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Supplementary Information

Engineering a chemoenzymatic cascade for sustainable photobiological hydrogen production with green algae

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1. Experimental Section

1.1 Materials
$Mg(OH)_2$, $Al(OH)_3$, $Ca(OH)_2$, $Fe(OH)_3$, $MgCl_2$, and glucose were purchased from Sinopharm Chemical Reagent Co., Ltd. WO_3 was purchased from Shanghai Aladdin Bio-Chem Technology Co., Ltd. Tris-acetate-phosphate liquid medium (TAP, (1×), pH 6.8) was purchased from Shanghai Guangyu Biological Technology Co., Ltd. Dimethyl sulfoxide (DMSO, Invitrogen™), phosphate buffered saline (PBS, (1×), pH 7.4, Gibco®), Fluorescein Diacetate (FDA, Invitrogen™) ,Propidium Iodide (PI, Invitrogen™), and Rhodamine 123 (Rh123, Invitrogen™) were purchased from Thermo Fisher Scientific. Glucose oxidase (GOx, Sigma-Aldrich, product number: G7141-50KU) and catalase (CAT, Sigma-Aldrich, product number: C40-100MG) were purchased from Sigma-Aldrich. Chlorophyll Analytical kit (Product number: GL3175) purchased from Beijing BioRab Technology Co. Ltd. CellTiter 96® AQueous One Solution Reagent purchased from Promega (Product number: G3582). *C. reinhardtii* was obtained from Freshwater Algae Culture Collection at the Institute of Hydrobiology, Chinese Academy of Sciences (FACHB, collection number: 479). *C. pyrenoidosa* (collection number: GY-D12) was obtained from Shanghai Guangyu Biological Technology Co., Ltd.

1.2 Culture of *C. reinhardtii*
C. reinhardtii cells were cultured photo-autotrophically in TAP liquid medium, that is, cells were incubated under illumination of 1000 Lux with cool-white fluorescent light for 12 h and then incubated in the dark for another 12 h at 25°C. The growth of *C. reinhardtii* cells was checked by measuring the cultures absorbance at 750 nm (OD750) using a 2600 UV-Vis spectrophotometer (Shimadzu, Japan). *C. pyrenoidosa* cells were cultured using the same culture conditions.

1.3 Sample preparation for photobiological H_2 production
When OD750 reached 0.25, 3 mL cultures of *C. reinhardtii* were transferred to 8 mL gastight glass tubes, leaving 5 mL of headspace. Then glucose (final concentration 50 mM), $Mg(OH)_2$ (150 μmol), GOx (final concentration 1 $mgmL^{-1}$ and enzyme activity circa 0.1 $KUmL^{-1}$) and CAT (final concentration 1 $mgmL^{-1}$ and enzyme activity circa 10 $KUmL^{-1}$) were added. As control groups, the same dosage of $Al(OH)_3$, $Ca(OH)_2$, $Fe(OH)_3$, and $MgCl_2$ (replacement of $Mg(OH)_2$) were added to gastight glass tubes, respectively. Then gastight glass tubes were sealed with rubber stoppers and incubated at 25°C under illumination of 6300 Lux with cool-white fluorescent light to induce photobiological H_2 production. Light intensity was measured by a MS6612 multi-functional light meter (Shenzhen Huayi Peakmeter Technology Co., Ltd., China). WO_3 powders (50 mg) were added to cell cultures as an indicator of H_2 production.

1.4 Gas quantitative analysis
To measure the amount of H_2 and O_2 content in headspace of sealed gastight glass tubes, 100 μL of gas was withdrawn from the headspace at predetermined time intervals with a gastight syringe, then injected into an Agilent 7890A gas chromatograph (Agilent Technologies Inc., USA) with a thermal conductivity detector

(TCD) for determining the concentrations of H_2 and O_2 simultaneously. The packed column was J&W CP-Molsieve 5Å (length 50 m, diameter 0.53 mm, film 50.00 μm) and was used in splitless mode. Highly pure N_2 gas was used as the carrier gas at a flow rate of 10 $mLmin^{-1}$. The temperatures of the injector, the TCD detector and column were kept at 100°C, 200°C and 100°C, respectively. The amount of H_2 and O_2 content were calculated according to the peak area, which was pre-calibrated by injecting known concentrations of standard gases.

1.5 pH analysis

The pH values of *C. reinhardtii* cultures were determined by using a pH meter (Thermo Orion 3-Star pH Benchtop, Thermo Scientific™) equipped with a micro sensor (Mettler Toledo, InLab® Micro) calibrated with pH 4.01, 7.00 and 10.01 buffers (Thermo Scientific™).

1.6 Chlorophyll content measurements and absorption spectra analysis

3 mL *C. reinhardtii* cultures from the H_2 production sample was centrifuged (2500 g, 5 min), and the algae cells were harvested, weighed and then mixed up with 6 mL chlorophyll assay buffer (from the Chlorophyll Analytical kit). The mixture was incubated for 10 minutes, then shook for 5 minutes, and incubated for 10 minutes again. The whole process was carried out at room temperature and in the dark. Next, the product mixture was filtered with filter papers, leaving the filtrate as the crude chlorophyll extract. The crude chlorophyll extract was then added to a 96-well plate, zeroed with the chlorophyll assay buffer, and the absorbance of the crude extract at 665 nm and 649 nm was measured by the Bio Tek Synergy H1 MD microplate reader (BioTek Instruments, Inc., USA). Chlorophyll content was then quantified using the equation as follows:

total chlorophyll content (mg/g) = $CT \times V \times N/(W \times 1000)$; $CT = 6.63 \times A665 + 18.08 \times A649$

V = chlorophyll crude extract volume (ml); N = dilution factor; W = sample fresh or dry weight (g)

Absorption spectrum of *C. reinhardtii* was measured with the 2600 UV-Vis spectrophotometer from 500 nm to 820 nm. All presented data were mean values of triplicate experiments.

1.7 Cell viability analysis

Cell viability analysis was performed by using the CellTiter 96® AQueous One Solution Cell Proliferation Assay with some modifications [1]. Briefly, 500 μL *C. reinhardtii* cultures from the H_2 production sample was centrifuged (2500 g, 5 min) and harvested, then the algae cells were washed for three times and re-suspended in 200 μL PBS buffer. Subsequently, 20 μl of the CellTiter 96® Solution were added, after an hour incubation under dark conditions at 25°C, the sample solution was transferred to a black 96-well plate and recorded the absorbance at 490 nm with the Bio Tek Synergy H1 MD microplate reader.

In addition, the fluorescence images about the algae cells were obtained by using FDA, PI, and Rh123 co-staining methods with some modifications [2-4]. 1 mL *C. reinhardtii* cultures from the H_2 production sample was centrifuged (2500 g, 5 min). The algae cells were harvested and added to fresh TAP liquid medium (1 mL)

containing 50 μgmL^{-1} of FDA and Rh123 (pre-dissolved in DMSO), 50 μgmL^{-1} of PI (pre-dissolved in PBS), then incubated for 30 min under dark conditions at 37°C. Thereafter, the samples were washed three times and re-suspended in 1 mL PBS buffer. After a 100 times dilution, these samples were recorded by a Leica SP8 confocal microscope (Leica Microsystems, Germany).

1.8 Microscopy analyses of flocculation

To analyze the flocculation of *C. reinhardtii*, 10 μL of *C. reinhardtii* cultures was withdrawn from the H_2 production samples at predetermined time intervals with a gastight syringe, then observed by Zeiss AXIOSKOP 2 plus fluorescence microscope (ZEISS, Germany).

2. Supplementary Figures

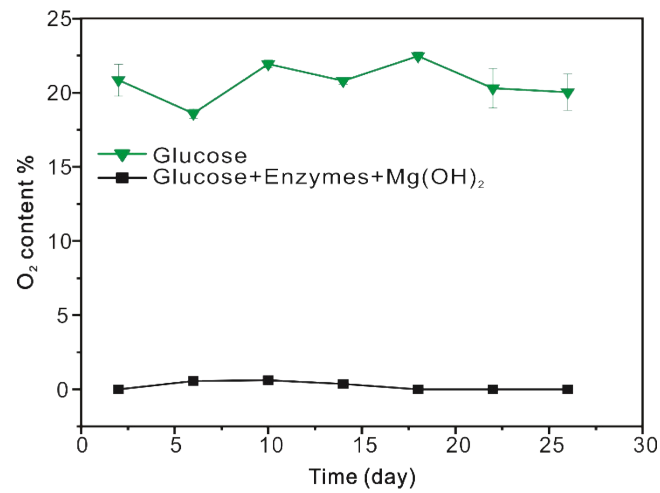

Fig. S1. Curves of O_2 content in the headspace of *C. reinhardtii* cultures with different additives (n=3). O_2 content in the headspace of *C. reinhardtii* cultures with glucose alone was remained at ca. 21%, due to the absence of the enzymes (green line). O_2 content in the headspace of *C. reinhardtii* cultures with glucose, enzymes, and $Mg(OH)_2$ was basically zero, because of the significant *C. reinhardtii* flocculation (dark line).

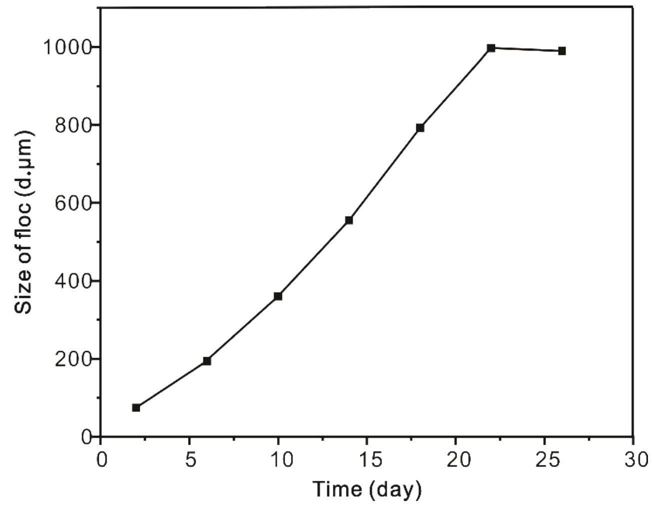

Fig. S2. Curve of *C. reinhardtii* floc size over time. Within the first 22 days, the floc size increased significantly over time.

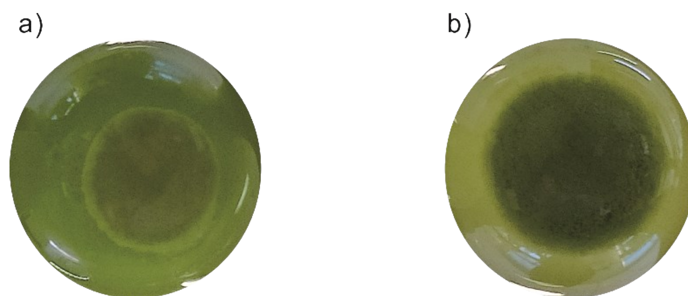

Fig. S3. Photographs of *C. reinhardtii* cultures without enzymes. a) *C. reinhardtii* cultures with $Mg(OH)_2$ alone. The green color of culture suggests *C. reinhardtii* cells were uniformly dispersed. WO_3 precipitates at the bottom with a lighter colour indicate no H_2 production. b) *C. reinhardtii* cultures with $Mg(OH)_2$ together with glucose. The color of cultures is much clearer, suggesting that some *C. reinhardtii* cells had settled. WO_3 precipitates at the bottom with a deeper colour indicate H_2 production.

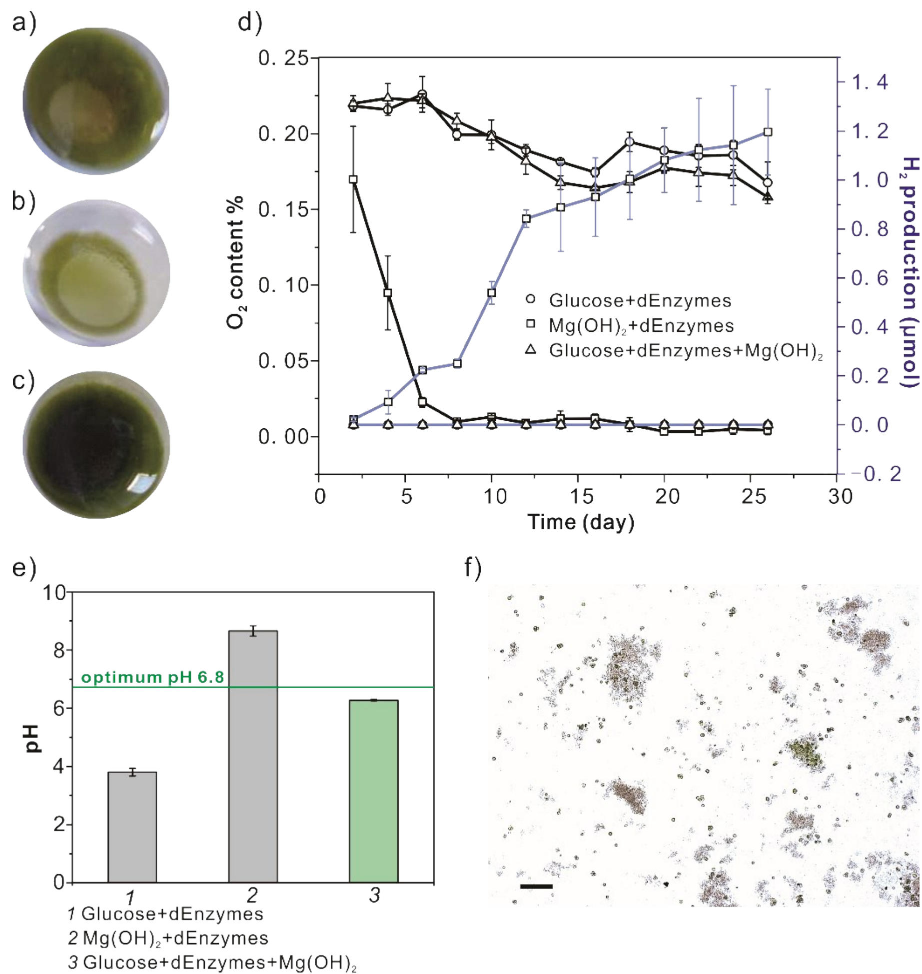

Fig. S4. Tests with denatured enzymes. a) Photograph of the *C. reinhardtii* cultures with glucose combined with denatured glucose oxidase and denatured catalase (denatured enzymes). WO_3 precipitates at the bottom with a lighter colour suggests no H_2 production. b) Photograph of the *C. reinhardtii* cultures with $Mg(OH)_2$ combined with denatured enzymes, the WO_3 with a yellow colour indicates no H_2 production. c) *C. reinhardtii* cultures with $Mg(OH)_2$ together with glucose and combined with denatured enzymes. The colour of WO_3 precipitates is much deeper that indicates H_2 production. d) Curves of H_2 production (Baby blue line) and O_2 (black line) content in the headspace of the culture systems with different additives (n=3). In the system that $Mg(OH)_2$ together with glucose and combined with denatured enzymes, O_2 continues to decrease, and H_2 continues to be produced. e) The pH values of the culture systems containing different additives (n=3). The pH of *C. reinhardtii* cultures with $Mg(OH)_2$ together with glucose and combined with denatured enzymes is near the optimum pH for H_2ase. f) Optical microscope photographs of *C. reinhardtii* with $Mg(OH)_2$ together with glucose and combined with denatured enzymes. There are obvious *C. reinhardtii* flocs in the system. Scale bar: 100 μm.

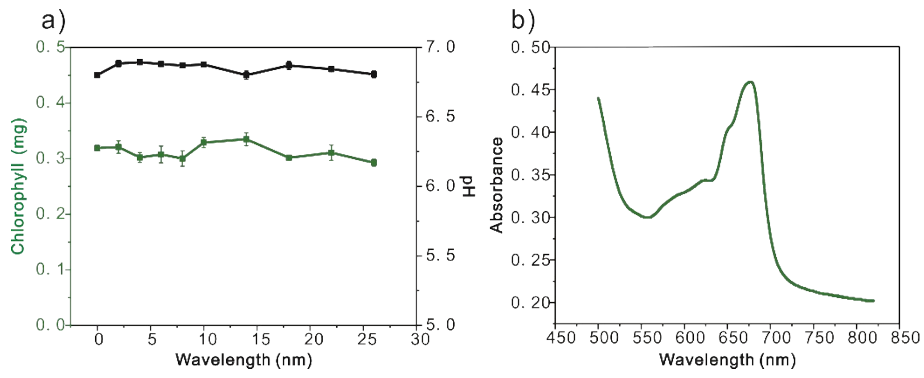

Fig. S5. Characterizations of *C. reinhardtii* cultured in normal condition. a) Curves of total chlorophyll contents and pH values of *C. reinhardtii* cultured in normal condition (n=3). The total chlorophyll contents and pH values were basically stable. b) Absorption spectrum of *C. reinhardtii* cultured in normal condition. The absorption peak of PSll is at 680 nm.

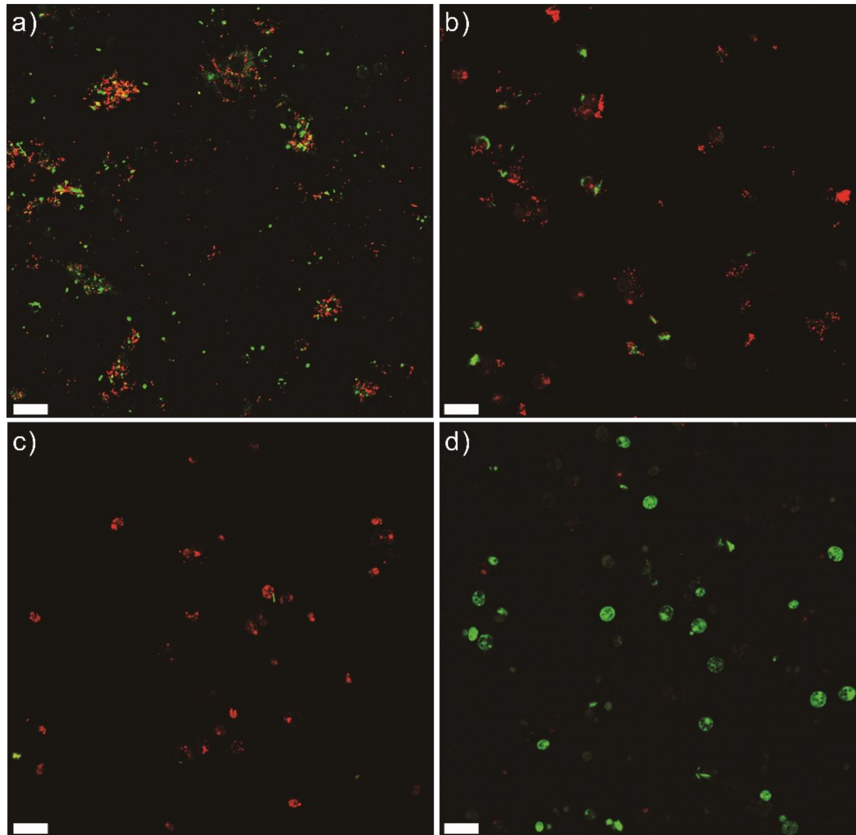

Fig. S6. Confocal fluorescence microscopy images reveal cell viability. a) *C. reinhardtii* cultures with glucose, enzymes, and $Mg(OH)_2$. There were both living (green) and dead (red) cells in the cultures, but due to cells flocculation, a large number of cells failed to stain. b) *C. reinhardtii* cultures with glucose, enzymes, and $MgCl_2$. More dead cells were observed than living cells in the cultures. c) *C. reinhardtii* cultures with glucose and enzymes. Living cells could hardly be observed in the cultures. d) *C. reinhardtii* cultures with glucose alone. Living cells existed dominantly in the cultures. But the number of cells was not as high as in the *C. reinhardtii* cultures with glucose, enzymes, and $Mg(OH)_2$. Scale bar: 20 μm.

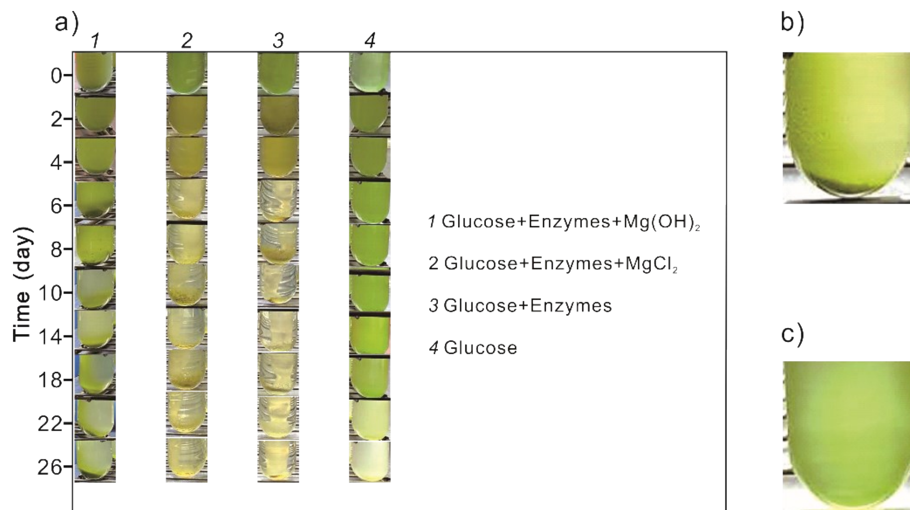

Fig. S7. Photographs of *C. reinhardtii* culture systems with different additives. a) Photographs taken on different days. In those culture systems with glucose, enzymes, but no $Mg(OH)_2$, they eventually lost their green colour. b) Photograph of the *C. reinhardtii* cultures with glucose, enzymes, and $Mg(OH)_2$ on day 26. After shaking, the culture system was still light green. c) Photograph of the *C. reinhardtii* grown normally for 26 days. The system showed a uniform light green colour.

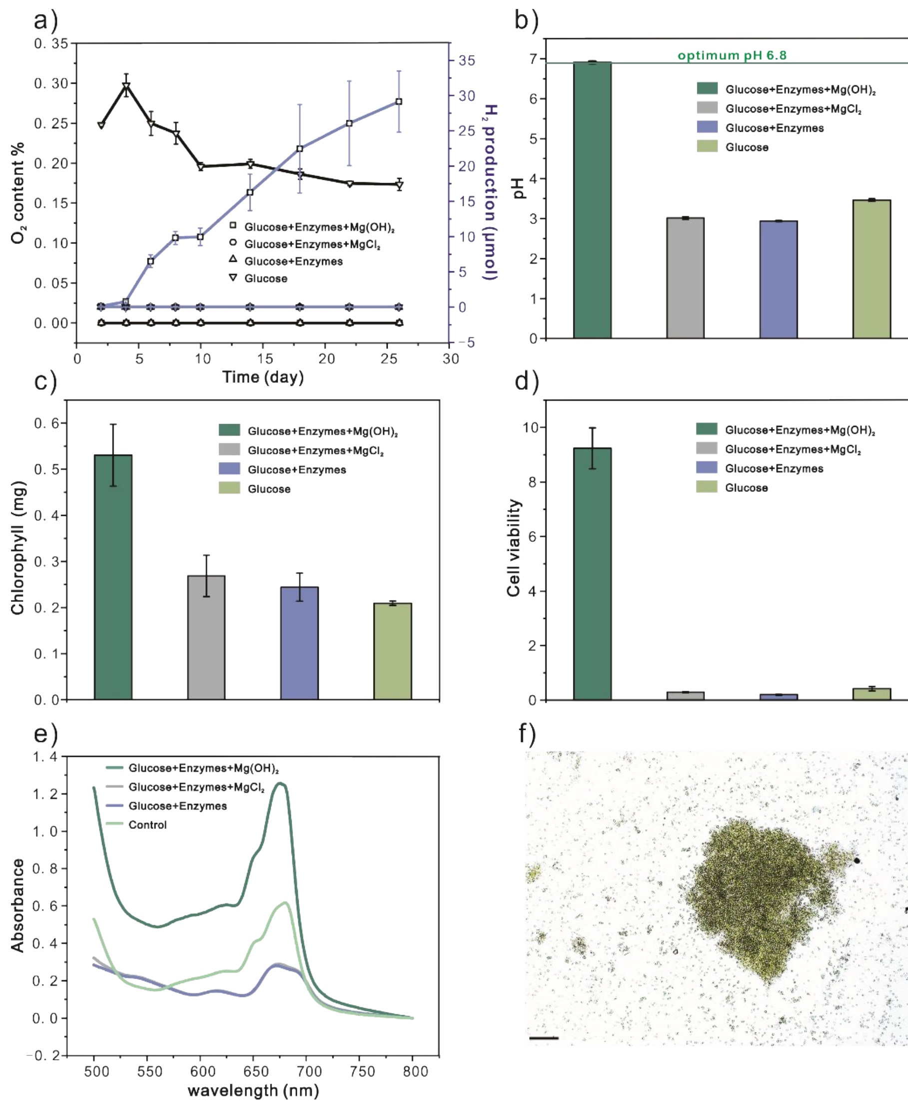

Fig. S8. H_2 production tests with *C. pyrenoidosa*. a) Curves of H_2 production and O_2 content in the headspace of the *C. pyrenoidosa* culture systems with different additives (n=3). *C. pyrenoidosa* cultures with glucose, enzymes, and $Mg(OH)_2$ (CEC strategy) sustained to produce 29 μmol H_2 under 26 days of continuous light. b) The pH values of *C. pyrenoidosa* culture systems containing different additives (n=3). The pH of *C. pyrenoidosa* cultures with CEC strategy is near the optimum pH for H_2ase. c) The chlorophyll content of *C. pyrenoidosa* culture systems containing different additives (n=3). The chlorophyll content of *C. pyrenoidosa* cultures with CEC strategy is the highest. d) The cell viability of the *C. pyrenoidosa* culture systems with different additives (n=3). The cell viability of *C. pyrenoidosa* cultures with CEC strategy is significantly higher than other groups. e) Absorption spectrum of *C. pyrenoidosa* culture systems with different additives and normal grown *C. pyrenoidosa* (control) (n=3). The absorption peaks of *C. pyrenoidosa* cultures with CEC strategy are almost same as normal grown *C. pyrenoidosa*. f) Optical microscope photographs of *C. pyrenoidosa* with CEC strategy. There are obvious *C. pyrenoidosa* flocs in the culture system. Scale bar: 100 μm.

3. Supplementary References

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