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Short Communication

The enhancement of hydrogen photoproduction in marine *Chlorella pyrenoidosa* under nitrogen deprivation

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ABSTRACT

Previously, we found that marine *Chlorella pyrenoidosa* strain IOAC707S could generate hydrogen (H_2) under nitrogen deprivation in seawater medium, without dark incubation, N_2 flushing or addition of protonophore. In this work, the H_2 yield of *C. pyrenoidosa* was enhanced, and the mechanism of H_2 photoproduction was investigated. It was found that *C. pyrenoidosa* could produce H_2 under hypoxic conditions, which indicated the hydrogenase of *C.pyrenoidosa* could tolerate a low concentration of O_2. Nitrogen deprivation could lower the efficiency of photosyntem (PS) II photochemical activity and PSII oxygenic activities, which were favorable for rapid establishment of anoxia and efficient H_2 photoproduction, and thus significantly enhance H_2 photoproduction of *C. pyrenoidosa*. Preculture in nitrogen-deprived medium could further increase the H_2 yield of *C. pyrenoidosa*, for its more severe inhibition of the oxygen-evolving complex, electron transport and photochemical efficiency of PSII, and PSII oxygenic activities, which facilitated the establishment of anaerobiosis and activation of hydrogenase.

Introduction

Concerns about the depletion of fossil fuel reserves, and aggravation of global warming, strongly encourage the exploration of alternative energy sources that are clean and renewable. H_2 gas is considered to be the ideal fuel for its high energy content per unit mass and its carbon-free composition [1]. Among the numerous methods for producing H_2, H_2 photoproduction by green algae is very attractive. First of all, green algae can produce significant amounts of H_2 using sunlight and water, which are the two most abundant resources on our planet [2]. Secondly, this method does not contribute harmful or polluting byproducts to the

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environment. In addition, algae offer valuable byproducts, such as polysaccharides, protein, fatty acids and so on. Research on H_2 photoproduction by green algae has mainly focused on fresh water strains, such as *Chlamydomonas reinhardtii* [3], *Chlamydomonas moewusii* [4], *Chlorella vulgaris* [5], *Scenedesmus obliquus* [6] and so on. A two-stage protocol based on sulfur-deprivation is widely used to induce photoproduction of H_2 by green algae. In stage 1, the cells are cultured in nutrient-replete medium to accumulate biomass. In stage 2, the cells are transferred to sulfur-deprived medium to induce H_2 photoproduction by the algae. Sulfur deprivation inhibits oxygenic photosynthesis greatly [7], but has little effect on the respiration rate [8]. As a result, an anaerobic environment is established in the sealed culture, leading to the expression of hydrogenases and to sustained H_2 photoproduction [9,10].

Compared with fresh-water algae, H_2 photoproduction by marine algae growing in seawater might be an effective method for scaling-up bio-hydrogen production, because it has the advantage of saving freshwater resources. However, as seawater contains substantial amounts of sulfur, it is hard to induce H_2 photoproduction by marine algae in natural seawater medium. In order to achieve sustained H_2 photoproduction by marine algae, artificial seawater and some supplementary means, such as dark anaerobic incubation, N_2 flushing, addition of special chemicals and so on, were usually used [11,12]. For instance, *Tetraselmis subcordiformis* cultured in natural seawater medium produced only ~20 μL/L H_2 in continuous illumination after an anaerobic condition was achieved by dark incubation and N_2 flushing. Addition of the PSII inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCUM) and protonophore carbonylcyanide m-chlorophenylhydrazone (CCCP) increased the H_2 yield, but the maximum H_2 yield was still low (4.9 mL/L culture using a 15 μM CCCP treatment) [13]. With dark incubation and CCCP treatment, the H_2 yield of *Tetraselmis subcordiformis* reached 15.2 and 55.8 mL/L culture in sulfur and nitrogen deprived artificial seawater medium, respectively [12]. However, the use of artificial seawater and protonophore would raise the cost of bio-hydrogen production.

In our previous work, a marine strain of the green alga *Chlorella pyrenoidosa* (IOAC707S), was found to generate H_2 in nitrogen-free natural seawater medium, without dark incubation, N_2 flushing or addition of protonophore, which made it possible to produce H_2 using seawater at relatively low cost [14]. However, the exact mechanism of H_2 photoproducion under these conditions remained unclear, and the H_2 yield still needed to be enhanced. In this study, we attempted to further improve H_2 photoproduction of *Chlorella pyrenoidosa* strain IOAC707S in seawater medium by nitrogen deprivation during growth period and sealed incubation, and explore the mechanism of its H_2 photoproduction under nitrogen-deprived conditions.

Materials and methods

Growth conditions

Marine *Chlorella pyrenoidosa* strain IOAC707S was obtained from the algal collection (IOAC) of the Institute of Oceanology, Chinese Academy of Sciences (IOCAS). The algae were cultured in L1 [15] or nitrogen-deprived L1 medium, with the addition of 1 mL/L acetic acid (named as L1+HAc or L1+HAc-N medium). Nitrogen-deprived L1 medium was prepared by excluding $NaNO_3$ from the L1 medium. The pH of both media was adjusted to 7.8 by adding 2.42 g/L of Tris base. The culture conditions were 25 ± 1 °C in 14:10 light:dark cycle under 25–30 μmol photon m^{-2} s^{-1} of illumination provided from the top by cool-white fluorescent lamps. During the growth periods, the cultures were manually shaken 2–3 times each day to avoid sticking.

H_2 production procedure

When reaching the late logarithmic phase, cells grown in L1+HAc or L1+HAc-N medium were harvested by centrifugation (3800 × g, 6 min), washed twice, and then both were resuspended in L1+HAc-N medium. These two treatments were named as the L1-N and L1-NN groups, respectively. Cells grown in L1+HAc medium and resuspended in the same medium were set as the control group (CK). The above cultures were incubated in ~150 mL glass bottles and sealed with butyl rubber stoppers. The headspace volume in each bottle was approximately 30 mL. The sealed bottles were placed into an incubator at 150 rpm to allow the adequate exchange between gas phase and algal culture. The incubation conditions were 25 ± 1 °C under continuous illumination of 60–65 μmol photon m^{-2} s^{-1} from two sides. Each treatment was done in triplicate.

H_2 and O_2 measurement

One fifth milliliter of headspace gas was sampled from each bottle every 24 h. H_2 and O_2 concentrations were determined using a gas chromatograph (GC112A, Precision & Scientific Instrument Co., LTD, Shanghai, China) according to He et al. [16].

Analytical procedures

The total chlorophyll contents were measured spectrophotometrically in 96% ethanol extracts, and calculated according to the following equation: Chlorophyll a + b (mg/L) = $6.63 \times A_{665} + 18.08 \times A_{649}$, where A_{665} and A_{649} correspond to the absorbance of extracted supernatant at 665 nm and 649 nm wavelength, respectively. The maximal efficiency of PSII photochemical activity (Fv/Fm) of cultures were measured with a FMS-2 pulse modulated fluorometer (Hansatech Instruments, Norfolk, UK) according to Zhang et al. [17]. The relative variable fluorescence at the K-step (W_K) and the number of active PSII reaction centers (RC) per excited cross-section (RC/CSo) were measured with a Plant Efficiency Analyzer (Hansatech Instruments, Norfolk, UK) according to Wen et al. [18]. The photosynthetic O_2 evolution and dark respiration capacities were measured at room temperature with a Clark-type O_2 electrode (Hansatech Instruments, Norfolk, UK) according to He et al. [16].

The statistical analyses were performed using SPSS 16.0 software. All data throughout the paper are given as mean ± SE.