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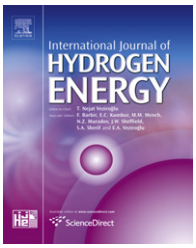

Effect of inactivation of genes involved in ammonium regulation on the biohydrogen production of *Rhodobacter capsulatus*

Gülşah Pekgöz [a,*], Ufuk Gündüz [a], Inci Eroğlu [b], Meral Yücel [a], Kornél Kovács [c], Gábor Rákhely [c]

[a] Department of Biological Sciences, Middle East Technical University, 06531 Ankara, Turkey
[b] Department of Chemical Engineering, Middle East Technical University, 06531 Ankara, Turkey
[c] Department of Biotechnology, University of Szeged, Hungary

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ABSTRACT

Hydrogen production by nitrogenase is an energetically expensive process for the cell, hence strictly controlled at different levels. Ammonium is one of the substances regulating nitrogenase activity. The key proteins in the regulation of nitrogenase by ammonium are two regulatory proteins; GlnB and GlnK. In order to increase hydrogen production of *Rhodobacter capsulatus* DSM1710 (wild type strain) grown on agricultural materials/wastes, ammonium inhibition of nitrogenase enzyme has to be eliminated. In this study, GlnB and GlnK were targeted to be inactivated by in frame site-directed mutagenesis. The *glnB* mutant *R. capsulatus* (GP1 strain) was obtained at the end of mutagenesis studies. In the case of *glnK*, the suicide vector was constructed and delivered into the cells. However, *glnK* mutant could not be obtained.

The effect of ammonium on the growth and hydrogen production of *R. capsulatus* GP1 was investigated and compared with DSM1710. Both DSM1710 and GP1 strains were effectively utilized acetate. The mutation did not affect cell growth significantly at different ammonium levels. Ammonium negatively affected hydrogen production of GP1 strain as well as the DMS1710. However, hydrogen production was significantly low in GP1 strain. The ammonium inhibition of hydrogen production could not be removed in *glnB* mutant probably due to the presence of an active GlnK protein in the cell. Therefore, GlnK has much more important role in the ammonium dependent control of nitrogenase than GlnB does. The growth and hydrogen production kinetics of *R. capsulatus* DSM1710 and GP1 were modelled. They were shown to fit to Logistic Model and Modified Gompertz Model, respectively.

1. Introduction

Due to rapid industrialization and urbanization, alternative energy carriers such as bioethanol, biogas or biohydrogen are expected to replace the fossil fuels in the near future. Among the energy carriers, hydrogen energy can be regarded as the fuel of future, since it can be efficiently utilized and completely recycled without CO_2 emission, which contributes global warming [1,2].

Different biohydrogen production mechanisms exist, e.g. dark fermentation and photofermentation. In dark fermentation, the carbon source can only be reduced to organic acids (acetic acid, butyric acid, lactic acid etc.) by fermentative

* *Corresponding author*. Tel.: +90 312 2105184; fax: +90 312 2107976.
E-mail address: gpekgoz@gmail.com (G. Pekgöz).
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bacteria [3]. It is very well known that organic acids are utilized by photosynthetic bacteria via photofermentation to H_2 and CO_2. The processes, in which the dark fermentation sequentially followed by photofermentation (or light fermentation), are called "integrated systems". With this application, the hydrogen is produced from both of the processes; hence the overall hydrogen yield can be improved to a great extent [3,4]. The overall equations of dark fermentation (1) and photofermentation (2) are given below, respectively:

$$C_6H_{12}O_6 + 2H_2O \rightarrow 2CH_3COOH + 2CO_2 + 4H_2 \quad (1)$$

$$2CH_3COOH + 4H_2O \rightarrow 8H_2 + 4CO_2 \quad (2)$$

The Hyvolution Project, "nonthermal production of pure hydrogen from biomass", is an integrated project, funded by European Union in the Sixth Framework Programme. In the first stage of the process, the biomass, which is pretreated properly, is used in thermophilic dark fermentation process. The carbon compounds are converted into acetate, H_2 and CO_2. In the second stage, the effluent of dark fermentation (DFE), which is rich in acetate and contains ammonium ions as well, is delivered into a photobioreactor. In the photofermentation, acetate is converted into H_2 and CO_2 by PNS bacteria, *Rhodobacter capsulatus* [5].

In the photofermentation mode of *R. capsulatus*, the hydrogen is produced by the nitrogenase enzyme in, which is a two-component enzyme system catalyzing the biological reduction of dinitrogen (N_2) into ammonium with the hydrolysis of ATP (nitrogen fixation) [6]. Hydrogen is produced as the byproduct of this reaction. In order to catalyze reaction, nitrogenase requires a great deal of chemical energy in the form of ATPs and reducing agents. There is strong regulation both on the expression and activity of nitrogenase by the end product -ammonium- [7].

In *R. capsulatus*, there are three proposed levels of ammonium regulation on nitrogenase enzyme complex [8,9]. The first level of control is the "NtrB/NtrC two-component system", which is responsible for measurement of cellular nitrogen status of the cell. At this level of control, transcription of *nifA1*, *nifA2* and *anfA* genes, which are the transcriptional activators of structural genes of nitrogenases, is regulated by GlnB regulatory protein. The second level of ammonium regulation is on the control of the activity of NifA transcriptional activators; both regulatory proteins (GlnB and GlnK) inhibit the activity of NifA1 and NifA2 in the presence of ammonium [10]. Third level of regulation is based on the post-translational control of nitrogenase activity. Similar to other levels, this level of regulation is also a GlnB-GlnK dependent regulatory process. The cell is capable to switch off the nitrogenase activity in response to ammonium addition [11,12]. With this multilevel regulation by ammonium, nitrogen fixation and hydrogen production completely ceases in the presence of ammonium. GlnB and GlnK proteins are the important elements of this multilevel regulation.

GlnB and GlnK are proteins of the P_{II} signal transduction superfamily, which play a major role in coordinating the regulation of central metabolic processes. Signals from the carbon, nitrogen and energy status of the cells are converted into different conformational (and modification) states of the P_{II} proteins. Depending on these states, the P_{II} proteins interact with various target proteins, most of which perform or regulate crucial reactions in nitrogen and carbon assimilatory pathways [13]. Their functions are known to be critical for the cell. There is intense ongoing research about P_{II} proteins, in order to reveal their functions completely, to understand how they interact with various target proteins in the cell and to comprehend the whole picture of interactions of P_{II} proteins. Mutational analysis of P_{II} proteins in various organisms, such as *Cyanobacteria* sp., *Rhodobacter* sp., *Rhizobium* sp., *Rhodospirillum* sp. and more, have been carried out to understand the functional characteristics of P_{II} proteins and to reveal the nature of interactions [8,9,14–19].

DFE, which is used for further hydrogen production in the photofermentation stage, generally contains high concentrations of ammonium. This phenomenon decreases hydrogen production efficiency of the overall system, since ammonium (>1–5 mM) inhibits hydrogen production by photofermentation due to the repression of nitrogenase enzyme activity and synthesis [20]. To increase overall hydrogen production efficiency in integrated biohydrogen production, the inhibitory effect of ammonium ions in DFE, on photofermentative hydrogen production should be overcome. Ammonium concentration might be decreased to a tolerable value by adsorption, however this will require to introducing additional separation processes between the dark fermentation and photofermentation units. This approach may decrease the feasibility of the process in industrial scale.

In the present study, another approach was followed: GlnB and GlnK proteins of *R. capsulatus*, which are critical elements of ammonium dependent regulation on the nitrogenase, were targeted to be inactivated in order to achieve an ammonium insensitive strain. *glnB* and *glnK* genes of the wild type strain were internally deleted. The deletions were done in frame, in order to keep downstream genes functional. The results of this inactivation process were examined with respect to biomass and hydrogen production.

2. Material and methods

2.1. Strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study were listed in Table 1. The microorganism used for hydrogen production and for genetic manipulations is *R. capsulatus* DSM1710 (wild type) strain. The genetic manipulations in this organism were performed with the help of two *Escherichia coli* strains; for general cloning purposes, *E. coli* XL1Blue strain was used and for delivery of plasmids into *R. capsulatus*, *E. coli* S17-1 (λpir) was used.

For cloning, the pBluescript SK (+) vector was used; deletion mutagenesis was performed with the pK18*mobsacB* suicide vector.

R. capsulatus was grown under continuous illumination at 30 °C in Biebl and Pfennig (BP) minimal medium [21]. Malate (7.5 mM) and glutamate (10.0 mM) were used as carbon and nitrogen sources, respectively. For hydrogen production, BP media containing acetate as the carbon source was used with 30 mM concentration. Different concentrations of ammonium