Bioresource Technology 118 (2012) 265–271

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Redirecting the electron flow towards the nitrogenase and bidirectional Hox-hydrogenase by using specific inhibitors results in enhanced H_2 production in the cyanobacterium *Anabaena siamensis* TISTR 8012

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HIGHLIGHTS

- Photosynthetic cyanobacterial based H_2 production is limited by electron supply.
- Redirecting competing pathways for electron flow results in increased H_2 production.
- Metabolic engineering leads to increased production of desired product.

ARTICLE INFO

Article history:
Received 5 March 2012
Received in revised form 8 May 2012
Accepted 11 May 2012
Available online 18 May 2012

Keywords:
H_2 production
Anabaena
Nitrogenase
Bidirectional Hox-hydrogenase
Metabolic inhibitors

ABSTRACT

The inhibition of competitive metabolic pathways by various inhibitors in order to redirect electron flow towards nitrogenase and bidirectional Hox-hydrogenase was investigated in *Anabaena siamensis* TISTR 8012. Cells grown in $BG11_0$ supplemented with KCN, rotenone, DCMU, and DL-glyceraldehyde under light condition for 24 h showed enhanced H_2 production. Cells grown in BG11 medium showed only marginal H_2 production and its production was hardly increased by the inhibitors tested. H_2 production with either 20 mM KCN or 50 μM DCMU in $BG11_0$ medium was 22 μmol H_2 mg chl a^{-1} h^{-1}, threefold higher than the control. The increased H_2 production caused by inhibitors was consistent with the increase in the respective Hox-hydrogenase activities and *nifD* transcript levels, as well as the decrease in *hupL* transcript levels. The results suggested that interruption of metabolic pathways essential for growth could redirect electrons flow towards nitrogenase and bidirectional Hox-hydrogenase resulting in increased H_2 production.

1. Introduction

H_2 metabolism in N_2-fixing cyanobacteria may involve three enzymes: nitrogenase, the uptake hydrogenase and the bidirectional Hox-hydrogenase. The nitrogenase complex consists of two complexes, dinitrogenase (encoded by *nifD* and *nifK*) and dinitrogenase reductase (encoded by *nifH*). Nitrogenase catalyzes H_2 production concomitantly with N_2-fixation with a minimum of 25% of the electrons used for H_2 production. The uptake hydrogenase consists of at least two subunits (encoded by *hupS* and *hupL*) oxidizing the H_2 evolved by nitrogenase. The bidirectional Hox-hydrogenase consists of a hydrogenase part (encoded by *hoxY* and *hoxH*) and a diaphorase part (encoded by *hoxE*, *hoxU* and *hoxF*). The enzyme may catalyze both the production and utilization of H_2 (Tamagnini et al., 2007; Phunpruch et al., 2006). N_2-fixing cyanobacteria are suitable for photobiological hydrogen production. They can use solar energy to split water and shuttle electrons through the electron transport chain to the terminal electron acceptor ferredoxin (Fd) via the plastoquinone pool. This will generate strong reductants such as NADPH and reduced ferredoxin, which can be utilized as substrates for H_2 production by either nitrogenase or hydrogenase (Lubitz et al., 2008 and Tamagnini et al., 2007) (Fig. 1). However, major obstacles for a sustainable H_2 production in N_2-fixing cyanobacteria are the irreversible inhibition of the enzymes by oxygen, H_2 consumption by the uptake hydrogenase and an overall low H_2 productivity due to the competition for electrons by numerous other assimilatory pathways. The

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http://dx.doi.org/10.1016/j.biortech.2012.05.052

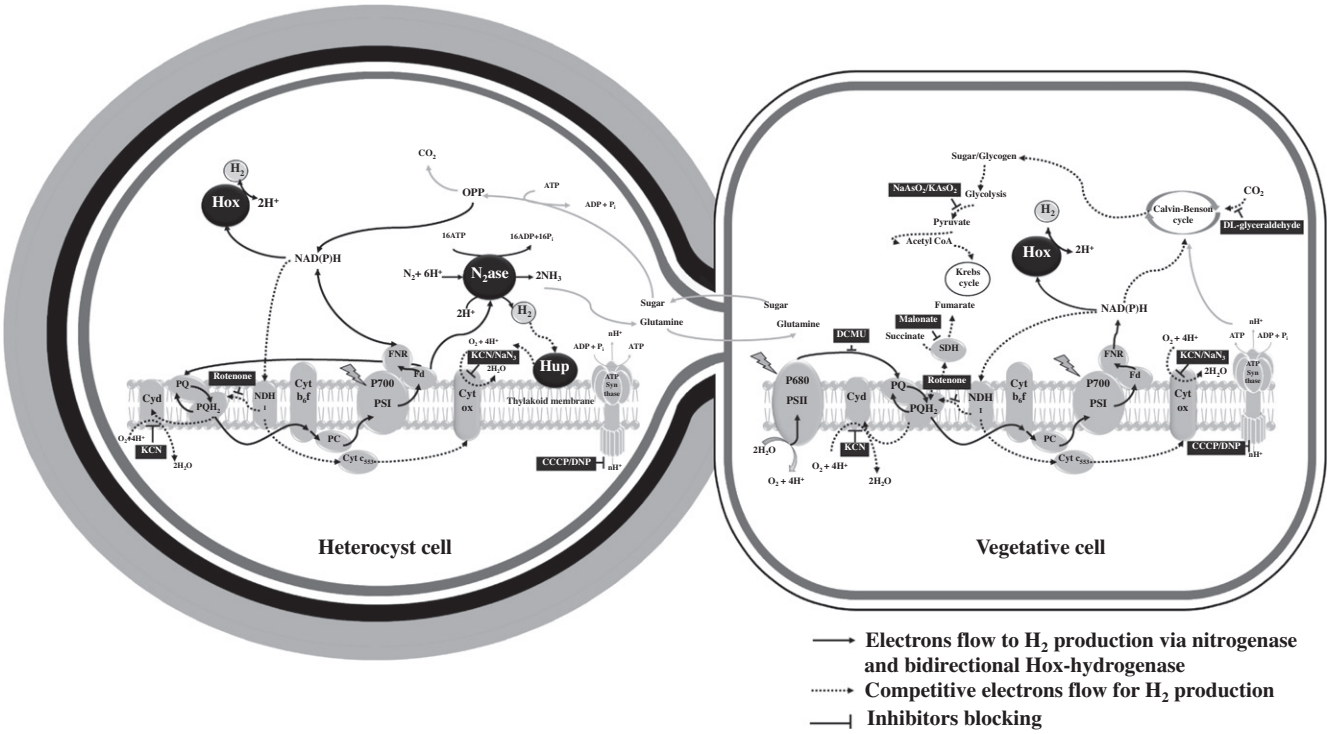

Fig. 1. A schematic presentation of different pathways of electron flow which involve H_2 metabolism of N_2-fixing cyanobacteria with specific inhibitors indicated. The bold arrows represent the electron flow to hydrogen metabolism through nitrogenase and hydrogenase associated with photosynthesis and carbohydrate catabolism. The dotted arrows represent competing pathways for electrons for H_2 production. Abbreviations; N_2ase: nitrogenase, Hox: bidirectional Hox-hydrogenase, Hup: uptake hydrogenase, PSII: photosystem II, PSI: photosystem I; PQ/PQH$_2$: plastoquinone pool, Cyt b_6f: cytochrome b_6f, PC: plastocyanin, Cyt c_{553}: cytochrome c_{553}, Cyd: quinol oxidase, Fd: ferridoxin, FNR: ferridoxin–NADP reductase, SDH: succinate dehydrogenase, NDH-I: NADPH dehydrogenase (complex I), Cyt ox: cytochrome c oxidase, OPP: oxidative pentose phosphate pathway. This scheme was modified from Meek and Elhai (2002) and Gutthann et al. (2007).

electrons generated through biophotolysis are not entirely shuttled to H_2 metabolism. Instead, these electrons can be used in other pathways involved in CO_2-fixation, generation of water through cytochrome c oxidase, and in the respiratory electron transport chain including NADPH dehydrogenase complex I (NDH-I), succinate dehydrogenase (SDH) and quinol oxidase (Eroglu and Melis, 2011 and Srirangan et al., 2011).

Previous studies using the unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 (*Synechocystis* PCC 6803) demonstrated enhanced H_2 production by inactivating the nitrate assimilation pathway (Baebprasert et al., 2011). The nitrate assimilation pathway competes directly for electrons available inside the cells. When this pathway is disrupted electrons are re-directed to flow towards the Hox-hydrogenase resulting in increased H_2 evolution. In addition, metabolic inhibitors have recently been used to redirect electrons to improve H_2 production in cyanobacteria. Burrows et al. (2011) showed that when using optimal concentrations and combinations of KCN and malonate a significantly higher H_2 production over 3 days of light/dark cycling was observed in *Synechocystis* PCC 6803. Miyamoto et al. (1979) reported that H_2 production was stimulated by 50% in the presence of DCMU under nitrogen limiting conditions. However, there have been only very limited studies on the inhibition of competing pathways for H_2 production in N_2-fixing cyanobacteria. Moreover, very little is known about the relationship of photosynthesis, respiration and H_2 metabolism in heterocysts and vegetative cells.

In the present study, we attempted to enhance H_2 production by inhibiting competing pathways for electrons so that significant flow of electrons is directed towards the cyanobacterial nitrogenase and/or bidirectional Hox-hydrogenase using selected inhibitors. Ten inhibitors were investigated for their effect on H_2 production. KCN (potassium cyanide) inhibits the electrons flow to all respiratory terminal oxidase such as quinol oxidase and cytochrome c oxidase whereas NaN_3 (sodium azide) inhibits only cytochrome c oxidases (Pils and Schmetterer, 2001). Malonate is a competitive inhibitor of succinate dehydrogenase (Peschek et al., 2004) and Rotenone blocks the electrons transfer from NDH-I to plastoquinone pool (Teicher and Scheller, 1998). DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea) is a photosystem II (PSII) inhibitor to prevent electron transfer from PSII to plastoquinone and also inhibit generation of oxygen through water splitting in PSII. DL-glyceraldehyde blocks the electron transfer via Calvin–Benson cycle to CO_2-fixation (Stokes and Walker, 1972) whereas CCCP (carbonyl cyanide m-chlorophenyl hydrazone) and DNP (2,4-dinitrophenol) uncouple the proton pumping from ATP resulting in the disruption of the proton gradient by carrying protons across a membrane (Heytler, 1979). Finally, $NaAsO_2$ (Sodium arsenate) and $KAsO_2$ (potassium arsenate) specifically inhibit some enzymes in Krebs cycle including pyruvate dehydrogenase (Tretter and Vizi, 2000).

The filamentous heterocyst forming N_2-fixing cyanobacterium *Anabaena siamensis* TISTR 8012, originally isolated from a rice field in Thailand (Antarikanonda, 1985) was used to study the effect of various inhibitors on H_2 production. This strain was selected due to its high potential for H_2 production as well as its resistance to environmental stresses (Khetkorn et al., 2010). In addition, relative transcription levels of genes involved in H_2 metabolism, and the activity of bidirectional Hox-bidirectional were also analyzed.

2. Methods

2.1. Strain and growth conditions

The filamentous heterocyst-forming cyanobacterium *A. siamensis* TISTR 8012 was cultured in 50 mL of either BG11 (containing 18 mM $NaNO_3$ as N-source) or $BG11_0$ medium (without N-source)