Nonmetallic Abiotic-Biological Hybrid Photocatalyst for Visible Water Splitting and Carbon Dioxide Reduction

**HIGHLIGHTS**

- H$_2$O$_2$-degrading enzymes from *R. eutropha* enable visible-light water splitting by C$_3$N$_4$
- C$_3$N$_4$ coupled with bovine catalase has a solar-to-hydrogen efficiency of 3.4%
- C$_3$N$_4$-catalase increases CO$_2$ conversion into bioplastic under light by *R. eutropha*
- Heterotrophic bioplastic production by *R. eutropha* is also improved by C$_3$N$_4$-catalase
Nonmetallic Abiotic-Biological Hybrid Photocatalyst for Visible Water Splitting and Carbon Dioxide Reduction

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SUMMARY

Both artificial photosystems and natural photosynthesis have not reached their full potential for the sustainable conversion of solar energy into specific chemicals. A promising approach is hybrid photosynthesis combining efficient, non-toxic, and low-cost abiotic photocatalysts capable of water splitting with metabolically versatile non-photosynthetic microbes. Here, we report the development of a water-splitting enzymatic photocatalyst made of graphitic carbon nitride (g-C3N4) coupled with H2O2-degrading catalase and its utilization for hybrid photosynthesis with the non-photosynthetic bacterium Ralstonia eutropha for bioplastic production. The g-C3N4-catalase system has an excellent solar-to-hydrogen efficiency of 3.4% with a H2 evolution rate up to 55.72 μmol h⁻¹ while evolving O2 stoichiometrically. The hybrid photosynthesis system built with the water-splitting g-C3N4-catalase photocatalyst doubles the production of the bioplastic polyhydroxybutyrate by R. eutropha from CO2 and increases it by 1.84-fold from fructose. These results illustrate how synergy between abiotic non-metallic photocatalyst, enzyme, and bacteria can augment solar-to-multicarbon chemical conversion.

INTRODUCTION

Multiple technologies are being developed to convert and store solar energy, which is not only ubiquitous, sustainable, and clean but also intermittent. Artificial water-splitting photosystems have attracted interest mainly for the production of storable H2 from sunlight (Chen et al., 2017). The three main types of abiotic photosystems for H2 production are photovoltaic cell coupled with electrolyzer (PV-E), photoelectrochemical cell (PEC), and particulate photocatalyst (PC) (Chen et al., 2017). PV-E has shown the highest solar-to-hydrogen (STH) conversion efficiency, but H2 production cost by PV-E is not competitive with already established industrial processes. Between PEC and PC, techno-economical assessments conclude that PC would have a lower H2 production cost, as little as $1.60 kg⁻¹ when similar STH efficiencies are considered (Pinaud et al., 2013). Compared with PV-E and PEC, PC is a simpler system more suitable for low-cost scaling up. However, STH efficiencies reported until now for PC and PEC are lower than those reported for PV-E (Chen et al., 2017; Prévot and Sivula, 2013). Another important challenge is that, with scarce exceptions, the most efficient PC systems are made with expensive rare-earth elements (Liu et al., 2015a).

A promising solar technology is hybrid photosynthesis where an abiotic photosystem converts sunlight into chemical energy and transfers it to microbes for the production of multicarbon chemicals (Zhang and Tremblay, 2017). In these systems, chemical energy from the abiotic photosystem can drive or accelerate either autotrophic or heterotrophic bioproduction processes (Xu et al., 2019; Zhang et al., 2018; Liu et al., 2015b, 2016; Sakimoto et al., 2016a, 2016b; Guo et al., 2018; Nichols et al., 2015; Ye et al., 2019). In its autotrophic configuration where the greenhouse gas CO2 is the carbon source, hybrid photosynthesis will mirror natural photosynthesis carried out by living organisms if electrons come from water splitting. Hybrid photosynthesis is being pursued owing to the abiotic photosystems that are significantly more efficient at converting solar energy into chemical energy than natural photosynthesis. Thus, coupling abiotic photosystems with productive microbial catalysts may outcompete natural photosynthesis in terms of solar-to-specific chemicals efficiency (Zhang, 2015; Tremblay et al., 2017; Claessens et al., 2016).

Graphitic carbon nitride (g-C3N4) is a metal-free low-cost PC, easy to fabricate, and made of earth-abundant elements with a band gap of 2.7 eV that can convert solar energy into H2 (Liu et al., 2015a; Wang et al., 2009; Yan et al., 2010; Rao et al., 2019). Other applications of g-C3N4 include light-driven pollutant remediation and CO2 reduction into other C1 compounds (Hu et al., 2014; Ye et al., 2015; Zhang et al., 2019).
Most pristine g-C3N4 materials without modifications are unable to evolve H2 in the absence of sacrificial electron donors (SED) such as triethanolamine (TEOA), methanol, or lactic acid (Wang et al., 2017). Photocatalytic water splitting by g-C3N4 and resulting H2 and O2 evolution is theoretically possible but is hindered by sluggish kinetics (Zhang et al., 2016; Mishra et al., 2019b). Recently, a study by Fang et al. described a g-C3N4 photocatalyst prepared by an alternative method from a cocrystal precursor capable of using H2O as electron donor for H2 evolution (Fang et al., 2019). The authors attributed the water-splitting capacity of their PC to improved generation and mobility of the photoexcited charge carriers. One of the main obstacles for light-driven water splitting by g-C3N4 involves the O2 evolution reaction, which necessitates the transfer of four electrons and four protons for the formation of a double bond between two oxygen molecules (2H2O → 4H+ + 4e− + O2, 1.23 eV). With g-C3N4, the four-electron reaction for O2 evolution has been shown to be outcompeted kinetically by a two-electron reaction wherein H2O is oxidized into H2O2 (2H2O → 2H+ + 2e− + H2O2, 1.78 eV) (Liu et al., 2012). This phenomenon inhibits O2 and H2 evolution reactions because H2O2 deactivates g-C3N4 by staying attached to it and by occupying active surface sites. A solution reported in the past to overcome H2O2 poisoning and complete O2 and H2 evolution is to modify g-C3N4 PC with carbon nanodots (CDots), which have good catalytic activity for H2O2 degradation (Liu et al., 2015a).

In a previous study, we showed that no SED was required for unmodified g-C3N4 to augment heterotrophic PHB production by R. eutropha under light (Xu et al., 2019). Possible explanations are that R. eutropha modifies the growth medium surrounding g-C3N4 or its surface in a way that improves g-C3N4’s photocatalytic performance and/or enables water splitting. To understand the mechanisms involved, we investigated the photocatalytic performance and behavior of g-C3N4 suspended in cell-free spent medium from R. eutropha. The results indicated that H2O2-degrading enzymes present in the spent medium were degrading the H2O2 generated by g-C3N4 under light, and thus enabled photocatalytic water splitting. Based on these observations, we developed a novel PC system coupling g-C3N4 with pure bovine liver catalase, which is an enzyme specialized in the degradation of H2O2 into O2 and H2O. This novel g-C3N4-catalase PC system was employed to build a hybrid photosynthesis system where the production of PHB from either CO2 or fructose by R. eutropha was driven by water-splitting photocatalysis.

RESULTS

H2O2-Degrading Enzymes from the Spent Medium of R. eutropha and Faster Hydrogen Evolution by Unmodified g-C3N4

To establish how g-C3N4 was active under light when coupled with R. eutropha for PHB production in the absence of SEDs (Xu et al., 2019), fructose-grown R. eutropha cells were removed from the growth medium by centrifugation, g-C3N4 (Figures S1 and S2) was added to the cell-free spent medium, and light-driven H2 evolution was monitored under light-emitting diode (LED) light (Figure 1A). g-C3N4 in the cell-free spent medium had a Hz evolution rate (HER) of 0.57 ± 0.05 μmol h−1 (57.4 ± 4.7 μmol g−1 h−1), which was 16.4 times higher than that of pristine growth medium and 2.1 times higher than that of pristine growth medium with 0.5% TEOA. Next, cell-free spent medium was treated with the protease mixture pronase to establish if the compound responsible for improved HER was a protein or a group of proteins (Figure 1B). With pronase-treated cell-free spent medium, HER was significantly reduced to 0.06 ± 0.01 μmol h−1 (5.8 ± 1.2 μmol g−1 h−1), which is comparable to HER observed with pristine medium. Thus, the compound(s) in cell-free spent medium accelerating light-driven HER by g-C3N4 is proteinaceous.

Besides HER, concentrations of the O2 evolution inhibitor H2O2 were also monitored when g-C3N4 was exposed to light in pristine growth medium and cell-free spent medium (Figure 2A). In pristine medium containing g-C3N4, H2O2 accumulated in a light-dependent manner up to 20.9 ± 4.7 μmol after 1 h. In
comparison, no H$_2$O$_2$ accumulated with cell-free spent medium, which probably explained why HER was significantly higher under this condition.

Many organisms including bacteria have an enzymatic mechanism to defend themselves against H$_2$O$_2$, which can cause extensive oxidative damages (Mishra and Imlay, 2012). Two enzymes ubiquitous in aerobic organisms are responsible for biological H$_2$O$_2$ degradation: the catalase and the peroxidase. Catalases degrade H$_2$O$_2$ according to the following equation: 2 H$_2$O$_2$ $\rightarrow$ 2 H$_2$O + O$_2$ (Mishra and Imlay, 2012). Peroxidases require a physiological electron donor such as thioredoxins, glutathione NAD(P)H, or cytochrome c to reduce H$_2$O$_2$ according to the following equation: RH$_2$ + H$_2$O$_2$ $\rightarrow$ R + 2H$_2$O. The genome of R. eutropha H16 comprises at least nine genes coding for catalases and peroxidases (Table S1) (Pohlmann et al., 2006). Results presented here strongly suggest that some of these enzymes are located in the cell-free spent medium of R. eutropha, either because of excretion or cell lysis, where they degraded H$_2$O$_2$ and increased HER by g-C$_3$N$_4$. In fact, when exogenous H$_2$O$_2$ was added to cell-free spent medium, it was degraded at a rate of 203.6 ± 16.7 nmol min$^{-1}$ mL$^{-1}$ or 9.1 ± 0.3 µmol min$^{-1}$ per mg protein in the spent medium, whereas no degradation was observed in pristine medium (Figure 2B).
A Water-Splitting Photocatalytic System Coupling g-C3N4 and Pure Catalase

To investigate further the impact of H2O2-degrading enzymes on the photocatalytic activity of g-C3N4, the performance of g-C3N4 loaded with different quantity of bovine liver catalase was evaluated in ultrapure water, in catalase buffer (CAT buffer; 50 mM KH2PO4 pH 7.4), and in pristine R. eutropha growth medium (Figures 3 and S3–S5). Under LED light, increasing the quantity of catalase within the three types of suspension resulted in significant H2 production by g-C3N4 up to 14.26 ± 1.00 μmol h−1 (1,426.4 ± 95.9 μmol g−1 h−1) with 2,000 U mL−1 catalase in CAT buffer. H2 production was accompanied by nearly stoichiometric O2 evolution of 7.29 ± 0.19 μmol h−1 (729.5 ± 18.5 μmol g−1 h−1) demonstrating light-driven water splitting by the enzymatic PC system (Figure 3B). In the absence of catalase, O2 evolution was not detected in ultrapure water, CAT buffer, or growth medium, indicating that the presence of the enzyme not only accelerated H2 evolution but also enabled O2 evolution. Besides O2 and H2 evolution, H2O2 accumulation was also monitored with the PC system coupling g-C3N4 with 2,000 U ml−1 catalase under light. With all three different suspensions, no H2O2 was detected, showing that the catalase degraded its substrate and prevented the buildup of this reactive oxygen species.

The addition of 100 U ml−1 superoxide dismutase (SOD) to g-C3N4 instead of catalase did not improve H2 production with a rate of 0.01 ± 0.00 μmol h−1 (1.5 ± 0.2 and 1.2 ± 0.2 μmol g−1 h−1) in both ultrapure water and growth medium (Figure S6). Under similar light condition, pure g-C3N4 had a H2 production rate of 0.02 μmol h−1 (2.0 μmol g−1 h−1) in both ultrapure water and growth medium (Figure 3A). At the same concentration of 100 U ml−1, catalase already significantly improved H2 evolution by g-C3N4 to 0.81 ± 0.09 and 0.87 ± 0.15 μmol h−1 (81.2 ± 8.6 and 87.1 ± 14.5 μmol g−1 h−1) in ultrapure water and growth medium, respectively. Like the catalase, SOD is an enzyme participating in living cells’ defense against oxidative stress (Johnson and Hug, 2019). SOD catalyzes the conversion of superoxide into O2 and H2O2. These results clearly suggest that the increased H2 production observed with the g-C3N4-catalase PC system was caused by the enzymatic activity of the catalase and not by an unspecific effect related to the addition of enzymes or proteins independently of their capacity to degrade H2O2.

The energetic efficiency of the novel g-C3N4-catalase system was evaluated with 2,000, 5,000, and 10,000 U ml−1 bovine liver catalase in CAT buffer under an AM 1.5G solar simulator with a light intensity of 200 or 800 W m−2 (ca. 0.2 or 0.8 sun) (Figure 4). The highest STH efficiency of 3.40% ± 0.2% was recorded with 10,000 U ml−1 catalase at a light intensity of 200 W m−2. For this experiment, the irradiated surface was 4.6 cm2 for a total input energy in 1 h of 331.2 J. H2 production was 47.49 ± 3.22 μmol h−1, which amounted to 11.26 J. At 5,000 U ml−1 catalase, the STH efficiency was statistically similar at 3.12% ± 0.69%, but it became 1.88 times lower when catalase concentration was reduced to 2,000 U ml−1. Increasing light intensity to 800 W m−2 augmented HER while reducing STH efficiency. The best PC system at 800 W m−2 had a catalase concentration of 5,000 U ml−1 with an STH efficiency of 1.00% ± 0.19% and a normalized HER of 55.72 ± 10.40 μmol h−1 (1,857.16 ± 346.72 μmol g−1 h−1). O2 evolution for all tested conditions
was nearly stoichiometric (Figures 4A and 4B). Quantum efficiency at a wavelength of 420 nm, which is in the optimal absorption spectrum for g-C3N4, was 23.48% with 2,000 U mL\(^{-1}\) catalase, 29.72% with 5,000 U mL\(^{-1}\) catalase, and 27.90% with 10,000 U mL\(^{-1}\) catalase (Figure 4D). STH efficiencies at low light intensity for the g-C3N4-catalase PC system described here are higher than Cdots-C3N4 PC, which is, with an efficiency of 2%, one of the best reported water-splitting photocatalysts (Table S2) (Liu et al., 2015a). In addition, the g-C3N4-based PC system was stable when tested with 10,000 U mL\(^{-1}\) catalase under a light intensity of 200 W m\(^{-2}\) (Figure S7). After three cycles, H\(_2\) and O\(_2\) evolution rates were comparable to the initial rates.

**Hybrid Photosynthesis with g-C3N4-Catalase and *R. eutropha* for CO\(_2\) Reduction**

Autotrophic hybrid photosynthesis apparatuses developed until now rely on metallic inorganic PC harvesting visible light to drive biological CO\(_2\) reduction by non-photosynthetic bacteria (Table S3) (Zhang and Tremblay, 2017; Zhang et al., 2018; Sakimoto et al., 2016a; Ye et al., 2019). Most of these systems photogenerated the reducing equivalents ultimately transferred to the autotrophic microbe by the oxidation of an SED such as cysteine. In these circumstances, the requirement for an SED diminishes significantly the sustainability prospect of PC-based bioprocesses. Only one hybrid photosynthesis system reported until now relies on water splitting and not on an SED (Sakimoto et al., 2016b). This tandem “Z-scheme” platform included potentially toxic TiO\(_2\) combined with the cocatalyst Mn(II) phthalocyanine photo-oxidizing water and transferring the electrons indirectly to CdS via a cysteine shuttle. CdS then photogenerated reducing equivalents, which were carried out to the acetogen *Moorella thermoacetica* for the reduction of CO\(_2\) into acetate.

To establish if the water-splitting g-C3N4-catalase PC system developed here could provide energy for autotrophic bioproduction process, it was coupled with *R. eutropha* growing autotrophically under a N\(_2\):H\(_2\):CO\(_2\):O\(_2\) atmosphere and synthesis of PHB was monitored (Figure 5A). With g-C3N4-catalase, PHB production from CO\(_2\) under light reached 41.02 ± 6.22 mg L\(^{-1}\) after 48 h compared

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**Figure 4. H\(_2\) Evolution and O\(_2\) Evolution by g-C3N4-Catalase under an AM1.5G**

(A) Gases evolution at a light intensity of 200 W m\(^{-2}\). (B) Gases evolution at a light intensity of 800 W m\(^{-2}\).

(C and D) (C) Solar-to-hydrogen efficiency and (D) quantum efficiency at 420 nm for the g-C3N4-catalase PC system. Each curve and bar is the average of at least three replicates with standard deviation.
with 18.72 ± 0.31 mg L⁻¹ for a light-exposed *R. eutropha* culture grown without the PC system (Figures 5B and 5C). Both scanning electron microscopy and transmission electron microscopy images showed the formation of large aggregates under autotrophic conditions with direct physical contact between g-C₃N₄ particles and *R. eutropha* cells (Figures 5D, 5E, and S8). Bovine liver catalase was not required for g-C₃N₄ to improve autotrophic PHB production, but the addition of the enzyme still had a significant positive effect. g-C₃N₄ without catalase augmented PHB production from CO₂ by *R. eutropha* to 28.05 ± 3.37 mg L⁻¹ under light. This can be explained by native H₂O₂-degrading enzymes possibly released by *R. eutropha* cells, which enables water splitting by g-C₃N₄, but not to the same level as with bovine liver catalase.

Production improvement observed with g-C₃N₄-catalase was light-dependent. *R. eutropha* with g-C₃N₄-catalase in the dark produced only 18.17 ± 3.29 mg L⁻¹ PHB after 48 h (Figures 5B and 5C). The addition of 5,000 U mL⁻¹ catalase alone without g-C₃N₄ did not improve PHB production, which demonstrates that *R. eutropha* cells could not use the enzyme as substrate for their metabolism.
The increase of PHB production observed with the photocatalytic system during autotrophic growth of *R. eutropha* may have three possible explanations (Figure 5A). First, H₂ initially provided in the gas phase to drive bacterial growth may become scarcer after biological oxidation. g-C₃N₄-catalase generates supplementary H₂ used by *R. eutropha* to keep its metabolism active, which results in additional PHB production. Second, O₂ may also become rarer in the gas phase after bacterial reduction during growth. While generating reducing equivalents, g-C₃N₄-catalase also evolves additional O₂ by water splitting that can be used by the bacterial cells as electron acceptor. Third, the photocatalytic system may transfer electrons directly to *R. eutropha* or generate reducing equivalents under a different form than H₂ that are preferentially used by the bacteria.

### Heterotrophic Production of PHB from Fructose with g-C₃N₄-Catalase

The impact of the g-C₃N₄-catalase system on heterotrophic production of PHB from fructose by *R. eutropha* was also evaluated (Figure S9). As reported previously (Xu et al., 2019), the addition of g-C₃N₄ alone is sufficient to improve PHB production from fructose under light with a 124% increase compared with an *R. eutropha* culture without g-C₃N₄ (Figures 6A, 6B, and S10). The only other example where heterotrophic bioproduction was enhanced by a light-harvesting photocatalyst coupled the rare element indium phosphide with a *Saccharomyces cerevisiae* strain producing shikimate from glucose (Guo et al., 2018). When the g-C₃N₄-catalase system developed here was combined with *R. eutropha*, PHB production reached 9.09 ± 0.71 g L⁻¹ after 96 h of growth under light, which was 1.84-fold higher compared with heterotrophic *R. eutropha* without the PC system. Large aggregates with direct physical contact between g-C₃N₄ and *R. eutropha* cells were also observed in the heterotrophic mode (Figures 6C and 6D). Fructose-grown *R. eutropha* control with catalase only and no g-C₃N₄ under light as well as...
a control grown in the dark with the g-C3N4-catalase system had PHB production comparable to *R. eutropha* without g-C3N4 and catalase (Figures 6A and 6B).

**Charge Transfer at the Interface in the Hybrid Photosynthesis System**

To gain additional insights into the mechanisms involved in the photocatalytic activity of the autotrophic hybrid photosynthesis systems described here, the photogenerated charge transfer process was evaluated via electrochemical impedance spectroscopy (EIS) (Figure 7). In Nyquist plots obtained from EIS study the semicircle at high frequency is related to the electron transfer-limiting process and its radius corresponds to the charge transfer resistance (Fu et al., 2019; Liu et al., 2020; Han et al., 2018). Thus, a Nyquist plot with a smaller radius indicates lower charge transfer impedance. Here, EIS under light showed that semicircles for g-C3N4-catalase (5,000 U mL\(^{-1}\)) with *R. eutropha* and pure g-C3N4 with *R. eutropha* had smaller radiiuses compared with *R. eutropha* alone and *R. eutropha* with catalase, indicating that adding g-C3N4 to the bacterial culture lowered the charge transfer impedance and promoted interfacial charge transfer. When catalase was added to either pure g-C3N4 or g-C3N4 with *R. eutropha*, both Nyquist plots exhibited the smallest semicircle radiiuses, which demonstrated that combining g-C3N4 with catalase can effectively lower charge transfer resistance and facilitate photogenerated charge transfer at the interface, thus improving H₂ or PHB generation. This is in accordance with the observation that the catalase prevents H₂O₂ poisoning at the surface of g-C3N4 and thus promotes charge transfer for proton reduction.

**DISCUSSION**

The discovery that H₂O₂-degrading enzymes present in the spent medium of *R. eutropha* complete the reaction series required for light-driven water splitting by g-C3N4 opens up possibilities for the development of efficient enzymatic PC systems for H₂ production. This new avenue for PC development began to be explored here with the coupling of bovine liver catalase with g-C3N4. Although the STH efficiency reported here is promising, it is below 5%–10%, which is considered to be the baseline for economically viable production of H₂ via PC (Chen et al., 2017; Pinaud et al., 2013). It is likely that the water-splitting performance of g-C3N4-H₂O₂-degrading enzyme PC can be further improved. Pure g-C3N4 has other drawbacks besides H₂O₂ poisoning for optimal photocatalytic activity such as narrow light absorption range below 470 nm, slow surface kinetics, and charge carrier transport as well as important activation energy requirement (Zhang et al., 2017a). Several studies described approaches relying on earth-abundant co-catalysts and on new structural designs to overcome these limitations (Zhang et al., 2017a; Niu et al., 2014). Other groups have improved the photocatalytic activity of g-C3N4 by fine-tuning its morphology, by increasing its crystallinity, or by optimizing its chemical arrangement and electronic properties (Lin et al., 2019; Li et al., 2019; Zhang et al., 2017b; Wu et al., 2019; Niu et al., 2012; Guo et al., 2016). Similar strategies could be employed to improve g-C3N4-catalase PC. In addition, other catalases or different H₂O₂-degrading enzymes such as peroxidases could be better suited for interacting with g-C3N4.

In its lower-cost configuration design, the g-C3N4-H₂O₂-degrading enzyme system can split water by coupling g-C3N4 with waste medium from microbial cultures. This strategy could create value and store energy under the form of H₂ by harnessing enzyme-rich wastes from bio-industrial processes. For this purpose, water-splitting performance of g-C3N4 coupled with biotechnological wastes from different sources containing unique profiles of H₂O₂-degrading enzymes should also be evaluated.
When immersed in an *R. eutropha* culture, g-C₃N₄ splits water and converts light energy into reducing equivalents, which are transferred to bacteria for PHB production. The addition of catalase to this hybrid photosynthesis system significantly improves autotrophic and heterotrophic productivity by curbing H₂O₂ poisoning of g-C₃N₄ and by accelerating water splitting. Simultaneously with H₂O₂ conversion into H₂O and O₂, the catalase may improve photocatalysis by g-C₃N₄ by other means. For instance, it has recently been shown that CDots enable water splitting by g-C₃N₄ and accelerate H₂ production by serving as a catalyst for H₂O₂ decomposition and at the same time as a co-catalyst for H₂ evolution (Qu et al., 2018). Further investigation is required to achieve a complete understanding of the functional interactions between g-C₃N₄ and the catalase enzyme.

The results presented here also show that coupling g-C₃N₄ with a bacterial catalyst significantly expands the photocatalytic products of the abiotic PC beyond H₂ including the conversion of CO₂ into carbon-chain polymers. However, to outcompete autotrophic bioproduction relying on natural photosynthesis, microbial CO₂ reduction driven by g-C₃N₄-catalase needs further improvement. Besides upgrading g-C₃N₄-catalase PC, this can be achieved by optimizing the metabolism of the microbial catalyst and by finding the best compromise in terms of growth conditions and bioreactor configuration to maximize microbial cell and PC activities.

In both the autotrophic and heterotrophic modes, g-C₃N₄-catalase doubled or nearly doubled PHB production by *R. eutropha*, which suggests great potential for CO₂ reduction by hybrid photosynthesis as well as for photoaugmentation of white biotechnologies designed for the conversion of organic substrate into valuable chemicals. In conclusion, the results demonstrate that g-C₃N₄ can harness the activity of H₂O₂-degrading enzymes such as the catalase to split water efficiently under sunlight and generate reducing equivalents for microbial production processes.

**Limitations of the Study**

A comprehensive study of the mechanisms involved in the transfer of electrons from g-C₃N₄ to *R. eutropha* is necessary to have a complete understanding of the hybrid photosynthesis system described here and be able to improve its performance in the future. A major optimization effort is also needed for practical applications.

**METHODS**

All methods can be found in the accompanying Transparent Methods supplemental file.

**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.isci.2019.100784.

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**AUTHOR CONTRIBUTIONS**

P.-L.T. and T.Z. conceived the study. M.X. carried out the enzymatic photocatalyst experiments and the hybrid photosynthesis experiments. P.-L.T. completed H₂O₂-degrading enzyme activity assay. Y.C. carried out scanning electron microscopy experiments. P.-L.T., M.X., and T.Z. interpreted the data and wrote the manuscript. All the authors reviewed and approved the final manuscript.

**DECLARATION OF INTERESTS**

The authors declare no competing interests.
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Supplemental Information

Nonmetallic Abiotic-Biological Hybrid Photocatalyst for Visible Water Splitting and Carbon Dioxide Reduction

Pier-Luc Tremblay, Mengying Xu, Yiming Chen, and Tian Zhang
**Figure S1. g-C₃N₄ characterization.** (A) SEM image, (B) XRD, (C) FT-IR, and (D) UV-visible diffuse reflectance spectra of synthesized g-C₃N₄. X-ray diffraction (XRD) shows the two main characteristic peaks associated with g-C₃N₄. The first peak at 13.2° corresponds to tri-s-triazine units packing in the lattice planes parallel to the c axis. The second peak at 27.6° is attributed to the interlayer stacking of aromatic segments (Mao et al., 2017; Zang et al., 2014; Cheng et al., 2016). The Fourier transform infrared spectrum (FT-IR) includes skeletal vibrations in the range of 1200-1650 cm⁻¹ for aromatic C-N heterocycles and a breathing vibration at 809.7 cm⁻¹ for tri-s-triazine units characteristic of g-C₃N₄. Between 3000-3500 cm⁻¹, a broad absorption peak is observed corresponding to stretching vibrational modes of the O-H or N-H groups (Tang et al., 2015; Wang et al., 2017). The UV-visible light absorption spectrum is also typical of g-C₃N₄ with an upper limit at ca. 450 nm (Patnaik et al., 2017). Related to Figures 1, 2, and 3.
Figure S2. TEM and AFM analysis of g-C₃N₄ particles. (A) TEM image shows typical layered sheet-like structure of g-C₃N₄. (B) Section analysis of the AFM image shows that the thickness of the g-C₃N₄ sheet-like particles was ca. 60 nm. Related to Figures 1, 2, and 3.
Figure S3. The crystal structure of the bovine liver catalase. The enzyme comprises four 60 kDa subunits. Each of these subunits contains an iron bound to a protoheme IX group (PDB ID: 1TGU; Sugadev, R., Balasundaresan, D., Ponnu Swamy, M.N., Kumaran, D., Swaminathan, S., Sekar, K. The crystal structure of bovine liver catalase.). One unit of bovine liver catalase will degrade 1.0 μmole H₂O₂ min⁻¹ at pH 7.0 at 25 °C according to the reaction 2H₂O₂ → 2H₂O + O₂. Related to Figures 3 and 4.
Figure S4. Schematic diagram of the g-C₃N₄-catalase enzymatic photocatalyst system. Water is oxidized to H₂O₂ by g-C₃N₄ under light. The bovine liver catalase decomposes H₂O₂ into O₂ and H₂O preventing H₂O₂ poisoning of g-C₃N₄. Concomitantly, protons are converted into H₂ by g-C₃N₄, thus completing the light-driven water-splitting reaction. CB: conduction band. VB: valence band. Related to Figures 3 and 4.
Figure S5. SEM images of pristine g-C₃N₄ and g-C₃N₄ loaded with 2000 U mL⁻¹ bovine liver catalase with iron elemental mapping and weight percentage for carbon, nitrogen, and iron. Elemental mapping and weight percentage were examined by energy dispersive X-Ray spectroscopy (EDS). Red dots on SEM images correspond to iron elements. Iron elements are bound to the four protoheme IX groups attached to the bovine liver catalase. Increased iron element weight percentage indicates the presence of catalase in the vicinity of g-C₃N₄. Related to Figures 3 and 4.
Figure S6. Light-driven water-splitting activity of g-C₃N₄ with catalase or SOD. Impact of 100 U mL⁻¹ bovine liver catalase or SOD on H₂ evolution rate by g-C₃N₄ suspended in ultrapure water or *R. eutropha* growth medium under 4200 lux LED light. Each bar is the average of at least three replicates with standard deviation. Related to Figures 3 and 4.
Figure S7. Cycling test for the g-C₃N₄-catalase PC system. H₂ and O₂ evolution by g-C₃N₄ with 10000 U mL⁻¹ catalase under a light intensity of 200 W m⁻² was monitored for several cycles of one hour. Related to Figures 3 and 4.
Figure S8. SEM image of *R. eutropha* H16 cells without g-C₃N₄. *R. eutropha* H16 is a Gram-negative rod-shape bacterium of the *Burkholderiaceae* family. *R. eutropha* H16 is also named *Cupriavidus necator* H16. Related to Figures 5 and 6.
Figure S9. Schematic diagram of heterotrophic hybrid photosynthesis with g-C₃N₄-catalase and *R. eutropha* producing PHB from fructose. Water-splitting g-C₃N₄-catalase augments heterotrophic PHB production either by providing H₂ and/or O₂ molecules for the bacterial metabolism or by transferring unknown reducing equivalents (Red) to *R. eutropha*. ETC: electron transport chain. MBH: membrane-bound hydrogenase. SH: soluble hydrogenase. Thyd: NAD(P)⁺ transhydrogenase. Related to Figure 6.
Figure S10. Impact of water-splitting g-C₃N₄-catalase on PHB to fructose yield of heterotrophically-grown *R. eutropha*. Yield gram PHB produced per gram fructose consumed (Yₚ/s) after 96 hours of growth. REH16: *R. eutropha* H16. Each bar is the mean of at least three replicates with standard deviation. Related to Figure 6.
**Table S1.** Gene in *R. eutropha* H16 coding for H$_2$O$_2$-degrading enzyme catalase and peroxidase.*

| Gene/locus  | Annotation                      |
|-------------|---------------------------------|
| **Catalase**|                                 |
| *katE1*/H16_A3109 | Catalase KatE1                |
| *katE2*/H16_B1428    | Catalase KatE2                 |
| *katG*/H16_A2777    | Catalase (peroxidase I) KatG   |
| **Peroxidase**    |                                 |
| H16_B0946          | Predicted iron-dependent peroxidase |
| *tpx*/H16_A3175    | Thiol peroxidase Tpx            |
| H16_A3102          | Glutathione peroxidase         |
| H16_A0065          | Glutathione peroxidase         |
| H16_A3267          | Peroxiredoxin                  |
| H16_A1460          | Peroxiredoxin                  |

*Related to Figure 2.
| System                        | Performance                                      | Comments                                      | Reference       |
|-------------------------------|--------------------------------------------------|-----------------------------------------------|-----------------|
| g-C₃N₄ with carbon nanodots   | H₂ evolution: 191.7 µmol h⁻¹                      | -80 mg PCs in 150 ml ultrapure water          | Liu et al., 2015|
|                               | O₂ evolution: nearly stoichiometric              | -AM 1.5G solar simulator                      |                 |
|                               | STH: 2.0%                                        |                                               |                 |
| carbon-rich g-C₃N₄            | H₂ evolution: 530 µmol h⁻¹ g⁻¹ catalyst           | -30 mg PC in 80 ml distilled water            | Fang et al., 2019|
|                               | O₂ evolution: nearly stoichiometric              | -300 W Xe lamp (≥ 300 nm)                     |                 |
|                               | STH: Not reported                                |                                               |                 |
| g-C₃N₄-catalase               | H₂ evolution: 55.72 µmol h⁻¹                      | -30 mg PC in 20 ml CAT buffer*               | This work       |
|                               | O₂ evolution: nearly stoichiometric              | -500 W Xe lamp with an AM1.5G filter          |                 |
|                               | STH: 3.40% (200 W m⁻²) and 1.00% (800 W m⁻²)    |                                               |                 |

*a*Related to Figure 4.

*b*STH: Solar-to-H₂ efficiency.

*c*PC: photocatalyst.

*d*Light intensity.

*e*CAT buffer: 50 mM KH₂PO₄ pH 7.4.

Table S2. Metal-free g-C₃N₄-based particulate photocatalyst systems splitting water under visible light.*
| Particulate photocatalyst | Microbe | Comments | Reference |
|--------------------------|---------|----------|-----------|
| CdS nanoparticles        | *Moorella thermoacetica* | -Cysteine as sacrificial e⁻ donor  
- Ca. 1.1 mM acetate produced in 36 hours | Sakimoto et al., 2016a |
| CdS and TiO₂ nanoparticles | *M. thermoacetica* | -Z-scheme, water splitting  
- Cysteine as e⁻ shuttle  
- Cocatalyst MnPc  
- Ca. 0.8 mM acetate produced in one day | Sakimoto et al., 2016b |
| CdS nanoparticles        | *Methanosarcina barkeri* | -Cysteine as sacrificial e⁻ donor  
- 0.19 μmol h⁻¹ methane | Ye et al., 2019 |
| CdS nanoparticles        | *Rhodopseudomonas palustris* | -Cysteine as sacrificial e⁻ donor  
- Biomass, carotenoids and polyhydroxybutyrate (PHB) production was increased to 148%, 122%, and 147% | Wang et al., 2019 |
| g-C₃N₄                    | *Ralstoniaeutropha H16* | - Water splitting  
- 1.5-fold increase of PHB production | This work |
| g-C₃N₄ with catalase      | *R. eutropha H16* | - Water splitting  
- 2.2-fold increase of PHB production | This work |

*Related to Figure 5.*
TRANSPARENT METHODS

g-C₃N₄ preparation

g-C₃N₄ particles were prepared by a facile thermal treatment of urea as described previously (Liu et al., 2011). 10 g urea was placed into a semi-closed quartz crucible and then calcined in a muffle furnace for two hours with a heating rate of 10 °C min⁻¹ up to 550 °C. The bulk product was filtered and washed three times with 20 mL 0.1 M HNO₃ and then washed five times with 20 mL ultrapure water to remove impurities from the sample surface. g-C₃N₄ was then dried at 55 °C for 24 h and ground into powder.

g-C₃N₄ characterization

The characterization of g-C₃N₄ was done with X-ray powder diffraction (XRD), Fourier-transform infrared spectroscopy (FT-IR), and UV-visible diffuse reflectance spectroscopy. The XRD spectrum of g-C₃N₄ was recorded within the scan range of 20 from 10⁰ to 80⁰ using a D8 ADVANCE powder X-ray diffractometer (Bruker, MA, USA) equipped with Cu Ka X-ray. The FT-IR spectrum of g-C₃N₄ was obtained with a Nicolet iS5 FT-IR spectrometer (Thermo Fisher Scientific, MA, USA) using the KBr pellet method within the 400–4000 cm⁻¹ range at a 0.8 cm⁻¹ resolution. The UV-Visible diffuse reflectance absorption spectrum of the photocatalyst (PC) was recorded with a Lambda 750s spectrophotometer (PerkinElmer, MA, USA).
Bacterial strain and growth medium

The bacterium *R. eutropha* H16 (DSM-428) was used for this study. All bacterial cultures were grown at 30 °C in a nitrogen-limiting minimal medium, which has a pH of 6.8 (Budde et al., 2010). Components of the minimal medium per liter of ultrapure water are: 1 mL L⁻¹ trace mineral mix, 1 g L⁻¹ NH₄Cl, 5.2 g L⁻¹ NaH₂PO₄·2H₂O, 11.6 g L⁻¹ Na₂HPO₄·12H₂O, 0.45 g L⁻¹ K₂SO₄, 0.8 g L⁻¹ MgSO₄·7H₂O, and 0.08 g L⁻¹ CaCl₂·2H₂O. Components of the trace mineral mix per liter of 0.1 M HCl are: 15 g L⁻¹ FeSO₄·7H₂O, 2.4 g L⁻¹ MnSO₄·H₂O, 2.4 g L⁻¹ ZnSO₄·7H₂O, and 0.48 g L⁻¹ CuSO₄·5H₂O.

Under heterotrophic conditions, *R. eutropha* was grown in flasks containing 50 mL minimal medium with 20 g L⁻¹ fructose. Under autotrophic conditions, *R. eutropha* H16 was grown in rubber stopper-sealed serum bottles containing 100 mL fructose-free minimal medium under a N₂:H₂:O₂:CO₂ (49:37:7:7) atmosphere.

Cell-free spent medium preparation, pronase treatment, H₂O₂-degrading enzymatic activity, and total protein concentration

For cell-free spent medium preparation, a *R. eutropha* culture grown with fructose for 40 hours was centrifuged at 10000 x g for 10 minutes at 4 °C. The supernatant was collected and centrifuged for a second time to ensure the complete removal of *R. eutropha* cells. The supernatant from the second centrifugation was considered as cell-free spent medium. For protein degradation treatment, 1 mg mL⁻¹ pronase was added to
the cell-free spent medium, which was then incubated overnight at 37 °C. Total protein concentration in cell-free spent medium was measured with the M5 BCA protein assay kit (Mei5 Biotechnology, China).

H₂O₂-degrading enzymatic activity was measured as described before with several modifications (Beers and Sizer, 1952). 10 mM H₂O₂ was added into sterile minimal medium or cell-free spent medium pre-incubated at 30 °C. 400 μL of the reaction was quickly transferred to a quartz cuvette and absorbance at 240 nm was monitored over time with an Evolution 220 UV-Visible spectrophotometer (Thermo Fisher Scientific, USA). H₂O₂ concentration was calculated according to equation (1).

\[ A = \varepsilon cl \]  

(1)

Where A is the absorbance, c is the concentration, l is the light path length (1 cm), and ε is the molar extinction coefficient, which is 43.6 M⁻¹ cm⁻¹ for H₂O₂ at a wavelength of 240 nm.

**Photocatalytic H₂, O₂, and H₂O₂ evolution under a 4200 lux LED light**

The photocatalytic evolution experiments of H₂, O₂, and H₂O₂ were carried out in rubber stopper-sealed 120 mL serum bottles incubated at 30 °C into a CZL-P280D LED light incubator (Hua De Li, China) set at 4200 lux. Serum bottles contained 10 mg g-C₃N₄ particles dispersed in 20 mL ultrapure water, 50 mM KH₂PO₄ buffer pH 7.4
(CAT), sterile minimal medium, or cell-free spent medium. Where indicated, 10, 100, 500, or 2000 U mL$^{-1}$ bovine liver catalase (Yuan Ye, China) or 100 U mL$^{-1}$ superoxide dismutase (Yuan Ye) was added to the PC suspension. Prior to photocatalytic experiments, g-C$_3$N$_4$ suspensions were flushed with pure nitrogen for 20 minutes. Where indicated, 5% (w/v) triethanolamine (TEOA) was added to the PC suspension.

H$_2$ and O$_2$ were measured with a GC9720 Plus gas chromatograph (FULI, China) equipped with a 5A molecular sieve column and a thermal conductivity detector (TCD). For H$_2$O$_2$ measurement, 1 mL g-C$_3$N$_4$ suspension was sampled and centrifuged at 7000 x g for 1 minute. The top 400 µl of the supernatant was transferred to a quartz spectrophotometer cuvette and H$_2$O$_2$ concentration was measured at 240 nm.

**g-C$_3$N$_4$-catalase activity under a solar simulator**

The photocatalytic activity of g-C$_3$N$_4$ suspended in CAT buffer containing 2000, 5000, or 10000 U mL$^{-1}$ bovine liver catalase was evaluated at room temperature under a PL-XQ 500 W Xe lamp (Pu Lin Sai Si, China) equipped with an air mass 1.5 global conditions filter (AM1.5G) with a light intensity of 200 W m$^{-2}$ or 800 W m$^{-2}$ (ca. 0.2 to 0.8 sun). For quantum efficiency ($\text{QE}_{420\text{nm}}$) determination, the Xe lamp was instead equipped with a band-pass filter of 420 ± 20 nm. The intensity of the light reaching g-C$_3$N$_4$-catalase was measured with a TES-132 light radiometer (TES, China). Nitrogen-flushed serum bottles with g-C$_3$N$_4$-catalase were prepared as described above with the exception that 30 mg PC was in suspension instead of 10 mg. For the cycling test, H$_2$
and O₂ evolution by g-C₃N₄ were measured three times with 10000 U mL⁻¹ catalase under a light intensity of 200 W m⁻². Between each run, the headspace of the serum bottle was flushed 20 minutes with pure nitrogen.

**Solar-to-hydrogen efficiency and quantum efficiency calculations**

The solar-to-hydrogen (STH) efficiency was evaluated with the 500 W Xe lamp equipped with an AM1.5G filter according to equation (2).

\[
\text{STH} \% = 100 \frac{E_{H_2}}{E_s A} \text{ or } 100\frac{\Delta G^0_{H_2}}{E_s A} \text{ (2)}
\]

Where \(E_{H_2}\) is the energy stored in H₂, \(E_s\) is the light energy radiation per m² per second (J m⁻² s⁻¹), \(A\) is the illumination area in m², \(\Delta G^0_{H_2}\) is the standard Gibbs energy of 237200 J mol⁻¹ at 297 K for the energy storage reaction generating H₂, and \(R_{H_2}\) is the H₂ evolution rate in mol s⁻¹.

\(\text{QE}_{420\text{nm}}\) was calculated according to equations (3) and (4).

\[
\text{QE}_{420\text{nm}} \% = \frac{2 \times \text{number of evolved H}_2 \text{ molecules}}{\text{number of incident photons}} \times 100 \text{ (3)}
\]

\[
\text{Number of incident photons} = I\lambda \frac{A t}{h c} \text{ (4)}
\]
Where $I$ is the light intensity in W m$^{-2}$, $A$ is the illumination area in m$^2$, $t$ is the time in second, $\lambda$ is the wavelength, $h$ is the Planck constant $6.626\times10^{-34}$ J s, and $c$ is the speed of light $3\times10^8$ m s$^{-1}$.

**Hybrid photosynthesis with R. eutropha and g-C$_3$N$_4$-catalase**

For autotrophic hybrid photosynthesis, *R. eutropha* grown to mid-log with CO$_2$ as sole carbon source was used as inoculum. A serum bottle containing 100 mL minimal medium with 0.5 g L$^{-1}$ g-C$_3$N$_4$ and 5000 U mL$^{-1}$ bovine liver catalase under a N$_2$:H$_2$:CO$_2$:O$_2$ atmosphere was inoculated with CO$_2$-grown *R. eutropha* at an initial optical density (OD$_{600\text{nm}}$) of 0.01, and then it was incubated at 30 °C under LED light at an intensity of 4200 lux. PHB production was monitored during 48 hours. For heterotrophic hybrid photosynthesis, a fructose-grown *R. eutropha* mid-log culture was used to inoculate a flask containing 0.5 g L$^{-1}$ g-C$_3$N$_4$ and 5000 U mL$^{-1}$ bovine liver catalase in 50 mL minimal medium with 20 g L$^{-1}$ fructose. The initial OD$_{600\text{nm}}$ was 0.05. Both PHB production and fructose consumption were monitored over 96 hours for the heterotrophic g-C$_3$N$_4$-catalase-*R. eutropha* system, which was incubated at 30 °C under LED light at an intensity of 4200 lux. Controls without g-C$_3$N$_4$, catalase, or light exposure were also tested for PHB production.

**PHB, fructose, and production yield**

PHB was extracted and measured as described previously (Xu et al., 2019; Braunegg et al., 1978). 10 mL of PHB-containing samples were centrifuged at 7000 x g for 10 minutes at 4 °C. Cell pellets were washed twice with ultrapure water before being dried overnight at 55 °C. Samples were added to 2 mL methanol acidified with 3% (v/v)
H$_2$SO$_4$. Extraction mixtures were then incubated for 4 hours in screw-capped glass tubes at 100 °C. After cooling of the samples at room temperature, 1 mL ultrapure water was added to the extraction mixtures, which were then shaken vigorously for 1 minute. The organic phase was removed after separation from the aqueous phase and transferred to a glass vial. Extracted PHB in the organic phase was measured with a GC9720 gas chromatograph (Fuli) equipped with a RB-FFAP column and a flame ionization detector (FID). The gas chromatograph column was maintained at an initial temperature of 50 °C for 2 minutes with subsequent temperature increase rate of 10 °C min$^{-1}$ up to 220 °C for 2 minutes.

Fructose concentration was measured with a Breeze 2 high-performance liquid chromatograph (Waters, MA, USA) equipped with a Refractive Index Detector and an Aminex HPX-87H anion exchange column (Bio-Rad, CA, USA) set at a temperature of 50 °C. The mobile phase was 5 mM H$_2$SO$_4$ at a flow rate of 0.6 ml min$^{-1}$. Yield of PHB production to fructose consumption is calculated according to equation (5).

\[ Y_{p/s} = \frac{\text{grams PHB ([C$_4$H$_6$O$_2$]$_n$) produced}}{\text{grams fructose (C$_6$H$_{12}$O$_6$) consumed}} \quad (5) \]

**Microscopy and energy-dispersive X-ray diffraction**

The structure of g-C$_3$N$_4$ particles was characterized by SEM, TEM, and atomic force microscopy (AFM). Elemental mapping of g-C$_3$N$_4$ samples with or without 2000 U mL$^{-1}$ bovine liver catalase was completed by energy dispersive X-ray spectroscopy (EDS).
Both SEM and EDS were performed with a S-4800 Field-emission scanning electron microscope (Hitachi, Japan). Samples were dropped on a silicon wafer and air-dried before being scanned at an accelerating voltage of 5 kV. For TEM, g-C₃N₄ samples were dispersed into ethanol by ultrasonic treatment and diluted before being dropped on a copper grid. TEM images were obtained with a JEM-1400Plus (JEOL, Japan) microscope at an accelerating voltage of 120 kV. AFM analysis was performed with a Nanoscope IV (Veeco, NY).

For SEM of hybrid photosynthesis system, 24-hours autotrophic or heterotrophic bacterial cultures with g-C₃N₄-catalase were collected and fixed overnight at 4 °C in a 0.1 M Na phosphate buffer (pH 7.0) containing 2.5% glutaraldehyde. Samples were then washed one time serially with 30%, 50%, 70%, 80%, 90% ethanol and two times with 100% ethanol. Samples suspended in 100% ethanol were dropped on a silicon wafer and air-dried, and then they were scanned at an accelerating voltage of 5 kV. The same procedure was employed to prepare the hybrid photosynthesis samples for TEM with the exception that g-C₃N₄-catalase with *R. eutropha* H16 was dropped on a copper grid instead of silicon wafer.

**Electrochemical impedance spectroscopy (EIS)**

Photoelectrochemical measurements were conducted with a CHI600E potentiostat (CH Instruments, TX, USA) using a three-electrode system with *R. eutropha* autotrophic growth medium as electrolyte. A glassy carbon electrode (diameter of 3 mm) served as
the working electrode, a platinum sheet was used as the counter electrode, and an Ag/AgCl electrode (3.5 M KCl) was employed as the reference electrode. EIS spectra were acquired with a sinusoidal perturbation of 5 mV amplitude and frequencies ranging from 100 kHz to 0.1 Hz at -1.2 V versus Ag/AgCl under illumination.

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