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Hydrogen production and transcriptional analysis of *nif*D, *nif*K and *hup*S genes in *Rhodobacter sphaeroides* O.U.001 grown in media with different concentrations of molybdenum and iron

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Abstract

Rhodobacter sphaeroides O.U.001 was grown in media with different concentrations of molybdenum and iron to study the expression level of *nif*D and *nif*K genes coding for the large subunit of Mo-nitrogenase and *hup*S gene coding for the small subunit of uptake hydrogenase. Hydrogen productions under the same growth conditions were also evaluated. Increasing concentrations of the molybdenum and iron stimulated hydrogen production and the highest total hydrogen accumulation was achieved in 16.5 μM sodium molybdate (0.84 l H_2/l culture at $t = 168$ h) and 0.1 mM ferric citrate (1.14 l H_2/l culture at $t = 240$ h) containing media. Maximal expressions of *nif*D and *nif*K were observed from the late log phase until the mid-stationary phase of growth and *hup*S expression was seen as soon as the hydrogen was produced in the cells. Moreover, *nif*K, *nif*D and *hup*S expressions were significantly reduced in the molybdenum and iron starved cells.

Keywords: Mo-nitrogenase; *nif*D; *nif*K; *hup*S; *Rhodobacter sphaeroides*; Uptake hydrogenase; Biological hydrogen

1. Introduction

Hydrogen is one of the future clean alternative energy sources. It can be produced by both biological and non-biological means. Among biological hydrogen production methods, photobiological hydrogen production by purple non-sulfur bacteria such as *Rhodobacter sphaeroides* is a potential candidate.

Hydrogen production and consumption in photosynthetic bacteria are mediated by nitrogenases and hydrogenases. While nitrogenase produces hydrogen, membrane-bound uptake hydrogenase consumes it [1]. In *R. sphaeroides*, there is a membrane-bound uptake hydrogenase catalyzing the conversion of molecular hydrogen to electrons and protons and it is encoded by *hup*SL genes. The genes for uptake hydrogenase are clustered in one region in *R. sphaeroides* similar to other Proteobacteria [2]. Nitrogenases are mainly responsible for hydrogen production but depending on the K_m values of hydrogenases for H_2 and the preferences of the bacteria, hydrogenases can also produce hydrogen [3]. Therefore, there is also some potential for the hydrogen production by hydrogenases [4].

Three types of nitrogenase systems (nif, vnf, anf) have been proved to exist and among them, Mo-nitrogenase that is encoded by *nif*HDK genes is commonly

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found in diazotrophs [5–8]. One mol of hydrogen and 2 mol of ammonia are formed by Mo-nitrogenase catalyzed reaction at the expense of 16 mol of ATP which might be considered a disadvantage of nitrogenase-based hydrogen production in addition to being slow with a turnover rate of $\sim 5\,s^{-1}$:

$$N_2 + 8e^- + 8H^+ + 16MgATP \rightarrow 2NH_3 + H_2 + 16MgADP + 16P_i. \quad (1)$$

Due to the slow turnover time making diazotrophs synthesize large amount of nitrogenase and the consumption of a considerable amount of ATP, the regulation of the nitrogenase genes at the transcriptional level in response to environmental stimuli is quite stringent [9]. Considerable amount of studies has been conducted to see the influence of various parameters like light/dark [10–15], oxygen [13,14], hydrogen [16], pH [17], nitrogen sources [10,18,19], and metal ions [16,17,20] on hydrogen production by bacteria. Light strongly stimulates nitrogenase synthesis [10] but oxygen and ammonia inhibit nitrogenases [9]. Availability of certain metal ions such as molybdenum and iron which are found in the structure of Mo-nitrogenase is also critical. In *Azotobacter vinelandii*, *nif*HDK transcription was repressed under Mo-deprived conditions [20]. Moreover, the presence of Mo was shown to repress alternative nitrogenases in *Rhdobacter capsulatus* [21].

In this study, the effect of various concentrations of sodium molybdate on the expression level of the *nif*K gene and of ferric citrate on the expression level of *nif*D and *hup*S genes were investigated. The hydrogen production was also evaluated under the same growth conditions. The gene expression analyses were performed with respect to time to understand the relations among the gene expression level, biohydrogen production and the growth phases.

2. Materials and methods

2.1. Media, culture conditions and measurements

R. sphaeroides O.U.001 was grown in Biebl and Pfenning [22] minimal medium in which D, L-malate and L-glutamate (15 mM/2 mM) were used as carbon and nitrogen sources. The cultures were incubated at 32 °C in 62 ml bottles under the irradiance of 940 $\mu E/m^2/s$. The bottles and the other glassware were washed with 30% (v/v) nitric acid and then ultrapure water several times to remove traces of metal ions. Furthermore, possible contaminants of molybdenum and iron accompanied with the inoculums (10%) were eliminated with the following treatments. The fresh cultures were first centrifuged and the pellets were dissolved in media with no molybdenum or iron. After several passages in their media, the final inoculations were done.

The medium named as control or standard (1X) contained 0.165 μM of sodium molybdate ($Na_2MoO_4{\cdot}2H_2O$) as a molybdenum source and 0.1 mM of iron citrate ($Fe(C_6H_5O_7){\cdot}H_2O$) as an iron source. The media denoted as 1/10X, 10X and 100X indicate how many times the medium was concentrated or diluted in terms of that metal content. For instance, 100X Mo meant that the concentration of molybdenum in this medium was 100 times higher than that of the standard/control medium (1X). No Mo and No Fe indicate the absence of these ions. At different time intervals, the absorbance at 660 nm, pH and hydrogen gas evolved were measured. In this setup, the hydrogen gas from 62 ml bottles in which bacteria were grown was collected into water-filled tubes and the composition of the gas was analyzed by gas chromatography in a Hewlett–Packard Series II system with a thermal conductivity detector and a Propak Q column [13].

2.2. RNA isolation

A Macherey–Nagel NucleoSpin® RNA II RNA isolation kit was used according to the manufacturer instructions. DNase I enzyme that was provided by the manufacturer was applied to the RNA samples to eliminate the contaminating DNA. In addition, all the solutions, tubes, and other materials used for the RNA extraction were treated with diethylpyrocarbonate (DEPC) which is an RNase inhibitor. The aliquots of the samples were first run in a native %1 agarose gel for 1 h at 90 V and the gel was visualized using gel image analyzer (Vilber Lourmat, France). Both the purity and the concentration of RNA samples were determined using the spectrophotometer (Shimadzu UV-1208, Japan) by measuring the absorbances at 260 and 280 nm.

2.3. Reverse transcription–polymerase chain reaction (RT–PCR)

For the expression analysis of the *nif*K gene, multiplex PCR was performed after cDNA synthesis to make a relative quantification of PCR products using 16S rRNA as an internal control. The RT reaction for the expression analysis of the *nif*K gene was performed as follows: 0.5 μg of total RNA and 0.2 μg of random hexamer were mixed in a sterile tube and the volume was completed to 11 μl with nuclease free water if needed.

Table 1
The primers used for the RT–PCR

Gene	Primer pairs for PCR (5′ → 3′)	Primer for RT (5′ → 3′)	Position
*nif*D	agaccgtgtggggcatctgac	agaccgtgtggggcatctgacggaacgggatg[a]	2,277,013–2,277,032
	cgtcgacgcctaccatgacc		2,277,229–2,277,248
*nif*K	gaggtgcgagcgatagtagg	random hexamer	2,276,476–2,276,495
	gatctgttcaaggaacccga		2,276,729–2,276,748
*hup*S	ccgtgcacaaggtcatcctc	agaccgtgtgggatcttgtagaggcagtagc	2,232,113–2,232,132
	agaccgtgtgggatcttgtag		2,232,353–2,232,371
16S rRNA	cagctcgtgtcgtgagatgt	random hexamer	34,683–34,702
	tagcacgtgtgtagcccaac		34,833–34,852

[a]The underlined sequence represents the tag.

The mixture was incubated at 70 °C for 5 min and then chilled on ice. Then 4 μl of 5X RT reaction buffer, 2 μl of 10 mM dNTP mixture and 2 μl of nuclease free water were added to the reaction mixture that was then incubated at 37 °C for 5 min. Forty units of M-MuLV reverse transcriptase (Fermentas) was added to the mixture and it was incubated at 42 °C for 1 h. Two microliters from this mixture was used for the multiplex PCR which was carried out in a total volume of 50 μl in the presence of 1X reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 25 pmol of each primer and 1.25 unit of *Taq* DNA polymerase (Fermentas). After an initial denaturation at 94 °C for 10 min, the reaction was carried out in 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s followed by a final extension at 72 °C for 10 min. The small aliquots from the samples were run in %1.5 agarose gel for 1 h at 90 V and the gel was visualized by gel image analyzer.

The *nif*D and *hup*S expression analyses were done using tag-extended RT primers [23] (Table 1). Due to this, multiplex PCR could not be applied but rather each band in agarose gel was compared to the others. In the RT reaction, 25 pmol of tagged primer was used instead of random hexamer which was the only difference from the RT reaction protocol followed for the *nif*K gene expression analysis. The PCR reaction conditions were also the same except for the annealing temperature which was 60 °C.

3. Results and discussion

3.1. The effect of different molybdenum concentrations on the pH, growth and hydrogen production

Fig. 1 illustrates the pH and absorbance changes during growth of *R. sphaeroides* O.U.001. Although the initial pH of the media was buffered to 7, it increased upto 9.0 in No Mo and 1/10X Mo media (Fig. 1A). On the other hand, in 1X Mo, 10X Mo and 100X Mo media, there was no significant rise of the pH which remained below 8.0. If we refer to the hydrogen production curve (Fig. 2), the rise in the pH started at $t = 24$ h corresponding to the beginning of the hydrogen production in 1X Mo, 10X Mo and 100X Mo media. This observation might suggest that the lack of molybdenum in No Mo medium and its deficiency in 1/10X Mo medium led to decreased hydrogen production and thus the reducing equivalents were used for the formation of products such as polyhydroxybutyrate (PHB) which resulted in the increase in pH.

In Fig. 1B it can be seen that molybdenum insufficiency did not interfere with growth. On the contrary, the bacteria in No Mo and 1/10X Mo media reached relatively higher absorbance values which were 1.74 and 1.90, respectively. Yet, the other three cultures reached relatively lower absorbance values which were below 1.5. Based on these observations, it could be concluded that the bacteria that did not spend their energy for hydrogen production reached relatively higher cell masses and densities.

Fig. 2 points out the influence of different concentrations of molybdenum on hydrogen production by *R. sphaeroides* O.U.001. Total hydrogen gas evolved was measured and the purity of the gas was analyzed by gas chromatography (GC) and the hydrogen constituted 97–99% (v/v) of the overall gas. There was almost no hydrogen production in No Mo medium and little hydrogen production in 1/10X Mo medium; however, there was an increase in total hydrogen production accompanied with elevated molybdenum concentrations. The maximal hydrogen accumulation was achieved in 100X Mo medium (0.84 l/l) followed by 10X Mo (0.74 l/l) and 1X Mo (0.64 l/l) media. The results demonstrate that increase in molybdenum concentration stimulated hydrogen production.

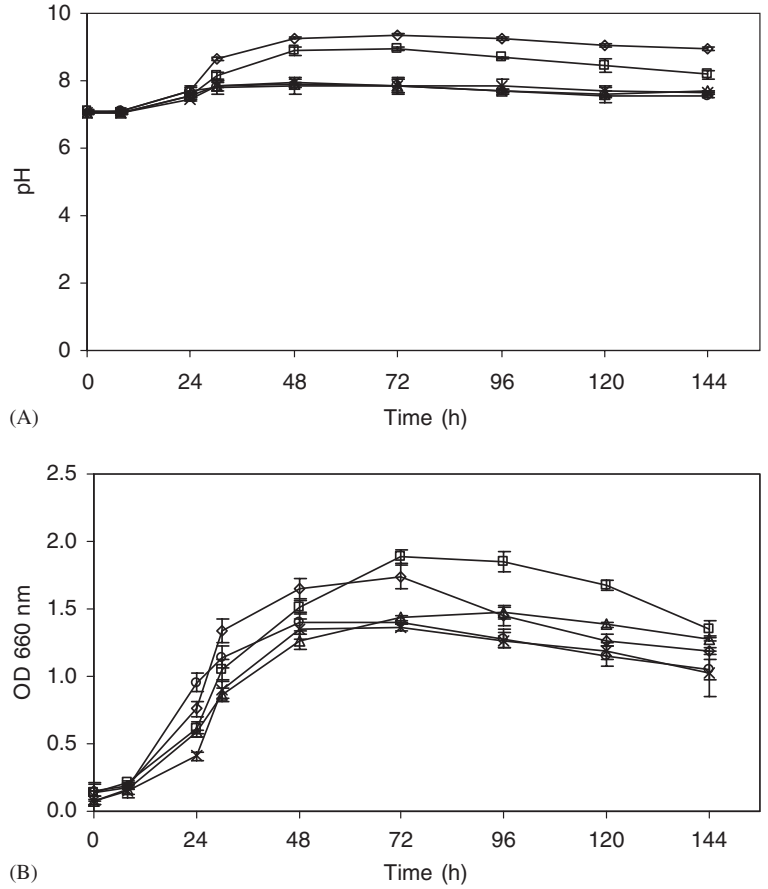

Fig. 1. pH (A) and the absorbance (B) changes during growth of *R. sphaeroides* O.U.001 in media with different molybenum concentrations. Initial pH of the cultures was buffered to 7 and was not controlled during incubation. Each value in the pH and growth curve is the mean of three times replication with ± standard deviation. (◊) No Mo; (□) 1/10X Mo; (△) 1X Mo; (×) 10X Mo; (○) 100X Mo.

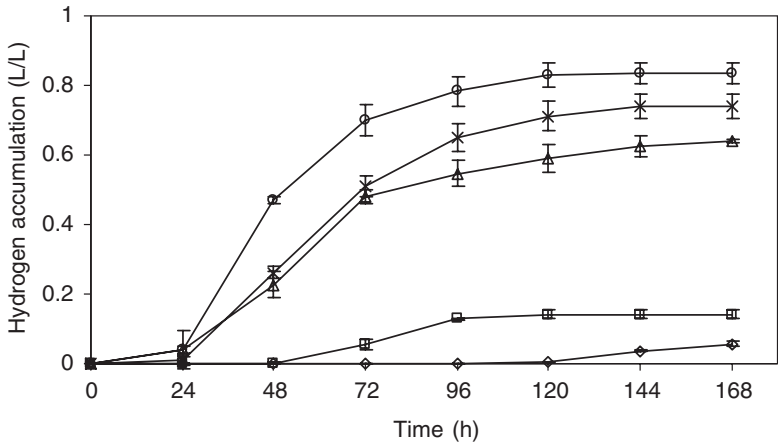

Fig. 2. Total hydrogen production by *R. sphaeroides* O.U.001 in media with different molybdenum concentrations. Each value in the curve is the mean of three times replication with ± standard deviation. (◊) No Mo; (□) 1/10X Mo; (△) 1X Mo; (×) 10X Mo; (○) 100X Mo.

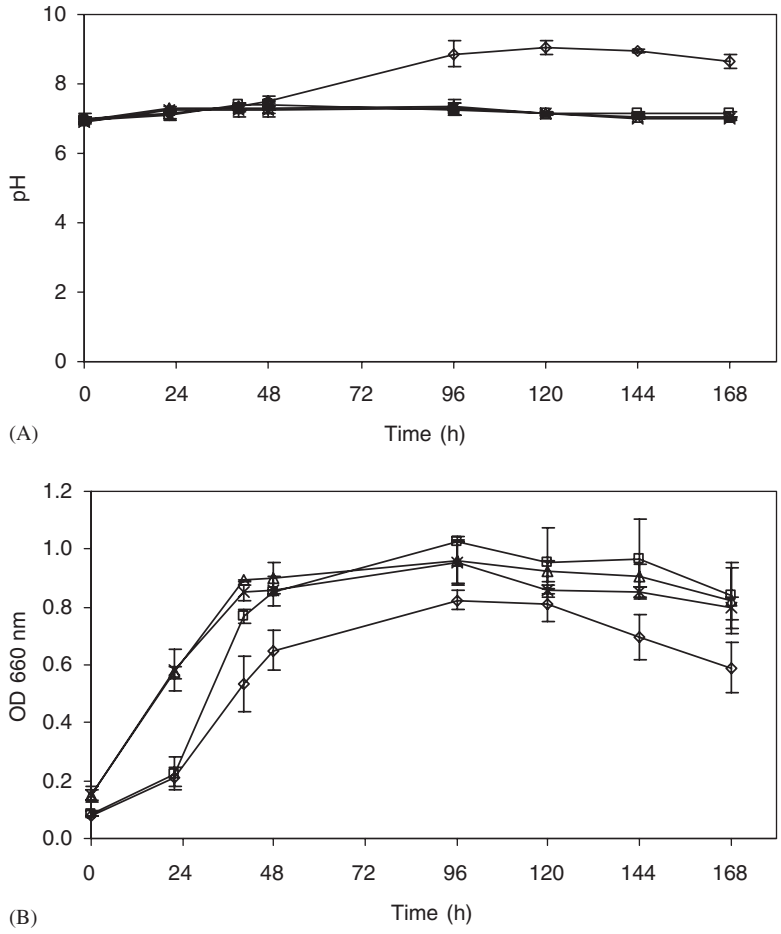

Fig. 3. pH (A) and the absorbance (B) changes during growth of *R. sphaeroides* O.U.001 in media with different iron concentrations. Initial pH of the cultures was buffered to 7 and was not controlled during incubation. Each value in the pH and growth curve is the mean of three times replication with ± standard deviation. (◊) No Fe; (□) 1/10X Fe; (△) 1X Fe; (×) 10X Fe.

3.2. The effect of different iron concentrations on the pH, growth and hydrogen production

In this study four types of media (No Fe, 1/10X Fe, 1X Fe, and 10X Fe) were prepared to see the effect of varying concentrations of iron on pH, growth, biohydrogen production and *nif*D expression. pH changes during growth of *R. sphaeroides* O.U.001 at various iron concentrations are illustrated in Fig. 3A. The pH values of 1/10X Fe, 1X Fe and 10X Fe media were all similar (∼ 7.0) and did not change considerably. However, the pH of No Fe medium started to rise after $t=46$ h and became alkaline (∼ 9.0). The rise in pH in media with reduced hydrogen production due to metal ion deficiency and the beginning of the hydrogen production were observed almost at the same time ($t = 24$ and 48 h in molybdenum and iron experiments, respectively). Therefore, this might imply that evolved hydrogen or the accumulation of some acidic compounds might keep the pH from rising in media with hydrogen production. There was a growth delay in No Fe and 1/10X Fe media indicating the vitality of iron for cellular functions (Fig. 3B). The fresh cultures in 1X Fe and 10X Fe media immediately entered the log phase yet this phase was only seen after $t=24$ h in No Fe and 1/10X Fe media. The observed colors of the iron starved cultures were also different from the others showing the interference of iron starvation with other cellular processes. The maximal absorbance value for the iron starved culture was 0.8 but the other three media reached optical densities circa 1.0.

GC analysis of the collected gas showed that the fraction of hydrogen was between 98–99% (v/v) in evolved gas. The highest total amount of hydrogen production was observed in 1X Fe medium (1.14 l/l). In addition,

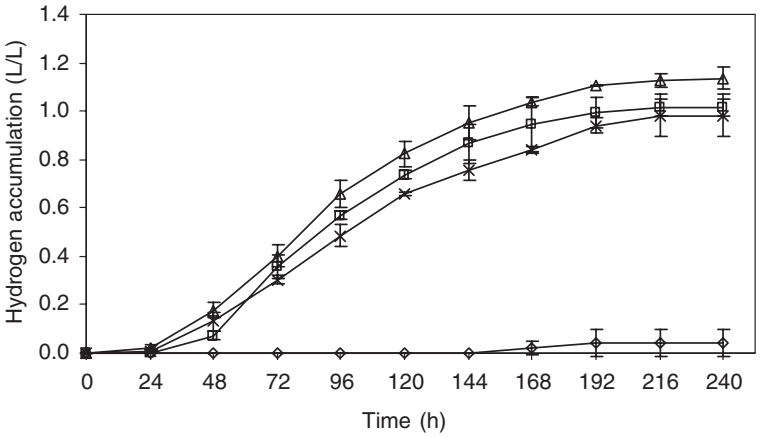

Fig. 4. Total hydrogen production by *R. sphaeroides* O.U.001 in media with different iron concentrations. Each value in the curve is the mean of three times replication with ± standard deviation. (◊) No Fe; (□) 1/10X Fe; (△) 1X Fe; (×) 10X Fe.

1.02 l/l and 0.98 l/l total hydrogen accumulation was achieved in 1/10X Fe and 10X Fe media. However, no hydrogen production was recorded in No Fe medium showing that iron is vital for hydrogen evolution. The highest iron concentration (1 mM) caused a decrease in hydrogen production which might be due to a toxic effect or due to intense color of the medium because of iron salt which might have limited the light penetration. Therefore, the optimum iron salt concentration is 0.1 mM (Fig. 4).

3.3. Time-dependent expression analysis of nifK at different molybdenum concentrations

The primers for the *nif*K and 16S rRNA genes were carefully designed and there was no complete non-specific match as a result of similarity search done using the BLAST at the NCBI web page (www.ncbi.nlm.nih.gov/BLAST/). False positive results due to the DNA contamination was also checked by a direct PCR from each RNA samples and no bands were observed at all. The expression of *nif*K with respect to time was followed during the growth of *R. sphaeroides* O.U.001 in No Mo, 1/10X Mo, 1X Mo and 100X Mo media (Fig. 5). There was no expression at t = 23 h and very low expression until $t = 120$ h in the cells grown in medium without molybdenum; however, there was some expression in the late stationary phase of the growth ($t = 120$ h). Hydrogen production also started in this phase in No Mo medium, although the amount of hydrogen was not considerable. This lag time might be the period for the cells to collect the traces of molybdenum to express nitrogenase genes. The expression in the cells grown in 1/10X Mo medium started at $t = 23$ h and reached the highest level at $t = 44$ h corresponding to the late log phase. Moreover, small amounts of expression was seen at $t = 65$ and 120 h. The expression level of the *nif*K in the cells grown in 1X Mo and 100X Mo media was almost the same in all phases of the growth. The expression started at t = 23 and reached the highest level in the late log phase ($t = 41$ h). Then, it decreased towards the end of the stationary phase of growth.

In general, the *nif*K expression was maximal in late log phase of bacterial growth (almost the beginning of the stationary phase) in 1/10X Mo, 1X Mo and 100X Mo media while it decreased towards the end of the stationary phase of growth. Colbeau et al. [24] and Krahn et al. [25] demonstrated that hydrogenase and nitrogenase activities were maximal in this phase. Although there was more hydrogen accumulation in 100X Mo medium than in 1X Mo and 1/10X Mo media, the gene expression levels were not different significantly. This might result from higher amount of properly assembled Mo-nitrogenase in 100X Mo medium due to higher molybdenum concentration.

3.4. Time-dependent expression analysis of the nifD gene at different iron concentrations

The stringent PCR conditions due to using carefully designed tagged primers did not allow false positive results that were confirmed by PCR using RNA as a template. The expression analyses were done at $t = 16$, 24, 44, 66, 118 and 191 h and the result is illustrated in Fig. 6. The *nif*D expression analysis in the cells grown in No Fe medium was started at $t = 44$ h and there was still no expression. Although a considerable amount of

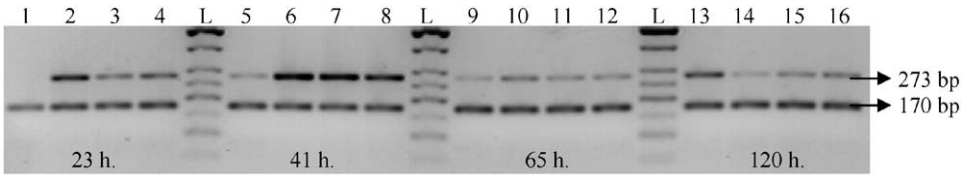

Fig. 5. Agarose gel of RT–PCR products of *nif*K in *R. sphaeroides* O.U.001 cultured in media with various concentrations of molybdenum ([$Na_2MoO_4 \cdot 2H_2O$]: without molybdenum (No Mo); 0.0165 μM (1/10X Mo); 0.165 μM (1X Mo); and 16.5 μM (100X Mo)). Products of RT–PCR were run on 1.5% agarose gel at 90 V for 1 h together with 100 bp DNA ladder (L). The analyses were performed at $t = 23$, 41, 65 and 120 h. Lanes 1–4, 5–8, 9–12 and 13–16 represent the results obtained from the cells grown in No Mo, 1/10X Mo, 1X Mo and 100X Mo media, respectively. In each lane, the *nif*K product is normalized using the internal control (16S rRNA) which was expressed uniformly throughout the growth phase.

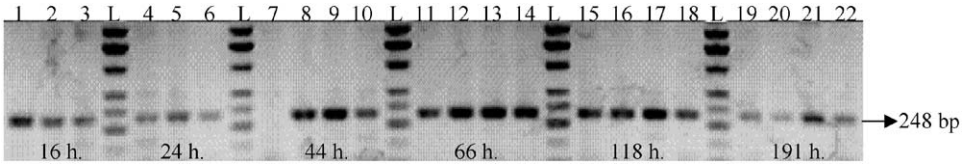

Fig. 6. Agarose gel of RT–PCR products of *nif*D in *R. sphaeroides* O.U.001 cultured in media with various concentrations of iron ([Fe(III) citrate.hydrate]: 0 mM (No Fe); 0.01 mM (1/10X Fe); 0.1 mM (1X Fe) and 1 mM (10X Fe)). Products of RT–PCR were run on 1.5% agarose gel at 90 V for 1 h, together with 100 bp DNA ladder (L). The analyses were performed at $t = 16$, 24, 44, 66, 118 and 191 h. Lanes 1–3, 4–6 represent the results obtained from the cells grown in 1/10X Fe, 1X Fe and 10X Fe media. Lanes 7–10, 11–14, 15–18 and 19–22 represent the results obtained from the cells grown in No Fe, 1/10X Fe, 1X Fe and 10X Fe media, respectively.

expression was detected at $t = 66$ and 118 h in the cells grown in No Fe medium, there was no hydrogen production at all. This could be due to fact that although the genes were transcribed, incorporation of sufficient amount of iron and correct assembly of fully active nitrogenase could not take place. There was a considerable amount of *nif*D expression in the cells grown in 1/10X Fe at $t = 16$ and 24 h. Moreover, the expression level increased at $t = 44$ and 66 h where the highest expression level was obtained. At $t = 118$ and 191 h, the *nif*D expression in the cells decreased considerably. The expression in the cells grown in 1X Fe medium has already started at $t = 16$ h and continued at the same level at $t = 24$ h. Furthermore, a significant amount of expression was observed at $t = 44$, 66, and 118 h and then the expression declined at $t = 191$ h. Considering the overall expression analysis of the *nif*D gene, it is seen that maximal expressions were obtained in the cells grown in 1X Fe medium throughout the growth phases. This result is in agreement with the hydrogen production experiment in that the maximal hydrogen production was achieved in 1X Fe medium implying that the optimum iron salt concentration for the hydrogen production is 0.1 mM. The expression of the *nif*D gene in the cells grown in 10X Fe medium has also started at $t = 16$ and 24 h and it increased until $t = 66$ h where the highest expression was observed. However, the amount of expression lessened towards the end of stationary phase. This result point out that further addition of iron does not increase the expression amount but rather it might cause a reduction in expression and hydrogen production. In general, the maximal expressions were observed around the beginning of the stationary phase. Also, maximal rates of hydrogen production were seen in the period starting from the beginning of the stationary phase to the mid-stationary phase.

3.5. Time-dependent expression analysis of the hupS gene at different iron concentrations

Membrane-bound uptake hydrogenase is known to oxidize molecular hydrogen thereby decreases the total hydrogen production. In addition to the nitrogenase genes, the expression of *hup*S with respect to time was also monitored and the result is given in Fig. 7. The specific primer strategy was also applied for *hup*S expression analysis and there were no false positive results. It was observed that *hup*S was not expressed considerably until $t = 23$ h in the iron starved cells. Although there was some expression at $t = 23$ h in the cells, it disappeared in the following analyses. This transient gene expression might have occurred as opposed to the physiological stress due to iron deficiency. There was very little amount of gene expression in the cells grown

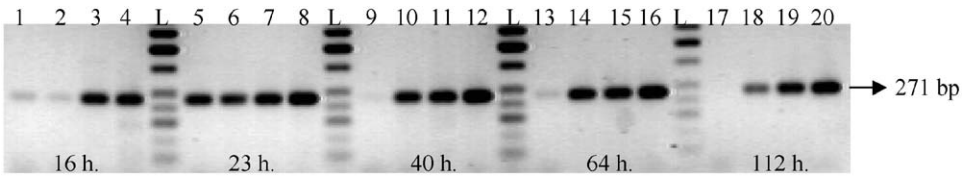

Fig. 7. Agarose gel of RT–PCR products of *hup*S at different iron concentrations ([Fe(III) citrate.hydrate]: 0 mM (No Fe); 0.01 mM (1/10X Fe); 0.1 mM (1X Fe) and 1 mM (10X Fe)). Products of RT–PCR were run on 1.5% agarose gel at 90 V for 1 h, together with 100 bp DNA ladder (L). The analyses were performed at $t = 16$, 23, 40, 64 and 112 h. Lanes 1–4, 5–8, 9–12, 13–16 and 17–20 represent the results obtained from the cells grown in No Fe, 0.1X Fe, 1X Fe and 10X Fe media, respectively.

in 1/10X Fe medium at $t = 16$ h but the expression increased at $t = 23$ h. Significant amount of *hup*S expression was seen until $t = 112$ h where the amount of expression decreased to a certain level. Moreover, the expression in the cells grown in 1X Fe and 10X Fe media was quite pronounced throughout the growth phases. If the Figs. 4 and 6 are referred, there were still significant amount of hydrogen production and *nif*D gene expression at this hour. Since there was still enough amount of hydrogen which could be used by the uptake hydrogenase, it was expected to see *hup*S expression until $t = 112$ h. The results emphasized that uptake hydrogenase gene was expressed significantly as soon as the hydrogen and the iron were available in the environment and iron limitation elicited significant reduction in the transcript amount.

4. Conclusion

This study demonstrates the metal (Mo and Fe) dependence of hydrogen production and the expression of Mo-nitrogenase and uptake hydrogenase genes (*nif*K, *nif*D and *hup*S). pH and the cell densities were also monitored under the same growth conditions. The cells grown in media without molybdenum and iron reached relatively higher densities since they do not spent energy for hydrogen production but the cells grown in metal ion containing media reached relatively lower absorbance values. Moreover, the rise in pH in media without hydrogen production could be attributed to the accumulation of cellular by-products such as PHB or the constant pH in media with hydrogen production could result from dissolved hydrogen gas or the acidic by-products preventing the pH to rise considerably. It is seen that the cells grown in media without molybdenum and iron could not produce significant amounts of hydrogen and the optimum concentrations of the molybdenum and iron salts were 16.5 μM and 0.1 mM, respectively. The gene expression analysis at different molybdenum concentrations showed that *nif*K expression in No Mo medium was strongly reduced but it was expressed significantly in the other media. The maximal gene expression expressions were observed in the late log phase of the growth in the cells grown in 1/10X Mo, 1X Mo and 100X Mo media.

The *nif*D and *hup*S expression analyses at various concentrations of iron showed that iron deficiency also led to pronounced reduction in the gene expression levels (Figs. 6 and 7). And, similar to the *nif*K expression, the highest *nif*D expression was observed between the very end of log phase and the mid-stationary phase. *hup*S gene was expressed significantly as soon as the hydrogen and the iron were available in the environment, and iron limitation elicited significant reduction in the transcript amount. In conclusion, if the gene expression analyses and the hydrogen production profiles of the cells were taken into account, it is suggested to keep the cells in the late log phase of the growth to get a high hydrogen production rate. And, inactivation of uptake hydrogenase might further increase hydrogen production in *R. sphaeroides* O.U.001.

Acknowledgments

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