Supporting information

Study on confined interface electron enhanced ethanol to hydrogen production of *Rhodopseudomonas palustris*

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Materials and methods

Bacterial strains and growth conditions.

The bacterial strain was *Rhodopseudomonas palustris* TIE-1. The cultured medium of *R. palustris* was K_2HPO_4 (1g/liter), $MgSO_4$ (0.5g/liter), and yeast extract (10g/liter). The pH of medium was adjusted to 7.4 using NaOH. The number of cells was determined by optical density measurement at 660 nm (OD_{660}) using UV-Visible spectroscopy (Eppendorf D30). The cultured light intensity was 5000 lux and initial OD_{660} was 0.2.

Coating of *R. palustris*.

The *R. palustris* were thoroughly washed with NaCl aqueous solution (0.01 M) and DI water for three times by a centrifugation method (5000×g, 5 min). The 5 mL of OD_{660} 3.0 *R. palustris* solution with 3 mg/mL Fe (III) chloride nonahydrate (pH 7.0), and left stirring at 200 rpm for 30 min, followed by centrifugation (5000×g, 5 min) to remove unbound Fe (III). The Fe (III)-treated *R. palustris* were re-suspended in 5 mL of DI water, and 20 μL of pyrrole was then added into the mixture. The mixture was stirred (200 rpm) at room temperature for 8 h, during which in situ Fe (III)-mediated oxidative polymerization of pyrrole occurred on the surface.

Hydrogen production conditions for native *R. palustris*, PPy-coated-*R. palustris* and aggregates.

The hydrogen-reaction medium was maintained at pH 7.0±0.2 and contained the following components (per liter): K_2HPO_4, 19.3 g; KH_2PO_4, 9.4 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; NaCl, 1.0 g; NH_4Cl, 1.6 g; $CaCl_2$, 1.0 g. Ethanol was sterilized separately and added in 30 $mmol \cdot L^{-1}$. Then the strains (the *R. palustris*@PPy required pretreatment centrifuge cleaning two to three times) were resuspended into 10 mL M9 of medium and subsequently was injected into 50 mL of serum bottle

(40 mL head air space) after centrifugation (10000×g, 1 min). The serum bottles were flushed with argon (Ar) gas (99.9%) for 30 min to ensure that the bottles were completely deprived of O_2. And then all the bottles were placed in a constant temperature (35 ℃) incubator with an adjustable plant light (Octopus 800X). The different light intensities were measured by HOPOOCOLOR (OHSP-350p). Subsequently, the Time-dependent change in H_2 concentration in the samples was monitored using a hydrogen detector (APES-B; capacity, 20% V; resolution ratio, 3%).

Construction and Electrochemical property of the aggregation system and other systems.

The *R. palustris*@PPy were re-suspended with 1.5% sodium alginate solution (500 μL M9 and 500 μL 3% sodium alginate). Inhale with a 1 mL needle and extrude into 1.5% Ca^{2+} solution. The diameter was 2 mm small sphere would form and transformed into hydrogen bottle.

The bio-electrodes were fabricated through a drop-coating process: carbon cloth electrodes (Innochem, 1 cm×2 cm, the thickness 0.35±0.02 mm, longitudinal resistance 0.12×10^{-2} Ω) were washed with acetone and ultrapure water and then were purified with 10 % H_2SO_4 and 10 % HNO_3 (v/v = 1:3) for 3 h. Then, the carbon cloth electrodes were washed with ultrapure water to pH = 7.0. Finally, the cells or micro-niches ink consisting of 0.5 % Nafion membrane solution was dropped on a carbon cloth electrode, and allowed to dry at 4 ℃.

Scanning electron microscopy (SEM) images were obtained on a SU8000 instrument with the samples sputter-coated with 10 nm platinum. The hydrogel aggregates were quick-frozen with liquid nitrogen and then freeze-dried and sliced. The cut samples were examined by SEM.

Cyclic voltammetry measurement of *R. palustris* and *R. palustris*@PPy

The electrochemical workstation CHI660F (CH Instruments) was used for the measurement. The CV test for different systems with a scan rate of 100 mV s^{-1} were implemented within the potential

region from - 0.5 V to 0.1 V. For the EIS test, frequency: 100 kHz-1Hz; amplitude: 5 mV. 40 mL of M9 buffer (Na_2HPO_4, 6 g/L; KH_2PO_4, 3 g/L; NaCl, 0.5 g/L; NH_4Cl, 1 g/L; $MgSO_4$, 1 mM; $CaCl_2$, 0.1 mM) was used as electrolyte for CV and EIS. Especially, the carbon cloth was soaked in sodium alginate solution with OD_{660} 3.0 bacteria (half the area of the carbon cloth is immersed in the 1 cm × 1 cm × 1 cm mold), and then the Ca^{2+} solution was added drop by drop until a condensate was formed. In comparison with the preparation aggregate electrodes, the free *R. palustris* were resuspended with 200 μL of H_2O (0.5% Nafion) and then was dropped on a carbon cloth electrode, and all allowed to dry at 4 ℃.

Fourier transform infrared spectroscopy (FTIR) measurement.

The samples PPy and *R. palustris*@PPy were dried overnight in a constant temperature drying oven (JC 101-0B, 50 ℃), and then the dried sample was ground into powder. KBr were placed in a vacuum drying oven (120 ℃, 2 h), and appropriate amount of KBr was mixed with the samples to make a thin tablet.

X-ray photoelectron spectroscopy (XPS) measurement

The instrument specification of XPS (KARTOS-AXISULTRA DLD) was microanalysis 15 μm, sensitivity 0.1%, power 450 W, and 0.5-4 kV ion sputtering. The *R. palustris*@PPy were cleaned with deionized water and were dried at 50 ℃ constant temperature drying oven. The dried sample was ground into powder for the measurement.

Hydrogen production for the aggregation systems.

The hydrogen produced medium was 10 mL pH 7.0 M9 solution with 30 mM ethanol. The light intensity was 7000 lux. All experiments were conducted in a serum bottle (50 mL; working volume, 10 mL) at 35℃.

The assay on the content of ethanol and NADH/NAD^+.

Quantification kits for ethanol (ACMEC) and NADH/NAD^+ (MAK037, Sigma-Aldrich) were used to determine the concentrations of the related intracellular compounds during the hydrogen production process. All the experiments were conducted following the procedures provided in the technical bulletins of the respective assay kits. The absorbance was measured using a UV-Vis Spectrophotometer (Tecan Safire Abs, Switzerland), and the concentrations of the individual compounds were calculated using standard curves.

Hydrogenase activity assay.

Hydrogenase activity in whole cells of photosynthetic bacteria is commonly assayed by following the reduction of methylene blue linked to H_2 oxidation. For the H_2 production activity assay of different systems, reaction solutions supplemented with 150 mM NaCl, 100 mM Tris-HCl (pH = 7.0), 5 mM methyl viologen, and 25 mM Na-dithionite were prepared in a 5 mL of thick-walled vial with a suction fitting and sealed with parafilm to hydrogen production under 30 ℃ for 20 min. The produced H_2 was monitored using a hydrogen detector (APES-B; capacity, 20000 ppm; resolution ratio, 1 ppm).

Cell viability tests.

Confocal scanning laser microscopy (CSLM) images were obtained on a Leica SP8 confocal laser scanning microscope attached to a Leica DMI 6000 inverted epifluorescence microscope. Stock solution of fluorescein diacetate (FDA) was prepared by dissolving FDA in acetone at a concentration of 5 mg/mL. To label the viable *R. palustris*, 5 μL of FDA solution was added into 1 mL of aqueous *R. palustris* dispersions. After incubation in dark at room temperature, the labeled cells were washed with DI water for three times and imaged in a CLSM. And the excitation

wavelength of FDA and PI was 488 nm and 532 nm, respectively. The sample of aggregates at different time were dissolved in 0.1 M sodium citrate solution, then centrifugation (10000×g, 1 min), discard the supernatant, and resuspend with 1 mL of water (repeat three times). Finally, the samples were used to measure the activity of biohybrid aggregation.

Microsensor-based measurement.

The O_2 microsensor was an OX-10 microsensor with a 10-μm tip diameter. To test the O_2 concentration, H_2 concentration, and redox potential within the different systems, the aggregate with a size of 2 mm in diameter and the same density (OD_{660} 3.0) as the micro-niches used for quantification of hydrogen evolution. The step size of the micromanipulator was 5 μm for O_2, H_2, and redox potential measurement. The freshly prepared micro-niche was used for measurements of H_2 production for at least 6 h. Then, the aggregates were harvested from the reaction system. A layer of micro-niches was fixed on the agar substrate (1.5 wt% agar). The diameter of microelectrodes was only 10 μm, and to protect the microelectrodes from damage during the downward movement, the samples must be placed on an agar base.

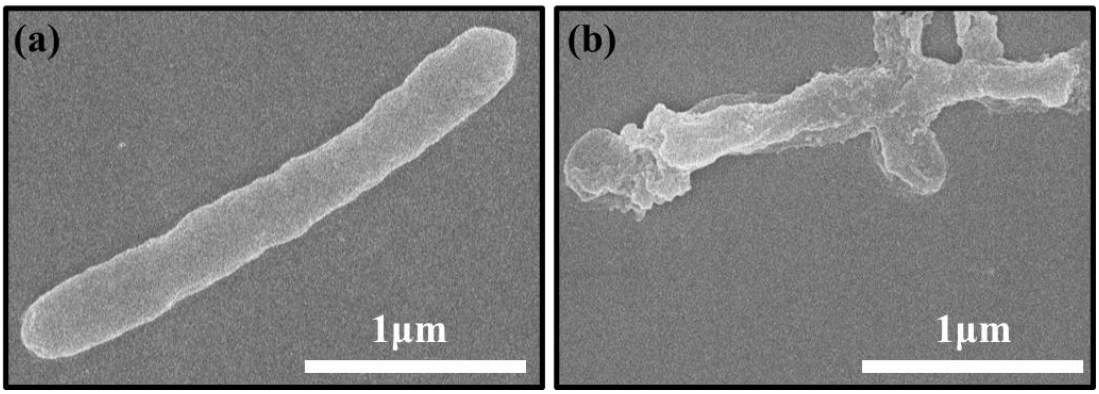

Figure S1. The SEM measurement of *R. palustris* and *R. palustris*@PPy. (a) native *R. palustris*; (b) *R. palustirs*@PPy.

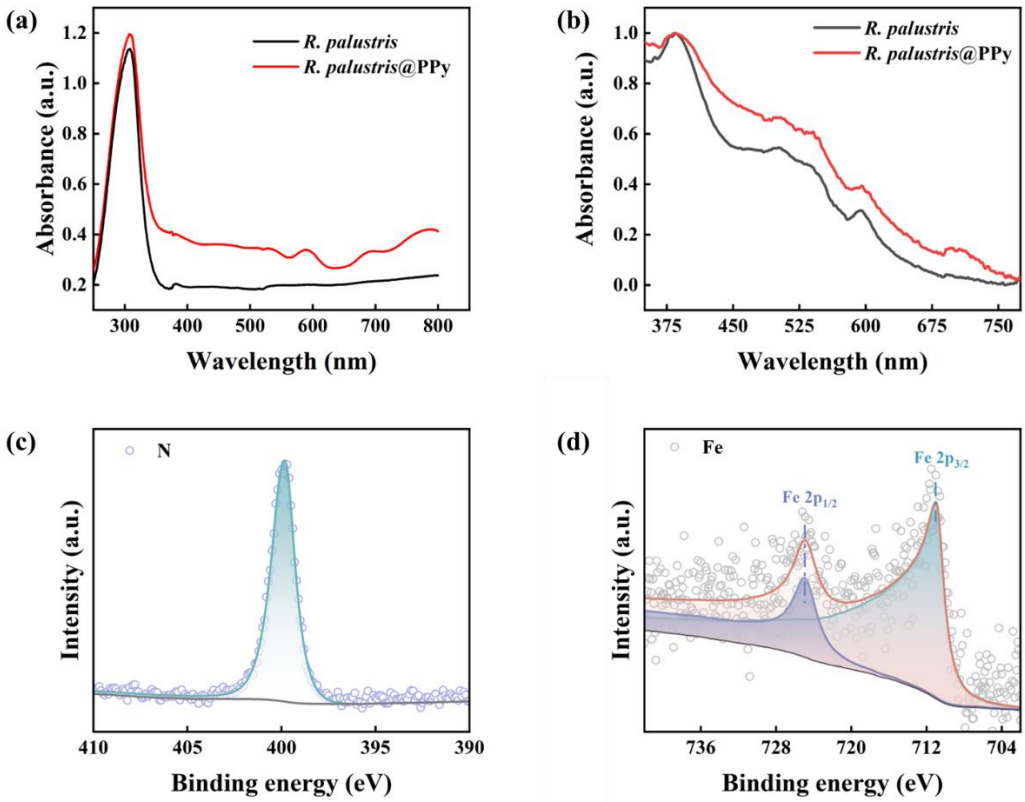

Figure S2. The characterization of bio-abiotic interface system. (a) The UV-Vis diffuse reflection of native *R. palustris* and *R. palustris*@PPy. The values of PPy-coated cells were much higher than native in the visible light range. (b) UV–Vis spectra of native and PPy-coated *R. palustris*. (c) and (d) The XPS measurement of *R. palustris*@PPy. The N element was detected. Elemental analysis for the *R. palustris*@PPy. Peaks for Fe are observed for PPy-coated *R. palustris*, which indicating the presence of a Fe (II)-doped PPy layer.

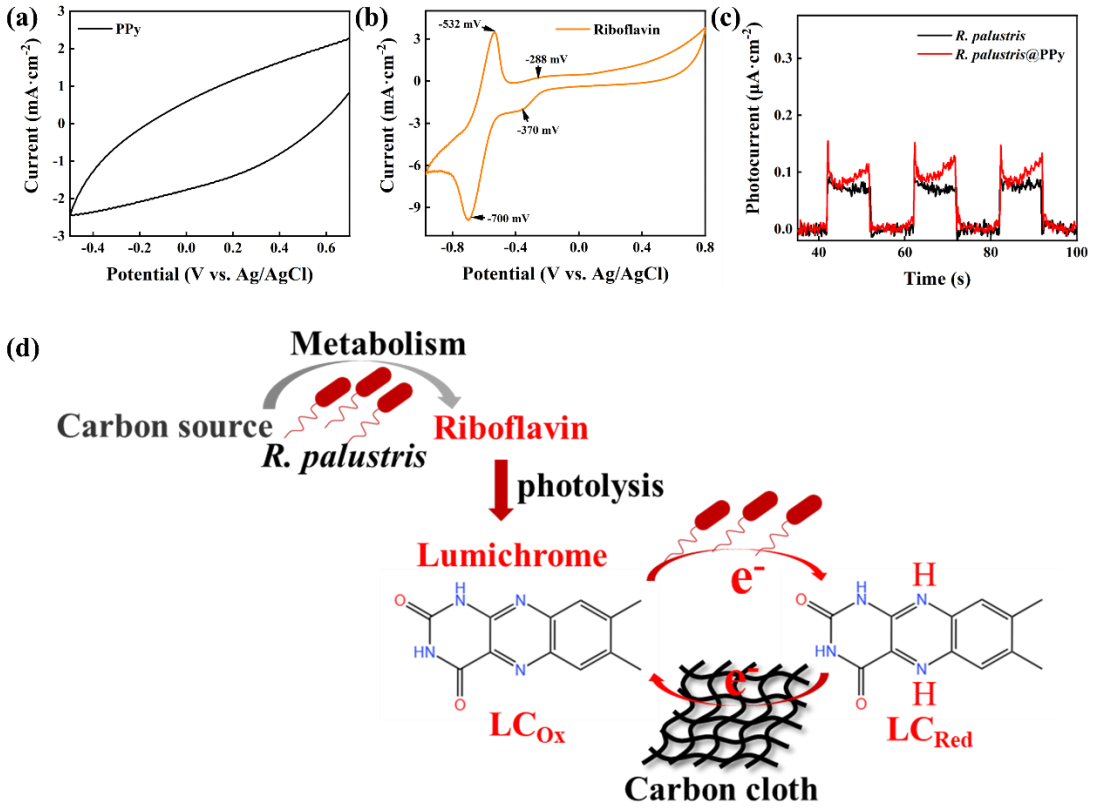

Figure S3. (a) and (b) The CV curves of PPy and riboflavin. The oxidation peaks of riboflavin were -288 mV and -532 mV. The reduction peaks of riboflavin were -370 mV and -700 mV. (c) Transient photocurrent densities of native cells and *R. palustris*@PPy electrodes with light on/off cycles under visible light irradiation in 0.1 M Na_2SO_4 electrolyte solution at an applied potential of 0.5 V versus Ag/AgCl reference electrode. (d) Schematic illustration showing the photocurrent generation of *R. palustris*@PPy. The *R. palustris*@PPy were attached to the carbon cloth and worked as electrode. With the metabolic growth of bacteria, the riboflavin could be secreted into extracellular environment and subsequently photolysis to produce lumichrome (LC_{Ox}) which played a crucial role in interface electron transfer. And the change of the state between the REDOX of the lumichrome would transfer electrons to the carbon cloth electrode, and then form the photocurrent effect.

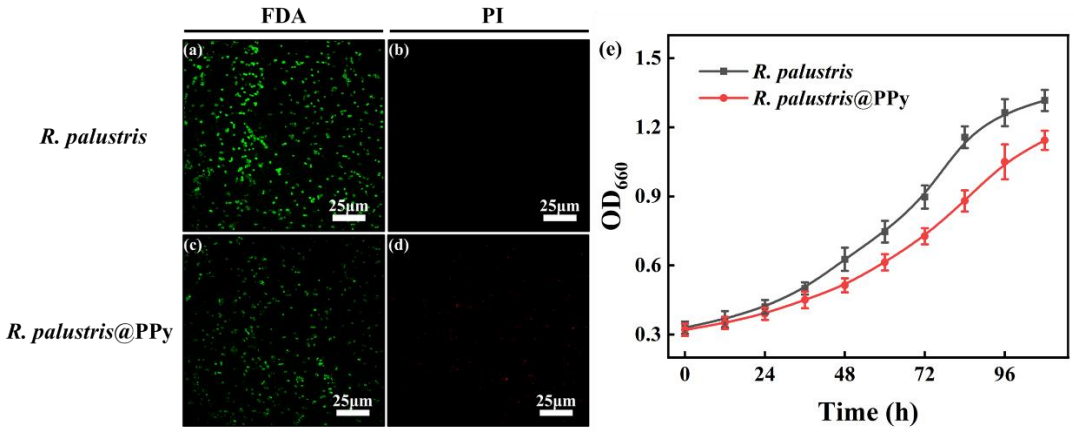

Figure S4. (a to d) The confocal scanning laser microscopy (CLSM) analysis of cell viability. Fluorescein diacetate (FDA) was prepared for live cells and propidium iodide (PI) was employed to dead cells. The 60 h *R. palustris* in culture medium were used as control. And the *R. palustris*@PPy (8 h total reaction time) were dyed by FDA and PI. (e) The OD_{660} growth curves of *R. palustris*@PPy and *R. palustris*. The initial OD_{660} of *R. palustris*@PPy and *R. palustris* were diluted to 0.3. The error bar indicated that the experiment takes three parallel measurements.

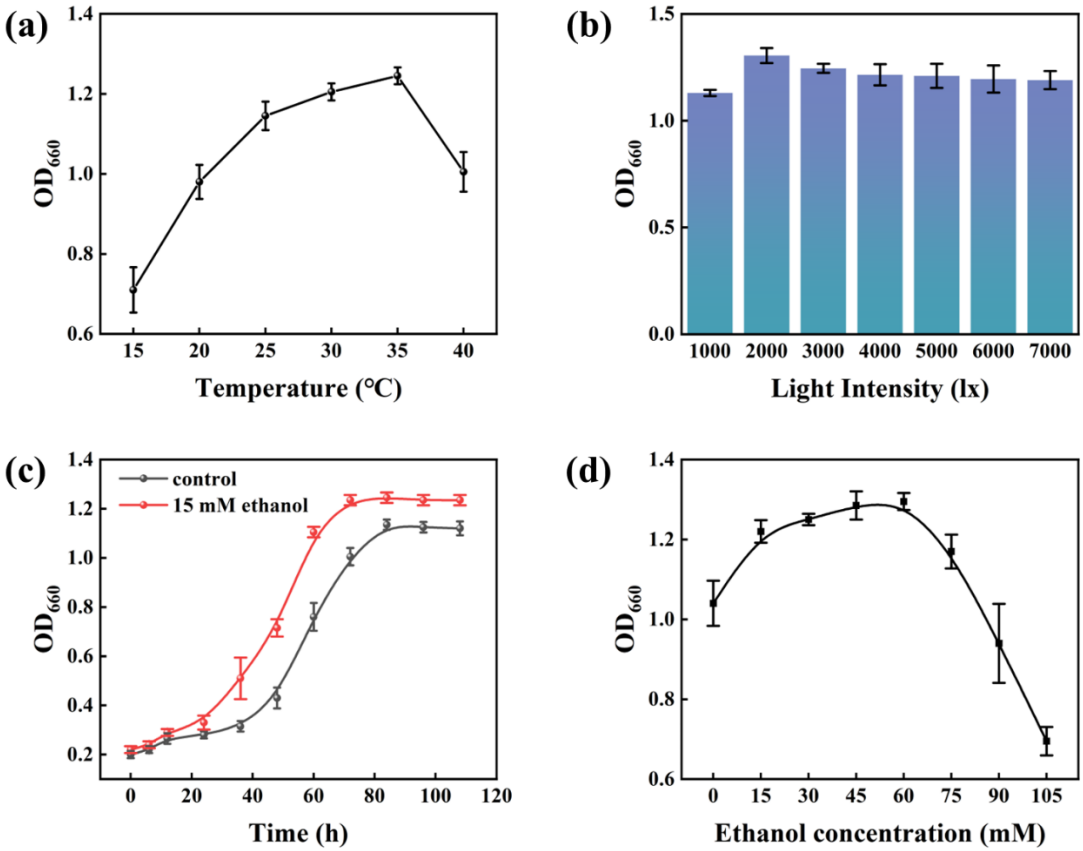

Figure S5. Growth characteristics of *R. palustris*. (a) The effect of temperature on *R. palustris* growth. The initial OD_{660} was 0.2 and the cultured light was 5000 lux. (b) The effect of light intensity on *R. palustris* growth. The initial OD_{660} was 0.2 and cultured temperature was 35 °C. The 2000-7000 range of light intensity has slightly influence on cell growth. (c) The effect of ethanol as only carbon source on cell growth. The initial OD_{660} was 0.2, temperature 35 °C and 5000 lux. (d) The effect of different ethanol concentration on cell growth. The initial OD_{660} was 0.2, temperature 35 °C and 5000 lux. The error bar indicated that the experiment takes three parallel measurements.

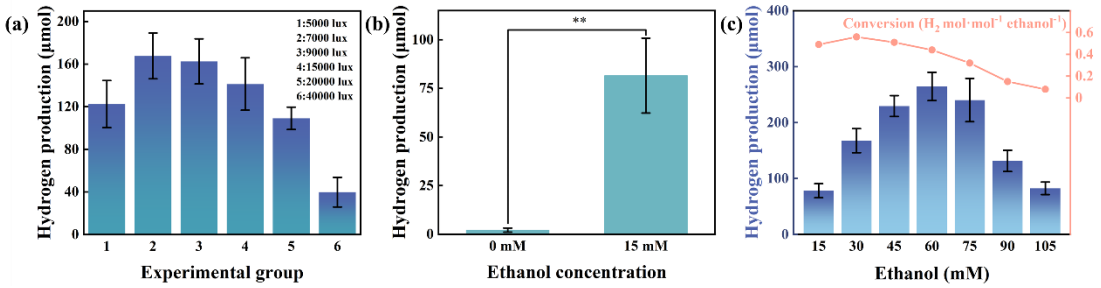

Figure S6. The ethanol to hydrogen characterization of native *R. palustris*. (a) The optimum light intensity for hydrogen production *of R. palustris*. The cultured 60 h *R. palustris* was used to produced hydrogen and 30 mM ethanol were used as substrate. The hydrogen produced medium was pH 7.0 M9 medium, initial OD_{660} 3.0, 35 ℃ and 7000 lux. (b) Analysis of *R. palustris* hydrogen production performance with or without ethanol. Two asterisks indicated a significant difference in the results. The hydrogen produced medium was pH 7.0 M9 medium, initial OD_{660} 3.0, 35 ℃ and 7000 lux. The error bar indicated that the experiment takes three parallel measurements. *P* value was 0.002, which represented the significant difference. (c) The effect of different ethanol on hydrogen production of *R. palustris* and the conversion rate of ethanol to hydrogen were exhibited. The hydrogen production was 78.21 μmol (15 mM), 167.78 μmol (30 mM), 229.5 μmol (45 mM), 264.51 μmol (60 mM), 240.19 μmol (75mM), 131.47 μmol (90 mM), and 82.5 μmol (105 mM), respectively. The conversion rates were 0.49 (15mM), 0.56 (30 mM), 0.51 (45 mM), 0.44 (60 mM), 0.32 (75 mM), 0.15 (90 mM), and 0.08 (105 mM), respectively. The hydrogen produced medium was pH 7.0 M9 medium, initial OD_{660} 3.0, 35 ℃ and 7000 lux. The error bar indicated that the experiment takes three parallel measurements.

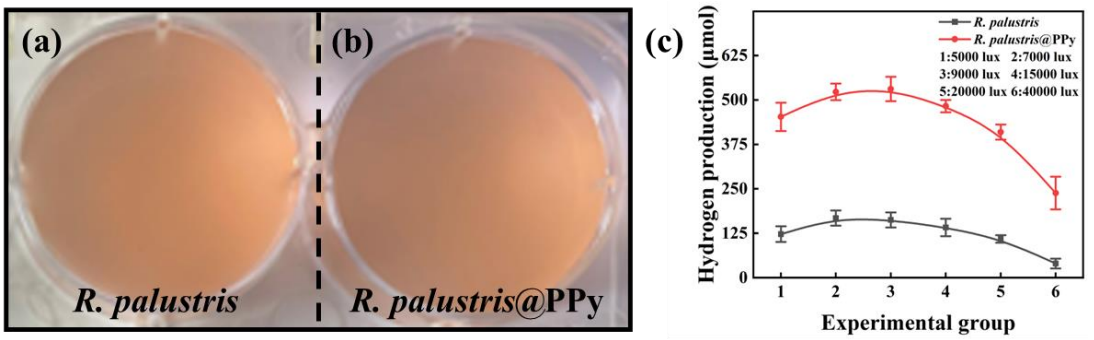

Figure S7. (a) and (b) The color change of bacteria after modification. (c) The effect of light intensity on hydrogen production in different systems. After modification, the *R. palustris*@PPy system still had obvious light response. The response trend was consistent with that of the control strains. The error bar indicated that the experiment takes three parallel measurements.

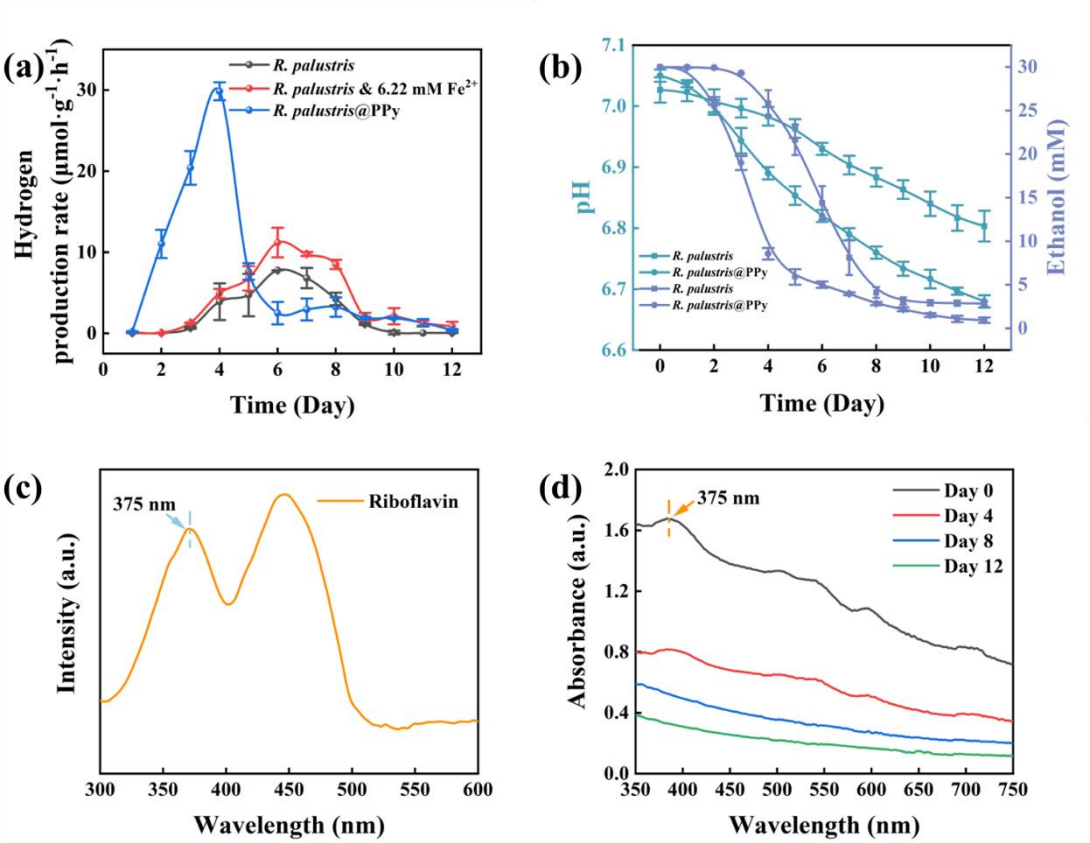

Figure S8. (a) The hydrogen production rate of native cell, Fe^{2+}, and *R. palustris*@PPy. The Fe^{2+} concentration of *R. palustris*@PPy were detected (about 6.22 mM) and used in *R. palustris* hydrogen system. The initial OD_{660} was 3.0, and the ethanol substrate was 30 mM. (b) The pH and ethanol change trends in hydrogen metabolism. (c) The standard UV-Vis spectra of riboflavin. The special peaks were 375 nm and 445 nm, respectively. (d) The UV-Vis spectra of *R. palustris*@PPy in different photo-fermentation stage. A 50 μL solution was removed from the reaction system, diluted 10 times and examined range from 300 to 750 nm. The error bar indicated that the experiment takes three parallel measurements.

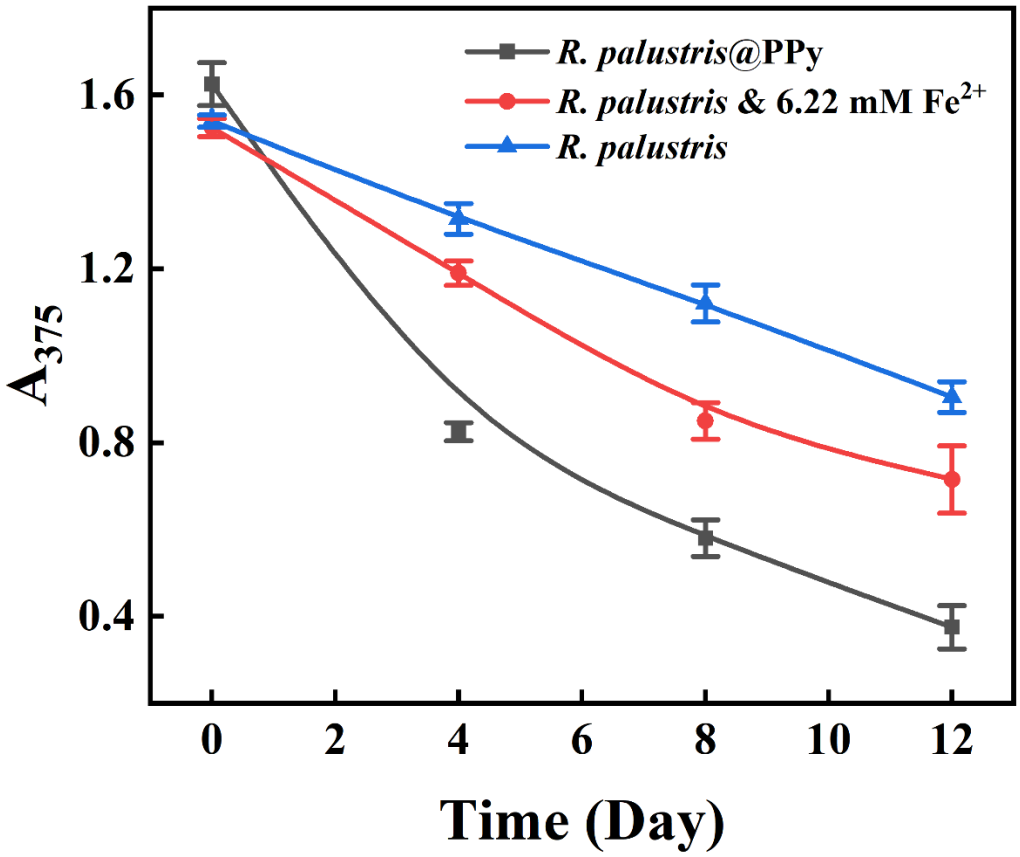

Figure S9. The riboflavin variation curves of different conditions in hydrogen produced system. The value measured stage was day 0, 4, 8, and 12, respectively. A 50 μL solution was removed from the reaction system, diluted 10 times and examined range from 300 to 750 nm. The error bar indicated that the experiment takes three parallel measurements.

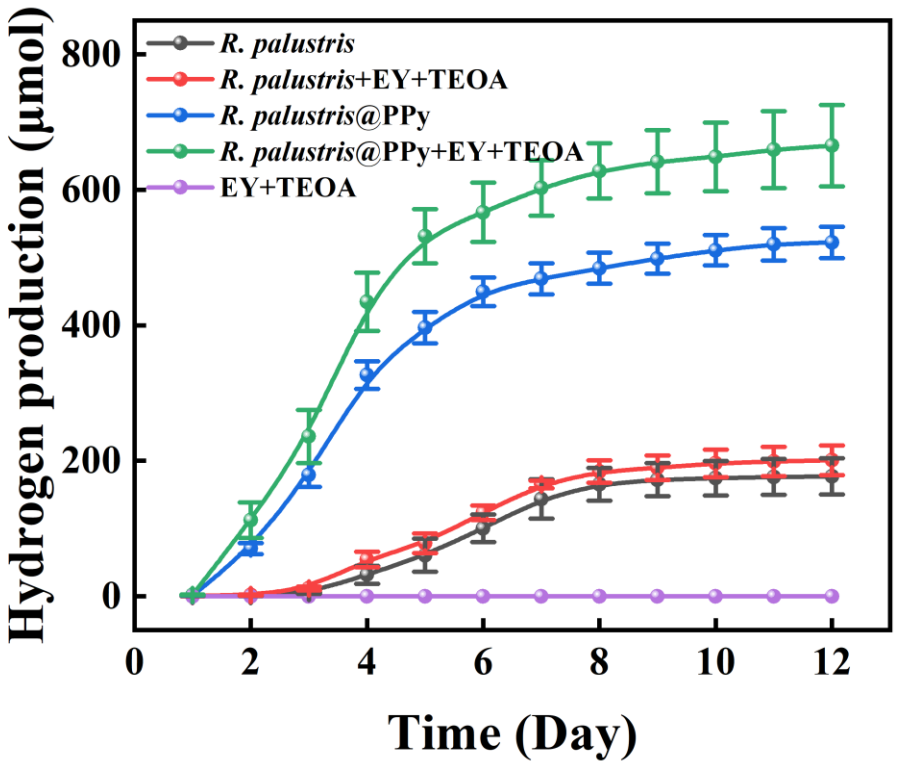

Figure S10. Time-dependent measurements of hydrogen production for *R. palustris*@PPy with or without Eosin Y disodium salt (EY) and triethanolamine (TEOA). EY and TEOA were added at the point of 1d, and the final-used concentration of EY and TEOA were 0.24 and 1.65 mg/mL. Samples were cultivated in seal vials with 30 mM ethanol containing pH 7.0 M9 medium, initial OD_{660} 3.0, 35 ℃ and exposed to daylight with an intensity of 7000 lux. The total hydrogen production was 177.01 μmol (*R. palustris*), 200.8 μmol (*R. palustris* + EY + TEOA), 522.4 μmol (*R. palustris*@PPy), and 665.4 μmol (*R. palustris*@PPy + EY + TEOA) respectively. For the wild type, the increase degree was 13.4% (calculation formula: (200.8-177.01)/177.01). The increase degree of PPy after modification was 25.5% (calculation formula: (655.4-522.4)/522.4), indicating that the introduction of PPy contributed to the uptake and conduction of external electrons. The error bar indicated that the experiment takes three parallel measurements.

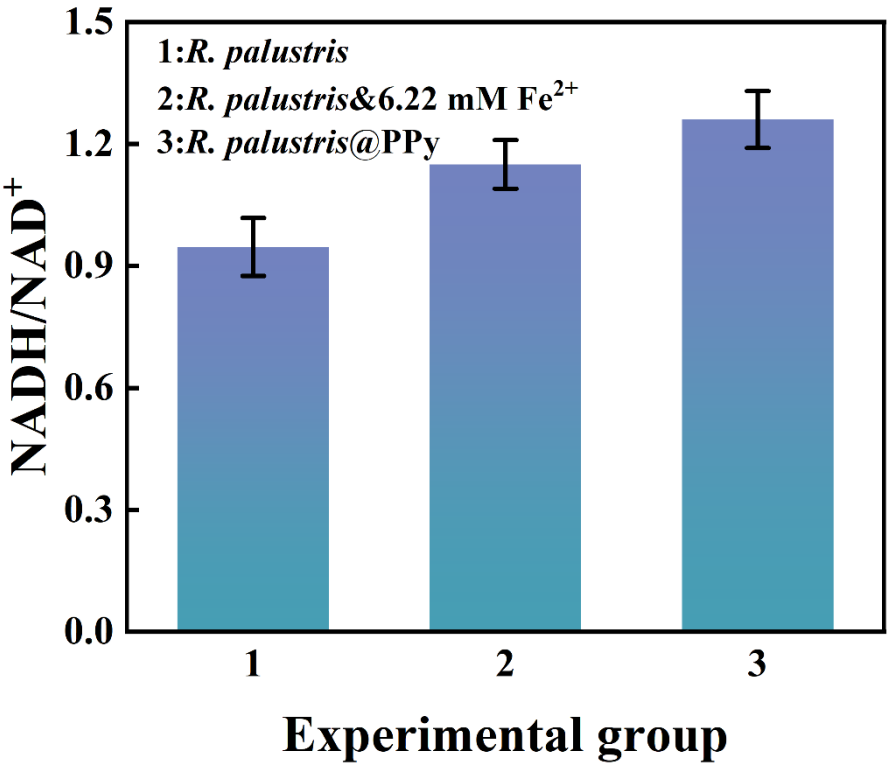

Figure S11. The NADH/NAD^+ ratio of different conditions in hydrogen produced system. The day 4 samples were used to analysis. The NADH/NAD^+ ratio was 0.95 (native), 1.15 (Fe^{2+}), and 1.26 (PPy), respectively. The error bar indicated that the experiment takes three parallel measurements.

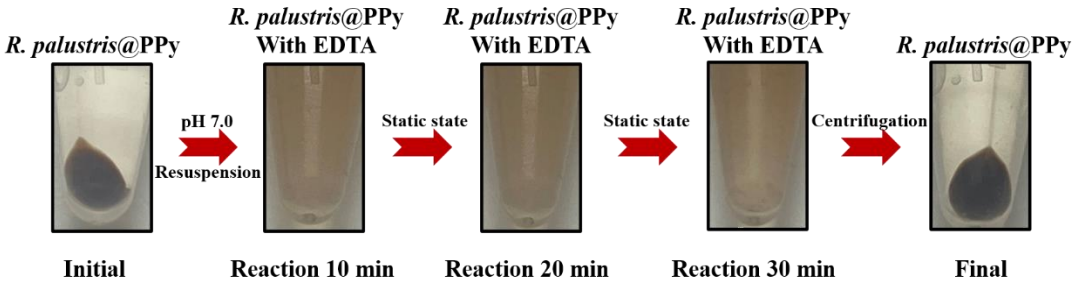

Figure S12. The structural stability verification of *R. palustris*@PPy biohybrid system. Add 0.5 M EDTA metal chelating agent to *R. palustris*@PPy solution. Add EDTA metal chelating agent and the reaction time was set to 30 min. There were no obvious changes in the characteristics of bacteria before and after the reaction, nor in the solution during the reaction, indicating that the structure of *R. palustris*@PPy was stable.

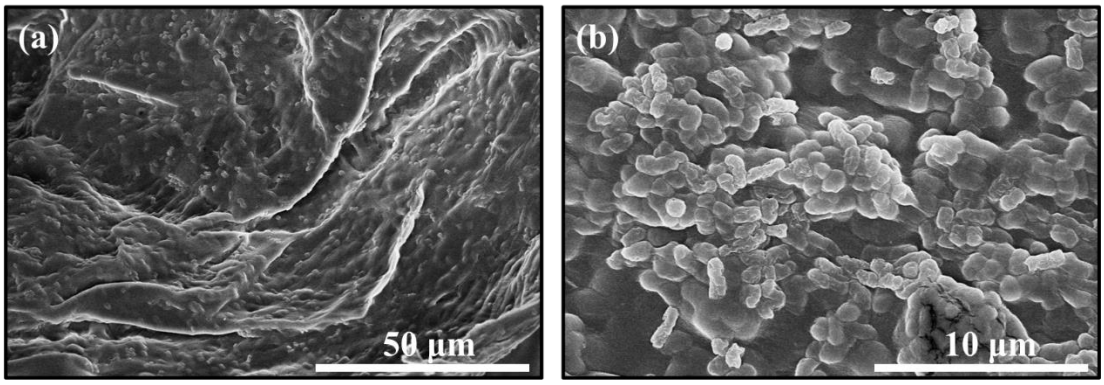

Figure S13. The SEM image of hydrogel aggregates. (a) Freeze-dried sheets of hydrogel aggregates. (b) The internal morphology of aggregates. The *R. palustris*@PPy aggregate samples were collected at day 4.

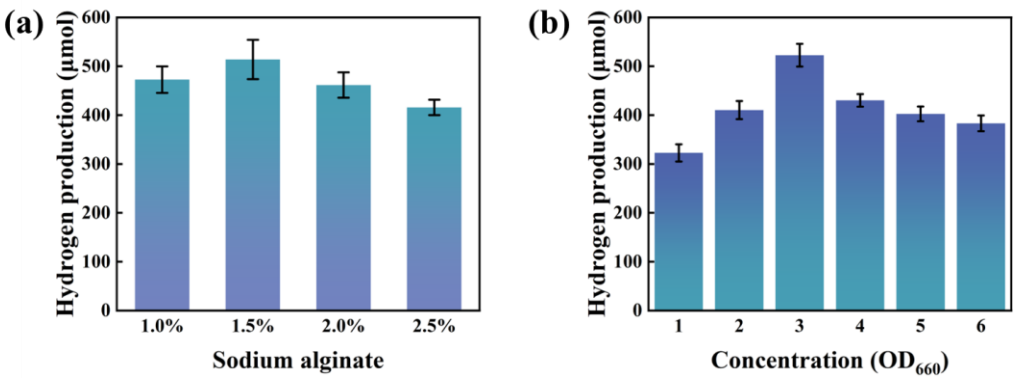

Figure S14. The effect of sodium alginate concentration and OD_{660} on hydrogen production of aggregates. (a) The sodium alginate concentration was 1.5%. (b) The suitable OD_{660} of aggregates was 3. The hydrogen produced conditions were 10 mL pH 7.0 M9, 30 mM ethanol, 35 ℃ and 7000 lux of light intensity. The hydrogen production was total 12 days. The error bar indicated that the experiment takes three parallel measurements.

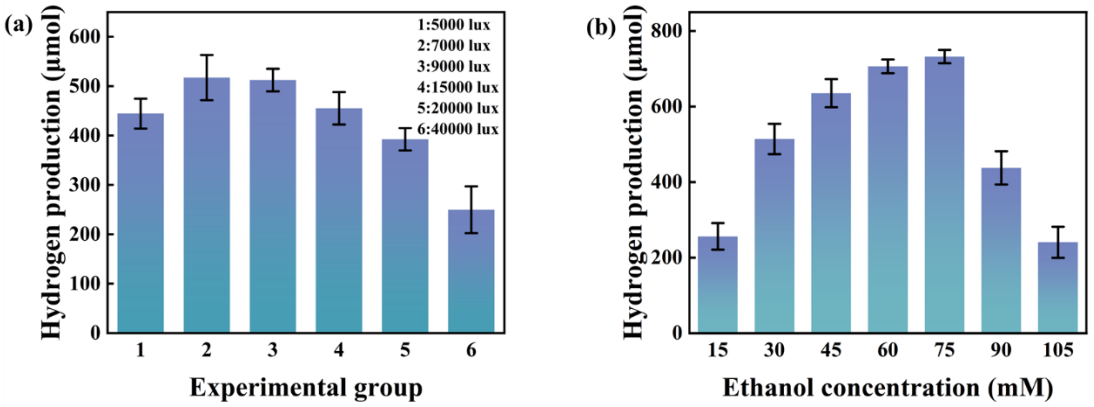

Figure S15. (a) The effect of light intensity on the hydrogen production in *R. palustris* augmentation. The *R. palustris* aggregates had obviously light response, the change trend was basically consistent with that of wild-type *R. palustris*. All the experimental group with 30 mM ethanol as carbon source. (b) The effect of ethanol on the hydrogen production in *R. palustris* augmentation. The error bar indicated that the experiment takes three parallel measurements.

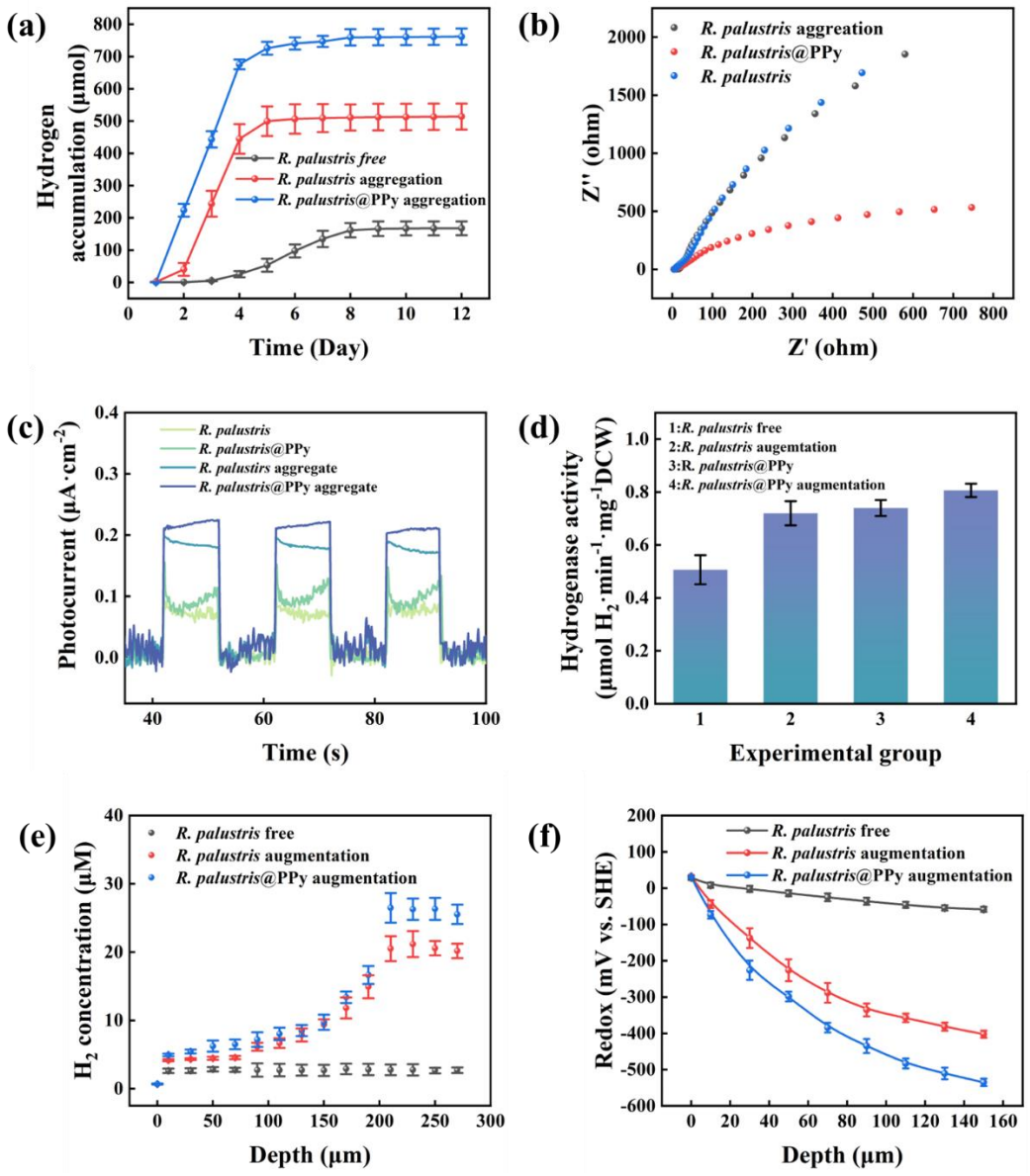

Figure S16. (a) The hydrogen production of different systems. The *R. palustris* free required deaeration, aggregation systems were at air. The hydrogen produced system were 10 mL pH 7.0 M9 medium with 30 mM ethanol, 35 ℃ and 7000 lux. (b) Electrochemical impedance spectroscopy (EIS) spectra of different systems in 0.1 M Na_2SO_4 solution at 0.5 V potentials. (c) Transient photocurrent densities of different systems electrodes with light on/off cycles under visible light irradiation in 0.1M Na_2SO_4 electrolyte solution at an applied potential of 0.5 V versus Ag/AgCl reference electrode. (d) The hydrogenase activity was measured of different systems. The dry cell weight was 164 mg, 171 mg, 166 mg, and 175 mg, respectively. And the day 4 samples were

collected for measurement. The error bar indicated that the experiment takes three parallel measurements. (e) The H_2 inside the systems at different depths. (f) Redox potential curves of different systems at different depths.

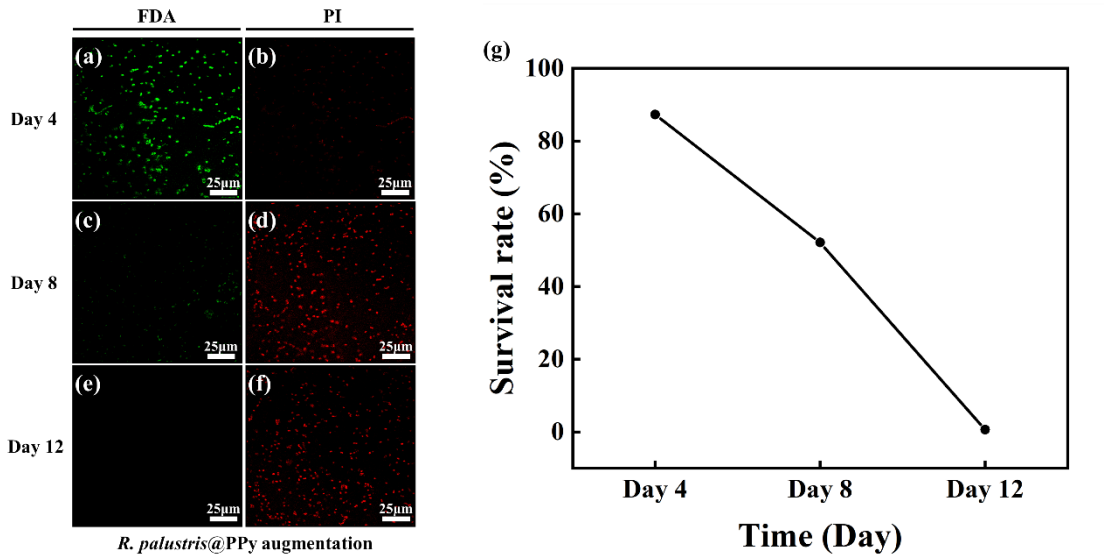

Figure S17. (a) to (f) Cell activity of *R. palustris*@PPy augmentation was identified by staining FDA/PI. The sample time nodes were day 4, day 8 and day 12. The live cells showed green fluorescence and the dead cells showed red fluorescence. (g) Trends in survival rate of *R. palustris*@PPy augmentation. Survival rates were analyzed by staining the proportion of live and dead cells shown on the left images. The day 4 survival rate was 87.3%, day 8 was 52.1%, and day 12 was 0.6%.

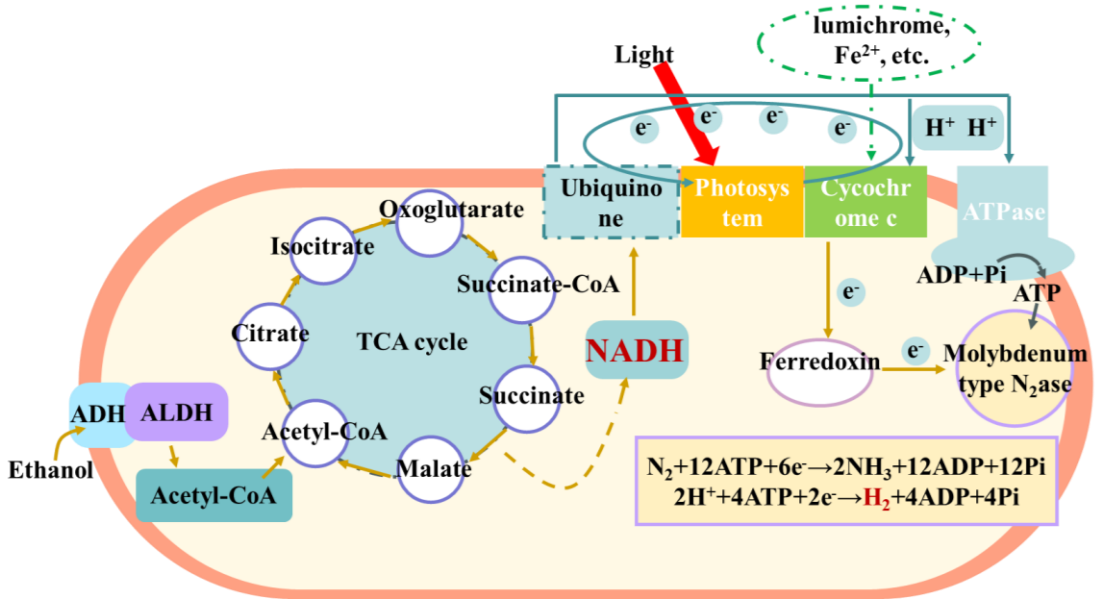

Figure S18. Overview of the ethanol to hydrogen metabolic process of *R. palustris* under photoheterotrophic nitrogen fixing conditions. Abbreviations: ADH: Alcohol dehydrogenase; ALDH: Acetaldehyde dehydrogenase; TCA cycle: Tricarboxylic acid cycle; ATPase: ATP synthesis; N_2ase: Molybdenum−iron nitrogenase. The green cycle represented the exogenous electron donors. The dotted yellow arrows represented all NADH produced by the TCA cycle: Malate→Acetyl-CoA, Isocitrate→Oxoglutrate, and Oxoglutarate→Succinate-CoA. The theoretical formula for ethanol to hydrogen: $CH_3CH_2OH+3H_2O\rightarrow 2CO_2+6H_2$.

Table S1 The primer for RT-PCR analysis

Primers	Sequence 5’ to 3’
RT-pioA-F	AGTFATGGACACCTGCTTC
RT-pioA-R	ACGCAGGTGATTTTCGTTTC
RT-pioB-F	GCCTGAAGAGCAACACC
RT-pioB-R	GCATAGCCGAGCTTGAAATC
RT-pioC-F	GAACGACAAACGCAACGAC
RT-pioC-R	AGGCCTTCTTGGTGACCTG
RT-FNR-F	TGGAAAAGGGTGTCCTACGC
RT-FNR-R	ACAGATACTCCGGCACATCG
16s rRNA-F	GCGGTAATACGGAGGGTG
16s rRNA-R	AATGCAATTCCCAGGTTG

Table S2 The ethanol to hydrogen conversion rate

Strains	Substrate	Conversion rate	Reference
Rhodobacter sphaeroides	lactate & ethanol	2.2 mol H_2/mol lactate & ethanol	14
Rhodopseudomonas palustris CGA009	ethanol	2 mol H_2/mol ethanol	15
Rhodopseudomonas palustris TIE-1	ethanol	2.54 mol H_2/mol ethanol	This work