. F. Massive multiplication of genome and ribosomes in dormant cells (akinetes) of *Aphanizomenon ovalisporum* (Cyanobacteria). *ISME J.* **6**, 670–679 (2012).
53. Mi, H., Endo, T., Ogawa, T. & Asada, K. Thylakoid Membrane-Bound, NADPH-Specific Pyridine Nucleotide Dehydrogenase Complex Mediates Cyclic Electron Transport in the Cyanobacterium *Synechocystis* sp. PCC 6803. *Plant and Cell Physiol.* **36**, 661–668 (1995).
54. Schreiber, U., Schliwa, U. & Bilger, W. Continuous recording of photochemical and non-photochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. *Photosynth. Res.* **10**, 51–62 (1986).
55. Schreiber, U., Endo, T., Mi, H. & Asada, K. Quenching Analysis of Chlorophyll Fluorescence by the Saturation Pulse Method: Particular Aspects Relating to the Study of Eukaryotic Algae and Cyanobacteria. *Plant Cell Physiol.* **36**, 873–882 (1995).
56. Campbell, D., Hurry, V., Clarke, A. K., Gustafsson, P. & Oquist, G. Chlorophyll Fluorescence Analysis of Cyanobacterial Photosynthesis and Acclimation. *Microbiol. Mol. Biol. Rev.* **62**, 667–683 (1998).
57. Kuhl, M., Chen, M., Ralph, P. J., Schreiber, U. & Larkum, A. W. D. Ecology: A niche for cyanobacteria containing chlorophyll d. *Nature* **433**, 820–820 (2005).
58. Schreiber, U., Quayle, P., Schmidt, S., Escher, B. I. & Mueller, J. F. Methodology and evaluation of a highly sensitive algae toxicity test based on multiwell chlorophyll fluorescence imaging. *Biosens. and Bioelectron.* **22**, 2554–2563 (2007).
59. Liu, J., Bukatin, V. E. & Tsygankov, A. A. Light energy conversion into H₂ by *Anabaena variabilis* mutant PK84 dense cultures exposed to nitrogen limitations. *Int. J. Hydrogen Energy* **31**, 1591–1596 (2006).
60. Yoon, J. H., Shin, J. H., Kim, M. S., Sim, S. J. & Park, T. H. Evaluation of conversion efficiency of light to hydrogen energy by *Anabaena variabilis*. *Int. J. Hydrogen Energy* **31**, 721–727 (2006).

Acknowledgments

We are grateful to Prof. James W. Golden (University of California, San Diego) for his kindly gift of mutant AMC-100; to Prof. Weimin Ma (Research Center of Spore Plant Biology, Shanghai Normal University) for providing us the microalgae species. We thank Dr. Zengjuan Fu (Institute of Botany, CAS) for providing us the excellent technical support on analysis of PSII activity. We thank Dr. Rui Shi (Center of Biomedical Analysis, Tsinghua University) for providing us the excellent microscopy technical service. The authors thank Prof. Feng Chen (Peking University) for his critical reading of the paper. This work was supported by National Key Technology R&D Program (Grant No. 2012 BAC18B01) and International scientific and technological cooperation projects (Grant No. 2010DFB64040) of the Ministry of Science and Technology of China.

Author contributions

M.C. performed the experiments with input from C.L. and J.L. M.C., J.L., L.Z. and S.C. designed the experiments, analysed the data and wrote the paper. S.L. and J.W. provided the overall guidance on study design, execution and wrote the paper.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Chen, M. *et al.* Auto-flotation of heterocyst enables the efficient production of renewable energy in cyanobacteria. *Sci. Rep.* **4**, 3998; DOI:10.1038/srep03998 (2014).

This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 3.0 Unported license. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/3.0>