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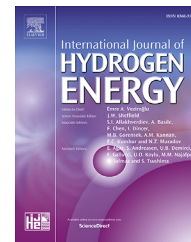

The effect of cycA overexpression on hydrogen production performance of *Rhodobacter sphaeroides* HY01

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ABSTRACT

The gene *cycA* encodes a periplasmic protein, cytochrome c_2 (cyt c_2), which dominates electron transfer from the membrane-bound ubiquinol: cyt c_2 oxidoreductase (cyt bc_1) to the photosynthetic reaction center, contributing to the production of transmembrane proton potential and then the synthesis of ATP. For photosynthetic bacteria, the total energy supply for light anaerobic growth and hydrogen production comes from photophosphorylation. As a result, the key protein encoding gene plays an important role in hydrogen production. To figure out the specific effect of cycA expression level on H_2 production ability of *Rhodobacter sphaeroides* HY01, cycA-expression plasmids derived from pRK415 and pBBR1MCS-2 were constructed and then crossed into the parent strain *R. sphaeroides* HY01 for H_2 production test. And further verification by RT-PCR suggested that there was about 20% enhancement of *cycA* expression level by pBBR1MCS-2 where the H_2 production performance of corresponding strain was improved by 6–8% compared with blank control. In contrast, *cycA* expression level was about 3.4 folds by vector pRK415 compared with control strain, but corresponding strains showed slightly depressed H_2 production performance. Besides, the mutant XJ01 with *cycA* gene overexpressing by 70% in the genome of HY01(*hupSL:cycA*) also showed positive effect on hydrogen production performance. The results demonstrated that slightly overexpression of *cycA* could enhance the hydrogen production rate, but too much higher level of *cycA*-expression could show negative effect on H_2 production performance of *R. sphaeroides* HY01.
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Introduction

Hydrogen has been accepted as one of the most promising alternative fuel for its high energy density and nonpolluting characteristics [1]. Among the hydrogen production pathways, bio-hydrogen production [2–5] and water splitting [6,7] are accepted to be potential strategies for sustainable hydrogen generation. In bio-hydrogen production pathway, some organic compounds in wastewater and solid wastes could serve as carbon source, and it is a sustainable and moderate H_2 production pathway. Photo-fermentative H_2 production, as an important branch of biological H_2 production process, is attracting much attention for its high substrate

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conversion efficiency. Many photosynthetic bacteria, such as *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, and *Rhodopseudomonas palustris* [8,9], were reported to be good candidates for photo-fermentation. However, the H_2 production rate of photo-fermentation is still low compared with the dark-fermentation [10]. Recently, much attention has been paid to improve the H_2 production rate of photo-fermentation [11–15].

As shown in Fig. 1, it suggested that H_2 production by photosynthetic bacteria is an integrated process associated with multiple metabolic pathways and enzymes, such as light absorption and ATP synthesis, substrate conversion (TCA) and H_2 production enzyme (nitrogenase). As a result, H_2 production rate of *R. sphaeroides* is significantly affected by ATP synthesis rate as well as the level of substrate converting and enzymes activity [16].

Under the anaerobic illumination condition, ATP synthesis, which is dominated by photophosphorylation, is of great importance for H_2 production [17]. The photo-induced cyclic electron transfer is one of the major components of photophosphorylation. And it depends on the function of three multimeric transmembrane protein complexes, including the reaction center and light-harvesting complexes (RC-LH), the cytochrome (cyt) bc_1 complex and a periplasmic protein cyt c_2 which functions as a mobile electron carrier in both aerobic and photosynthetic electron transport chains [18,19]. The mobility of cyt c_2 and its interaction with membrane bound partners are important in the function of the photosynthetic apparatus [20]. The function and association of cyt c_2 with RC and cyt bc_1 [20–22] have been studied for decades. It is demonstrated that cyt c_2 encoded by *cycA* transports electrons from cyt bc_1 to RC and binds with RC in short-range hydrogen bond interactions [20]. Cyt c_2 is proved to be indispensable in cyclic electron transfer of *Rhodobacter sphaeroides*, where *Rhodobacter sphaeroides* mutant lacking cyt c_2 was incapable of photosynthetic growth [23]. It is undoubted that cyt c_2 plays an important role in photo-induced electron transfer of *Rhodobacter sphaeroides*.

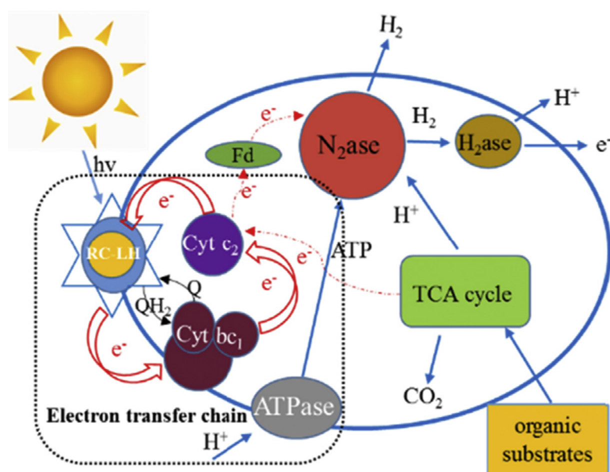

Fig. 1 – Schematic representation of the photo-induced cyclic electron transfer chain in *Rhodobacter sphaeroides*. LH: light-harvesting complexes, RC: reaction center.

Many studies proved that overexpression of genes could enhance the encoded enzymes level, and therefore promotes the reaction dominated by these enzymes. Overexpression of genes encoding for *Rnf* complex could enhance the nitrogenase activity [24]. Increasing the NAD(H) pool could decrease the NADH/NAD^+ ratio, and further increase the substrate utilization rate, leading to the improvement of hydrogen production performance [25]. Moreover, overexpressing the structural genes of ATPase also promoted ATP synthesis and therefore enhanced nitrogenase activity and H_2 production performance [16,26]. Besides, it was reported that the relatively low content of cyt c_2 limited the turnover rate of cyt bc_1 and therefore limited the cyclic electron transfer rate, and further limited the photophosphorylation rate [27,28]. Logically, overexpressing the gene, *cycA*, encoding cyt c_2, could increase the content of this protein and further promote cyclic electron transfer rate and ATP synthesis. To the best of our knowledge, there was no study on the relationship between *cycA* overexpression and photo-fermentative hydrogen production performance.

In this work, in order to study the effect of *cycA* expression level on the photosynthetic growth and H_2 production performance, expression vectors pRK415 and pBBR1MCS-2 were used to overexpress the native *cycA* in *Rhodobacter sphaeroides* HY01, which is a purple non-sulfur photosynthetic bacterium (PNSB) with relatively high H_2 production rate [29]. Strains with duplicated *cycA* in the genome derived from *R. sphaeroides* HY01 were also constructed and characterized on growth, *Bchl* content and H_2 production performance under different culture conditions.

Material and methods

Bacterial strains and plasmids

Bacterial strains, including *R. sphaeroides* and *E. coli*, plasmids and primers used in this study were listed in Table 1. *R. sphaeroides* HY01 was used as the parental strain. HY01 and derived strains were incubated in sistrom's mineral medium (Med) [30] or MPYE [31] medium at 35 ± 0.5 °C. *E. coli* strains were cultured in Luriae Bertani medium (LB) at 37 ± 0.5 °C medium supplemented with proper antibiotics when needed. For pre-culture, *R. sphaeroides* and *E. coli* strains were cultured in 50 mL sterile screwed tubes with 10 mL Med or 5 mL LB medium in a shaker, respectively. Antibiotics were used at the following concentrations: tetracycline 12.5 mg/L, kanamycin 50 mg/L, ampicillin 100 mg/L for *E. coli* and tetracycline 2.5 mg/L, kanamycin 10 mg/L for *R. sphaeroides*, respectively.

Construction of *cycA* overexpression strains

The *cycA* gene and around 500 bp flank sequences were amplified by polymerase chain reaction (PCR) with the genomic DNA of *R. sphaeroides* HY01 as the template. The sequences of primers were listed in Table 1. A 5′ *Kpn*I site or 3′ *Xba*I site was added in each primer for the ligation sites with the vectors. The PCR product, *cycA* DNA (1.5 kp) was ligated into pBlu2SKP via *Xba*I and *Kpn*I to obtain a new plasmid pcycA01 for DNA sequencing. In order to avoid mutation

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