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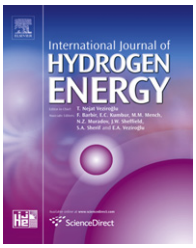

Effects of ammonium ion, acetate and aerobic conditions on hydrogen production and expression levels of nitrogenase genes in *Rhodobacter sphaeroides* O.U.001

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ABSTRACT

In the present study, expression levels of nitrogenase encoding *nifH* and control genes *nifA* and *prrA* were examined at different physiological conditions in *Rhodobacter sphaeroides* O.U.001. In addition to variations in expression levels, changes in hydrogen production and growth were also investigated in response to different concentrations of ammonium source, acetate and aerobic conditions.

In the present study, increasing concentration of ammonium chloride was found to be caused decrease in hydrogen production. Glutamate containing medium had the capacity for higher hydrogen production. Hydrogen production was observed even in aerobic conditions. The expression levels of *nif*H and *nif*A genes decreased with the increasing concentrations of ammonium chloride. Although the expression of *nif*A was present in the highest concentrations of NH_4Cl under anaerobic conditions, no expression was observed under aerobic conditions of the same culture conditions. This was likely due to transcriptional level inhibition of nitrogenase in the presence of ammonium ion. Negative correlation was observed between the expression levels of *prr*A gene and its target, *nif*A gene.

1. Introduction

Biological hydrogen production has several advantages such as low energy requirements, reduced initial investment costs and high-energy conversion efficiency. The hydrogen production by photosynthetic bacteria is found to be the most promising as compared to other microbial systems due to; high substrate to product conversion yield, lack of oxygen evolving activity, ability to use a wide wavelength range, and capability to use organic substrates derived from wastes [1]. Purple non-sulfur (PNS) bacteria produce hydrogen under light in anaerobic conditions from the utilization of organic acids such as malate, acetate and lactate. *Rhodobacter sphaeroides*, which is PNS bacteria, can use light energy to overcome the free energy of activation and produce hydrogen by utilizing organic acids as substrates [1]. The hydrogen production is catalyzed by nitrogenase enzyme in *R. sphaeroides*.

Nitrogenases, which catalyze the biological reduction of dinitrogen to ammonia, contain two components that are named according to their metal composition [2]. Site of substrate reduction is nitrogenase molybdenum-iron (MoFe) protein, also known as dinitrogenase. The obligate electron donor to MoFe protein is nitrogenase iron protein (Fe protein),

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also known as dinitrogenase reductase [3]. MoFe protein is composed of two different polypeptides encoded by *nif*D and *nif*K genes; the Fe protein is encoded by the *nif*H gene [4].

Since nitrogenase activity requires large amounts of ATP and reducing power, synthesis and activity of nitrogenase is strictly regulated at transcriptional level and post-translational level in response to environmental stimuli. Oxygen and fixed nitrogen such as ammonium are involved in the regulation of the expression of *nif* genes.

The absence or the presence of fixed nitrogen is one of the factors involved in the control of nitrogenase. Three levels of regulation of nitrogenase in *Rhodobacter capsulatus* were briefly reviewed by Tremblay et al. [5]. At the transcriptional level, the NtrB/NtrC two-component system controls *nif*A transcription. In turn, NifA induces the expression of the other *nif* genes, including the Mo-nitrogenase structural genes. At the post-translational level, NifA activity is regulated, being active only in the absence of fixed nitrogen. As well, at a third level, Mo-nitrogenase activity is regulated, being switched off within a short time after an ammonium shock.

Oxygen interferes with biological nitrogen fixation at different levels. At genetic level, oxygen represses the nitrogenase synthesis. At enzyme activity level, oxygen causes a reversible inhibition (switch-off) of nitrogenase activity in *R. sphaeroides* [6]. Photosynthetic purple non-sulfur bacteria contain NifA protein that carries CXXXXC motif. The four conserved cysteine residues could coordinate a metal cofactor that senses the redox status of the cell and a bound metal would respond to redox by triggering the conformational changes in NifA structure that modulate its function [7]. Genetic evidence has suggested that phosphorylation of *R. sphaeroides* PrrA by PrrB stimulates transcription of target genes when oxygen becomes limiting [8]. The global redox-responsive PrrB–PrrA two-component system directly regulates nif transcription in *R. capsulatus* [2]. Under aerobic conditions, PrrA is unphosphorylated and less active as a result of decreased PrrB kinase activity or increased ability of PrrB to dephosphorylate phosphor-PrrA.

Relationship between genes that are effective in nitrogenase synthesis and regulation should be understood well to increase the yield of hydrogen. In this study, growth, hydrogen production and expression levels of *nif*H, *nif*A, and *prr*A genes were investigated at different conditions. The effects of ammonium chloride and acetate, which are the components of dark fermentor effluent in two-step process of dark and photofermentation used for higher yields of hydrogen, were examined under anaerobic and aerobic conditions.

2. Materials and methods

2.1. Media, culture conditions and measurements

R. sphaeroides O.U.001 (DSM 5864) was grown in Biebl and Pfenning minimal medium [9] which did not involve ammonium chloride and yeast extract. 15 mM D,L-malate and 2 mM L-glutamate containing medium was used as control due to its high capacity for producing hydrogen in maximum rate [10]. Anaerobic cultures of *R. sphaeroides* were grown in 55 ml glass bottles at 30–32 °C, under illumination that is provided by 100-watt tungsten lamp from a distance of 15–20 cm. Pure argon gas (99.995% purity) was flushed into the bottles to obtain anaerobic atmosphere. Aerobic cultures were maintained by filling 65% of the glass bottles with the media and leaving the air on the top of the bottles for aerobic growth. Initial pH of the media in all bioreactors was 6.7.

Nitrogen and carbon sources were replaced with ammonium chloride and acetate to investigate their effects on growth, hydrogen production and expressions of *nif*H, *nif*A and *prr*A genes. Effect of ammonium chloride was determined by media containing 15 mM D,L-malate as carbon source and different concentrations of (1, 2, 3, 5, 10 mM) NH_4Cl as nitrogen source. Carbon source was replaced from D,L-malate to acetate and different concentrations of glutamate were used (1, 2, 3, 5 mM glutamate) to determine optimum culture conditions with acetate containing medium.

The bacterial cell concentration was determined spectrophotometrically. The absorbance values were converted to dry cell weight where optical density (OD) of 1 at 660 nm corresponded to 0.59 g dry cell weight per liter of medium ([11], p.157). Hydrogen gas was collected and measured volumetrically by graduated-glass burettes with the replacement of water. The purity of collected gas was measured by gas chromatography (Agilent 6890N). Gas samples were analyzed with Carboxen 1010 column by Supelco with thermal conductivity detector.

2.2. RNA isolation

RNA isolation was performed by TRI REAGENT™ (Sigma) according to the procedure provided by manufacturer. Cells from 10 to 15 ml *R. sphaeroides* culture grown for 36 h were used for RNA isolation. DNase treatment was performed according to the protocol given by DNA-free™ kit from Ambion. Purity and concentration of DNA-free RNA samples were determined by the absorbance measurements at 260 nm and 280 nm on spectrophotometer (Shimadzu UV-1208).

2.3. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Reverse transcription reaction for the expression analyses of *nif*H, *nif*A and *prr*A genes were initiated by cDNA synthesis. DNase treated 1 μg of total RNA template, 20 pmol/μl of sequence-specific primer was added into a sterile tube and the volume of the mixture was completed to 11 μl with DEPC-treated water. The mixture was incubated at 70 °C for 5 min and then, chilled on ice. 4 μl of 5× reaction buffer, 2 μl of 10 mM dNTP mix (1.0 mM-final concentration) and DEPC-treated water were added to the tube. The mixture was incubated at 37 °C for 5 min. 60 units (0.3 μl) of RevertAid TM M-MuLV Reverse Transcriptase was added and the reaction mixture was incubated at 42 °C for 60 min. The reaction was stopped by heating the mixture at 70 °C for 5 min and chilled on ice.

Contamination of RNA with chromosomal DNA is often encountered in RT-PCR on prokaryotic RNA and contaminated DNA generates false-positive products [12]. Primers that generate 5′-tagged cDNA during RT that is used as specific

Table 1 – Primers in RT-PCR amplification.

Genes	Primer types	Primer sequences
*nif*H	cDNA primer	5′-AGACCGTGTGGGGCGTGCTGCACGATATT-3′[a]
	Forward primer	5′-GGCGCCTATGACGACGTCGAC-3′
	Reverse primer	5′-AGACCGTGTGGGGCGTGCTGC-3′
*nif*A	cDNA primer	5′-AGACCGTGTGGGGATGCAGCTTCTTCTTC-3′
	Forward primer	5′-GGCCAAGCTCCTGCGCATCC-3′
	Reverse primer	5′-AGACCGTGTGGGGATGCAGCT-3′
*prr*A	cDNA primer	5′-AGACCGTGTGGGCGAGCGAATTTCGTGCT-3′
	Forward primer	5′-CCCCGAGTTCGAATACCAG-3′
	Reverse primer	5′-AGACCGTGTGGGCGAGCGAAT-3′
16S rRNA	cDNA primer	5′-AGACCGTGTGGGCCGCGTTGGATTAGGTA-3′
	Forward primer	5′-CGCCACTGGTGTTCCTCCGAA-3′
	Reverse primer	5′-AGACCGTGTGGGCCGCGTTG-3′

a The underlined sequences represent the tag.

template during PCR are generated to improve the reliability of RT-PCR. GC-rich tag sequence on the 5′-end of the cDNA provides a means for PCR amplification of cDNA at relatively high annealing temperature [13]. No DNA-derived product is amplified during PCR at relatively high annealing temperature since the sequence of 5′ tag is not present in chromosomal DNA.

Primers were designed to amplify a specific region from nitrogenase structural gene *nif*H, nif-specific transcriptional activator *nif*A and response regulator coding *prr*A. 16S rRNA was used as internal control since it is constitutively expressed. Sequences of primers for RT-PCR amplifications are given in Table 1.

PCR reactions were prepared in 50 μl aliquots and reaction was performed in Thermal Cycler (ApolloTM ATC 401 Thermal Cycler and Thermo Thermal Cycler). PCR conditions and compositions were as follows: 1× reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.2 μM of each primer and 1.25 unit of Taq DNA polymerase (Fermentas) were added to 100–300 ng cDNA. PCR reaction was initiated at 94 °C for 6 min. After initial denaturation, reaction was carried out in 30 cycles with denaturation at 94 °C for 30 s, annealing at 60–65 °C for 30 s and extension at 72 °C for 30 s. The reaction was terminated with the final extension at 72 °C for 10 min. The samples of PCR were run in 2% agarose gel for 1 h at 90 V and gel was visualized on a software UV transilluminator and photographed by Vilber Lourmat Gel Imaging System. The gel photographs were processed with ImageJ software. Relative expression levels (REL) of genes were calculated by formula given below:

$$REL = (S_{GOI}/S_{IC})/(CM_{GOI}/CM_{IC})$$

Densitometric band intensities were designated by S and CM for samples and control medium, respectively. GOI stands for the gene of interest and IC stands for internal control (16S rRNA).

False-positive products were controlled for each expression analyses of all genes. False-positive control is necessary to be sure that there is no DNA contamination in RNA used for cDNA synthesis.

Real-time PCR was performed according to the procedure given by LightCycler® TaqMan® Master from Roche. Hydrolysis probes (TaqMan) were used to detect and evaluate PCR products fluorimetrically. Reaction was prepared by adding the following components in a 1.5 ml tube; 7 μl of PCR grade water, 2 μl of primer/probe mixture (0.5 μM of forward primer, 0.5 μM of reverse primer and 0.2 μM of probe) and 4 μl of master mix. After pipetting 15 μl PCR mix into pre-cooled LightCycler® Capillaries, 7 μl of the DNA template was added. Capillary was sealed with a stopper and centrifuged at 700×g for 5 s. Then the capillary was transferred to the sample carousel of the LightCycler® Instrument. The sample was pre-incubated at 95 °C for 15 min and cycled in amplification step by denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s and extension at 72 °C for 1 s for 45 cycle. The sample as cooled at 40 °C for 30 s.

The results were subjected to statistical tests by using Minitab Statistical Software (Minitab Inc., USA) to determine significant difference between means of groups ($\alpha = 0.05$)

3. Results and discussion

3.1. *Effect of ammonium ion on growth and hydrogen production of R. sphaeroides O.U.001*

Growth media containing different concentrations of ammonium (1, 2, 3, 5, 10 mM) were used to examine anaerobic growth and hydrogen production of *R. sphaeroides* O.U.001. Medium composed of 15 mM malate and 2 mM glutamate was used as a control since previous studies showed that the maximum hydrogen production rate was obtained in the growth medium containing 15 mM L-malic acid and 2 mM sodium glutamate concentrations [10]. While glutamate is a good source of nitrogen for hydrogen production, it is important to examine the effect of ammonium chloride on growth and hydrogen production of photosynthetic bacteria. When hydrogen is produced by two-step process of dark and photofermentation, the composition of dark fermentation effluent, which contains ammonium as nitrogen source, affects the growth and hydrogen production of photosynthetic bacteria. In a study by Afşar et al. [14], it was mentioned that two different dark fermenter effluents of potato steam peel hydrolysates included 3.1 mM and 4.0 mM NH_4Cl. These concentrations of NH4Cl may have an inhibitory effect on

activity and synthesis of nitrogenase. Therefore, it is important to determine limiting concentrations of ammonium chloride for growth and hydrogen production.

Nitrogen concentration has an important role in growth because the most abundant element in the cell is nitrogen following carbon. Therefore, as ammonium chloride concentration increased, the dry cell weight also increased under anaerobic conditions (Fig. 1a). Maximum biomass in 10 mM NH_4Cl medium was 1.6 folds higher than maximum biomass in 1 mM NH_4Cl medium.

Medium containing 15 mM malate as carbon source and 2 mM glutamate as nitrogen source was used as control. The maximum biomass in 15 mM malate/2 mM glutamate containing medium and 15 mM malate/10 mM NH_4Cl containing medium was approximately the same. Glutamate ($C_5H_9NO_4$) which is used as nitrogen source has also considerable amount of carbon that may result in higher growth.

Substrate involved in culture medium is one of the important factors influencing the yield of hydrogen production. Although a wide variety of substrates can be utilized by *R. sphaeroides* for growth, only a portion of them is suitable for hydrogen production [15]. Ammonium chloride, an excellent source of nitrogen for bacterial growth, may act as an inhibitor in hydrogen generation process [16] since nitrogenase, which produces hydrogen gas while catalyzing the reduction of dinitrogen to ammonia, have developed complex regulatory systems for the control of nitrogenase synthesis and activity [17]. Inhibiting the activity or synthesis of nitrogenase system requiring large amounts of ATP and reducing power is advantageous when the cellular level of fixed nitrogen is high [3]. As shown in Fig. 1a, the growth of bacteria in all ammonium chloride concentrations was close to the growth in glutamate containing medium. However, it is demonstrated in Fig. 1b that the highest hydrogen production was obtained in glutamate containing medium, and the hydrogen production decreased at increasing NH_4Cl concentrations. There was no hydrogen production at higher concentrations of NH_4Cl than 2 mM NH_4Cl. Hydrogen production was inhibited by high (3 mM and above) concentrations of ammonium chloride due to activity level and transcriptional level control of nitrogenase.

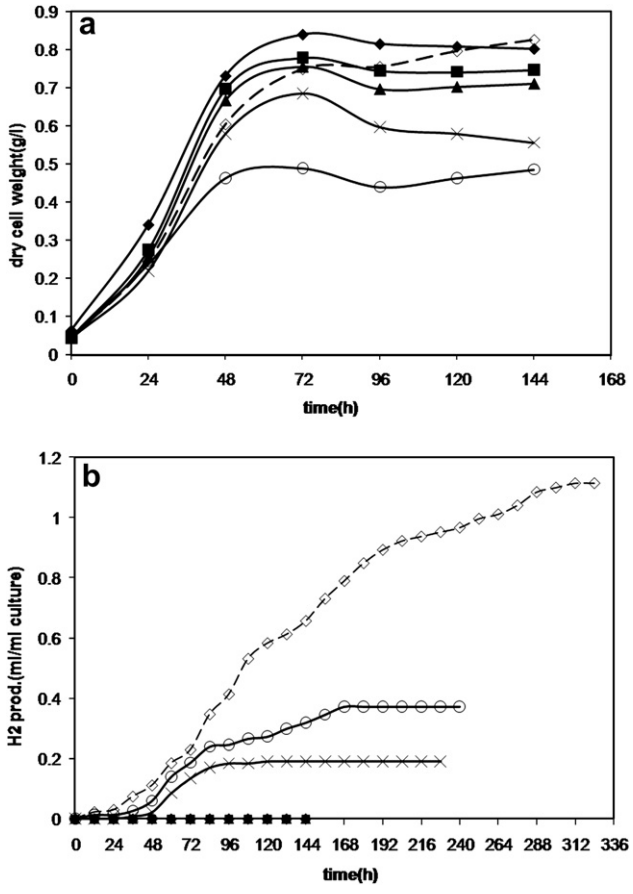

Fig. 1 – a) Growth and b) hydrogen production of *R. sphaeroides* O.U.001 in 15 mM malate and different concentrations of ammonium chloride containing medium. (◆) 10 mM NH_4Cl; (■) 5 mM NH_4Cl; (▲) 3 mM NH_4Cl; (×) 2 mM NH_4Cl; (○) 1 mM NH_4Cl;(-◇-) 2 mM glutamate.

Cumulative hydrogen production and H_2 production rate decreased as the concentration of ammonium chloride increased. H_2 yield in 1 mM NH_4Cl containing medium was approximately 2.5 folds higher than H_2 yield in 2 mM NH_4Cl containing medium (Table 2). However, the maximum biomass was increased as the ammonium chloride concentration increased. As a result, the hydrogen production was inversely and growth was directly proportional to ammonium chloride concentration. The result of hydrogen production data was comparable with the results of a study by Waligórska et al. [18]. In that study, it was also observed that an increase of ammonium ions concentration resulted in a decrease of maximal specific hydrogen potential production. At higher ammonium ion concentrations, no hydrogen evolution was observed.

3.2. *Effect of aerobic conditions on growth and hydrogen production of R. sphaeroides O.U.001*

R. sphaeroides has flexible metabolic capabilities such as photoheterotrophy, aerobic or anaerobic respiration, and fermentation. Growth media containing different concentrations of ammonium chloride (1, 2, 3, 5, 10 mM) were used to examine how aerobic conditions affect the growth and hydrogen production of bacteria. As shown in Fig. 2a, the highest growth was observed in the medium containing 15 mM malate, 2 mM glutamate. Aerobic growth in all ammonium chloride containing media had approximately the same pattern and was lower than the growth in control medium. When the growths under aerobic and anaerobic conditions were compared, higher growth was observed in anaerobic conditions of NH_4Cl containing media. This may be due to toxic effect of ammonium and oxygen combination.

Treatment of cultures of *R. capsulatus* with low concentrations of oxygen has been shown to cause an immediate and complete inhibition of nitrogenase activity that was fully reversible upon return to anoxic conditions [19].

The hydrogen production that was much lower compared to anaerobic conditions was observed in aerobic cultures of *R. sphaeroides* (Fig. 2b). The total hydrogen production was nearly 3 folds higher under anaerobic conditions in control

Table 2 – C/N ratio, efficiency, cumulative hydrogen production, maximum biomass and hydrogen production rates in media containing different concentrations of ammonium chloride.

Substrates (mM/mM)	Concentration (mM/mM)	C/N Ratio	Max biomass ($g/l_{culture}$)	Light conversion efficiency	Substrate conversion efficiency	Yield ($g_{H_2}/g_{substrate}$)	H_2 prod. rate ($ml/l_{culture}$ h)
Malate/NH_4Cl	15/1	60	0.49	0.46	0.18	0.0182	4.6
Malate/NH_4Cl	15/2	30	0.55	0.24	0.09	0.0075	3.3
Malate/NH_4Cl	15/3	20	0.71	0	0	0	0
Malate/NH_4Cl	15/5	12	0.75	0	0	0	0
Malate/NH_4Cl	15/10	6	0.8	0	0	0	0
Malate/Glutamate	15/2	35	0.83	1.03	0.45	0.0440	5.1

medium than under aerobic conditions. The total hydrogen production in 1 mM NH_4Cl containing medium under aerobic conditions was 1.6 fold lower than anaerobic conditions. There was no hydrogen production in 5 mM and 10 mM NH_4Cl containing medium probably due to the inhibition of nitrogenase activity by both ammonium chloride and oxygen. The hydrogen evolution observed in aerobic cultures may be explained by the removal of oxygen by respiration. Nitrogenase can tolerate oxygen concentrations lower than 4.6 μM [20]. This can be explained by the competition between nitrogen fixation and respiration for electrons. The preferential flow of electrons into the respiratory chain causes reversible inactivation of nitrogenase [20]. Therefore, bacteria can produce hydrogen after the removal of inhibitory level of oxygen by respiration.

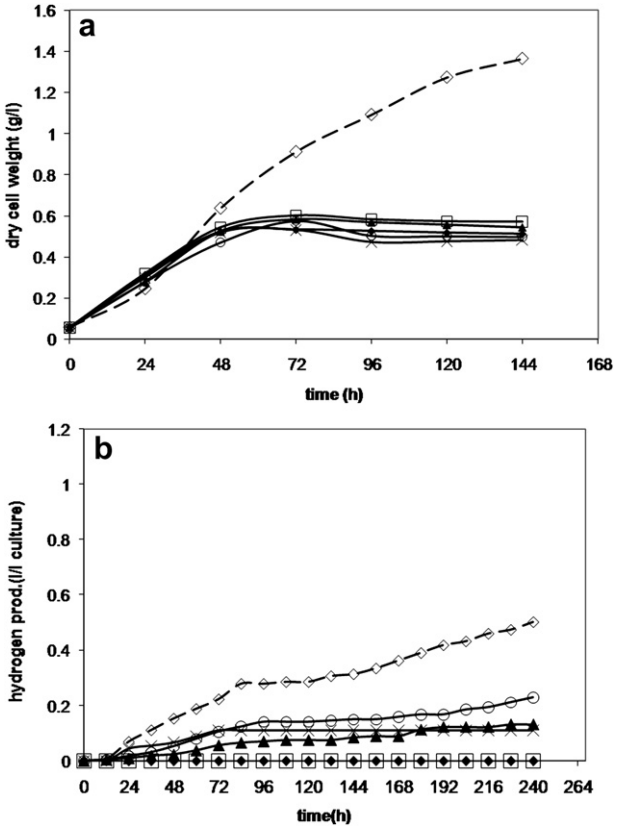

Fig. 2 – a) Growth and b) hydrogen production of *R. sphaeroides* O.U.001 in different concentrations of ammonium chloride containing medium under aerobic conditions. (◆) 10 mM NH_4Cl; (□) 5 mM NH_4Cl; (▲) 3 mM NH_4Cl; (×) 2 mM NH_4Cl; (○) 1 mM NH_4Cl; (- ◇ -) 2 mM glutamate.

3.3. *Effect of acetate on growth and hydrogen production of* R. sphaeroides *O.U.001*

Anaerobic fermentation of organic wastes produces intermediate low-molecular-weight organic acids in a first step, which are then converted to hydrogen by photosynthetic bacteria using light energy, in the second step. Therefore, the conversion of low-molecular-weight acetic acid would be advantageous in order to couple energy production with organic waste treatment [21]. Media containing 30 mM acetate and 1, 2, 3, 5 mM glutamate were used to investigate growth and hydrogen production. Control medium was composed of 15 mM malate and 2 mM glutamate.

As seen in Fig. 3, higher hydrogen production was obtained in lower glutamate concentrations in acetate containing medium since as the nitrogen concentration increases, the nitrogenase enzyme was inhibited at transcriptional level and enzyme activity level [3,22]. The highest hydrogen production was observed in the control medium containing malate as carbon source. In the study by Uyar et al. [23], the highest substrate conversion efficiency and hydrogen production rate

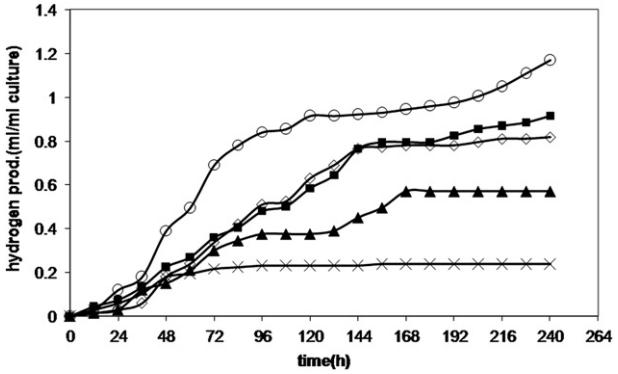

Fig. 3 – Hydrogen production in 30 mM acetate and different concentrations of glutamate containing media. (◇) 1 mM glutamate; (■) 2 mM glutamate; (▲) 3 mM glutamate; (×) 5 mM glutamate; (○) 15 mM malate–2 mM glutamate.

Table 3 – C/N ratio, efficiency, yield, maximum biomass and hydrogen production rates in media containing different concentrations of glutamate in 30 mM acetate.

Substrates (mM/mM)	Conc. (mM/mM)	C/N Ratio	Max biomass ($g/l_{culture}$)	Light conversion efficiency (%)	Substrate conversion efficiency	Yield($g_{H_2}/g_{substrate}$)	H2 Production rate (ml H_2/L_c h)
Acetate/Glutamate	30/1	65	0.97	1.03	0.28	0.038	5.8
Acetate/Glutamate	30/2	35	1.29	1.15	0.29	0.042	5.2
Acetate/Glutamate	30/3	25	1.78	0.71	0.17	0.026	4.4
Acetate/Glutamate	30/5	17	2.1	0.30	0.06	0.011	3.3

and highest amount of hydrogen was obtained in 15 mM malate containing media among the carbon sources; malate, acetate, propionate, lactate and butyrate.

As glutamate concentration increases in 30 mM acetate containing medium, maximum biomass was increasing. However, the yield of hydrogen and hydrogen production rate were decreasing in increasing concentrations of glutamate (Table 3). 2 mM glutamate was the optimum concentration to obtain higher H_2 yield in the presence of acetate as carbon source. *C/N* ratio is important parameter for producing maximum amount of hydrogen. In the present study, the best *C/N* ratio was 35, which results in highest hydrogen yield as stated in the study by Eroglu et al. [10].

3.4. *The effect of ammonium chloride on expression levels of nitrogenase genes under anaerobic conditions*

The expression levels of nitrogenase structural gene, *nif*H, nif-specific transcriptional activator, *nif*A and response regulator, *prr*A were investigated in ammonium chloride containing media under anaerobic conditions in *R. sphaeroides* O.U.001. The reason for the selection of nitrogenase controlling genes for the expression analysis is that although hydrogenase of photosynthetic bacteria is capable of both hydrogen production and consumption, the maximum activity for hydrogenase occurs at conditions favorable for H_2 uptake only [15].

Expression level of *nif*H gene, which is the structural gene of nitrogenase, decreased with the increasing NH_4Cl concentrations under anaerobic conditions as shown in Figs. 4a and 5a. *nif*H was not expressed at all in 5 mM and 10 mM NH_4Cl containing medium (Figs. 4a and 5a). In addition, no hydrogen production was observed in these media (Fig. 1b). Although the expression was observed in 3 mM NH_4Cl containing medium, there was no hydrogen production either. It can be considered that the inhibition of nitrogenase activity is not at transcriptional level in 3 mM ammonium chloride containing medium. Higher concentrations of ammonium cause the loss of expression of the structural gene.

*nif*A gene, which is the transcriptional activator of *nif*HDK, expressed in all concentrations of ammonium chloride in the present study under argon atmosphere however the expression levels decreased at high concentrations of ammonium (Fig. 5b). The expression level of *nif*A gene was 1.5 fold higher in glutamate containing medium than in 5 mM NH_4Cl containing medium. Although *nif*A gene was expressed in, 5 and 10 mM NH_4Cl containing media, no *nif*H gene expression was observed in the same media. Inhibition of NifA may be at the translational or post-translational levels, so that inactive NifA prevents the expression of *nif*H in 5 mM and 10 mM NH_4Cl containing media.

In the presence of ammonium ion, nitrogenase is inhibited at transcriptional or activity level due to the concentration. Expression of the nitrogenase structural genes *nifHDK* is under the direct control of the transcriptional activator NifA that is in turn under positive control of phosphorylated NtrC [24]. The second level of ammonium control is mediated again by GlnB and GlnK which regulate NifA activity. The presence of *nifA* expression despite the absence of *nifH* expression in high concentrations of NH_4Cl (5 mM and 10 mM) can be explained by the second level of ammonium control in nitrogenase.

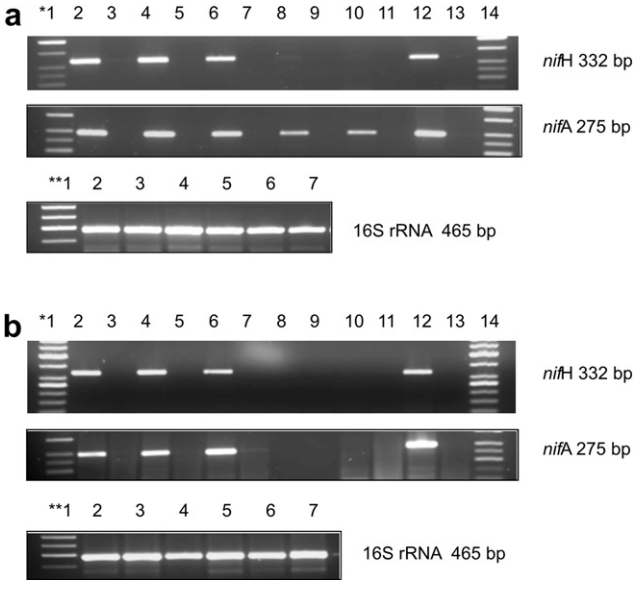

Fig. 4 – PCR products of a) nitrogenase related genes (nifH, nifA) under anaerobic and b) aerobic conditions on 2% agarose gel. *Lanes 1,14: DNA ladder (50 base pair); lanes 2, 4, 6, 8, 10, 12: Samples from 1 mM, 2 mM, 3 mM, 5 mM, 10 mM NH4Cl and 2 mM glutamate containing medium as nitrogen source, respectively. Lanes 3, 5, 7, 9, 11, 13: False-positive control of samples. **Lane 1: DNA ladder; lanes 2, 3, 4, 5, 6, 7: Samples from 1 mM, 2 mM, 3 mM, 5 mM, 10 mM NH4Cl and 2 mM glutamate containing medium as nitrogen source, respectively.

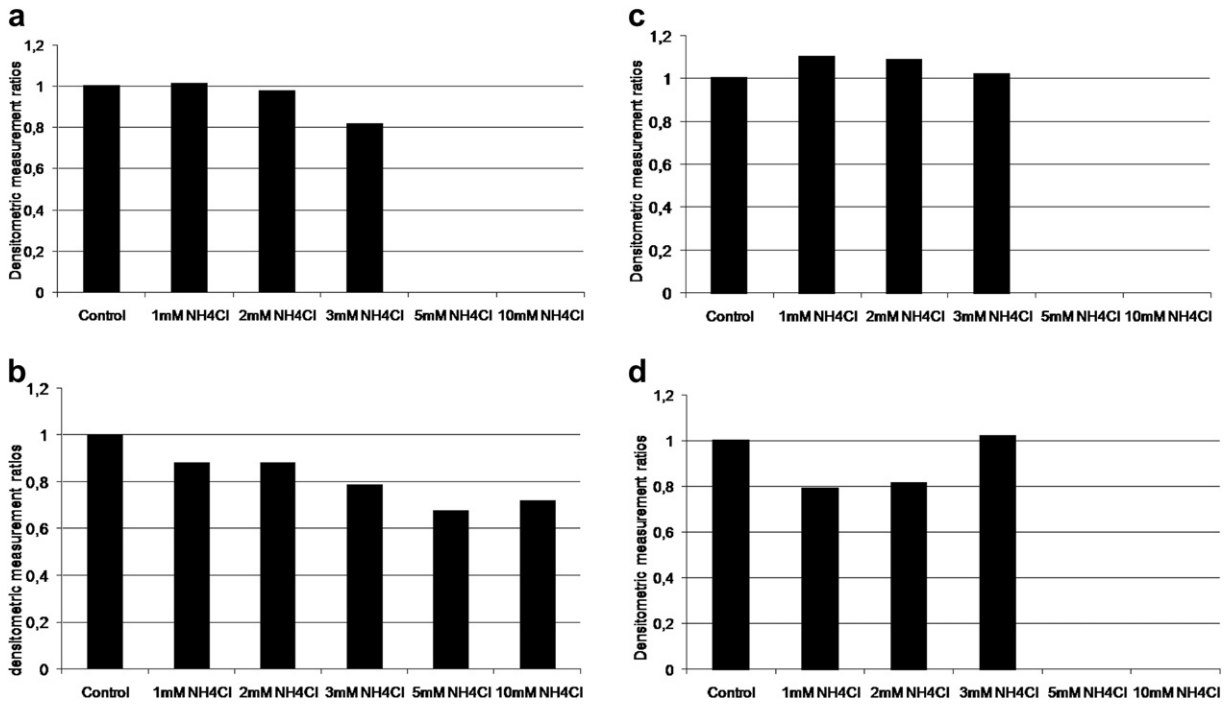

Fig. 5 – The expression levels of a) *nif*H gene and b) *nif*A gene at various concentrations of NH_4Cl under anaerobic conditions and c) *nif*H; d) *nif*A under aerobic conditions. Serials refer to NH_4Cl samples/control (2 mM glutamate).

3.5. *The effect of ammonium chloride on expression levels of nitrogenase genes under aerobic conditions*

Oxygen is one of the environmental stimuli to which regulatory genes respond. The expression levels of *prr*A, *nif*A and *nif*H genes were examined in aerobic conditions to examine the response of genes found in regulatory pathway to oxygen.

As seen in Figs. 4b and 5c, no expression of *nif*H was observed in 5 mM and 10 mM NH_4Cl containing medium under aerobic conditions. There was decrease in expression levels of *nif*H gene as NH_4Cl concentration increased. However, this decrease was not in significant levels. When the expression levels of *nifH* gene in aerobic and anaerobic media compared, there was not much difference between them. According to H_2 production data, the oxygen effect on nitrogenase activity was more obvious (Fig. 2b).

Aerobic conditions totally inhibited *nif*A expression in 5 mM and 10 mM NH_4Cl containing medium (Fig. 5d). Oxygen has inhibitory effect on both NifA activity by changing its conformation and on transcription of *nif*A [25]. The transcriptional inhibition of *nif*A was observed in only higher ammonium chloride containing media (Fig. 5d). Among the three levels of regulation of nitrogenase in *R. capsulatus* reviewed by Tremblay et al. [5], the NtrB/NtrC two-component system controls *nif*A transcription at the first level. In the present study, the total inhibition of *nif*A expression in high concentrations of ammonium (5 mM and 10 mM) can be explained as the transcriptional level of inhibition of nitrogenase. Both the presence of high concentration of NH_4Cl and oxygen caused total inhibition of expression of *nif*A gene. The expression levels of *nif*A did not change proportionally according to the concentration of NH_4Cl in aerobic conditions. The highest expression level of *nif*A was observed in 3 mM NH_4Cl containing medium among aerobic cultures. The complex regulatory network of nitrogenase synthesis has many control points. Strains expressing either *nif*A1 or *nif*A2 from a constitutive promoter in an ntrC mutant still show inhibition of *nif*H transcription in ammonium, suggesting post-translational control of NifA activity [26]. Thus, it can be concluded that the high expression levels in *nif*A may not result in high expression levels of its target gene, *nif*H. Therefore, it can indirectly be considered that the higher expression of *nif*A did not cause higher activity in nitrogenase.

The effect of ammonium chloride on expression levels of *prr*A were examined by real-time PCR. As seen in Fig. 6 expression levels in 1 mM, 2 mM and 3 mM NH_4Cl containing aerobic media were higher than anaerobic conditions of the same media. Although a decrease was observed in expression levels of *nif*A in NH_4Cl containing medium in comparison to glutamate containing medium, *prr*A gene expression significantly increased in all concentrations of NH_4Cl except 10 mM under aerobic conditions.

The expression levels of *prr*A gene changed in opposite directions in aerobic and anaerobic conditions in ammonium chloride containing media. As the expression increased in aerobic conditions, a decrease was observed in anaerobic conditions in 1 mM, 2 mM and 3 mM ammonium chloride containing media. In a study by Elsen et al. [27], it has been identified that RegA in *R. capsulatus*, the homologous of PrrA in *R. sphaeroides*, enhanced *nif*A2 expression under all growth

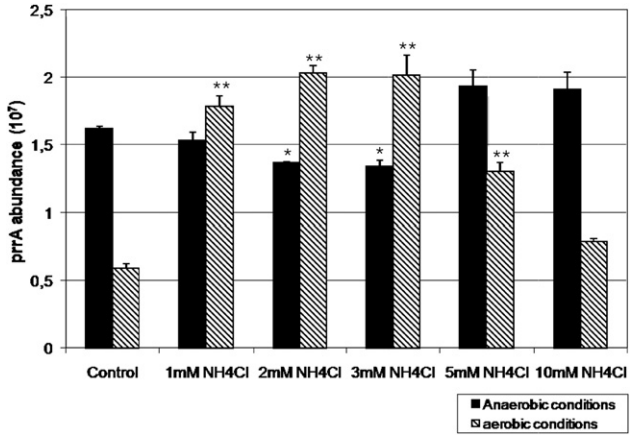

Fig. 6 – The expression levels of *prrA* gene (real-time PCR results). *Represents the significant difference between groups with respect to control in anaerobic conditions and **Represents the significant difference with respect to control in aerobic conditions ($p < 0.05$).

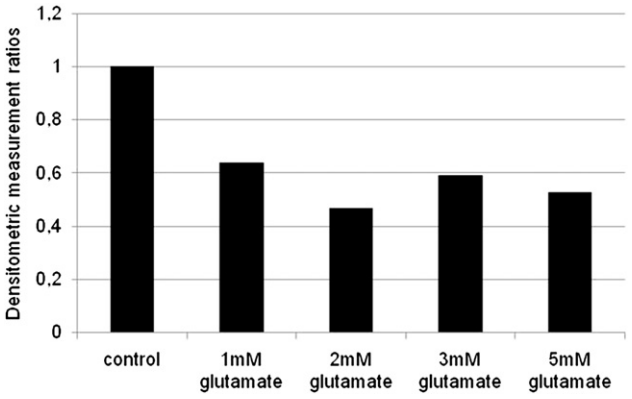

Fig. 7 – The expression levels of *nifH* gene in 30 mM acetate and different concentrations of glutamate containing medium under anaerobic conditions. Serials refers to densitometric measurements of NH4Cl samples/control (2 mM glutamate/15 mM malate).

conditions including aerobic, anaerobic, ammonium and glutamate containing media. RegA appears to function as a coactivator with NtrC to ensure optimal *nifA2* expression. Correspondingly, unphosphorylated NtrC inhibits the expression of *nifA* under aerobic conditions and in 1 mM, 2 mM and 3 mM NH_4Cl containing media in the present study. When *nifA* was not expressed optimally, *prrA* expression may have increased to enhance *nifA* expression in our case. Control medium that did not contain NH_4Cl allow NtrC to enhance *nifA* expression, therefore *prrA* was not required to reach optimum *nifA* expression. As a result, the level of expression of *prrA* was higher in 1, 2, 3 mM NH_4Cl containing media than in control medium. When anaerobic conditions were evaluated, *nifA* was expressed in all concentrations of NH_4Cl containing media. The lower expression of *prrA* in 1 mM, 2 mM and 3 mM NH_4Cl under anaerobic conditions may be due to tolerability of NtrC to NH_4Cl concentrations lower than or equal to 3 mM. High level of expression in 5 mM and 10 mM NH_4Cl containing media may be the reason of inhibitory effect of NtrC. Expression of *nifA* may be observed in these conditions due to the activator effect of such high expression level of *prrA*.

3.6. *The effect of acetate and different concentrations of glutamate on expression levels of nitrogenase genes*

Acetate is one of the carbon sources that *R. sphaeroides* can utilize to grow and produce hydrogen. The expression levels of structural *nifH* gene were investigated in 30 mM acetate and different concentrations of glutamate (1, 2, 3, 5 mM) containing medium.

The expression levels of *nifH* gene in acetate containing medium were lower than malate containing control medium. The expression in control medium was 2 folds higher than the expression in 2 mM glutamate and 30 mM acetate containing medium. There was not significant change among the expression levels of *nifH* gene in acetate containing medium (Fig. 7). The expressions were nearly the same regardless of glutamate concentration. However, unlike expression levels, there was higher difference between the hydrogen yields in different concentrations of glutamate containing medium. Hydrogen production was inhibited in enzyme activity level as glutamate concentration increased up to 5 mM but there was no significant change in expression levels.

4. Conclusion

Relationship between genes that are effective in nitrogenase synthesis and regulation should be understood well to increase the yield of hydrogen. In the present study, expression levels of nitrogenase encoding *nifH* and control genes *nifA*, *prrA* were examined at different physiological conditions. In addition to variations in expression levels, changes in hydrogen production and growth were also investigated in response to different concentrations of ammonium source, acetate and aerobic conditions.

Biomass amount of *R. sphaeroides* O.U.001 increases in parallel with ammonium chloride concentration at anaerobic conditions. However, the hydrogen production decreased as the concentration of ammonium chloride increased. Glutamate is a better source of nitrogen for hydrogen production. Yield of hydrogen in 1 mM NH_4Cl containing medium was 2.4 folds lower than in 2 mM glutamate containing medium. There was no hydrogen production when ammonium chloride concentration above 2 mM due to transcriptional level control of nitrogenase synthesis.

The expression levels of *nifH* and *nifA* showed a decreasing tendency as the concentration of NH_4Cl increased at anaerobic conditions. The expression of *nifH* was lost in 5 mM and 10 mM ammonium chloride containing medium since such high concentrations of ammonium chloride inhibited the transcription of *nifA* and provides the transcriptional level of control among three levels of regulation of nitrogenase

reviewed by Tremblay et al. [5]. *prr*A gene was expressed at higher levels with increasing concentrations of NH_4Cl.

There was no significant difference in the growth of bacteria in different concentrations of ammonium chloride containing media under aerobic conditions. Total biomass in 2 mM glutamate–15 mM malate containing medium reached 2.7 folds higher values than the total biomass in 2 mM NH_4Cl containing media since glutamate can also be used as carbon source. Hydrogen production was also observed in aerobic conditions of 1 mM, 2 mM NH_4Cl and 2 mM glutamate containing media. Total yield of hydrogen in malate–glutamate containing medium under aerobic conditions was 3 folds lower than under anaerobic conditions.

The expressions of *nif*H decreased as the concentration of ammonium chloride increased in aerobic conditions and the expression was lost at 5 mM and 10 mM NH_4Cl. There was expression of *nif*A gene under anaerobic conditions however; it was lost in 5 mM and 10 mM NH_4Cl containing media under aerobic conditions. This can be explained by the transcriptional level control of nitrogenase synthesis in higher concentrations of nitrogen and oxygen combination.

Expression of *prr*A gene was decreased as the expression level of *nif*A gene increased in both anaerobic and aerobic conditions. This negative correlation requires further investigations.

When acetate was the sole carbon source, total biomass of the bacteria increased as the concentration of glutamate increased. Total growth in 30 mM acetate-2 mM glutamate containing medium was 2 folds higher than the total growth in 15 mM malate–2 mM glutamate containing medium. However, the hydrogen production was highest in malate–glutamate containing medium. As the glutamate concentration increased, the hydrogen production decreased in acetate containing medium.

In the present study, transcriptional level control of nitrogenase was shown in different conditions. Further investigations were required to detect the relationship between *prr*A and nitrogenase in the control of nitrogenase activity and synthesis.

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