EXPRESSION ANALYSIS OF NITROGENASE GENES IN *RHODOBACTER SPHAEROIDES* O.U.001 GROWN UNDER DIFFERENT PHYSIOLOGICAL CONDITIONS


A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY


BY


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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOLOGY


FEBRUARY 2008

Approval of the thesis:

**EXPRESSION ANALYSIS OF NITROGENASE GENES IN *RHODOBACTER SPHAEROIDES* O.U.001 GROWN UNDER DIFFERENT PHYSIOLOGICAL CONDITIONS**

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# ABSTRACT

## EXPRESSION ANALYSIS OF NITROGENASE GENES IN *RHODOBACTER SPHAEROIDES* O.U.001 GROWN UNDER DIFFERENT PHYSIOLOGICAL CONDITIONS

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February 2008, 114 pages

Hydrogen has an extensive potential as a clean and renewable energy source. Photosynthetic, non-sulphur, purple bacteria, *Rhodobacter sphaeroides* O.U.001 produces molecular hydrogen by nitrogenase enzyme. Nitrogenase enzyme is encoded by *nifHDK* genes and expression of the structural genes, *nifHDK,* is controlled by NifA which is encoded by *nifA* gene. The transcription of *nifA* is under the control of Ntr system and product of *prrA* gene.

Relationship between the genes that have roles in nitrogenase synthesis should be understood well to increase biological hydrogen production. In this work, expression levels of nitrogenase encoding *nifH* and control genes *nifA*, *prrA* were examined at different physiological conditions. In addition to modifications in expression levels, changes in hydrogen production and growth capacity were also investigated in response to different concentrations of ammonium source, oxygen and different light intensities.

In this study, it was found that increasing concentrations of ammonium chloride caused decrease in hydrogen production. Glutamate containing medium had the capacity for higher hydrogen production. The expression levels of *nifH* and *nifA* genes decreased with the increase in concentrations of ammonium chloride. There was a negative correlation between the expression levels of *prrA* gene and its target, *nifA* gene. Hydrogen production was observed even in aerobic conditions of the same media compositions.

It was observed that different culture media had changing growth and hydrogen production capabilities at different light intensities. There was no direct proportion between the expression levels of *nifH* gene and amount of hydrogen at different light intensities.

Keywords: *Rhodobacter sphaeroides* O.U.001; Biohydrogen; Nitrogenase; *nif* Gene Expression

# ÖZ

## FARKLI FİZYOLOJİK KOŞULLARDA ÜRETİLEN *RHODOBACTER SPHAEROIDES* O.U 001'DE NİTROJENAZ GENLERİNİN İFADESİ

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Şubat 2008, 114 sayfa

Hidrojen temiz, yenilenebilir bir enerji kaynağı olarak büyük potansiyele sahiptir. Fotosentetik bir bakteri olan *Rhodobacter sphaeroides* O.U.001'de hidrojen üretimi nitrojenaz enzimi ile gerçekleşir. Nitrojenazın yapısal proteinlerini *nifHDK* genleri kodlamaktadır. Yapısal genlerin ifadesini kontrol eden NifA proteini, *nifA* geni tarafından kodlanmaktadır. Bu genin ifadesi ise *prrA* geninin ürünü olan protein ve Ntr sisteminin kontrolü altındadır.

Biyolojik hidrojen üretimini artırmak için nitrojenaz üretiminde rol alan genlerin birbirleriyle olan ilişkisinin iyi anlaşılması gerekir. Bu tez çalışmasında nitrojenazı kodlayan *nifH* geni ve bu genleri kontrol eden *nifA*, *prrA* genlerinin ifade düzeyleri farklı fizyolojik ortamlarda incelenmiştir. Farklı derişimlerdeki amonyum kaynağına, oksijene ve değişik ışık şiddetlerine bağlı gen ifadesi düzeylerindeki değişimlerle birlikte, hidrojen üretimi ve üreme farklılıkları da incelenmiştir.

Bu çalışmada, artan amonyum klorür derişimlerinin hidrojen üretiminde düşüşe neden olduğu görülmüştür. Glutamat içeren besiyeri, daha fazla hidrojen üretme kapasitesine sahiptir. *nifH* ve *nifA* genlerinin ifadesi artan amonyum klorür

derişimlerinde azalmıştır. *prrA* geni ile kontrolündeki *nifA* genlerinin ifadesi birbiriyle ters orantılıdır. Aynı nitelikteki besiyerlerinde oksijenli ortamda da bir ölçüde hidrojen üretimi gözlenmiştir.

Değişik besiyerlerinin ve farklı ışık şiddetlerinin, hidrojen üretimini ve üremeyi etkilediği görülmüştür. Farklı ışık şiddetlerinde üretilen hidrojen miktarıyla, *nifH* geninin ifade düzeyleri arasında doğru bir orantı saptanamamıştır.

Anahtar Kelimeler: *Rhodobacter sphaeroides* O.U.001; Biyohidrojen; Nitrojenaz; *nif* Genlerinin İfadesi

*To My Family*

# ACKNOWLEDGEMENT

First of all I would like to thank to my supervisor Prof. Dr. Ufuk Gündüz for her guidance, support and suggestions in preparing this thesis. I would like to express my gratitude to Prof. Dr. İnci Eroğlu and Prof. Dr. Meral Yücel for their valuable ideas, understanding and trust in me.

I also feel great appreciation to the examining committee members for their contribution and comments.

I am also grateful to Gökhan Kars, Özlem İşeri and Meltem Kars for their continous help, support and friendship.

Başar Uyar, Ebru Özgür, Elif Genç and all the other members of METU Biohydrogen group deserve special thanks for their interest and valuable hints.

I would like to thank deeply to Esra Güç, Mine Nuyan, Yaprak Dönmez, Pelin Sevinç and Ömer Faruk Gerdan for their friendship, support in difficult times, and encouragement to complete this thesis.

I am deeply indebted to my family whose moral support, patience and love help me in every step of my life.

Last but not least, I would like to give my special thanks to Derya for his support, encouragement and patient love through all of those years.

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| | |
|---|---|
| ADP | Adenosine di-phosphate |
| ATP | Adenosine tri-phosphate |
| BP | Biebl Pfenning |
| DEPC | Diethyl pyrocarbonate |
| EDTA | Ethylene diamine tetra-acetic acid |
| EtBr | Ethidium bromide |
| GC | Gas choromatography |
| ICM | Intracytolasmic membrane system |
| M-MuLV-RT | Moloney Murine Leukemia Virus Reverse Transcriptase |
| NCBI | National Center of Biotechnology Information |
| *nifA* | Nitrogen Fixation Gene A |
| *nifH* | Nitrogen Fixation Gene H |
| OD | Optical Density |
| PCR | Polymerase Chain Reaction |
| *prrA* | Gene encoding response regulator PrrA |
| PS | Photosystem |
| *R. capsulatus* | *Rhodobacter capsulatus* |
| *R. sphaeroides* | *Rhodobacter sphaeroides* |
| RT-PCR | Reverse Transcriptase – PCR |
| TCA | TriCarboxylic Acid |
| 16S rRNA | 16S ribosomal RNA |

# CHAPTER 1

# INTRODUCTION

## 1.1 Hydrogen

Hydrogen is the simplest and most plentiful element in the world. Hydrogen gas is very rare in the Earth's atmosphere (1 ppm by volume) because of its light weight, which enables it to escape from gravity more easily than heavier gases.

Hydrogen has the potential to be a clean fuel when used in energy applications such as fuel cells or modified internal combustion engines. As a potential energy carrier of future, hydrogen can be used in powering nonpolluting vehicles, heating homes and fueling aircraft.

Hydrogen is a potential fuel for many reasons such as its production uses a variety of methods and feedstocks (such as methanol, natural gas, ethanol, petroleum and renewables) allowing most regions of the world to produce hydrogen. Hydrogen is also safe to produce, store, transport and use in fuel cells and internal combustion engines (Hydrogen and Fuel Cells Canada, 2008).

## 1.2 Production of Hydrogen

Hydrogen can be produced from a variety of feedstock; from fossil resources such as natural gas and coal, and from renewable resources such as biomass and water with input from renewable energy sources (e.g. sunlight, wind, wave or hydro-power)

(Riis et al., 2005). There are many hydrogen production methods including chemical, biological, electrolytic, photolytic and thermo-chemical. The methods are given below: (Wikipedia the Free Encyclopdia, 2007):

- Steam reforming: Commercial bulk hydrogen is usually produced by the steam reforming of natural gas.
- Carbon monoxide: Additional hydrogen can be recovered from the carbon monoxide through the lower-temperature water gas shift reaction, performed at about 130 °C.
- Coal: Coal can be converted into syngas and methane, also known as town gas, via coal gasification.
- Electrolysis of water is used to produce hydrogen. Electrolysis of water is the decomposition of water into oxygen and hydrogen gas due to an electric current being passed through the water.
- Photoelectrochemical Water Splitting: Water is broken into hydrogen and oxygen by electrolysis—a photoelectrochemical (PEC) process.Using electricity produced by photovoltaic systems offers the cleanest way to produce hydrogen.
- High temprature electrolysis: High-temperature electrolysis (also HTE or steam electrolysis) is a method currently being investigated for the production of hydrogen from water with oxygen as a by-product. High temperature electrolysis is more efficient economically than traditional room-temperature electrolysis because some of the energy is supplied as heat, which is cheaper than electricity, and because the electrolysis reaction is more efficient at higher temperatures.

## 1.2.1. Biological Hydrogen Production

All processes of biological hydrogen production are dependent on the presence of a hydrogen- producing enzyme (Hallenbeck and Benemann, 2002). Biological

hydrogen production processes are classified as follows by Das and Veziroglu (2001):

1. Biophotolysis of water using algae and cyanobacteria.
2. Photodecomposition of organic compounds by photosynthetic bacteria.
3. Fermentative hydrogen production from organic compounds
4. Hybrid systems using photosynthetic and fermentative bacteria.

Cyanobacteria and green algae produce both hydrogen and oxygen by light-driven biophotolysis processes involving both nitrogenase and hydrogenase enzymes (Basak and Das, 2007). Water is used as electron donor in this process. Production of oxygen causes the reduction in hydrogen production because of the inhibitory effect on hydrogenase.
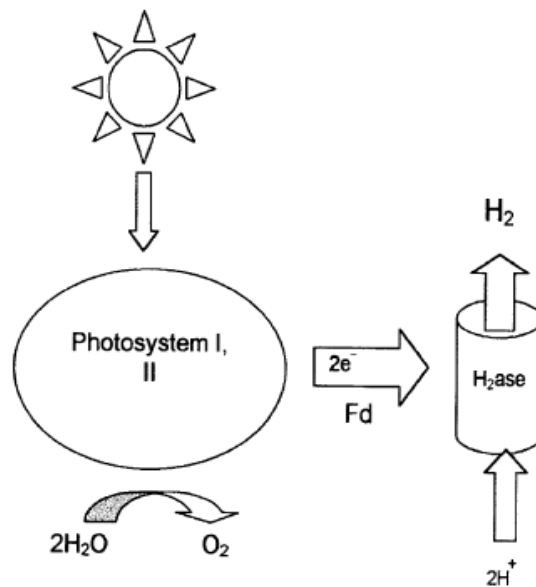


**Figure 1.1 Direct biophotolysis (Hallenbeck and Benemann, 2002)**

Photosynthetic bacteria produce hydrogen from various organic acids by using light energy and nitrogenase enzyme. Purple non-sulfur (*Rhodobacter*) and purple sulfur bacteria (*Chromatium*) produce hydrogen by photofermentation.(Basak and Das, 2007).

Hydrogen production by fermentative bacteria was catalyzed by hydrogenase. Dark fermentation has many advantages such as: The high rate of hydrogen evolution of fermentative bacteria and they can produce hydrogen constantly through day and night from organic substrates (Das and Veziroglu, 2001).

The hybrid system is a combination of dark fermentation by fermentative bacteria followed by photofermentation by purple non-sulfur photosynthetic bacteria, wherein the overall hydrogen yield can be enhanced to a great extent (Basak and Das, 2007).

## 1.3. Hydrogen Production by Photosynthetic Bacteria

Photosynthetic bacteria carry out anoxygenic photosynthesis using organic compounds and reduced sulfur compounds as electron donors, which are categorized as non-sulfur and sulfur photosynthetic bacteria (Asada and Miyake, 1999). The hydrogen production by photosynthetic bacteria is found to be the most promising as compared to other microbial systems because of; high substrate to product conversion yield, lack of oxygen evolving activity, ability to use a wide wavelength of light, and capability to use organic substrates derived from wastes (Basak and Das, 2007).

Nitrogenase enzyme catalyzes the hydrogen production whereas hydrogenases may be active for hydrogen uptake in many photosynthetic bacteria (Asada and Miyake, 1999). Figure 1.2 is a brief summary of hydrogen production by photofermentation. Purple non-sulfur (PNS) bacteria produce hydrogen under light in anaerobic conditions from the utilization of organic acids such as malate, acetate and lactate.
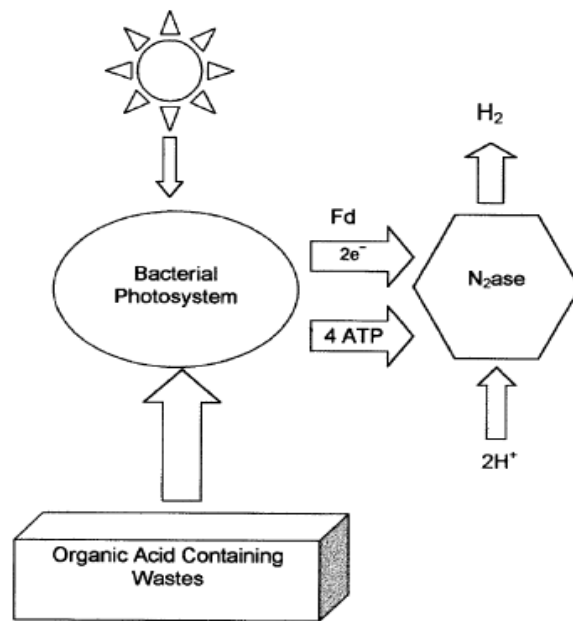
**Figure 1.2. Photofermentation (Hallenbeck and Benemann, 2002)**

### 1.3.1. Photosynthetic Purple Non-Sulfur Bacteria

Purple non-sulfur (PNS) photosynthetic bacteria constitute a non-taxonomic group of versatile organisms which can grow as photoheterotrophs, photoautotrophs or chemoheterotrophs—switching from one mode to another depending on available conditions such as: degree of anaerobiosis, availability of carbon source ($CO_2$ for autotrophic growth, organic compounds for heterotrophic growth), and availability of a light source (Basak and Das, 2007).

### 1.3.1.1. *Rhodobacter sphaeroides*

The bacterium *Rhodobacter sphaeroides* belongs to the α-subdivision of the *Proteobacteria*. The bacteria of this group are the most nearly spherical of any of the non-sulfur purple bacteria. pH range is wide and extends from at least pH 6.0 to

8.5. Optimum temperature is low and lies below 30°C (Niel, 1944). Color of *R.sphaeroides* O.U.001 cultures is brown to dark brown when they are grown anaerobically. Aerobically grown cultures change their color into red gradually during the exposure to air (Holt et al., 1984).

*R. sphaeroides* possesses an extensive range of energy acquiring mechanisms including photosynthesis, lithotrophy, aerobic and anaerobic respiration. It can also fix molecular nitrogen; synthesize important tetrapyrroles, chlorophylls, heme, and vitamin B12. (Department of Microbiology and Molecular Genetics at the University of Texas Health Science Center, 2007).

### 1.3.1.2. Growth Modes of *R.sphaeroides*

*Rhodobacter sphaeroides* can grow aerobically and anaerobically by respiration, fermentatively, and it can also grow photoautotrophically and photoheterotrophically under anaerobic conditions in the light (Eraso and Kaplan, 2000).

### 1.3.1.2.1. Photoheterotrophic Growth

Illumination, anaerobic atmosphere and organic substrates are required for photoheterotrophic growth in *R.sphaeroides*. The energy is obtained from light and carbon source is organic substrates. This growth mode results in high growth rate and hydrogen production. The other modes of growth are not very efficient in hydrogen production.

### 1.3.1.2.2. Photoautotrophic Growth

Photoautotrophy is the process by which organisms convert radiant energy into biologically useful energy and synthesize metabolic compounds using only carbon dioxide or carbonates as a source of carbon (Erosa and Kaplan, 2001). *R.sphaeroides* is able to grow photoautotrophically. However this mode growth is

undesirable for hydrogen production because hydrogen is consumed in this process. Cells require highly reduced external electron donor compounds to dissimilate $CO_2$. Hydrogen is split into protons and electrons by membrane bound hydrogenase and thus producing the reducing equivalents for $CO_2$ fixation (Koku, 2001).

### 1.3.1.2.3. Aerobic Respiration

*R.sphaeroides* is able grow under aerobic conditions with the supplementation of organic carbon. Aerobic respiration has an importance in hydrogen production because hydrogen producing nitrogenase enzyme is highly oxygen sensitive and photosynthetic apparatus is induced when oxygen tension falls. If high levels of oxygen are present, *Rhodobacter* gains energy by aerobic respiration and synthesizes only low amounts of photosynthetic complexes.

### 1.3.1.2.4. Anaerobic Respiration and Fermentation

*Rhodospirillum rubrum and Rhodopseudomonas capsulata* are capable of anaerobic, dark growth using energy generated either from the fermentation of sugars to mixed acid products or from nonfermentable organic substrates in electron transport-coupled, anaerobic respiration. Nonfermentable substrates, such as succinate, malate, or acetate, supported growth only in the presence of an electron acceptor such as dimethyl sulfoxide or trimethylamine oxide (Schultz and Weaver, 1982). When light is scarce or completely absent in the presence of fermentable sugars, the bacteria can grow fermentatively. Both anaerobic respiration and fermentation are undesirable for hydrogen production. These pathways help the bacteria to maintain their metabolic activity under a serious shortage of energy (Koku, 2001).

## 1.4. Hydrogen Production by *Rhodobacter sphaeroides*

Major route for hydrogen production is biological nitrogen fixation in photosynthetic microorganisms (Rey et al, 2007). *R. sphaeroides* which is PNS bacteria can use light energy to overcome the positive free energy of activation and

produce hydrogen by utilizing organic acids as substrates (Basak and Das, 2007). The carbon substrate is oxidized to produce $CO_2$ and electrons in TCA cycle. TCA cycle works in parallel with photosynthetic membrane apparatus which converts light energy into ATP. ATP is channeled into nitrogenase along with the protons and electrons which are supplied by the TCA cycle. Nitrogenase reduces the protons to molecular hydrogen (Koku et al., 2002).

The hydrogen production is catalyzed by nitrogenase enzyme in *R. sphaeroides*. Membrane-bound uptake hydrogenase consumes $H_2$ produced by nitrogenase.
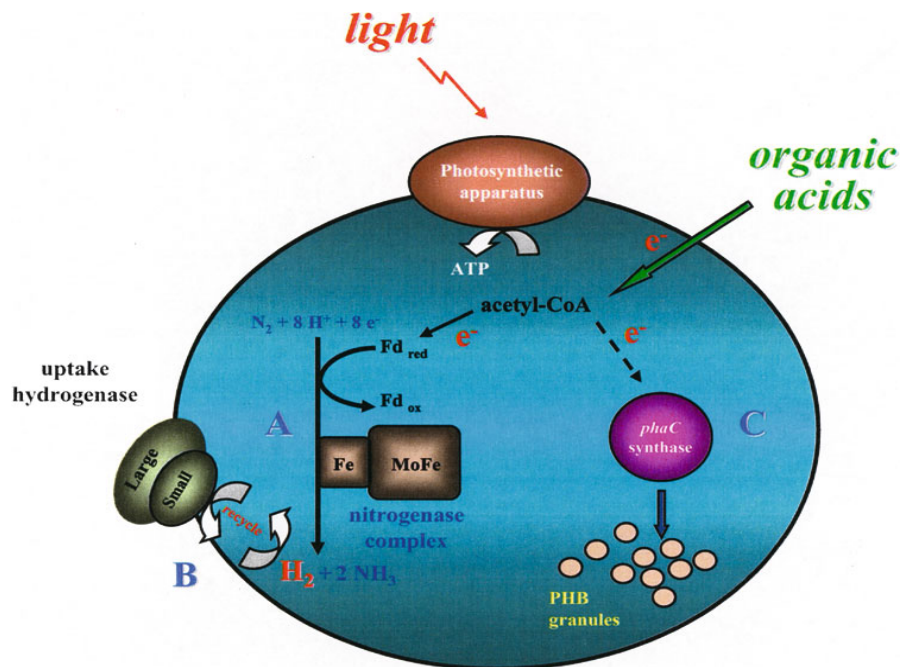


**Figure 1.3. Metabolic pathway of H₂ production and uptake (Franchi et al., 2004)**

The amount of hydrogen evolved is determined by the interaction of several metabolic pathways as seen in Figure 1.3. $H_2$ evolution, mediated by the enzyme

nitrogenase; H₂ uptake (recycling), catalyzed by a membrane-bound uptake hydrogenase that reduces the net amount of gas evolved (Vignais et al., 1985); and biosynthesis of alternative electron sinks for reductants, in particular, poly-3-hydroxybutyrate (PHB) in the form of cytoplasmic granules (Franchi et al., 2004).

### 1.4.1. Enzymes in Hydrogen Production

Two enzymes named hydrogenase and nitrogenase are involved in hydrogen production *in Rhodobacter sphaeroides*.

### 1.4.1.1. Hydrogenase

Three different classes of hydrogenases have been identified so far: [Fe]-hydrogenase which catalyses the hydrogen production, [NiFe]-hydrogenase which is responsible for hydrogen uptake and [NiFeSe]-hydrogenase (Basak and Das, 2007). Hydrogen utilization is typically mediated by uptake hydrogenases that catalyze the oxidation of hydrogen (Rey et al., 2006). Electrons derived from this process are then transferred to ferredoxins and cytochromes. Hydrogenases play a significant role in the membrane bioenergetic process by coupling oxidation of H₂ to ATP synthesis through generation of membrane potential (Kovacs and Bagyinka, 1990). Hydrogenases protect the nitrogenase from inhibitory effect of nitrogenase. In *R.capsulatus*, hydrogen is oxidized in the presence of oxygen and oxygen is assimilated into water by the aid of hydrogenase which provides electrons (Meyer et al., 1978). Membrane-bound nickel-iron [NiFe] uptake hydrogenase which is encoded by *hupSL* genes is found in *R. sphaeroides* and catalyzes the reaction below:

$$H_2 \leftrightarrow 2H^+ + 2e^- \qquad\qquad (1.1)$$

Hydrogenases are usually sensitive to inhibition by oxygen. Oxygen does not affect the structural integrity of [NiFe] hydrogenases but reversibly inactivates their catalytic function (Buhrke et al, 2005).

**1.4.1.2 Nitrogenase**

Nitrogenases, which catalyze the biological reduction of dinitrogen to ammonia, contain two components that are named according to their metal composition (Dixon and Kahn, 2004). Nitrogenases come in many different forms; with the most common categorization having four different families based on the type of metal cluster constituting the N2 binding site (*i.e.*, the active site) (Igarashi and Seefeldt, 2003). One family of nitrogenases which is found in *Streptomyces thermoautotrophicus* contains two component proteins. One component, a CO-dehydrogenase, oxidizes CO to $CO_2$ and reduces $O_2$ to the superoxide anion radical ($O_2^-$). The second component is a manganese-dependent oxidoreductase that oxidizes $O_2^-$, providing electrons to the N2 and reducing the MoFeS active site (Ribbe *et al.*, 1997). The other three families of nitrogenase share many similarities to each other, with a distinguishing feature being the metal content of the active site (including Mo, V, or Fe only) (Igarashi and Seefeldt, 2003). Nitrogen fixation genes (*nif*) system is responsible for encoding the conventional molybdenum (Mo)-containing nitrogenase. Other two nitrogenase systems are closely related to the Mo nitrogenase, but Mo-independent. One is the vanadium (V)-dependent nitrogen fixation (*vnf*) system encoding a nitrogenase which contains V instead of Mo in the cofactor (vanadium nitrogenase), whereas the other, represented by the alternative nitrogen fixation (*anf*) gene system, encodes a nitrogenase containing neither Mo, V nor any other heterometal atom, and has therefore been designated as the Fe nitrogenase or Fe-only nitrogenase (Siemann et al., 2002).

The protein containing the 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), also known as dinitrogenase reductase (Halbleib and Ludden, 2000). MoFe protein is composed of two different polypeptides encoded by *nifD* and *nifK* genes that are arranged as two dimers ($\alpha2\beta2$) (Igarashi and Seefeldt, 2003). Each MoFe protein tetramer contains two pairs of metalloclusters unique to MoFe protein, i.e., two molydenum-iron-sulfur-

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homocitrate clusters (FeMo-co) and two [Fe$_8$S$_7$] clusters (P-cluster) (Halbleib and Ludden, 2000). FeMo-cofactor is nitrogen reduction site and P-cluster participates in electron transfer from Fe-protein to FeMo-cofactor (Rey et al., 2007).

The Fe protein (also called dinitrogenase reductase), is coded for by the *nifH* gene, and is composed of two identical subunits (α2) with a single [4Fe-4S] cluster bound between the subunits. The Fe protein also contains two MgATP binding sites, one on each subunit (Igarashi and Seefeldt, 2003).
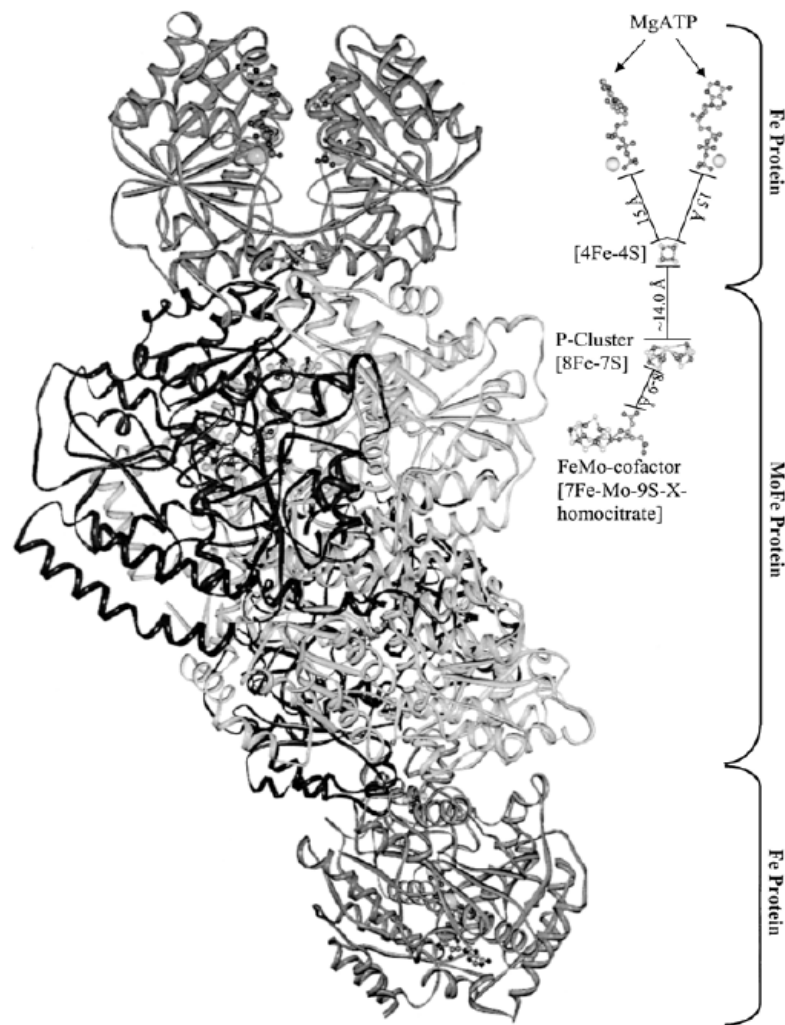


**Figure 1.4. The overall structure of nitrogenase (Igarashi and Seefeldt, 2003).**

The transfer of electrons from dinitrogenase reductase to dinitrogenase requires the mediation of MgATP (Burris, 1991). General sequence of events in the catalytic cycle of nitrogenase is as follows (Halbleib and Ludden, 2000):

Binding of MgATP at $[Fe_4S_4]$ active sites causes a conformational change in Fe protein. MgATP–induced conformational change promotes interaction of Fe protein with MoFe protein. Upon complex formation, an additional conformational change of Fe protein shifts the redox potential of $[Fe_4S_4]$ cluster to value, making the transfer of single electron from the Fe protein to MoFe protein energetically favorable. The hydrolysis of MgATP, which is bound to Fe protein, to MgADP and $P_i$ is coupled to this electron transfer. One electron is transferred for each 2MgATP hydrolyzed. As single electron is inadequate to reduce $N_2$, the cycle must be repeated until the dinitrogenase has accumulated adequate electrons to reduce $N_2$ (Burris, 1991). The overall reaction to reduce $N_2$ to ammonia (Vignais et al., 2006)

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16\ ADP + 16P_i \qquad (1.2)$$
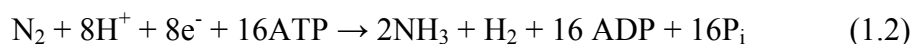
Nitrogenase activity requires large amounts of ATP and reducing power. Therefore synthesis and activity of nitrogenase is strictly regulated at transcriptional level and post-translational level in response to environmental stimuli.
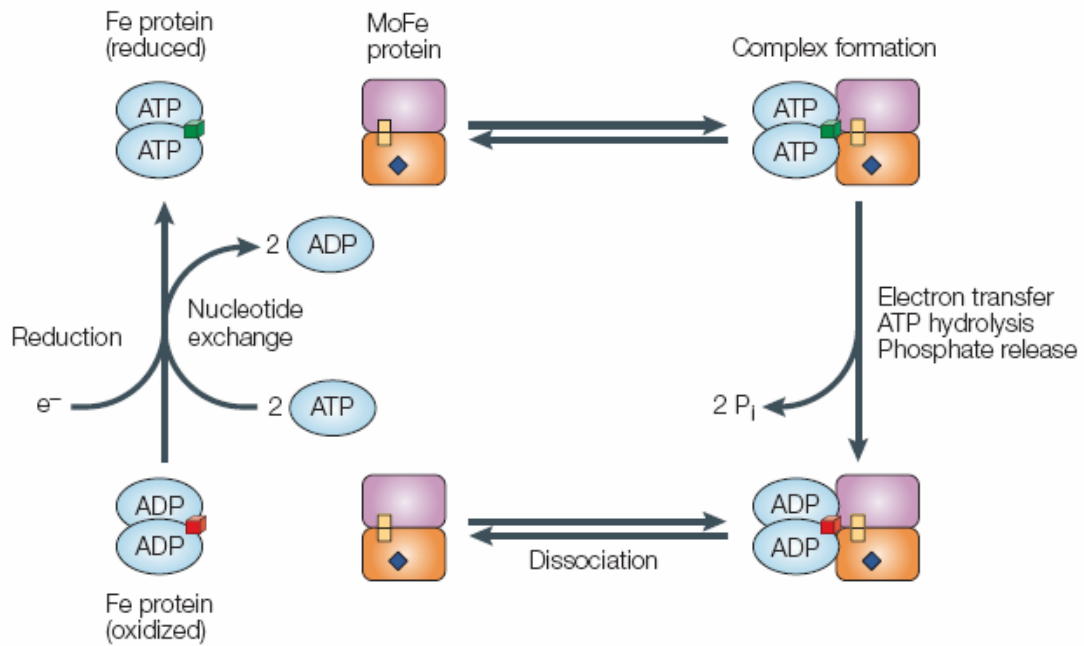
**Figure 1.5. Nitrogenase turnover cycle** (Dixon and Kahn, 2004) (The α and β subunits of the MoFe protein are depicted as orange and pink, respectively, the yellow squares represent the P cluster and the blue diamond represents the FeMo cofactor.)

## 1.5. Transcriptional Regulation of Nitrogenase

Nitrogen fixation is high energy-demanding process. Therefore, synthesis of nitrogenases is regulated at transcriptional level by complex regulatory systems. Expression of the structural genes of nitrogenase *nifHDK* is controlled by the σ54 (NtrA)-dependent activator NifA (Paschen et al, 2001). Transcription of *nif* genes is dependent on NtrBC two-component system (Merrick, 2004). The global nitrogen regulatory (*ntr*) system is composed of four enzymes: a uridylyltransferase/uridylyl-removing enzyme (UTase/UR), encoded by the *glnD* gene, a small trimeric protein, PII, encoded by *glnB*, and a two-component regulatory system composed of the histidine protein kinase NtrB and the response regulator NtrC (Merrick and Edwards, 1995). ntr system responds to nitrogen status of the cell. When cells are nitrogen limited, UTase covelently modifies $P_{II}$ by addition of UMP group and the

resultant uridylylated form of $P_{II}$ promotes deadenylylation of GS by ATase. When GlnB is modified, GlnB-UMP no longer interacts with NtrB and the kinase activity predominates so that NtrC is phosphorylated and transcriptionally active (Merrick, 2004). In nitrogen excess conditions, the uridylyl-removing activity of GlnD predominates and the deuridylylated PII promotes adenylylation of GS by ATase and unmodified GlnB stimulates dephosphorylation of NtrC by NtrB because the binding of GlnB to the kinase domain of NtrB inhibits kinase activity.
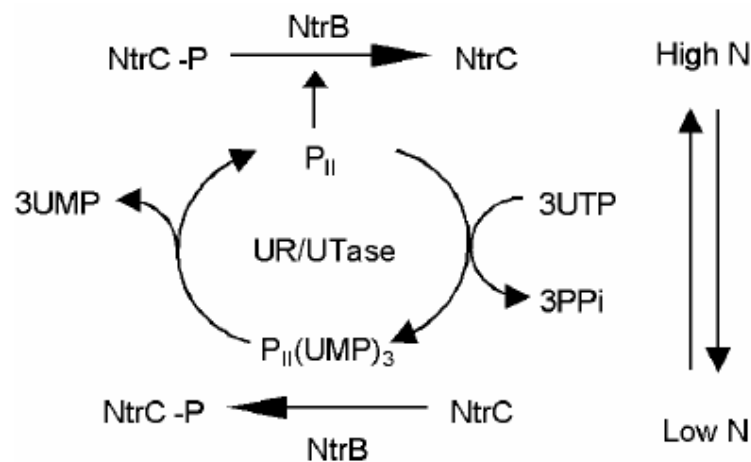


**Figure 1.6 The nitrogen regulation system (Ntr) of enteric bacteria (Merrick, 2004).**

The transcription of *nifA* is under the control of *ntrBC* gene products (Halbleib and Ludden, 2000). The *nifA* promoter provides the interface between the global and the *nif*-specific regulatory circuits and this promoter is activated by the NtrC protein under nitrogen-limiting conditions (Merrick, 2004). Both the expression and the activity of NifA can be regulated in response to the cellular nitrogen status, and the mechanism of this regulation varies according to the organism (Merrick and Edwards, 1995).

Nitrogen and carbon metabolism must invariably be kept in balance and in *R. capsulatus* and in *Rhodobacter sphaeroides* they are coordinated through the actions of the RegB-RegA two-component system. (Merrick, 2004). Two-component system homologous to RegB-RegA is PrrB-PrrA two component system in *Rhodobacter sphaeroides*. Reg/PrrA global regulator system is involved in the control of $CO_2$ assimilation, nitrogen fixation, hydrogen metabolism and energy-generation pathways (Dubbs and Tabita, 2004). This system found to be involved in regulation of nitrogen fixation in a *cbb* mutant of *R. sphaeroides* that derepresses the nitrogenase synthesis in the presence of ammonium and this derepression requires RegB (Qian and Tabita, 1996). In *R. capsulatus,* RegA acts as a coactivator, together with NtrC, of *nifA2* expression but the precise mechanism of this co-activation is not known (Elsen *et al*., 2000). Expression studies in *R. sphaeroides* confirmed that the Reg/Prr system functions as a positive regulator of *nif*HDK expression in addition to the *nif*-specific regulator NifA. The positive regulation is a direct result of PrrA binding to the *nif*HDK promoter (Dubbs and Tabita, 2004).

### 1.5.1. *nif*-Specific Control of Nitrogenase

*nif* structural genes are controlled by a *nif*-specific regulator and the expression of this protein is subjected to global nitrogen control. In the proteobacteria *nif* genes are invariably subject to transcriptional activation by NifA (a member of the enhancer-binding protein (EBP) family), together with the RNA polymerase sigma factor, $\sigma^{54}$ (Dixon and Kahn, 2004). The NifA protein, like NtrC, is a $\sigma^{N}$-dependent transcriptional activator and consequently all *nifA*-dependent promoters are characterized by the recognition site for $\sigma^{N}$ RNA polymerase and these promoters also contain binding sites for NifA (Merrick, 2004). Dixon and Kahn gave brief information about EBP as in the following:

Interaction of the EBP with the $\sigma^{54}$ RNA polymerase holoenzyme is facilitated by the binding of the activator to DNA sequences (upstream activator sequences,UAS) usually located at least 100 bp upstream of the transcription initiation site. DNA looping is required to establish productive interactions between the DNA-bound

activator and the polymerase. In some cases this is assisted by other DNA-binding proteins, such as integration host factor (IHF). Nucleotide hydrolysis by the activator promotes remodelling of the closed complex through a series of protein–protein and protein–DNA interactions that favour conversion to the open promoter complex (in which the DNA strands surrounding the transcription start site are locally denatured) (Dixon and Kahn, 2004).

The NifA proteins consist of at least three distinct domains, namely a regulatory N-terminal domain, a central ATP-binding activator domain, a DNA-binding C terminal domain (Paschen et al., 2001). N-terminal domain is a member of GAF domain family, and central domain is characteristic of all $\sigma^{54}$-dependent activators or Enhancer Binding Proteins (EBP) (Merrick, 2004). NifA protein is not a classical response regulator protein because there was no evidence that NifA is phosphorylated under any conditions. Between the central domain and the C-terminal domain is a variable region that characteristically divides the NifA proteins into two sub-families which are from γ proteobacteria i.e. *Klebsiella, Enterobacter* and *Azotobacter* and α, β proteobacteria i.e. *Azospirillum, Rhodobacter, Rhodospirillum* and *Herbaspirillum*. NifA proteins in α, β proteobacteria carry two conserved cysteine residues, in a CXXXXC motif, and these proteins are distinguished from the proteins without the motif by the oxygen sensitivity (Merrick, 2004).

Nitrogen control of oxygen sensitive NifA protein is mediated directly by $P_{II}$ protein. The photosynthetic bacteria *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* are metabolically very similar and closely related species whose in vivo nitrogenase activities are among the highest in the diazotrophic world (Madigan et al, 1984). *Rhodobacter capsulatus* possesses two genes (*glnB* and *glnK* ) encoding PII-like proteins. A mutation in *glnB* results in the constitutive expression of *nifA* and the post-traslational ammonium inhibition of NifA is completely abolished in *glnB-glnK* double mutant (Drepper et al., 2003). Neither GlnB nor GlnK is essential for the activation of NifA, but both PII-like proteins are involved in inactivation of NifA in the presence of ammonium (Pawlowski et al., 2003). Although a *glnB* mutation

results in expression of *nifA* in the presence of ammonium, NifA-mediated *nifH* gene expression is inhibited under ammonium-sufficient conditions (Drepper et al., 2003).

As a conclusion, expression of nitrogen fixation genes (*nif*) is activated by the transcriptional activator, NifA, and PII-like proteins, GlnB and GlnK, are the immediate regulators of NifA activity. Transcription of nifA gene is under the control of phosphorylated NtrC protein.

## 1.6. Factors Affecting Hydrogen Production

Several environmental factors influence the hydrogen production and so nitrogenase activity. Temperature, pH, light intensity, C/N ratio, availability of fixed nitrogen and presence of molecular oxygen are some of these factors.

Photoproduction of hydrogen is optimum at a temperature range of 30-40°C and at pH 7.0 for *Rhodobacter sphaeroides* (Sasikala et al.,1991). High C/N ratio forces the bacteria to use the excess energy and reducing power for hydrogen production (Koku et al., 2002). Nitrogenase activity is dependent on energy which is supplied from the conversion of light energy into chemical bond energy (ATP). Therefore light is absolute requirement for growth and production of hydrogen by photosynthetic bacteria (Sasikala et al., 1991). Different light intensities and alternating periods of light and dark affect the nitrogenase activity. Fixed nitrogen causes nitrogenase activity level and transcriptional level of inhibition. There are several regulatory mechanisms in response to ammonium. Oxygen treatment with low concentrations of oxygen causes the immediate, complete inhibition of nitrogenase activitywhich was fully reversible upon return to anoxic conditions (Yakunin and Hallenbeck, 2000).

Those environmental factors may affect the regulation of nitrogenase either in transcriptional or in posttranslational level.

## 1.6.1. Effect of Fixed Nitrogen on Nitrogenase Synthesis and Activity

The absence or the presence of fixed nitrogen is the factor involved in the control of nitrogenase. Three levels of regulation of nitrogenase in *R. capsulatus* were briefly reviewed by Tremblay *et al* (2007). At the transcriptional level, the NtrB/NtrC two-component system controls *nifA* transcription. In turn, NifA induces the expression of the other *nif* genes, including the Mo-nitrogenase structural genes. At the posttranslational 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 5 minutes after an ammonium shock by either a dinitrogenase reductase ADP-ribosylation-dependent or -independent mechanism. ADP-ribosylation-dependent switch-off effect is not seen in *R.sphaeroides*. While an in vivo nitrogenase switch-off response to $NH4^+$ addition has been observed in *R.sphaeroides*, no evidence of Fe-protein ADP-ribosylation was found (Yakunin et al., 2001). Figure 1.7 shows these three levels of regulation schematically.
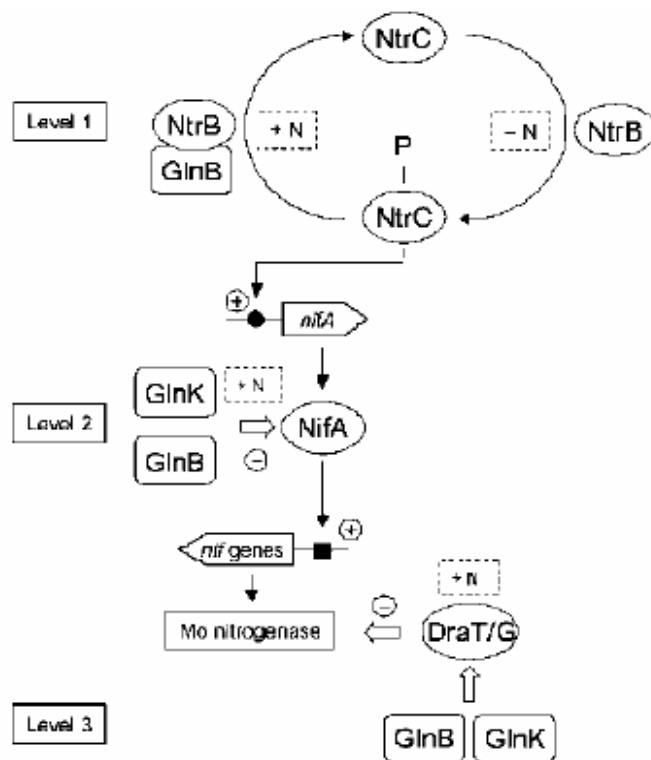


**Figure 1.7. Model of signal transduction by P$_{II}$-like proteins in *R. capsulatus* (Drepper et al., 2003).**

PII signal-transduction proteins are important for communicating the nitrogen status to various regulatory targets to control *nif* gene transcription in response to the availability of fixed nitrogen (Dixon and Kahn, 2004). 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 (Dubbs and Tabita, 2004). Under nitrogen limiting conditions, NtrB can no longer interact with GlnB and its kinase activity predominates so NtrB phosphorylates NtrC.

The second level of ammonium control is mediated again by GlnB and GlnK which regulate NifA activity. It was found that ammonium control of NifA activity is completely abolished in a *glnB–glnK* double mutant (Drepper et al., 2003). Studies on post-translational ammonium control of NifA activity showed that constitutive (NtrC-independent) expression of *nifA1* in R.capsulatus leads to a high-level accumulation of the NifA1protein in both absence and presence of ammonium, however NifA1-mediated *nifH* transcription was still inhibited by ammonium (Paschen et al., 2001).

Several diverse types of diazotrophs are capable of the short-term (<10 min), reversible modulation of their in vivo nitrogenase activity in response to various environmental stimuli, e.g., $NH_4^+$, darkness, oxygen and anaerobiosis. This type of regulation has been termed nitrogenase "switchoff/ switch-on" (Yakunin et al., 2001). Nitrogenase switch-off is mediated by a two-enzyme system, dinitrogenase reductase ADP-ribosyltransferase (DRAT) and dinitrogenase reductase-activating glycohydrolase (DRAG), which causes a covalent modification/demodification of Fe protein via ADP-ribosylation (Yakunin and Hallenbeck, 1998). In the photosynthetic bacterium *Rhodospirillum rubrum,* ADP-ribosylation appears to be the sole mechanism responsible for the in vivo switch-off of nitrogenase activity. However, *R.capsulatus* has two different switch-off responses: an ADP-ribosylation dependent and an ADP-ribosylation independent process (Yakunin and Hallenbeck, 2000). ADP-ribosylation independent response is also called magnitude response in which the quantity of added ammonium affects the magnitude of the inhibition (Yakunin and Hallenbeck, 1998). Although nitrogenase switch-off response to

ammonium addition is observed in *R. sphaeroides*, no evidence of Fe-protein ADP-ribosylation and the presence of *draTG* genes were found under a variety of growth and incubation conditions (Yakunin et al., 2001). Yakunin et al. observed that introduction of draTG had no appreciable effect on the kinetics of the switch-off/on process so ADP-ribosylation independent nitrogenase regulatory pathway which is found in *R.sphaeroides* is sufficient for nitrogenase regulation.

### 1.6.2. Effect of Light on Hydrogen Production

The energy required for nitrogenase-mediated $H_2$ evolution is provided from anoxygenic photophosphorylation under anaerobic conditions in the light. Photosynthetic apparatus converts the light energy into ATP. High demand for ATP and redox equivalents of nitrogenase, which are necessary to reduce nitrogen to ammonia, are involved in the up-regulation of photosynthetic apparatus (Kern et al., 1998). When grown anaerobically in the light or dark*, R.sphaeroides* synthesizes an intracytoplasmic membrane (ICM) system, which constitutes the photosynthetic apparatus and possesses the structural components necessary for light energy capture, subsequent electron transport, and energy transduction (Roh et al., 2004). ICM houses the integral membrane pigment-protein complexes constituting the photosystem (PS), comprised of the reaction center (RC), and two light harvesting (LH) complexes (Ryalls et al., 1998). LH complexes act as antenna to funnel photons to reaction center (RC) Bchl-protein complexes in which light energy is converted to chemical energy by photo-induced oxidation reduction reactions (Kiley and Kaplan, 1988). The cellular levels of ICM are inversely related to light intensity (Roh et al., 2004). Genes encoding the reaction center, the light-harvesting complex and proteins involved in synthesis of the corresponding pigments, are only expressed under anaerobic conditions (Kern et al., 1998). In *R.sphaeroides*, structural genes that encode components for the photosynthetic apparatus are organized into three operons denoted *puf, puh,* which encode structural proteins of the photosynthetic reaction center and the light harvesting I complex, and the *puc* operon which encodes the structural proteins of light harvesting II complex (Dubbs and Tabita, 2004). The PrrB-PrrA two-component regulatory system positively regulates the

*puf, puc* and *puh* operons in anaerobic conditions. The RegB/RegA system is not only involved in the transmission of oxygen-dependent redox signals but also in the transmission of light-dependent signals that are mediated by the electron transport chain (Happ et al., 2005).

A mutation in *hvrA,* which is known to be involved in low-light activation of the photosynthetic apparatus, released both ammonium and oxygen control of *nifH* expression, demonstrating a regulatory link of nitrogen fixation and photosynthesis via HvrA (Kern et al., 1998). Regulatory genes involved in nitrogen fixation and photosynthesis control include two-component regulatory system (*regA-regB*) responsible for oxygen regulation and two genes (*hvrA* and *hvrB)* necessary for low-light activation (Kern et al, 1998).

Light is critical environmental signal that regulate the formation of the photosynthetic apparatus. High light intensity causes a decrease in the amount of photosynthetic complexes in the phototrophically grown *Rhodobacter* cultures (Happ et al, 2005).

## Photosystem

## N₂ase

Electron pool

e⁻*

Bacterial type    PS I

Photons

e⁻*

b-f

e⁻

ADP

ATP

e⁻

Organic → H⁺+CO₂
substrates

2Fdox

2Fdred

4ATP

4ADP

MoFe    Fe

H₂    H2

NiFe

L subunit    S subunit
(60kDa)    (25kDa)

uptake H₂ase

ATP

ATP pool
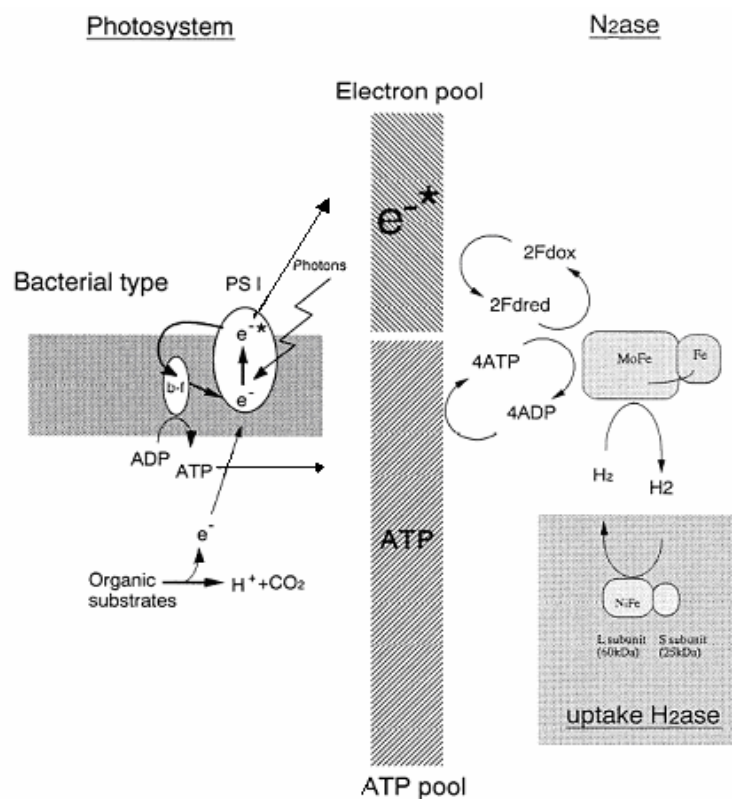
**Figure 1.8. Mechanism of light to hydrogen conversion (Miyake et al, 1999)**

The excess reducing equivalents generated by the oxidation of carbon substrates (as seen in Figure 1.8), such as malate, are consumed by the reduction of protons and by the production of hydrogen by dinitrogenase system (Tichi and Tabita, 2001). Light energy is transferred from antenna to the reaction center. Absorption of energy excites the special pair of bacteriochlorophyll a, converting it to a strong electron donor. After this strong donor is produced, the remaining steps in photosynthetic electron flow function to conserve the energy released when electrons are transported through a membrane from carriers of low reduction potential to high reduction potential. Synthesis of ATP during photosynthetic electron flow occurs as a result of the formation of a proton motive force generated by proton extrusion during electron transport and the activity of ATPases which uses the light –

generated proton gradient in the synthesis of ATP (Madigan et al, 2003). As a result, photosynthetic purple non-sulphur bacteria utilize light energy for carbon assimilation and also for nitrogen fixation.

Hydrogen production catalyzed by nitrogenase varies in direct proportion to the incident light intensity (Hillmer and Gest, 1977). Malate consumption by the organisms and derepression of nitrogenase significantly increased when illumination was increased from 3 klux up to the range of 15–20 klux (Maner and Oelze, 1999).

Light can become a growth limiting factor due to self shading and light absorption by the cells that are close to the illuminated surface at a certain biomass concentration (Barbosa et al., 2001).

### 1.6.3. Effect of Oxygen on Nitrogenase Synthesis and Activity

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* (Goldberg et al., 1987).

### 1.6.3.1. Effect of Oxygen on Synthesis of Nitrogenase

Photosynthetic purple non-sulfur bacteria contain NifA protein that carries CXXXXC motif. The activity of these proteins is oxygen sensitive. Oxygen affects not only the DNA-binding activity of NifA, but also its ability to stimulate $\sigma^{54}$-RNA polymerase to form an open complex (Bauer et al, 1999). The central/C terminal domains are responsible for oxygen control of NifA activity (Souza et al., 1999). 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 (Bauer et al, 1999). The effect of *hvrA* mutations on NifA regulation by oxygen was studied by

Kern et al. (1998) and it was found that HvrA influences the modulation of NifA activity by oxygen.
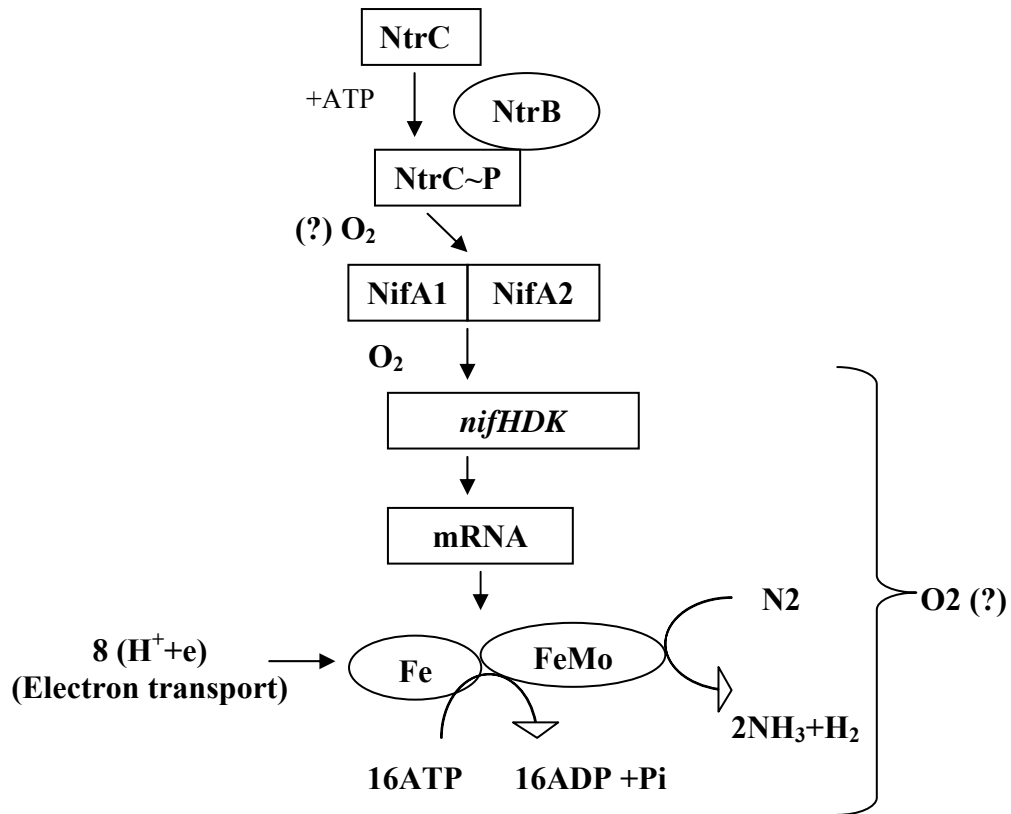


**Figure 1.9. Regulators of *nif* gene transcription and possible points of oxygen control in *Rhodobacter capsulatus* (Oelze and Klein, 1996)**

Genetic evidence has suggested that phosphorylation of *R.sphaeroides* PrrA by PrrB stimulates transcription of target genes when oxygen becomes limiting (Comolli et al, 2002). A redox signaling pathway in *R. sphaeroides* involving the *cbb*3 cytochrome *c* oxidase/Rdx protein(s) serve as the primary oxygen sensor to generate a signal inhibitory for PS gene expression. This signal is ultimately transmitted to the *prrBA* two-component activation system to regulate PS gene expression (Eraso and Kaplan, 2000).

24
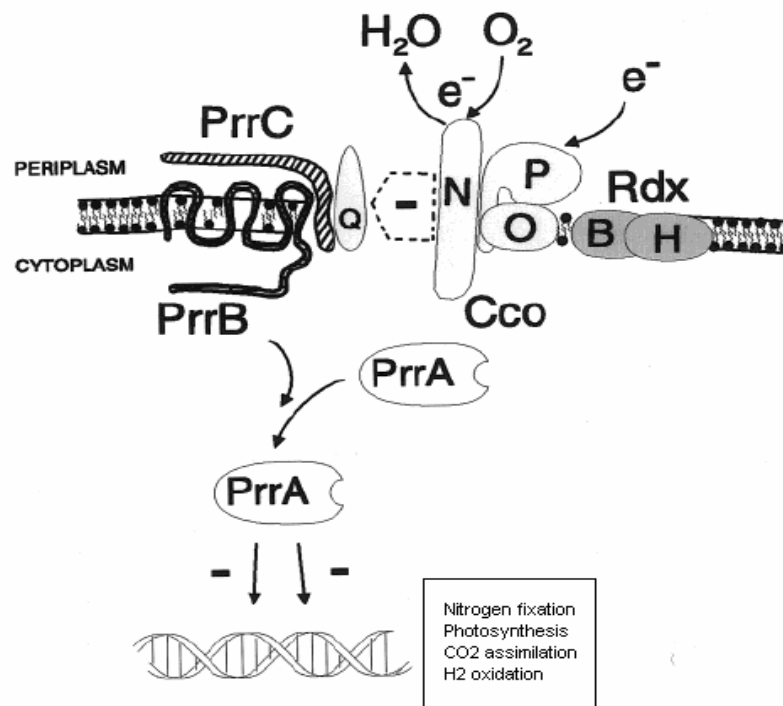
**Figure 1.10. Oxygen sensing and transduction of the inhibitory signal (Erosa and Kaplan, 2000)**

Figure 1.10 shows the sensory mechanism of oxygen and regulation of genes by oxygen. CcoQ, a component of the *cbb*3 oxidase, was shown to be involved in the transduction of the oxygen-sensing inhibitory signal from the *cbb*3 oxidase to a downstream regulatory element, proposed to be either PrrC or PrrB, a membrane-localized histidine kinase (Erosa and Kaplan, 2000). The signal coming through the *cbb*3- RdxBH sensor-signal generator will either inhibit the kinase activity of PrrB or increase its phosphatase activity or both, resulting in the lack of activation of PrrA under aerobic conditions (O'Gara et al., 1998).

The global redox-responsive PrrB-PrrA two-component system directly regulates *nif* transcription in *R.capsulatus* (Dixon and Kahn, 2004). 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. As the oxygen tension decreases, a signal which was thought to be generated by electron flow through the $cbb3$ respiratory oxidase sensed by PrrB, increases the kinase activity of PrrB to convert the pool of PrrA to phosphor-PrrA. phosphorylation of PrrA causes rapid induction of transcription of PrrA-dependent target genes (Comolli et al., 2002).

The $cbb3$-RdxBH oxygen sensor-signal generator also plays a role in the expression of uptake hydrogense, $CO_2$ fixation and nitrogen fixation pathways because of the role of PrrA in those pathways (O'Gara et al, 1998).

## 1.6.3.2. Oxygen Effect on Nitrogenase Activity

In *R. capsulatus,* low concentrations of oxygen causes rapid, complete inhibition of nitrogenase activity that is reversible upon return to anoxic condition (Hochman and Burris, 1981). Molecular oxygen caused a rapid inhibition of light-driven nitrogenase activity in highly nitrogen limited cultures of *R. capsulatus* grown anaerobically under

photoheterotrophic conditions with a full inhibition in the presence of 1% $O_2$ (Yakunin and Hallenbeck, 2000). The reason for this switch-off effect is not ADP-ribosylation of FE-protein. Even several hours of incubation of cells with 20% O2 in the light did not result in Fe-protein modification (Yakunin and Hallenbeck, 2000). Nitrogenase can tolerate oxygen concentrations lower than 4.6μM. 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 (Oelze and Klein, 1996).

## 1.6.4. Substrates for Hydrogen Production

While *Rhodobacter sphaeroides* can use variety of substrates for growth, only a few of them are suitable for hydrogen production (Koku et al., 2002). Hydrogen production performance of substrates depends on (Koku, 2001):

1. The activity of TCA cycle because the organic acids are assimilated by TCA cycle
2. The oxidation state of the substrate
3. The pathways that are alternative to hydrogen production, such as PHB synthesis

Organic acids such as malate and lactate can be converted to hydrogen by photosynthetic bacteria more efficiently. However hydrogen production capacity of sugars such as glucose and sucrose is not high. Sasikala et al. observed the highest hydrogen production with malate at concentration of 30mM (1991). L-malate is metabolized by TCA cycle and D-malate is converted to pyruvate. Pyruvate is also a substrate for hydrogen production. Cells grown photoheterotrophically on $C_4$ dicarboxylic acids with glutamate as N source evolve $H_2$ from the $C_4$ acids (fumarate, succinate, malate) and also from lactate and pyruvate (Hillmer and Gest, 1977).

In most bacteria acetate is assimilated by TCA cycle. *R. sphaeroides* can grow photoheterotrophically with acetate as sole carbon and energy source (Filatova et al., 2005). However, growth on acetate as the sole source of carbon is impossible if C4 acids drained from the TCA cycle for biosynthetic reactions are not replenished. The replenishment of $C_4$ acids is carried out by glyoxylate cycle with isocitrate lyase as the key enzyme (Ivanovsky et al., 1997). However *R.sphaeroides* and *R.rubrum* lack the isocitrate activity and the corresponding gene. *R. rubrum* and *R. sphaeroides* use citramalate cycle for acetate assimilation (Ivanovsky et al., 1997; Filatova et al., 2005). The three parts of this pathway is defined by Alber et al. (2006). In the first part pyruvate and acetyl-CoA are converted via citramalate to propionyl-CoA and glyoxylate. In a second part propionyl-CoA is carboxylated and converted conventionally to succinate. In a third part glyoxylate condenses with another molecule of acetyl-CoA to malate, from which the starting molecule pyruvate is reformed.

When *R.sphaeroides* uses the spent media of *Enterobacter cloacae* strain DM11, the yield of hydrogen is about 1.5–1.72 mol $H_2$/mol acetic acid. In photo-fermentation, acetic acid is presumably the sole substrate for hydrogen production (Nath et al., 2005). In *Rhodopseudomonas* sp., the highest hydrogen yield was obtained in acetate containing medium among the mediums containing other carbon sources, such as lactate, malate and butyrate (Barbosa et al., 2001).

## 1.7. Hyvolution Project

Hyvolution is a European Union 6th framework integrated Project with a full title "non-thermal production of hydrogen from biomass". The main scientific objective of the project is to produce pure hydrogen from variety of biomass feedstocks by two-stage bioprocess. The first step of bioprocess contains thermophilic fermentation of feedstock to hydrogen, $CO_2$ and intermediates. In the second step which is photo-heterotrophic fermentation, all intermediates will be converted to more hydrogen and $CO_2$. On the other hand, the main technological objective of this project is to construct prototype modules of the plant which, when assembled, form the basis of a blue print for the whole chain for converting biomass to pure hydrogen (Biohydrogen, 2008, pp. 6-7). The main aim of our work package is to optimize photofermentative hydrogen production from organic acids with high yields and to construct a prototype photobioreactor. The organic acids produced by dark fermenter bacteria is generally acetic acid. Ammonium chloride is used as nitrogen source of dark dark fermenter. Therefore it is important to make investigations on hydrogen production and growth of bacteria in ammonium chloride and acetate containing media.

## 1.8. Aim of the study

In this study, hydrogen production and expression levels of *nifH*, *nifA*, and *prrA* genes were investigated at different conditions. The effect of ammonium chloride on hydrogen production and the expression levels of genes of interest are examined under anaerobic and aerobic conditions. Ammonium chloride effect on hydrogen

production is important because when two-step process of dark and photofermentation is used to produce higher yield of hydrogen, dark fermentor effluent may contain NH$_4$Cl in some cases. The expression levels of *nif* genes and *prrA* gene give information about the transcriptional level regulation of nitrogenase in response to ammonium. The changes in expression levels will be determined by reverse-transcriptase PCR and real-time PCR.

Both the function and expression of nitrogenase enzyme that catalyzes hydrogen production in *Rhodobacter sphaeroides*, are strictly controlled by oxygen. Therefore hydrogen production and the expression levels of *nif* genes are examined. Light intensity is another factor that affects the efficiency of hydrogen production. Different light intensities and dark conditions are applied to different culture media which contain acetate or malate as carbon source and NH$_4$Cl or glutamate as nitrogen source.

Relationship between genes that are effective in nitrogenase synthesis and regulation should be understood well to increase the yield of hydrogen. If transcriptional level of control in response to external stimuli is determined, further genetic studies and manipulations can be done to improve hydrogen production capacity.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. Materials

*Rhodobacter sphaeroides* O. U. 001 (DSM 5864) was obtained from Deutsche Sammlung von Mikrooganismen und Zellkulturen GmbH, Germany as freeze-dried culture. This strain was used in hydrogen production and expression analysis. All chemicals and their suppliers were listed in Appendix A.

## 2.2. Methods

### 2.2.1. Growth Conditions and Media

*Rhodobacter sphaeroides* O. U. 001 was grown in Biebl and Pfenning minimal medium (Appendix B) which did not involve ammonium chloride and yeast extract. 15mM malate was used as carbon source and 2mM glutamate was used as nitrogen source with the supplementation of vitamin and trace element solutions (Appendix B). 5ml bacteria was inoculated into 45ml anaerobic, sterile, liquid growth medium to make 10% (v/v) . Anaerobic cultures of *R. sphaeroides* were grown in 50ml penicillin bottles at 30-32 °C, under illumination that is provided by 100 watt tungsten lamp from a distance of 15-20cm. Pure argon gas (99.995 % purity) was flushed into the bottles to obtain anaerobic atmosphere. The time of inoculation was considered as zero-time and samples were taken for determination of growth at 24 hours intervals. Bacterium was kept at -80°C for storage in 30% glycerol.

## 2.2.1.1. Growth Media Containing Different Concentrations of Ammonium Chloride

Concentrations of nitrogen source and carbon source used in growth media were shown in Table 2.1.

**Table 2.1. Different concentrations of nitrogen source in 15mM malate containing media.**

|  | Concentration of nitrogen source |
|---|---|
| 1 | 1mM NH$_4$Cl |
| 2 | 2mM NH$_4$Cl |
| 3 | 3mM NH$_4$Cl |
| 4 | 5mM NH$_4$Cl |
| 5 | 10mM NH$_4$Cl |
| 6 (control) | 2mM glutamate |

## 2.2.1.2. Growth Media Containing Acetate

Different concentrations of nitrogen source used in acetate containing media were given in Table 2.2

**Table 2.2. Different concentrations of nitrogen source in acetate containing media**.

|  | Nitrogen source | Carbon source |
|---|---|---|
| 1 | 1mM glutamate | 30mM acetate |
| 2 | 2mM glutamate | 30mM acetate |
| 3 | 3mM glutamate | 30mM acetate |
| 4 | 5mM glutamate | 30mM acetate |
| 5 (control) | 2mM glutamate | 15mM malate |

### 2.2.1.3. Aerobic Growth

Penicillin bottles were used as bioreactor for the growth of bacteria. Aerobic growth was performed by filling nearly 65% of the bottles with media given in Table 2.1. *R. sphaeroides* used the air left on the top of penicilin bottles for aerobic growth.

### 2.2.1.4. Growth under Different Light Intensity

*R. sphaeroides* was grown in media containing different concentrations of carbon and nitrogen (Table 2.3) under dark, and light intensities of 3500 and 6500 lux. Both aerobic and anaerobic growth at the same light conditions were examined.

**Table 2.3. Media compositions for light experiments**

|  | Nitrogen source | Carbon source |
|---|---|---|
| 1 | 2mM glutamate | 15mM malate |
| 2 | 2mM $NH_4Cl$ | 15mM malate |
| 3 | 2mM glutamate | 30mM acetate |

### 2.2.1.5. Growth Curves

As the bacterial growth occurs, samples were taken at time intervals and the optical density was measured at 660 nm by spectrophotometer (Shimadzu UV-1208). Time of inoculation was considered as zero in the growth curve. pH of the samples taken were also measured with pH meter (Inolab WTW series). Graph of dry cell weight versus $OD_{660}$ developed by Harun Koku was used to transform the absorbance to the dry cell weight.

### 2.2.2. Hydrogen Production

Hydrogen gas produced was measured by the set up shown in Figure 2.1 (Uyar *et al.*,2003) for the all of the conditions that was described above. Hydrogen gas was collected and measured volumetrically by graduated-glass burettes by the replacement of water. The purity of collected gas was measured by gas chromatography (Agilent 6890N). Gas samples withdrawn were analyzed with Carboxen 1010 column by Supelco. The device was Agilent Technologies 6890N with thermal conductivity detector. A sample gas analysis was given in Appendix E. Concentrations of hydrogen and carbondioxide were determined by peak area analysis. According to gas chromatography results, the amount of hydrogen in the total gas collected was around 96-99 % (v/v).
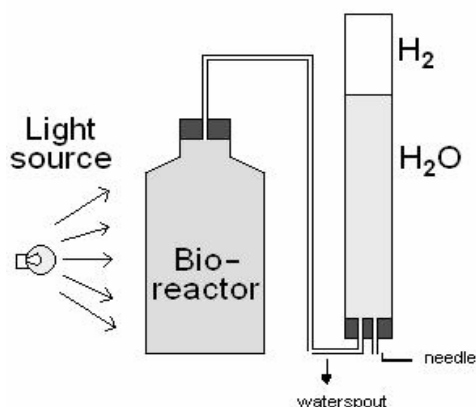


**Figure 2.1. Experimental set up for hydrogen production**

### 2.2.3. Total RNA Isolation

### 2.2.3.1. Diethyl Pyrocarbonate (DEPC) Treatment

All glassware and plastics were treated by % 0.1 DEPC solution overnight for RNA isolation. All of the materials treated with DEPC were autoclaved and dried in oven. Solutions used during the procedure were prepared with DEPC treated water.

### 2.2.3.2. Rapid Isolation of RNA from Gram-Negative Bacteria

Rapid isolation of RNA protocol was performed according to the protocol defined by Reddy *et al* (Ausubel et al., 1995). Bacterial cells from 10 ml gram negative culture grown up for 36 hours was centrifuged at 12,000xg for 10 min at 4°C in centrifuge (Thermo IEC). Then the pellet was resuspended in 10 ml of protoplasting buffer (Appendix B). 80μl of 50 mg/ml lysozyme was added before incubation on ice for 15 minutes. Protoplasts were centrifuged at 5900xg for 5 min at 4°C. The pellet was resuspended in 0.5 ml gram negative lysing buffer (Appendix B) and 15 μl of DEPC was added, mixed and transferred into microcentrifuge tube. The mixture was incubated at 37°C for 5 min and then chilled on ice. 250μl saturated NaCl (Appendix B) was added, mixed and incubated on ice for 10 min. The mixture was centrifuged for at 13,000 rpm for 10 min at room temperature. The supernatant was removed into two clean centrifuge tubes. 1 ml ice cold 100% ethanol was added to each tube, and precipitated overnight at -20°C. After centrifugation at 13,000 rpm for 15 min at 4°C, the pellet was rinsed with 500 μl ice cold 70% ethanol by centrifuge at 13,000 rpm for 10 min at 4°C. The pellet was dried and dissolved in 100μl DEPC treated water.

### 2.2.3.3. Isolation of Total RNA from bacteria by TRI REAGENT ™

RNA isolation was performed according to the procedure provided by manufacturer. Cells from 10-15 ml *R.sphaeroides* culture that was grown for 36 hours were centrifuged for 3 min at 12,000xg, at 4°C. Then 0.1g of precipitated bacterial cells

were resuspended in 80 µl TE containing 5mg/ml lysozyme and incubated for 15 minutes at 37°C. 1ml TRI REAGENT <sup>TM</sup> was added to the tubes to lyse the bacterial cells and allow samples to stand for 5 min at room temperature. 0.2 ml of chloroform per ml of TRI REAGENT <sup>TM</sup> used was added and the sample was shaken vigorously for 15 seconds. After incubation for 15 min at room temperature, the resulting mixture was centrifuged at 12,000xg for 15 min at 4°C centrifugation separated the mixture into 3 phases. The bottom phase was a red organic phase containing protein, the interphase was containing DNA, and the upper phase was aqueous colorless phase containing RNA.  The aqueous phase was transferred into a fresh tube and 0.5 ml of isopropanol per ml of TRI REAGENT <sup>TM</sup> was added. After 10 min incubation at room temperature, the sample was centrifuged at 12,000xg for 10 min at 4°C. the RNA precipitate formed a pellet on the side and the bottom of the tube. The supernatant was removed and the RNA pellet was washed by adding 1 ml of 75 % ethanol. The sample was centrifuged at 12,000xg for 5 min at 4°C. The supernatant was poured, the pellet was air dried and dissolved in 30µl water. To facilitate dissolution, mix by repeated pipetting at 55-60°C for 10 min.

### 2.2.3.4. Spectrophotometric Analysis of RNA

5µl of isolated RNA was diluted to 1000µl by adding 995µl DEPC-treated water in a quartz cuvette. The absorbance at 260 nm and 280 nm were measured (Shimadzu UV-1208 spectrophotometer) using DEPC-treated water as a blank.

The purity of RNA samples were determined by the ratio of $A_{260}$ to $A_{280}$. The ratio should be around 1.8-2.0 for sufficiently pure RNA.

The concentration of RNA was determined by the equation 2.1:

$$\text{RNA concentration} = A_{260} \times 40 \ \mu g \ /ml \times \text{Dilution factor} \qquad (2.1)$$

## 2.2.3.5. DNase Treatment

DNase treatment was performed according to the protocol given by DNA-free™ kit from Ambion. 10µg of isolated RNA was used for 50µl reaction mixture. 0.1 volume of 10X DNase I buffer and 1µl rDNase I were added to the RNA and it was mixed gently. The mixture was incubated at 37 ºC for 20-30 min. 5µl resuspended DNase Inactivation reagent was added and mixed well. The mixture was incubated 2 min at room temperature by mixing occasionally. It was centrifuged at 10,000xg for 1.5 min and the RNA transferred to a fresh tube.

## 2.2.4. Reverse Transcriptase – Polymerase Chain Reaction (RT-PCR)

### 2.2.4.1. Complementary DNA (cDNA) Synthesis

DNase treated 1µg 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 11µl with DEPC-treated water. The mixture was incubated at 70ºC for 5 min and then, chilled on ice. 4µl of 5X reaction buffer, 2µl of 10 mM 4 dNTP mix (1.0 mM-final concentration) and DEPC-treated water were added. The mixture was incubated at 37ºC for 5 min. 60 units (0.3µl) of RevertAid ™ M-MuLV Reverse Transcriptase was added and the reaction mixture was incubated at 42ºC for 60 min. The reaction was stopped by heating at 70ºC for 5 min and chilled on ice.

Introns do not exist in prokaryotic genomes so their mRNA sequences are the same with their DNA sequence. False positive controls are necessary to distinguish whether amplification product is originated from mRNA (in other words cDNA) or contaminated DNA. All cDNA samples were synthesized with their false positive controls. Reverse transcriptase was not added to the samples which were false positive controls. No band formation was expected on the gel in the samples without RT enzyme.

**2.2.4.2. Polymerase Chain Reaction (PCR)**

**2.2.4.2.1. Primer Design**

Contamination of RNA with chromosomal DNA is often encountered in RT-PCR on prokaryotic RNA and contaminated DNA generates false-positive products. (Sybesma *et al*., 2001). Primers that generate 5'-tagged cDNA during RT that is used as specific 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 (Cobley et al., 2002). No-DNA-derived product is amplified during PCR at relatively high annealing temperature because the sequence of 5' tag is not present in chromosomal DNA.
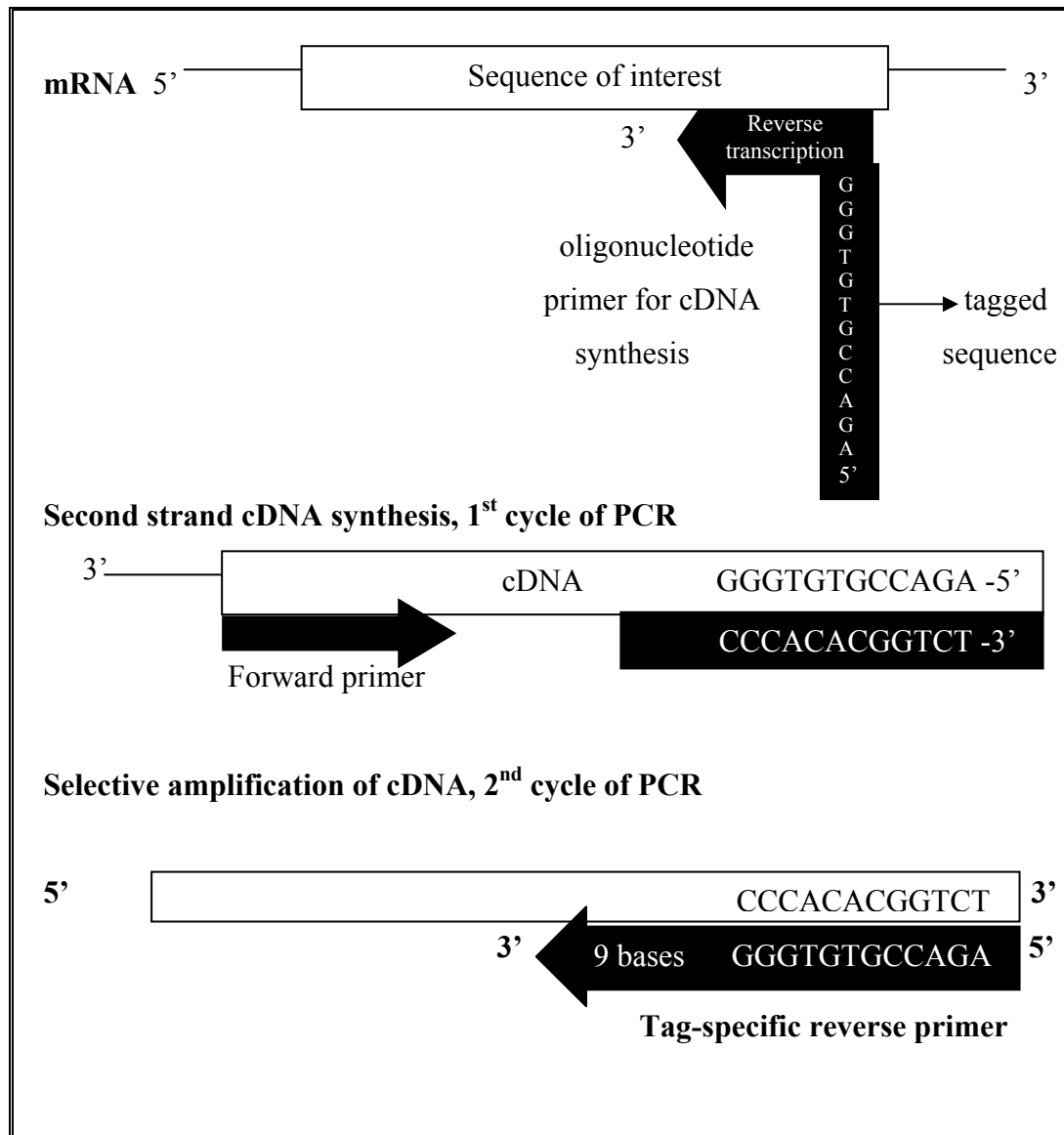
**Figure 2.2. Principle of RT-PCR with tagged primer for cDNA synthesis and tag-specific primer for amplification of cDNA.**

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 is constitutively expressed so it was used as internal control.

Sequence of *nif*H gene that expresses nitrogenase iron protein and the sequence of *nif*A that expresses NifA transcriptional activator were obtained from Integrated Microbial Genomes (IMG). The sequences were for another strain, *Rhodobacter sphaeroides* 2.4.1 . The sequence of *prr*A gene coding for response regulator and the sequence of 16S rRNA of *Rhodobacter sphaeroides* 2.4.1 were taken from the NCBI Gnome Database. Then, the GeneFisher software tool, provided by Bielefeld University Bioinformatics Server, was used to design degenerate primers. Self-dimer and heterodimer formations were tested for the reverse and forward primers by using Integrated DNA Technologies.  Appropriate primers were searched on BLAST to check whether they are specific for *R. sphaeroides* or not. The final pairs of primers were synthesized by Integrated DNA Technologies (IDT) and Iontek (İstanbul). The sequences of primers were given in Table 2.4.

**Table 2.4. Sequence of primers for RT-PCR amplifications**

| | | |
|---|---|---|
| ***nif*H** | **cDNA primer** | 5'-AGACCGTGTGGGGCGTGCTGCACGATATT-3' |
| | **Forward primer** | 5'-AGACCGTGTGGGGCGTGCTGC-3' |
| | **Reverse primer** | 5'-GGCGCCTATGACGACGTCGAC-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' |

### 2.2.4.2.2. PCR Conditions and Compositions

PCR reactions were prepared in 50μl aliquots and reaction was performed by Thermal Cycler (Apollo™ ATC 401 Thermal Cycler and Thermo Thermal Cycler). PCR conditions and compositions were given in the Table 2.5.

**Table 2.5. PCR conditions and compositions**

| PCR Ingredients | Final Concentrations | PCR Amplification Program | |
|---|---|---|---|
| ReactionBuffer | 1X | Initial denaturation at 94°C for 6 minutes | |
| MgCl2 | 1.5mM | | |
| dNTP mix | 0.2mM of each | Denaturation at 94°C for 30 seconds | 30 cycle |
| Forward Primer | 0.2μM | Annealing at 60°C-65°C for 30 seconds | |
| Reverse Primer | 0.2μM | Extension at 72°C for 30 seconds | |
| cDNA | 100-300ng/50μl | | |
| Taq DNA polymerase | 1.25 units/50μl | Final Extension at 72°C for 10 minutes Final hold at 4°C | |
| Pyrogen free sterile water | ___ | | |

## 2.2.5. Agarose Gel Electrophoresis of PCR Products

Agarose gel electrophoresis was performed according to the procedure in Short Protocols in Molecular Biology (Ausubel, F. M. et.al., 1990). Agarose gel was prepared by boiling the 2 g of agarose powder in 100 ml TAE Buffer (Appendix B) to have 2% (w/v) concentration for PCR products. On the other hand, RNA samples were visualized by 1% (w/v) concentration of gel. The homogenous, molten agarose is cooled to 50-60°C before pouring onto tray. Gels were stained by adding Ethidium Bromide (EtBr) (Appendix B) after cooling. Samples mixed with loading dye were loaded, after the gel was completely set. The gel was run at 90 V for one hour. DNA bands were visualized on a software UV transilluminator and photographed by Vilber Lourmat Gel Imaging System.

## 2.2.6. Densitometric Analysis

The gel photographs were processed with ImageJ software (National Institutes of Health, Maryland, USA) to determine band intensities. Relative expression levels (REL) of genes were calculated by formula given:

$$REL = \frac{(S_{GOI}/S_{IC})}{(CM_{GOI}/CM_{IC})} \tag{2.2}$$

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

## 2.2.7. Real Time PCR

Real time PCR was performed according to the procedure given by LightCycler® TaqMan® Master from Roche in METU Central Laboratory. Hydrolysis (TaqMan) probes were used to detect and evaluate PCR products fluorimetrically. Probe contains both fluorescence reporter and fluorescence quencher. When probe is intact the quencher dye is close enough to suppress reporter dye. During PCR, 5' nuclease activity of polymerase cleaves probe, seperates the reporter and quencher, permitting the reporter dye to emit fluorescence. One reaction was prepared by adding the following components in a 1.5 ml tube. 7µl of PCR grade water, 2µl of Primer/probe mixture (0.5µM of forward primer, 0.5µM of reverse primer and 0.2µM of probe) and 4µl of master mix. After pipetting 15µl PCR mix into precooled LightCycler® Capillaries, 7µl of the DNA template was added. Capillary was sealed with a stopper and centrifuged at 700xg for 5 seconds. Then the capillary was transferred to the sample carousel of the LightCycler® Instrument. The sample was pre-incubated at 95ºC for 15 min and cycled in amplification step by denaturation at 95ºC for 10 seconds, annealing at 60ºC for 30 seconds and extension at 72ºC for one second for 45 cycle. The sample as cooled at 40ºC for 30 seconds.

## 2.2.8. Statistical Analysis

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

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1. Growth and Hydrogen Production of *Rhodobacter sphaeroides*

### 3.1.1. Effect of Ammonium Ion on Growth of *Rhodobacter sphaeroides* O.U.001

Growth media containing different concentrations of ammonium were used to examine anaerobic growth of *R. sphaeroides* O.U.001 (Table 2.1). 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 affects the growth and hydrogen production in photosynthetic bacteria. Ammonium ions may be involved in effluent because large amounts of ammonium is used as nitrogen source in dark fermentation, therefore determining growth and hydrogen production limiting concentrations of ammonium chloride is important. The growth was observed by measuring the absorbance at 660 nm at time intervals. The absorbance values were converted to dry cell weight where 1 OD at 660nm corresponds to 0.59 g dry cell weight /l of medium (Koku H, 2001, p. 157).

Nitrogen concentration has an important role in growth because after carbon, the most abundant element in the cell is nitrogen. Therefore, as ammonium chloride concentration increased, the dry cell weight also increased under anaerobic conditions (Figure 3.1). Medium containing 15mM malate as carbon source and

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2mM glutamate as nitrogen source was used as control. The growth in 15/2 malate/glutamate containing medium and 15/10 malate/NH$_4$Cl containing medium was approximately the same. Glutamate (C$_5$H$_9$NO$_4$) which is used as nitrogen source has also considerable amount of carbon which may result in high growth. The difference in growth at different concentrations (2mM to10mM) of NH$_4$Cl containing medium was not very significant. However growth in 1mM NH$_4$Cl medium was 2 fold less than growth in 10mM NH$_4$Cl medium.
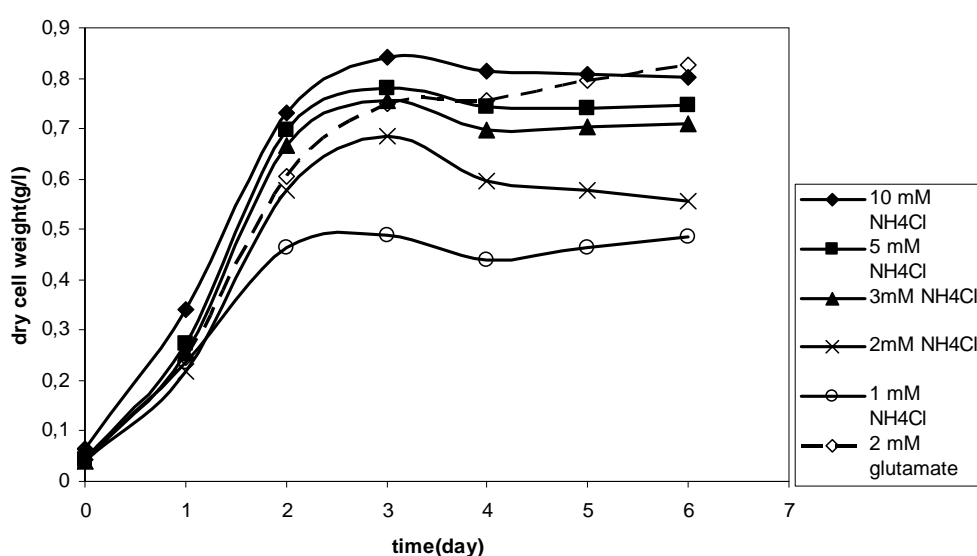


**Figure 3.1.Growth of *R. sphaeroides* O.U.001 in 15mM malate and different concentrations of ammonium chloride containing medium**

### 3.1.1.1. pH Changes during Growth of *R.sphaeroides* in Ammonium Chloride Containing Media

*R. sphaeroides* O.U.001 can grow in pH range of 6.0 to 9.0 and optimal growth was obtained at pH 6.8-7.0 (Sasikala et al., 1991). Figure 3.2 shows the pH changes during the growth of *R.sphaeroides* in different ammonium chloride containing media. As it is seen from the figure, pH was within the limits of optimum pH range needed for the growth of bacteria.

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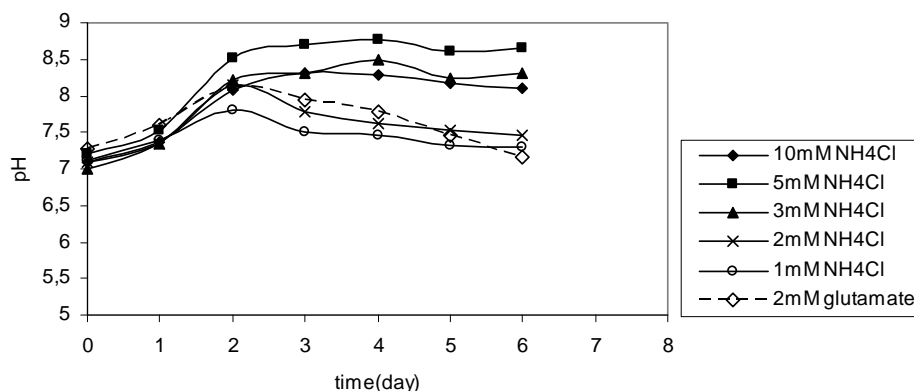
**Figure 3.2. pH change during anaerobic growth of *R.sphaeroides* O.U.001 in 15mM malate and different concentrations of NH₄Cl containing media**

## 3.1.2. Effect of Aerobic Conditions on Growth of *Rhodobacter sphaeroides* O.U.001

*R. sphaeroides* have flexible metabolic capabilities such as photoheterotrophy, aerobic or anaerobic respiration, and fermentation. Different sets of growth media containing different concentrations of ammonium chloride (in Table 2.1) were used to examine how aerobic conditions affect the growth of bacteria. As seen in Figure 3.3, the highest growth was observed in BP medium containing 15mM malate, 2mM glutamate. Aerobic growth in all ammonium chloride containing media had approximately same pattern and lower than the growth in BP medium which was used as control medium. Bacteria reached 0.5 g/l culture biomass value within 48 hours in 5mM NH₄Cl containing aerobic conditions. However it reached 0.7 g/l culture biomass value within the same time in 5mM NH₄Cl containing medium under anaerobic conditions. The growth was faster in anaerobic conditions than aerobic conditions in ammonium chloride containing media.
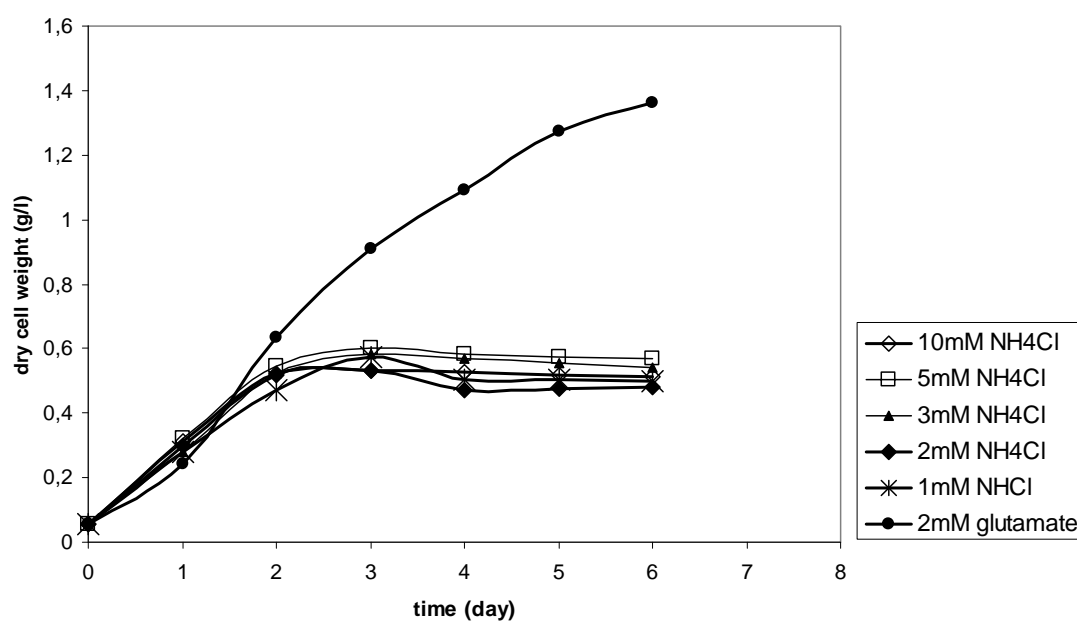
**Figure 3.3. Growth of *R. sphaeroides* O.U.001 in different concentrations of ammonium chloride containing medium under aerobic conditions**

When the growth profiles under aerobic and anaerobic conditions were compared, higher growth was observed in anaerobic conditions in all media except in 1mM and BP media (Figure 3.4). Ammonium and oxygen may have some toxic effect together. Bacteria could grow better in BP medium which does not contain ammonium.
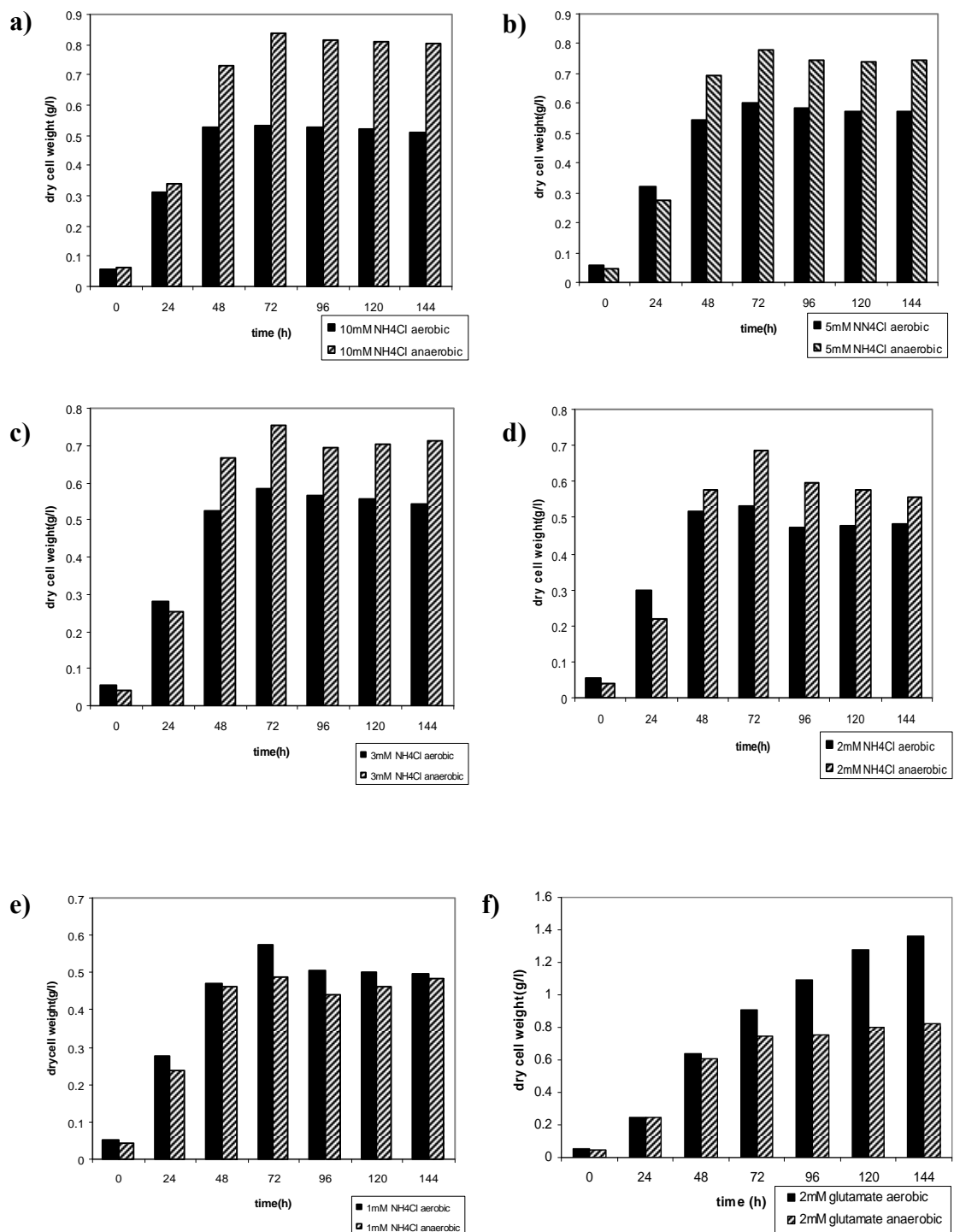
**Figure 3.4. Comparison of growth of *R. sphaeroides* O.U.001 under aerobic and anaerobic conditions in a) 10mM b) 5mM c) 3mM d) 2mM e) 1mM ammonium chloride and f) 2mM glutamate containing media.**

**3.1.2.1. pH changes during growth in ammonium chloride containing media under aerobic conditions**

As bacterial growth occurs, the pH of the culture medium was increased. The highest pH value was observed in 2mM glutamate containing medium where high degree of growth was obtained (Figure 3.3 and Figure 3.5). The media containing ammonium chloride had the same pH profiles and aerobic growth rates. The pH values were within the limits for normal growth.
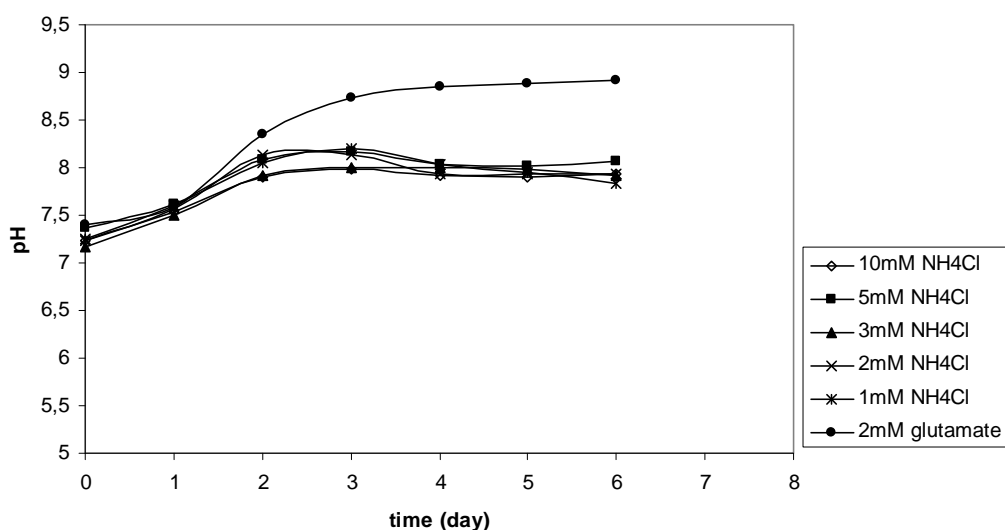


**Figure 3.5. pH change during the aerobic growth of *R.sphaeroides* in 15mM malate and different concentrations of NH$_4$Cl containing media**

**3.1.3. Growth of *R. sphaeroides* O.U.001 in acetate containing media at different glutamate concentrations**

*R.sphaeroides* can utilize a wide variety of substrates as carbon and nitrogen sources. This bacterium was found to be able to grow photoheterotrophically in the medium in which acetate was the sole source of carbon and energy (Filatova et al., 2005). Combined dark and photofermentation achieves higher yields of hydrogen by complete utilization of chemical energy stored in substrate (Nath et al., 2005). The

spent medium from dark fermentation contains unconverted metabolites, mainly acetic acid. The acetic acid is used by photosynthetic bacteria for producing hydrogen. For this reason growth of *R.sphaeroides* was tested by changing the carbon source from malate to acetate. Different concentrations of glutamate were used as nitrogen source (Table 2.2). Glutamate was chosen as nitrogen source because stable growth can not be determined by using ammonium chloride. The growth rate of bacteria increased with the increase in glutamate concentration under anaerobic photosynthetic conditions (Figure 3.6). The lowest growth was seen in BP (control) medium. Although malate has 4 carbons and acetate has 2 carbons, higher growth was observed in acetate containing medium. When 30mM malate/ 2mM glutamate containing medium was compared with BP, maximum biomass in acetate containing medium was twice as high as maximum biomass in BP medium. This may be the result of higher substrate conversion efficiency in acetate/glutamate containing medium.
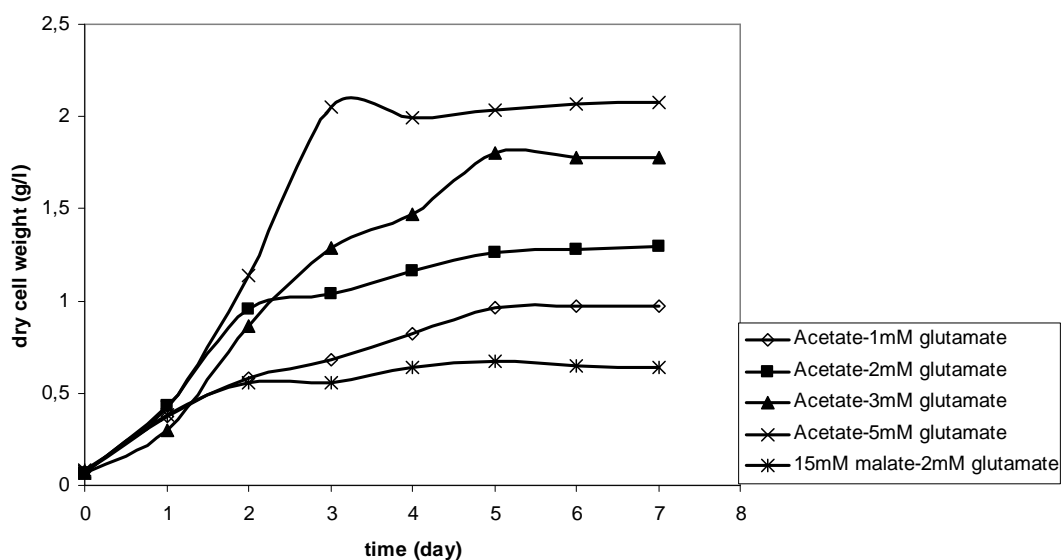


**Figure 3.6. Anaerobic growth of *R. sphaeroides* O.U.001 in 30mM acetate and different concentrations of glutamate containing medium.**

### 3.1.3.1. pH Changes in Acetate and Different Glutamate Concentrations Containing Media

The growth profile and pH change had almost the same pattern (Figure 3.6 and Figure 3.7). The highest pH value belongs to the medium that has highest growth and the lowest one belongs to the medium that has the lowest growth. Therefore the highest pH value was seen in 30mM acetate/5mM glutamate medium. Although pH rose up to 10 in this medium, the growth continued.
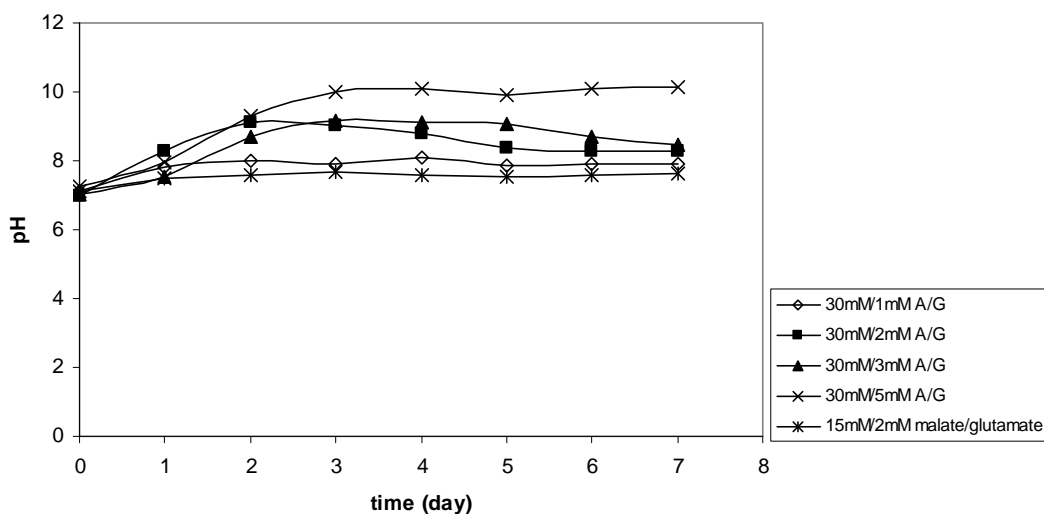


**Figure 3.7. pH change during the anaerobic growth of *R.sphaeroides* in 30mM acetate and different concentrations of glutamate containing media**

### 3.1.4. The Effect of Different Light Intensities on Anaerobic and Aerobic Growth of *R. sphaeroides* O.U.001

The energy required for growth, as well as for the hydrogen production activity of nitrogenase is provided by the photosynthetic apparatus, which converts light energy into chemical energy, ATP (Koku et al., 2002). Therefore continuity and the intensity of light are important for the growth of photosynthetic *R.sphaeroides*. The effect of different light intensities on growth was examined in BP medium, 15mM

malate and 1mM NH$_4$Cl containing medium, and 30mM acetate and 2mM glutamate containing medium under both aerobic and anaerobic conditions (Table 2.3). When the biomass yield in different light intensities under aerobic conditions was compared, the highest growth was seen at the light intensity of 6500 lux in BP medium (Figure 3.8). However biomass yield at 3500-4000 lux reached as high biomass yield as 6500lux at the end in aerobic conditions. A light intensity more than 3000 lux causes saturation in biomass yield (Sasikala et al., 1991)

As the intensity decreased, the biomass yield also decreased. However the highest growth under anaerobic conditions was observed at 3500 lux. Continuous high light intensity exposure may cause the limitation in biomass yield at 6500 lux. As seen in Figure 3.8, the biomass yield was higher under aerobic mode than anaerobic mode in all light conditions when glutamate is nitrogen and malate is carbon source.
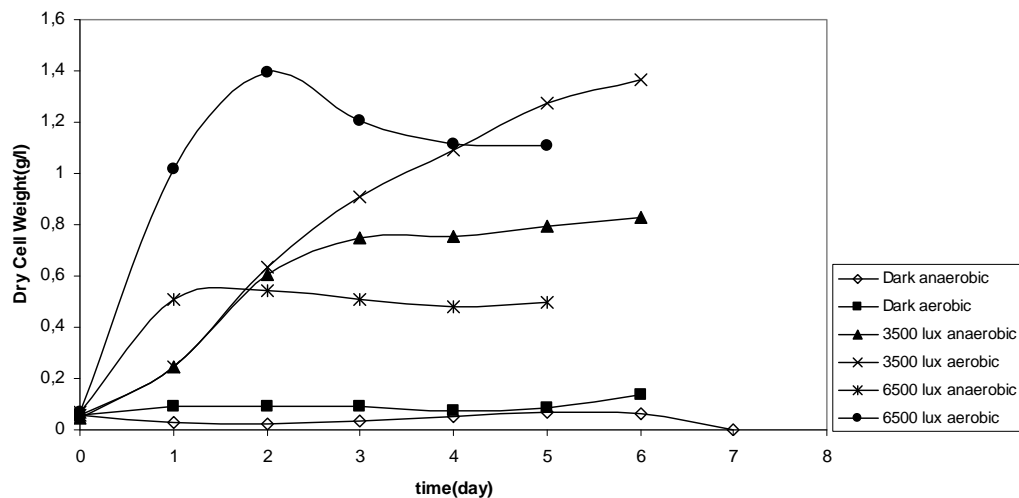


**Figure 3.8. Anaerobic and aerobic growth of *R. sphaeroides* O.U.001 in 15mM malate-2mM glutamate containing medium under dark and light intensities of 3500 lux and 6500 lux.**

Aerobic and anaerobic growth in 1mM NH$_4$Cl containing medium as nitrogen source was given in Figure 3.9 by changing light intensities. Biomass yield increased with the increase in light intensity under anaerobic conditions in contrast to BP medium. The highest growth was observed at 6500 lux anaerobic conditions. Under aerobic conditions, growth was approximately the same at 3500 lux and dark conditions. However the maximum aerobic biomass at 6500 lux was 2.5 times higher than the biomass at 3500 lux aerobic conditions.
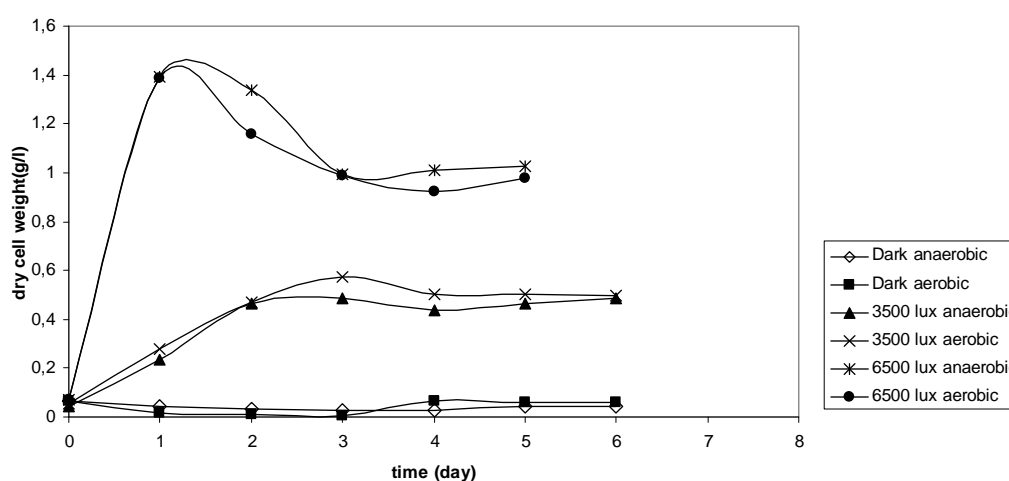


**Figure 3.9. Anaerobic and aerobic growth of *R. sphaeroides* O.U.001 in 1mM NH$_4$Cl containing BP medium under dark and light intensities of 3500 and 6500 lux.**

The growth was compared in 30mM acetate and 2mM glutamate containing medium under different light intensities (Figure 3.10). The growth pattern in this medium was like BP medium. Under aerobic conditions, growth was increased as the light intensity increased. However under anaerobic conditions, the highest growth was observed at 3500 lux intensity, not at 6500 lux. Light intensity higher than the optimum may saturate the growth so the bacteria can grow best under 3500-4000 lux. High light intensity may have inhibitory effect on growth.
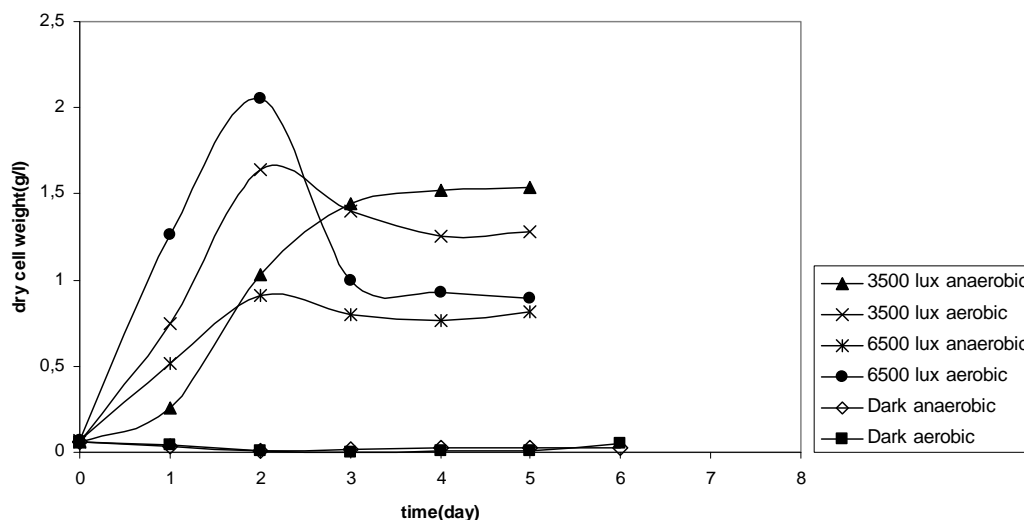
**Figure 3.10. Anaerobic and aerobic growth of _R. sphaeroides_ O.U.001 in 30mM acetate and 2mM glutamate containing medium under dark and light intensities of 3500, 6500 lux**

As a conclusion, growth was better at aerobic conditions of 6500 lux and 3500 lux in acetate containing medium. However aerobic condition is not suitable for hydrogen production, the best anaerobic growth was observed at 3500 lux in acetate containing medium. While aerobic conditions caused a sharp increase in growth, anaerobic conditions have a slower rate of increase in biomass. There was a sharp decrease in aerobic growth of 6500 lux after 2nd day. This may be due to rapid consumption of substrate which caused inhibition of growth and formation toxic materials. The pH of medium at 6500 lux aerobic conditions reached 10. Therefore this increase in pH may be caused the decrease in growth after 2nd day. The growth in malate-glutamate and malate- ammonium chloride containing media was also highest at 6500 lux aerobic conditions. However growth in these two media did not reach 2 g/ culture as in the case of acetate-glutamate containing medium. Therefore this sharp decrease was not observed in malate- glutamate and malate- ammonium chloride containing media.

## 3.1.5. Effect of Ammonium Ion on Hydrogen Production

The yield of hydrogen production may be affected by different factors. Substrate used in the culture medium is one of the important factors. Although a wide variety of substrates is used for growth, only a portion of these are suitable for hydrogen production (Koku et al., 2002). Ammonium chloride ($NH_4Cl$), an excellent source of nitrogen for bacteria growth may act as inhibitor in hydrogen generation process (Waligorska et al,. 2006). Figure 3.1 showed that the growth of the bacteria in all concentrations of ammonium chloride containing media was higher than the growth in glutamate. However from Figure 3.11 it is obvious that, the highest hydrogen production was in BP medium containing glutamate, and the hydrogen production decreased at increasing $NH_4Cl$ concentrations. There was no hydrogen production at higher concentrations than 2mM $NH_4Cl$. Hydrogen production inhibited by high (3mM and above) concentration of ammonium chloride. When pH values of ammonium chloride containing media were evaluated for hydrogen production data, pH values of hydrogen producing media (1mM, 2mM $NH_4Cl$ and 2mM glutamate containing) were lower than non-hydrogen producing media (3mM, 5mM and 10mM $NH_4Cl$ containing) (Figure 3.2). pH values of hydrogen producing media were closer to optimum pH value for hydrogen production which is 7.3-7.8 (Eroglu et al., 1999).
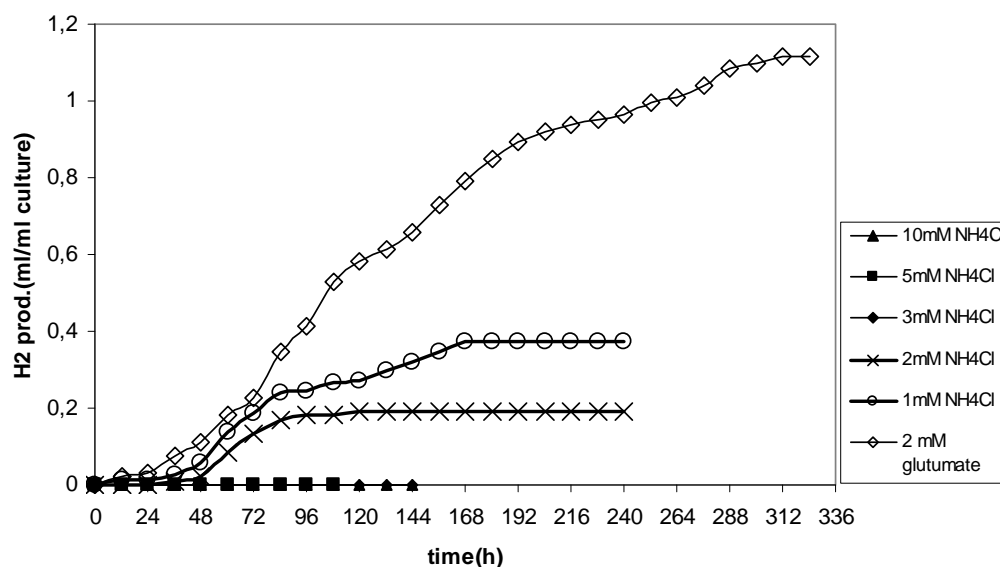


**Figure 3.11. Hydrogen production in 15mM malate medium containing different concentrations of ammonium chloride.**

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Table 3.1 shows the effect of the different concentrations of ammonium chloride on hydrogen yield (Appendix C), growth, substrate conversion efficiency (Appendix C), hydrogen production rate, and light conversion efficiency (Appendix C) in a summary. As seen in table, hydrogen yield and $H_2$ production rate decreased when the concentration of ammonium chloride increased. Hydrogen yield in 1mM $NH_4Cl$ containing medium was approximately 2.5 folds higher than the $H_2$ yield in 2mM $NH_4Cl$ containing medium. 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.

**Table 3.1. C/N ratio, efficiency, yield of hydrogen, 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 ($gH_2/ g_{substrate}$) | $H_2$ Production rate ($ml/l_{culture}.h$) |
|---|---|---|---|---|---|---|---|
| Malate/NH$_4$Cl | 15/1 | 60 | 0.49 | 0.46 | 0.18 | 0.0182 | 4.6 |
| Malate/NH$_4$Cl | 15/2 | 30 | 0.55 | 0.24 | 0.09 | 0.0075 | 3.3 |
| Malate/NH$_4$Cl | 15/3 | 20 | 0.71 | 0 | 0 | 0 | 0 |
| Malate/NH$_4$Cl | 15/5 | 12 | 0.75 | 0 | 0 | 0 | 0 |
| Malate/NH$_4$Cl | 15/10 | 6 | 0.8 | 0 | 0 | 0 | 0 |
| Malate/ Glutamate | 15/2 | 35 | 0.83 | 1.03 | 0.45 | 0.0440 | 5.1 |

## 3.1.6. The Effect of Oxygen on Hydrogen Production by *Rhodobacter sphaeroides*

Oxygen caused a reversible inhibition of nitrogenase activity (Goldberg et al., 1987). Treatment of cultures of *R.capsulatus* with low concentrations of oxygen has been

shown to cause an immediate and complete inhibition of nitrogenase activity which was fully reversible upon return to anoxic conditions (Hochman and Burris 1981).

The hydrogen production was observed in aerobic cultures of *R.sphaeroides* however it was lower under aerobic conditions than anaerobic conditions. The total hydrogen production was nearly 3 folds higher under anaerobic conditions in BP medium. There was still hydrogen production in 1mM and 2mM NH4Cl containing medium under aerobic conditions. However the total hydrogen production in 1mM NH4Cl containing medium under aerobic conditions was 1.6 fold lower than anaerobic conditions. A minimal hydrogen production was also observed in 3mM NH4Cl containing medium under aerobic conditions. There was no hydrogen production in 5mM and 10mM NH4Cl containing medium probably because of the inhibition of nitrogenase activity by both ammonium chloride and oxygen. The hydrogen evolution observed in aerobic cultures may be the result of the removal of oxygen by respiration. Therefore bacteria can produce hydrogen after the removal of inhibitory level of oxygen by respiration.
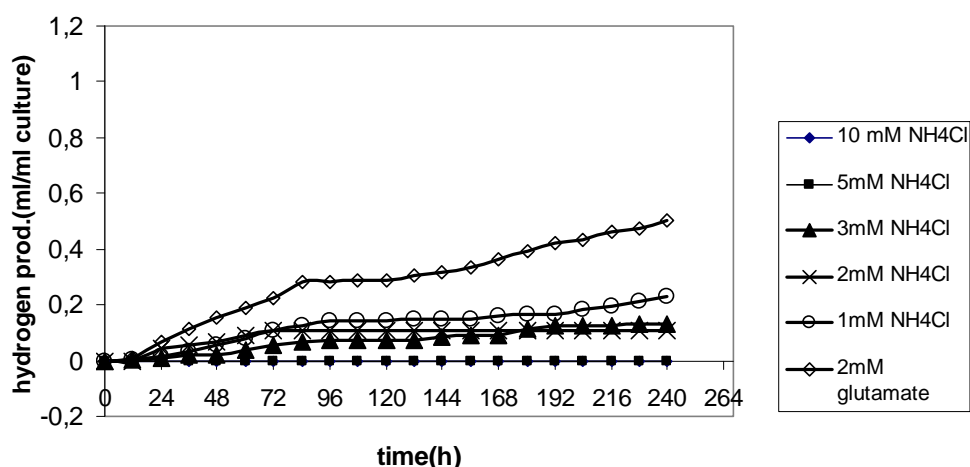


**Figure 3.12 The effect of different concentrations of ammonium chloride on hydrogen production under aerobic conditions (15mM malate as carbon source).**

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2.5-5.0 % O2 concentration in the gas phase was required for the partial inhibition of nitrogenase activity and 7.5% O$_2$ was required for complete inhibition (Yakunin et al., 2000). Nitrogenase can tolerate low concentrations of oxygen and enzyme is not inhibited completely. Therefore *R.sphaeroides* can produce hydrogen even in the presence of oxygen.

## 3.1.7. Hydrogen Production by *R. sphaeroides* O.U.001 in Media Containing Acetate and Different Glutamate Concentrations

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 (Barbosa et al,. 2001). 30mM acetate was used with 1, 2, 3, 5mM glutamate in culture media for growth and hydrogen production. BP medium which was composed of 15mM malate and 2mM glutamate was control.
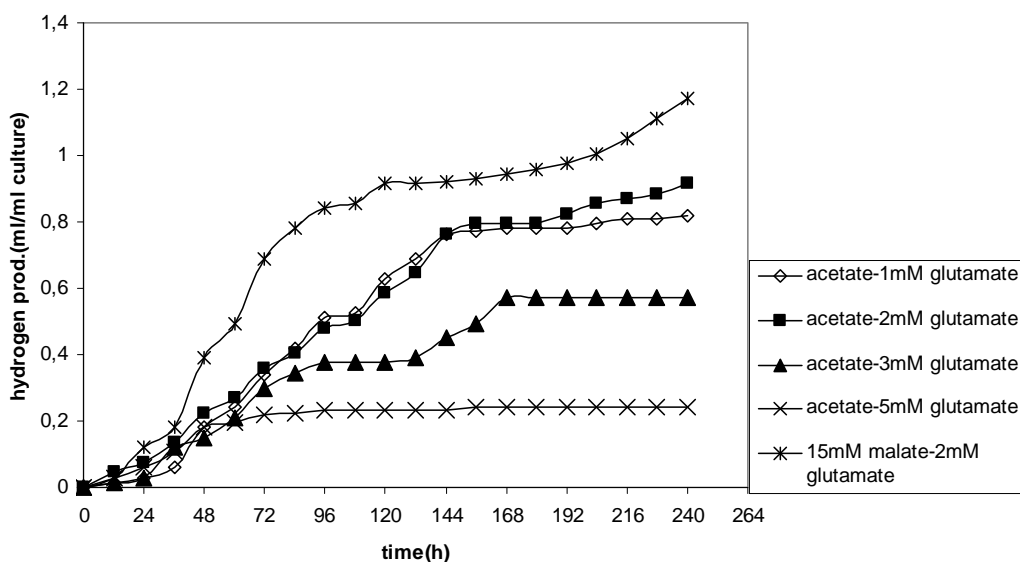


**Figure 3.13. Hydrogen production by *R.sphaeroides* O.U.001 in 30mM acetate and different concentrations of glutamate containing media**

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The best hydrogen producing medium was 2mM glutamate and 15mM malate containing medium however there was not much difference in between hydrogen producing capacity of the bacteria grown in acetate or malate. As seen in Figure 3.13, the total hydrogen yield in malate containing medium was 1.2 ml/ml culture and in acetate and 2mM glutamate containing medium it was 0.9 ml/ml culture. The best hydrogen production was obtained in lower glutamate concentrations in acetate containing medium because as the nitrogen concentration increases, the nitrogenase enzyme was inhibited at transcriptional level and enzyme activity level (Yakunin and Hallenbeck, 2000; Halbleib and Ludden 2000). The hydrogen production in malate containing medium was 5 folds higher than 5mM glutamate amd 30mM acetate containing medium. As a result increase in glutamate concentration in acetate containing media caused the decrease in hydrogen production.

The pH values of media in which hydrogen production was observed were lower than the pH values of media in which no hydrogen production was obtained (Figure 3.7). The hydrogen production may be inhibited with the increase in pH. In previous work (Fang et al., 2005), hydrogen was accumulated more at pH ranging 6.0 to 8.0 than those at other pH values which were 5, 9 and 10. In 5mM and 3mM glutamate containing media, the pH values exceeded 8. Therefore hydrogen production may have decreased in response to high values of pH.

**Table 3.2. C/N ratio, efficiency, yield, maximum biomass and hydrogen production rates in media containing different concentrations of glutamate in 30mM acetate.**

| Substrates (mM/mM) | Conc. (mM/mM) | C/N Ratio | Max Biomass $(g/L_c)$ | Light Conversion Efficiency (%) | Substrate Conversion Efficiency | Yield $(gH_2/g_{substrate})$ | H2 Production rate $(mlH_2/L_c.h)$ |
|---|---|---|---|---|---|---|---|
| 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 |

In Table 3.2, the effect of different concentrations of glutamate on substrate conversion efficiency, maximum biomass, yield, light conversion efficiency and H2 productivity were shown. As glutamate concentration increases, while maximum biomass was incresing, the yield of hydrogen and hydrogen production rate were decreasing. However 1mM glutamate containing medium had lower yield than 2mM glutamate containing medium. 2mM glutamate was optimum for higher H2 production in the presence of acetate as carbon source. C/N ratio is important parameter for producing maximum amount of hydrogen. The best C/N ratio was 35 which results in highest yield. In one of previous studies, the maximum hydrogen production rate is observed with 15mM malate and 2mM glutamate containing medium in which C/N ratio is 35 (Eroglu et al., 1999).

## 3.1.8. The Effect of Light Intensity on Hydrogen Production by *R. sphaeroides* O.U.001

Light is a major requirement for photoproduction of hydrogen in *R. sphaeroides*. Change in light intensity affects the hydrogen production. Three different light conditions, dark-3500 lux-6500 lux, were investigated to understand the hydrogen producing capacities of the bacteria under those conditions. The culture was incubated in standard conditions (30°C, 3500 lux) for 24 hours before taken to dark.

### 3.1.8.1 The effect of light intensity on hydrogen production in malate-glutamate containing medium

As seen in Figure 3.14, the highest hydrogen yield was obtained at 6500 lux under anaerobic conditions in BP containing medium followed by hydrogen production at 3500 lux under anaerobic conditions. There was also hydrogen evolution under aerobic conditions at 3500 lux and 6500 lux. The collected hydrogen volume reached to about the same value at 3500 and 6500 lux at 144th hour. No hydrogen was evolved in dark conditions as expected.
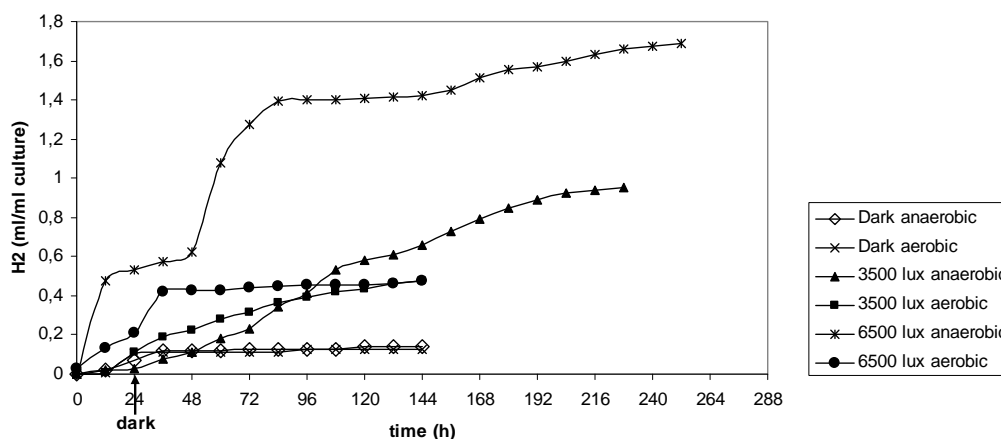
**Figure 3.14. Hydrogen production at different light intensities in 15mM malate and 2mM glutamate containing medium.**

When the hydrogen production in the first 48 hours was examined, the highest production was observed at 6500 lux again. However the lowest hydrogen production was seen at 3500 lux under anaerobic conditions. The lag time at 3500 lux was longer than 6500 lux under both anaerobic and aerobic conditions.

Total hydrogen yields under different illuminations in 15mM malate and 2mM glutamate containing medium (BP) showed that the hydrogen production increased with light intensity.

### 3.1.8.2 The effect of light intensity on hydrogen production in malate-ammonium chloride containing medium

Ammonium ion has inhibitory effect on nitrogenase activity and therefore on hydrogen production. The effect of light intensity in ammonium chloride containing medium was investigated. Although total hydrogen production was highest at 3500 lux, anaerobic conditions, hydrogen yield at 6500 lux was close to the yield at 3500 lux in 1mM $NH_4Cl$ containing medium (Figure 3.14). 0.2 ml/ml culture of was produced at 3500 lux under aerobic conditions. Oxygen in the gas phase was used by

aerobic respiration in *Rhodobacter sphaeroides* and excess amount of oxygen was consumed to the amount which nitrogenase enzyme can tolerate. Therefore bacteria were able to produce hydrogen in aerobic conditions.
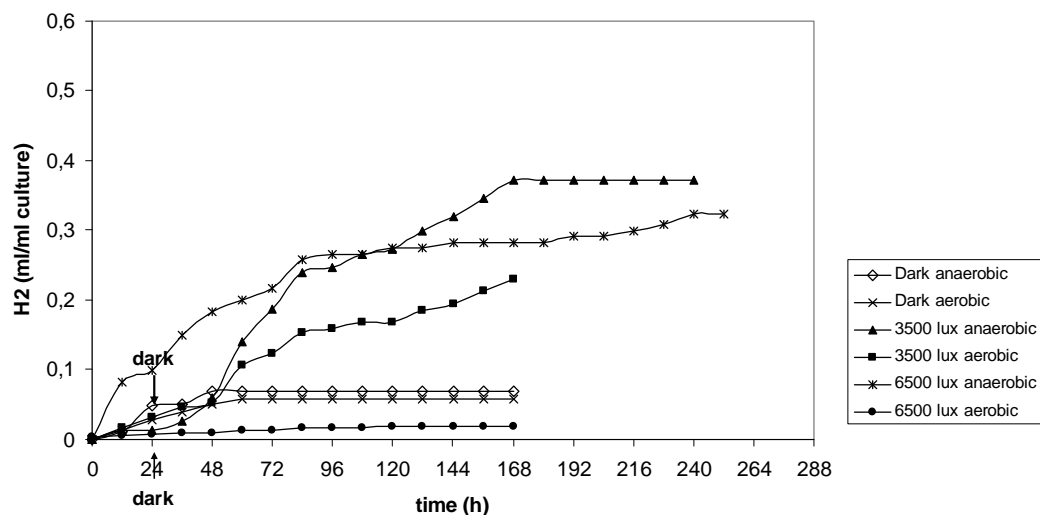


**Figure 3.15. Hydrogen production at different light intensities in 15mM malate and 1mM NH$_4$Cl containing medium.**

The hydrogen production at 6500 lux was 3 folds higher than the production in aerobic and anaerobic cultures of 3500 lux in 48 hours. However the rate of hydrogen production at 3500 lux was accelerated between 2$^{nd}$ and 3$^{rd}$ days and exceeds the hydrogen production at 6500 lux after 5$^{th}$ day. The hydrogen production rate at 6500 lux started to decrease earlier than the rate at 3500 lux. This can be due to the faster increase of biomass since cells grown at 6500 lux reached the highest biomass concentration (Figure 3.9). Light can be a limiting factor due to self shading and light absorption by the cells close to the illuminated surface (Barbosa et al., 2001).

No hydrogen evolution was observed under dark conditions and aerobic condition at 6500 lux. Apparently, both aerobic conditions and ammonium ion presence inhibited the nitrogenase activity. The high energy demand of the nitrogen fixation can be supplied by photosynthesis (Kern et al., 1998). Therefore no nitrogenase activity was expected at dark conditions.

### 3.1.8.3. The Effect of Light Intensity on Hydrogen Production in Acetate-Glutamate Containing Medium

Acetate is one of the carbon sources that *R. sphaeroides* can use for growth and hydrogen production. In the Figure 3.14 the hydrogen production at different illuminations were shown. The highest hydrogen yield was observed at 3500 lux under anaerobic conditions and it was 2 folds higher than the hydrogen production at 6500 lux at the end of the experiment. The hydrogen production at 6500 lux continued for 60 hours. However hydrogen was still produced at 264 hours at 3500 lux. High light intensity may have inhibitory effect on hydrogen production in acetate containing medium.

There was no hydrogen evolution under both aerobic and dark conditions in 30mM acetate containing medium.
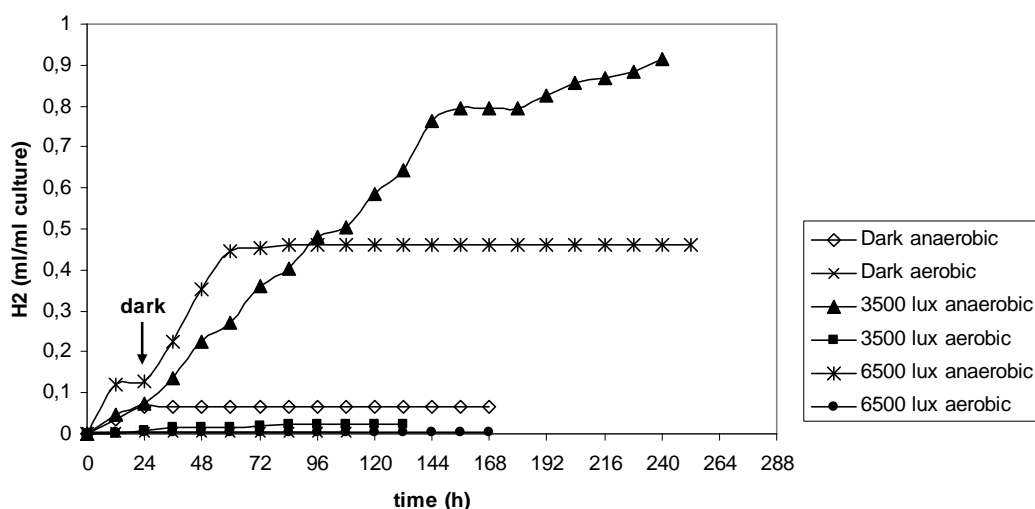


**Figure 3.16. Hydrogen production at different light intensities in 30mM acetate and 2mM glutamate containing medium.**

**Table 3.3. Carbon to nitrogen ratio, light conversion efficiency, yield of hydrogen and $H_2$ production rate in different media.**

| Substrates (mM/mM) | Concentration (mM/mM) | C/N Ratio | Light intensity | Light Conversion Efficiency | Yield (gH2/gsubstrate) | $H_2$ Production rate (ml/l$_{culture}$.h) |
|---|---|---|---|---|---|---|
| Malate/glutamate | 15/2 | 35 | 3500lux anaerobic | 1.07 | 0.044 | 5.0 |
| | | | 3500lux aerobic | 0.86 | 0.024 | 3.5 |
| | | | 6500lux anaerobic | 0.94 | 0.064 | 10.5 |
| | | | 6500lux aerobic | 0.45 | 0.019 | 15.2 |
| Malate/NH$_4$Cl | 15/1 | 60 | 3500lux anaerobic | 0.40 | 0.0182 | 2.2 |
| | | | 3500lux aerobic | 0.35 | 0.009 | 1.4 |
| | | | 6500lux anaerobic | 0.19 | 0.0120 | 1.0 |
| | | | 6500lux aerobic | 0.01 | 0.0007 | 0.0 |
| Acetate/glutamate | 30/2 | 35 | 3500lux anaerobic | 0.98 | 0.0400 | 4.6 |
| | | | 3500lux aerobic | 0.03 | 0.00097 | 0.0 |
| | | | 6500lux anaerobic | 0.67 | 0.0202 | 7.2 |
| | | | 6500lux aerobic | 0.00 | 0.0002 | 0.0 |

The effect of light intensity on hydrogen yield, $H_2$ production rate and light conversion efficiency of bacteria was given in Table 3.3. There was no hydrogen production under dark therefore Table 3.3 did not include any dark results. When the

hydrogen production yield in malate-glutamate containing medium under different light intensities are compared, the highest hydrogen yield was observed at 6500 lux anaerobic conditions and the lowest yield was at 6500 lux aerobic conditions. Aerobic conditions are very inhibitory for hydrogen production however total inhibition was not observed. Light conversion efficiency decreased as the light intensity increased. However this decrease was not as high as in a previous study in which there was 2 folds decrease in light conversion efficiency (Uyar et al., 2007).

In 1mM NH$_4$Cl-malate containing medium, the hydrogen yield was highest under 3500 lux anaerobic conditions. Higher light intensities inhibited hydrogen production in NH$_4$Cl containing medium. The lowest hydrogen yield and productivity were obtained under 6500 lux aerobic conditions as in the case of glutamate-malate containing medium.

The highest hydrogen yield was obtained again at 3500lux anaerobic conditions and the lowest was 6500 lux aerobic in acetate-glutamate containing medium. Light conversion efficiency in 3500 lux was 2 folds higher than in 6500 lux as in a study of Uyar et al. (2007).

## 3.2. Expression Analyses of Structural and Regulatory Genes of Nitrogenase

Biological nitrogen fixation in proteobacteria is catalyzed by the nitrogenase enzyme complex (Paschen et al., 2001). Fe protein (also called dinitrogenase reductase), is coded for by the *nifH* gene (Igarashi et al., 2003). Expression of the structural genes of nitrogenase *nif*HDK and other *nif* genes is controlled by the σ$^{54}$ (*rpoN* gene product) - dependent activator *nif*A (Paschen et al., 2001). NifA is the master regulator of nitrogen fixation although regulatory cascades differ; each regulatory circuit ultimately results in regulation of NifA expression or modulation of its activity in response to oxygen and/or fixed nitrogen (Dixon and Kahn, 2004). Photosynthesis, CO$_2$ fixation and N$_2$ assimilation catalyzed by photosynthetic bacteria are affected by the PrrA-PrrB global two-component transduction signal system in *Rhodobacter sphaeroides* (Elsen et al., 2000).

The expression levels of structural gene of nitrogenase, *nif*H, *nif*-specific transcriptional activator *nif*A and response regulator *prr*A were investigated in *Rhodobacter sphaeroides* O.U. 001 under different growth conditions. All the cultures were grown for 36 hours in the conditions that would be examined. After 36-hour growth RNA was isolated and RT-PCR was performed.

### 3.2.1. The Effect of Ammonium Chloride on Expression Levels of Nitrogenase Genes

Nitrogen fixation is regulated at the transcriptional level in response to environmental oxygen and ammonium levels. It is advantageous to repress the expression of the metabolically expensive nitrogenase system when the cellular level of fixed nitrogen is sufficiently high (Halbleib and Ludden, 2002). PII signal-transduction proteins are important for communicating the nitrogen status to various regulatory targets to control *nif* gene transcription in response to the availability of fixed nitrogen (Dixon and Kahn, 2004).

Different concentrations of ammonium chloride (1, 2, 3, 5, and 10mM) were used as nitrogen source in the media to examine the effect of ammonium on expression of *nif*H, *nif*A and *prr*A. The growth media compositions were given in Table 2.1. Biebl-Pfenning medium that contains 15mM malate as carbon source and 2mM glutamate as nitrogen source was used as control medium. RNA was isolated for RT-PCR after 36 hours incubation of bacteria in conditions of interest. Figure 3.15 shows the RT-PCR amplification products after gel electrophoresis (Chapter 2, Materials and Methods). The gel photographs were processed with ImageJ software. Relative expression levels (REL) of genes were calculated by formula given:

$$REL = \frac{(S_{GOI}/S_{IC})}{(CM_{GOI}/CM_{IC})} \qquad (3.1)$$

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

False positive control was performed for all expression analyses of all genes. False positive control is necessary to be sure that there is no DNA contamination in RNA which is used for cDNA synthesis.
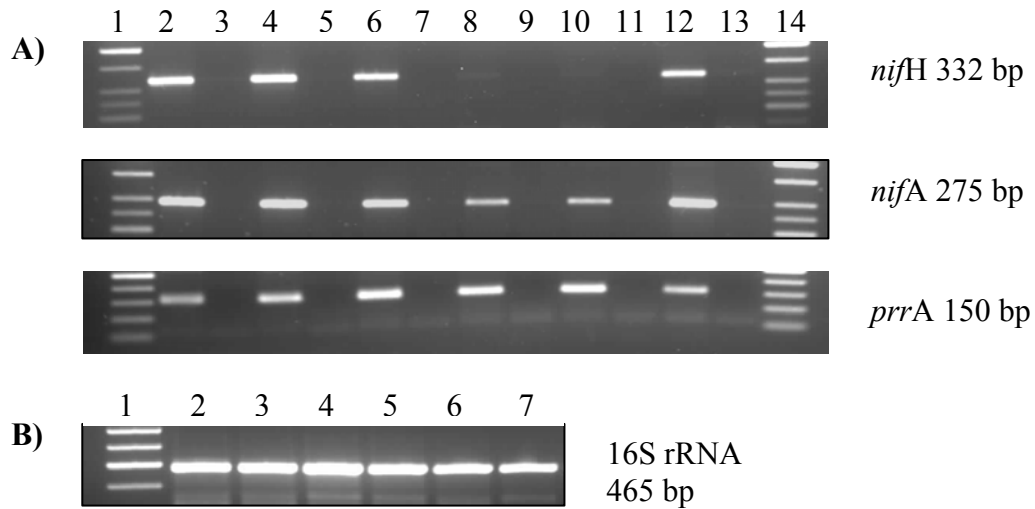


**Figure 3.17. PCR products of A) nitrogenase related genes (*nifH, nifA, prrA*) and B) 16S rRNA on 2% agarose gel**

A) Lane 1, 14: DNA ladder (50 base pair)
   Lane 2, 3: Samples from 1mM $NH_4Cl$ containing medium
      Lane 3: False positive control of sample in lane 2
   Lane 4, 5: Samples from 2mM $NH_4Cl$ containing medium
      Lane 5: False positive control of sample in lane 4
   Lane 6, 7: Samples from 3mM $NH_4Cl$ containing medium
      Lane 7: False positive control of sample in lane 6
   Lane 8, 9: Samples from 5mM $NH_4Cl$ containing medium
      Lane 9: False positive control of sample in lane 8
   Lane 10, 11: Samples from 10mM $NH_4Cl$ containing medium
      Lane 11: False positive control of sample in lane 10
   Lane 12, 13: Samples from 2mM glutamate containing (BP) medium

B) Lane 1: DNA ladder
   Lane 2: Samples from 1mM NH$_4$Cl containing medium
   Lane 3: Samples from 2mM NH$_4$Cl containing medium
   Lane 4: Samples from 3mM NH$_4$Cl containing medium
   Lane 5: Samples from 5mM NH$_4$Cl containing medium
   Lane 6: Samples from 10mM NH$_4$Cl containing medium
   Lane 7: Samples from 2mM glutamate containing (BP) medium

### 3.2.1.1. Change in Expression Levels of *nif*H gene

Expression level of *nifH* gene, which is the structural gene of nitrogenase, decreased with increased NH$_4$Cl concentration under anaerobic conditions as shown in Figure 3.18. *nifH* was not expressed at all in 5mM and 10mM NH$_4$Cl containing medium. There was no hydrogen production in these media (Figure 3.11). Although the expression was observed in 3mM NH$_4$Cl containing medium, there was no hydrogen production either. The inhibition of nitrogenase activity was not at transcriptional level in 3mM ammonium chloride containing medium. Higher concentrations of ammonium cause the loss of expression of the structural gene.
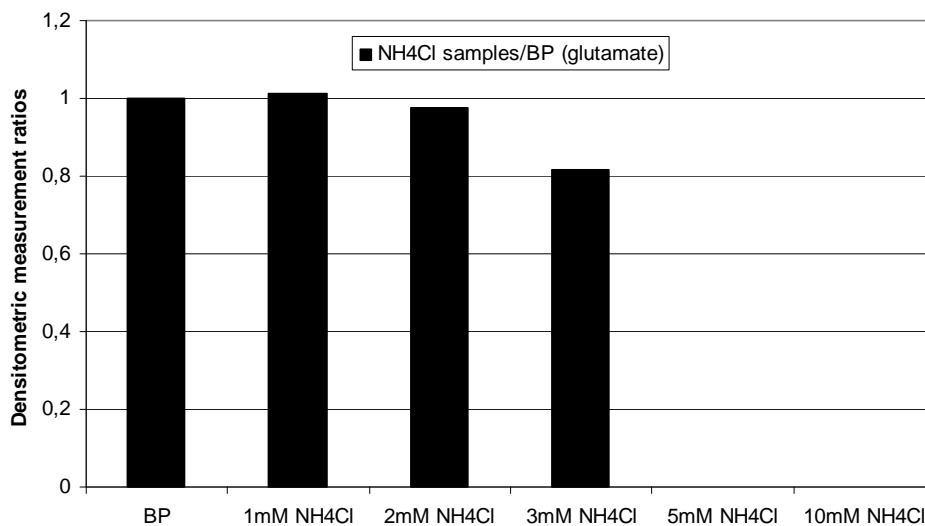


**Figure 3.18. The expression levels of *nif*H gene at various concentrations of NH$_4$Cl under anaerobic conditions**

### 3.2.1.2. Change in Expression Levels of *nif*A gene

*nifA* gene, which is the transcriptional activator of *nifHDK*, expressed in all concentrations of ammonium chloride under argon atmosphere but the expression levels decreased at high concentrations of ammonium. The expression level of nifA gene was 1.5 fold higher in glutamate containing BP medium than the level in 5mM NH$_4$Cl containing medium. Although there was some expression in *nifA* gene (transcriptional activator of *nifHDK*), no expression of nifH gene was observed in 5 and 10mM NH$_4$Cl containing media. Inhibition of NifA may be at the translational or post-translational levels, so that inactive NifA prevents the expression of *nifH*.
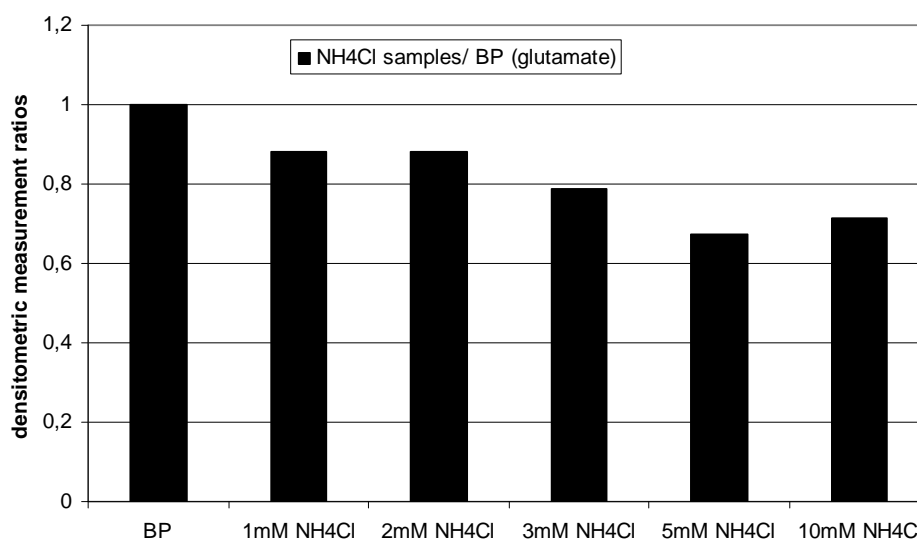


**Figure 3.19. The expression levels of *nif*A gene at different NH$_4$Cl concentrations under anaerobic conditions**

### 3.2.1.3. Change in Expression Levels of *prr*A gene

Although there was a tendency of increase in expression levels of prrA at higher NH$_4$Cl concentrations (Figure 3.20), this may not be significant. PrrA-PrrB two component global transduction signal system has function in N$_2$ assimilation (Elsen

et al., 2000). Therefore such an increase in expression level of *prr*A gene may be related to its role in $N_2$ assimilation. The expression level in 10mM $NH_4Cl$ containing medium was 1.6 fold higher than in 1mM $NH_4Cl$ containing medium (Figure 3.20). Comolli et al showed that PrrA levels were lower when target gene expression was stimulated in vivo. When the expression levels of *nifA* and *prrA* were compared, the expression levels of *prrA* decrease as the the expression levels of *nifA* increase.
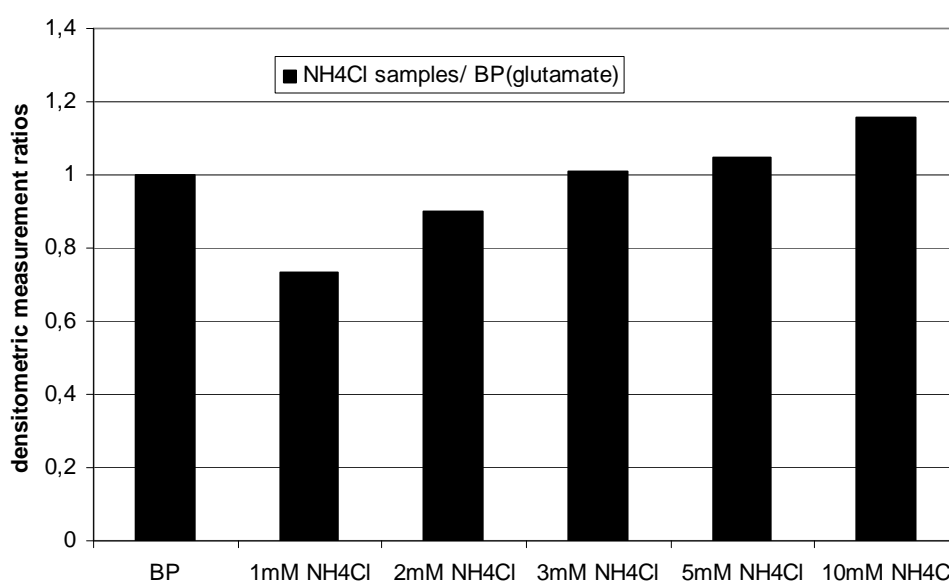


**Figure 3.20. The expression levels of *prr*A gene at different $NH_4Cl$ concentrations under anaerobic conditions**

## 3.2.2. The effect of ammonium chloride on expression levels of nitrogenase genes under aerobic conditions

*Rhodobacter sphaeroides* involves the $cbb_3$ cytochrome *c* oxidase/Rdx proteins which serve as the primary oxygen sensor to generate a signal inhibitory for photosystem (PS) gene expression. The signal generated is transmitted to the *prrBA* two-component activation system to regulate target gene expression (Eraso and

Kaplan, 2000). Joshi and Tabita reported that a specific two-component response regulator-sensor kinase signal transduction system (PrrBA) regulates biological nitrogen fixation. RegA indirectly activates nitrogenase synthesis by binding to and activating the expression of *nifA2* (Elsen et al, 2000). NifA is the transcriptional activator of nitrogenase structural genes. Oxygen has an inhibitory effect on NifA activity by the conformational changes that modulate its function. The synthesis of nitrogenase enzyme is controlled by many regulatory genes some of which are explained above. Oxygen is one of the environmental stimuli to which regulatory genes respond. The expression levels of *prrA*, *nifA* and *nifH* genes were examined in aerobic conditions to see the response of genes found in regulatory pathway to oxygen.
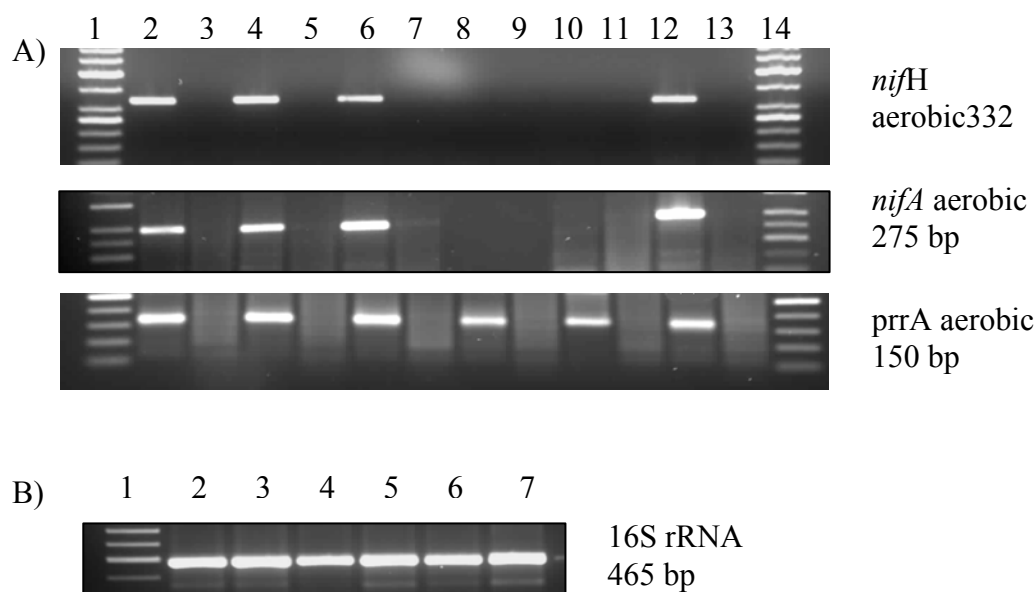
**Figure 3.21. PCR products of A) nitrogenase related genes (*nifH, nifA, prrA*) and B) 16S rRNA in aerobic conditions (2% agarose gel)**

A) Lane 1, 14: DNA ladder (50 base pair)
　　Lane 2, 3: 1mM NH₄Cl containing medium
　　　　Lane 3: False positive control of sample in lane 2
　　Lane 4, 5: 2mM NH₄Cl containing medium
　　　　Lane 5: False positive control of sample in lane 4
　　Lane 6, 7: 3mM NH₄Cl containing medium
　　　　Lane 7: False positive control of sample in lane 6
　　Lane 8, 9: 5mM NH₄Cl containing medium
　　　　Lane 9: False positive control of sample in lane 8
　　Lane 10, 11: 10mM NH₄Cl containing medium
　　　　Lane 11: False positive control of sample in lane 10
　　Lane 12, 13: 2mM glutamate containing (BP) medium
B) Lane 1: DNA ladder
　　Lane 2: 1mM NH₄Cl containing medium
　　Lane 3: 2mM NH₄Cl containing medium
　　Lane 4: 3mM NH₄Cl containing medium
　　Lane 5: 5mM NH₄Cl containing medium
　　Lane 6: 10mM NH₄Cl containing medium
　　Lane 7: 2mM glutamate containing (BP) medium

### 3.2.2.1. Change in Expression Levels of *nif*H Gene under Aerobic Conditions
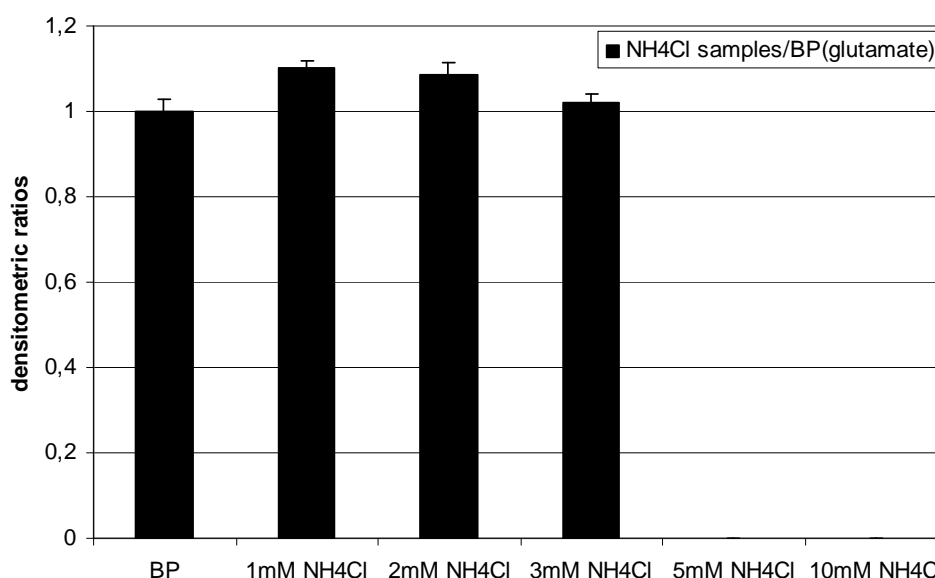


**Figure 3.22. The expression levels of *nif*H gene at different NH₄Cl concentrations under aerobic conditions**

As seen in Figure 3.22, no expression was observed in 5mM and 10mM NH₄Cl containing medium under aerobic conditions. There was a decrease in expression levels of *nifH* gene when the NH₄Cl concentration increased however this decrease was not in significant levels. Inhibition of the hydrogen production seen in Figure 3.12 can be explained by the inhibition of *nifH* transcription. There was no hydrogen production in 5mM and 10mM NH₄Cl containing medium and so no *nifH* expression was observed in those mediums. The same pattern of decrease was also observed in anaerobic cultures of the same media. When the expression levels in aerobic and anaerobic media compared, there was not much difference between them. The oxygen effect on nitrogenase activity was seen more obviously according to H2 production data (Figure 3.12).The absence of *nifH* expression in 5mM and 10mM media may depend on the ammonium control of regulatory genes because *nifH* transcription also lacks in anaerobic conditions.

### 3.2.2.2. Change in Expression Levels of *nif*A Gene under Aerobic Conditions

The expression levels of *nifA* gene under aerobic conditions were examined in NH₄Cl containing medium. Aerobic conditions totally inhibited *nifA* expression in 5mM and 10mM NH₄Cl containing medium (Figure 3.23). Oxygen has inhibitory effect on both NifA activity by changing its conformation and *nifA* transcription. The transcriptional inhibition of *nifA* was observed in only higher ammonium chloride containing media. Both the presence of NH₄Cl and oxygen caused total inhibition of expression of *nifA* gene. The expression levels of *nifA* did not change proportionally according to the concentration of NH₄Cl in aerobic conditions. Highest expression level of *nifA* was observed in 3mM NH₄Cl containing medium among aerobic cultures. Strains expressing either *nifA*₁ or *nifA*₂ from a constitutive promoter in an *ntrC* mutant still show inhibition of *nifH* transcription in ammonium, suggesting post-translational control of NifA activity (Hübner *et al*., 1993). According to this data, it can be concluded that the high expression levels in *nifA* may not mean the high expression levels of its target gene, *nifH*. The complex regulatory network of nitrogenase synthesis has many control points. Therefore it was observed that the higher expression of *nif*A did not cause higher activity in nitrogenase.
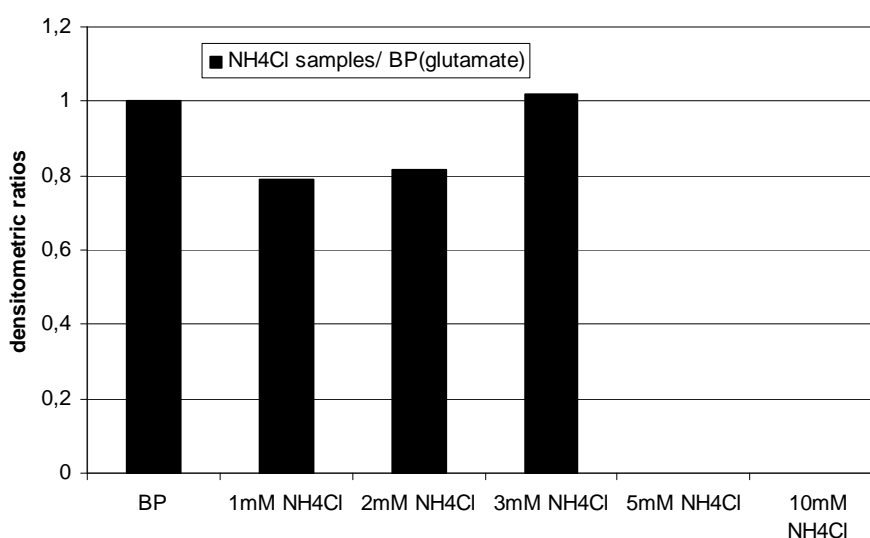


**Figure 3.23. The expression levels of *nif*A gene in different concentrations of NH₄Cl containing medium under aerobic conditions**

### 3.2.2.3. Change in expression levels of *prrA* gene under aerobic conditions

The comparison of expression levels of *prrA* gene under aerobic and anaerobic conditions was illustrated in Figure 3.24. As shown in figure, the expression levels of *prrA* were lower in BP, 5 and 10mM $NH_4Cl$ containing media insignificantly under aerobic conditions. The expressions in remaining media were approximately the same. When the expression levels of *prrA* in aerobic and anaerobic conditions were compared, *prrA* was expressed in lower values under anaerobic conditions in lower concentrations of $NH_4Cl$. This can be explained by the negative autoregulation of PrrA which means that less PrrA was present in the absence of oxygen, conditions in which the activities of many PrrA target genes increase (Comolli et al., 2002). The target gene of PrrA is *nifA* in nitrogenase regulatory network. *nifA* gene expression was higher in anaerobic conditions than aerobic conditions when the concentration of $NH_4Cl$ was low.
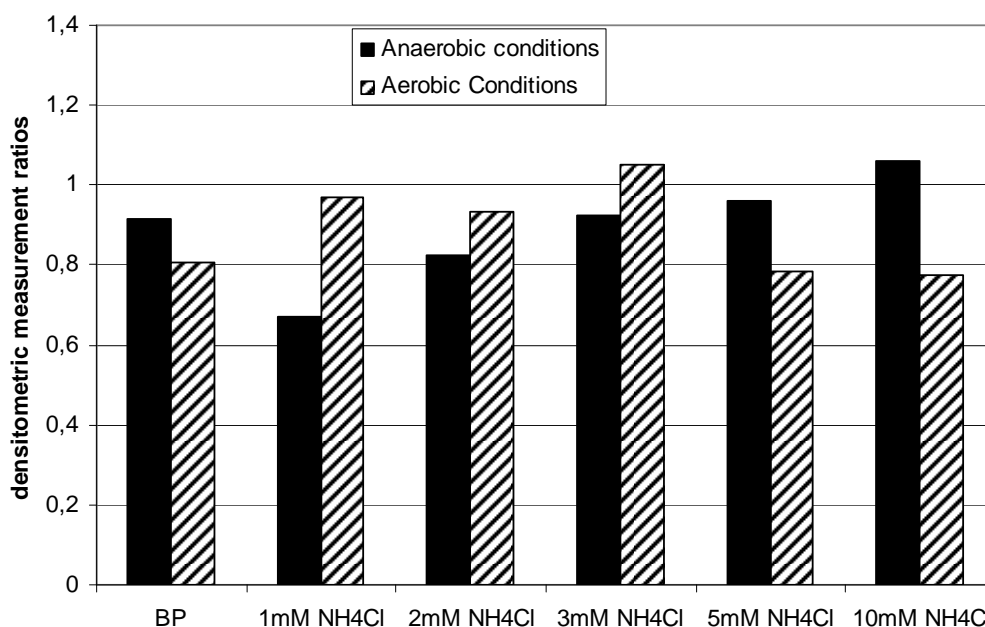


**Figure 3.24. The expression levels of *prrA* gene at different $NH_4Cl$ concentrations under aerobic conditions and anaerobic conditions**

Real-time PCR results supported the results in reverse transcriptase PCR. As seen in Figure 3.25 expression levels in 1mM, 2mM and 3mM $NH_4Cl$ containing aerobic media were higher than anaerobic conditions of the same media. This result was obtained also in reverse-transcriptase PCR. Although there was a decrease in expression levels of *nifA* in $NH_4Cl$ containing medium in comparison to glutamate containing medium, *prrA* gene expression significantly increased in all concentrations of $NH_4Cl$ except 10mM under aerobic conditions.

The expression levels of *prrA* gene change in opposite directions in aerobic and anaerobic conditions in ammonium chloride containing media. As the expression increases in aerobic conditions, a decrease was observed in anaerobic conditions in 1mM 2mM and 3mM ammonium chloride containing media.
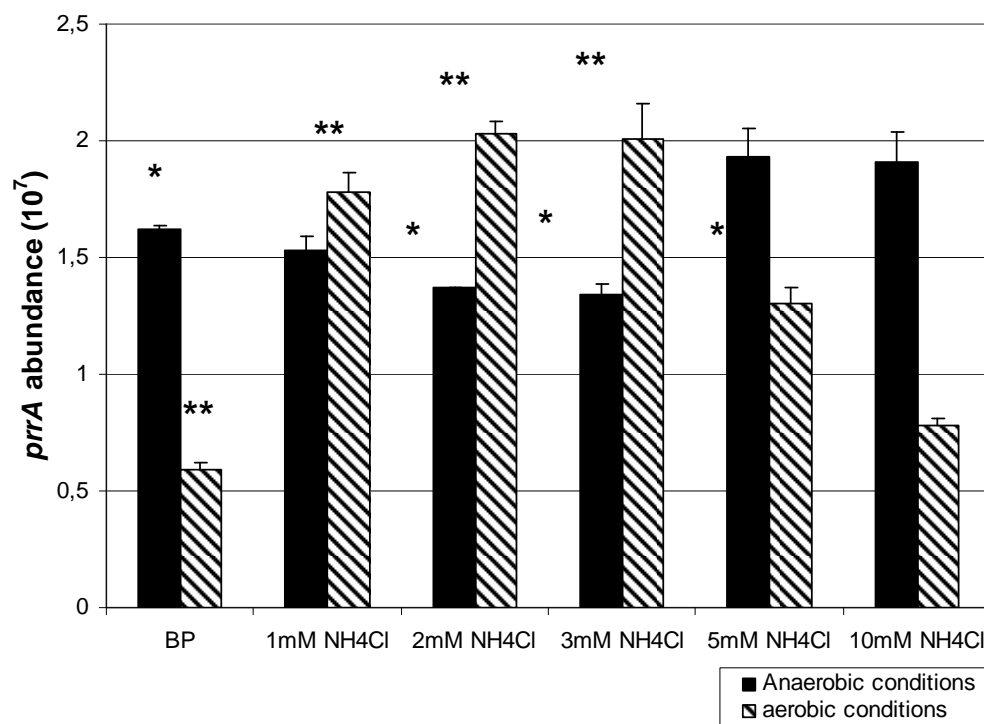


**Figure 3.25. The expression levels of *pprA* gene (real-time PCR results).**
* represents the significant difference between groups with respect to BP anaerobic and ** represents the significant difference with respect to BP aerobic $p < 0.05$

### 3.2.3. 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 *nif*H and regulatory gene *nif*A of nitrogenase were investigated in 30mM acetate and different concentrations of glutamate (1, 2, 3, 5mM) containing medium. The growth media compositions were given in Table 2.2. BP medium was used as control medium. Figure 3.26 shows the RT-PCR products of genes of interest on 2% agarose gel.
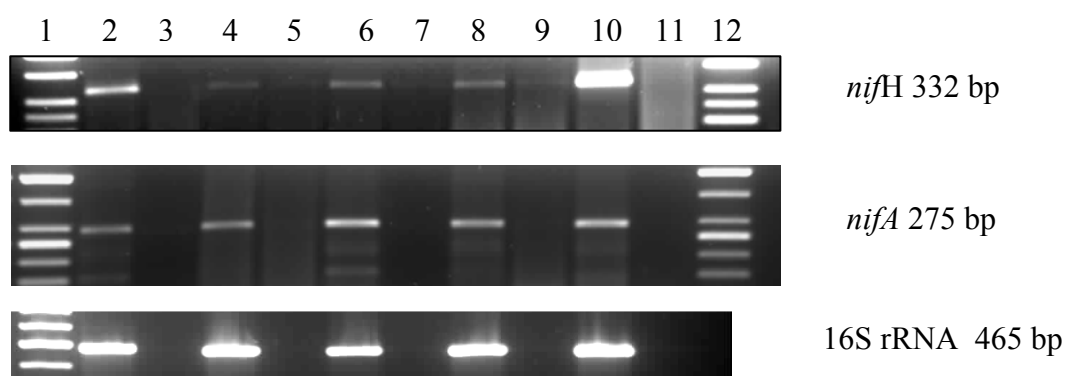


**Figure 3.26. RT-PCR amplification products of *nif*H, *nif*A and 16S rRNA genes in acetate and glutamate containing medium on 2% agarose gel.**

Lane 1, 12: DNA ladder (50 base pair)
Samples from;
Lane 2, 3: 1mM glutamate and 30mM acetate containing medium
     Lane 3: False positive control of sample in lane 2
Lane 4, 5: 2mM glutamate and 30mM acetate containing medium
     Lane 5: False positive control of sample in lane 4
Lane 6, 7: 3mM glutamate and 30mM acetate containing medium
     Lane 7: False positive control of sample in lane 6
Lane 8, 9: 5mM glutamate and 30mM acetate containing medium
     Lane 9: False positive control of sample in lane 8
Lane 10, 11: 2mM glutamate and 15mM malate containing medium
     Lane 11: False positive control of sample in lane 10

### 3.2.3.1. Change in Expression Levels of *nif*H gene

The expression levels of *nif*H gene in acetate containing medium were lower than malate containing BP medium. The expression in BP medium was 2 folds higher than the expression in 2mM glutamate and 30mM acetate containing medium. There was not significant change among the expression levels of *nif*H gene in acetate containing medium. The expressions were nearly the same regardless of glutamate concentration. When the hydrogen productions in the same media compared (Figure 3.13), the highest hydrogen production was seen in BP medium. However unlike expression levels, there was higher difference between the hydrogen yields in acetate medium. Hydrogen production was inhibited in enzyme activity level as glutamate concentration increased up to 5mM but there was no significant change in expression levels.

**nifH expression in acetate and glutamate containing medium**
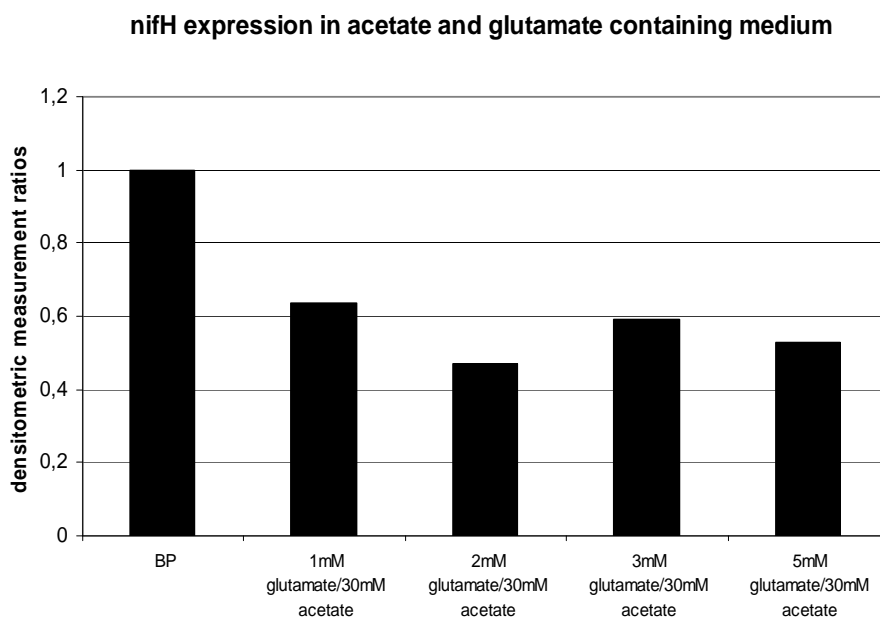


**Figure 3.27. The expression levels of *nif*H gene in 30mM acetate and different concentrations of glutamate containing medium under anaerobic conditions**

### 3.2.3.2. Change in Expression Levels of *nif*A gene

The expressions level of *nifA* gene in acetate-glutamate containing medium showed different pattern of change when compared with malate-NH$_4$Cl containing medium. Expression of *nifA* gene was highest in 3mM glutamate and 30mM acetate containing medium.

**nifA expression levels in acetate and glutamate containing medium**
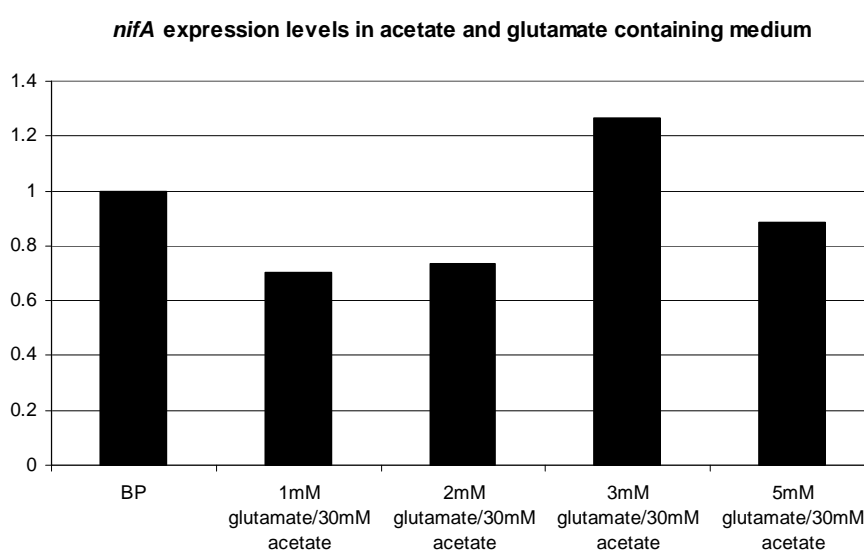


**Figure 3.28. The expression levels of *nif*A gene in 30mM acetate and different concentrations of glutamate containing medium under anaerobic conditions**

### 3.2.4. The Effect of Different Light Intensities on Expression of Nitrogenase Genes

The energy required for growth and hydrogen production activity of nitrogenase is provided by the photosynthetic apparatus, which converts light energy into chemical bond energy (ATP) (Koku et al., 2002). Synthesis of photosynthetic apparatus is regulated by light intensity (Kern et al., 1998). cbb$_3$-Prr system has role in  light

control of photosystem gene expression (Roh et al., 2004). Two component regulatory system (regA-regB) and hvrA and hvrB genes are regulatory genes involved in synthesis of photosynthetic apparatus (Kern et al., 1998). Kern et al (1998) presented evidence that HvrA, which was previously shown to be responsible for light regulation of the photosynthetic apparatus, is also involved in the ammonium control of *nif* gene expression. In addition to HvrA effect on nitrogenase genes, the RegB-RegA two component regulatory system was found to be involved in nitrogenase gene regulation by participating in the activation of *nifA2* gene expression in *R. capsulatus* (Elsen et al., 2000). The *nifA* gene product is the transcriptional activator of the nitrogenase structural genes (Bauer et al., 1998).

The structural gene of nitrogenase, *nifH*, the transcriptional activator of nitrogenase structural genes, *nifA*, and response regulator, *prrA* were examined for their expression levels at different light intensities. The light effect on the expression levels of the genes of interest were investigated in culture media which contains different carbon sources and nitrogen sources.

### 3.2.4.1 The Effect of Different Light Intensities on Expression Levels of Nitrogenase Genes in Malate-Glutamate Containing Medium

Expression levels of *nifH*, *nifA*, and *prrA* gene were examined in 15mM malate and 2mM glutamate containing medium under dark and two different light intensities, 3500lux and 6500lux. The effect of aerobic and anaerobic conditions on expression levels was also determined.

#### 3.2.4.1.1. The Expression of *nifH* Gene

RT-PCR products of malate-glutamate containing medium under aerobic and anaerobic conditions at different light intensities on 2% agarose gel were shown in Figure 3.29.

**Figure 3.29.  RT-PCR amplification products of *nifH* and 16S rRNA genes in malate-glutamate containing medium at different light intensities**

*nifH* gene product of;
Lane 2: Dark anaerobic system
Lane 3: Dark aerobic system
Lane 4: 3500 lux anaerobic system
Lane 5: 3500 lux aerobic system
Lane 6: 6500 lux anaerobic system
Lane 7: 6500 lux aerobic system
16S rRNA gene product of;
Lane 9: Dark anaerobic system
Lane 10: Dark aerobic system
Lane 11: 3500 lux anaerobic system
Lane 12: 3500 lux aerobic system
Lane 13: 6500 lux anaerobic system
Lane 14: 6500 lux aerobic system

Lane 1, 8, 15: DNA ladder (50 base pair)

The expression levels of *nifH* gene which encodes dinitrogenase reductase were shown schematically in Figure 3.30. The highest expression was seen in dark aerobic conditions. Dark nitrogenase activity would require an alternative energy source to light which is dark respiration (Meyer et al., 1978). As in the case of activity, dark respiration may induce the synthesis of nitrogenase. Therefore the expression level of *nifH* was higher under dark aerobic conditions. The expression level of nifH gene changed significantly at dark aerobic condition with a p-value of 0.013 and at 3500 lux aerobic conditions with a p-value of 0.015. The highest

hydrogen production was observed at 6500lux anaerobic conditions in malate-glutamate containing medium (Figure3.14). However the synthesis of nitrogenase was not the highest in that condition. This may be explained by post-translational control of nitrogenase. *nifH* gene expressed higher under aerobic conditions of the same light intensity, 6500 lux, however the hydrogen production was higher under anaerobic condition. This may be due to inhibition of nitrogenase activity by oxygen because both the nitrogenase component proteins are extremely oxygen sensitive.



**Figure 3.30. The expression levels of *nifH* gene in malate-glutamate containing medium at different light intensities under aerobic and anaerobic conditions** (The values shown are the means of four assays. Standard error of means are indicated as vertical bars. * represents the significant difference between control and other groups with $p < 0.05$; ** represents the significant difference between aerobic groups with $p < 0.05$)

### 3.2.4.1.2. The expression of *nifA* gene



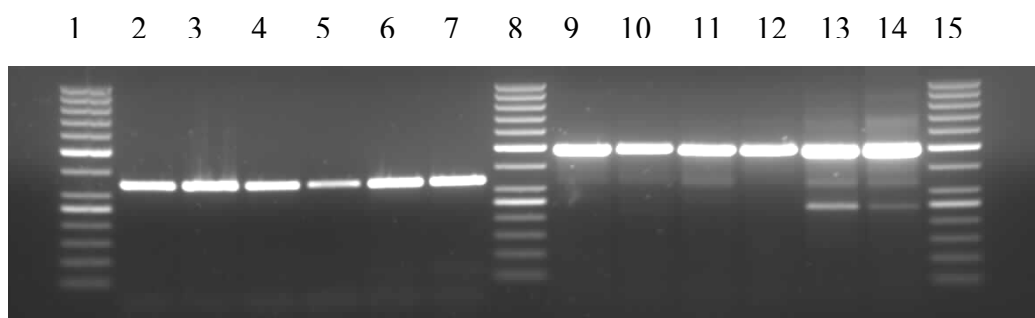**Figure 3.31. RT-PCR amplification products of *nifA* gene in malate-glutamate containing medium at different light intensities**

Lane 1, 14: DNA ladder (50 base pair)
Lane 2: *nifA* gene product of dark anaerobic grown bacteria
      Lane 3: False positive control of sample in lane 2
Lane4: *nifA* gene product of dark aerobic grown bacteria
      Lane 5: False positive control of sample in lane 4
Lane 6: *nifA* gene product of 3500 lux anaerobic grown bacteria
      Lane 7: False positive control of sample in lane 6
Lane 8: *nifA* gene product of 3500 lux aerobic grown bacteria
      Lane 9: False positive control of sample in lane 8
Lane 10: *nifA* gene product of 6500 lux anaerobic grown bacteria
      Lane 11: False positive control of sample in lane 10
Lane 12: *nifA* gene product of 6500 lux aerobic grown bacteria
      Lane 13: False positive control of sample in lane 12

*nifA* gene is the transcriptional activator of *nifHDK*. The expression levels of *nifA* under different growth conditions was shown in Figure 3.32 schematically. Expression levels were compared considering the expression level at 3500lux anaerobic conditions as a control condition. The highest expression level was obtained in dark anaerobic conditions and the lowest expression was observed at 6500 lux anaerobic condition. Under aerobic conditions, *nifA* is moderately expressed under the control of an unknown activator, whereas under microaerobic conditions, NifA protein activates its own expression (Hübner et al., 1991). In light experiments, microaerobic conditions were used; therefore NifA of *R.sphaeroides* may also activate its own synthesis. Negative autoregulation may cause higher expression levels in repressing conditions. When the expression levels under anaerobic conditions were compared, a decrease was observed as light intensity

increased. The hydrogen production at 6500 lux was highest however the expression level of *nifA* in the same conditions was lower. These results suggest that negative autoregulation of NifA in *R.sphaeroides*. There were some differences between the expression levels of *nifA* gene in these conditions, however there were no significant difference among groups.



**Figure 3.32. The expression levels of *nifA* gene in malate-glutamate containing medium at different light intensities under aerobic and anaerobic conditions**

**The expression of *prrA* gene**
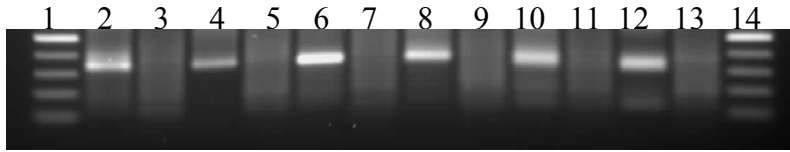


**Figure 3.33. RT-PCR amplification products of *prrA* gene in malate-glutamate containing medium at different light intensities**

Lane 1, 14: DNA ladder (50 base pair)
Lane 2: *prrA* gene product of dark anaerobic grown bacteria
     Lane 3: False positive control of sample in lane 2
Lane4: *prrA* gene product of dark aerobic grown bacteria
     Lane 5: False positive control of sample in lane 4
Lane 6: *prrA* gene product of 3500 lux anaerobic grown bacteria
     Lane 7: False positive control of sample in lane 6
Lane 8: *prrA* gene product of 3500 lux aerobic grown bacteria
     Lane 9: False positive control of sample in lane 8
Lane 10: *prrA* gene product of 6500 lux anaerobic grown bacteria
     Lane 11: False positive control of sample in lane 10
Lane 12: *prrA* gene product of 6500 lux aerobic grown bacteria
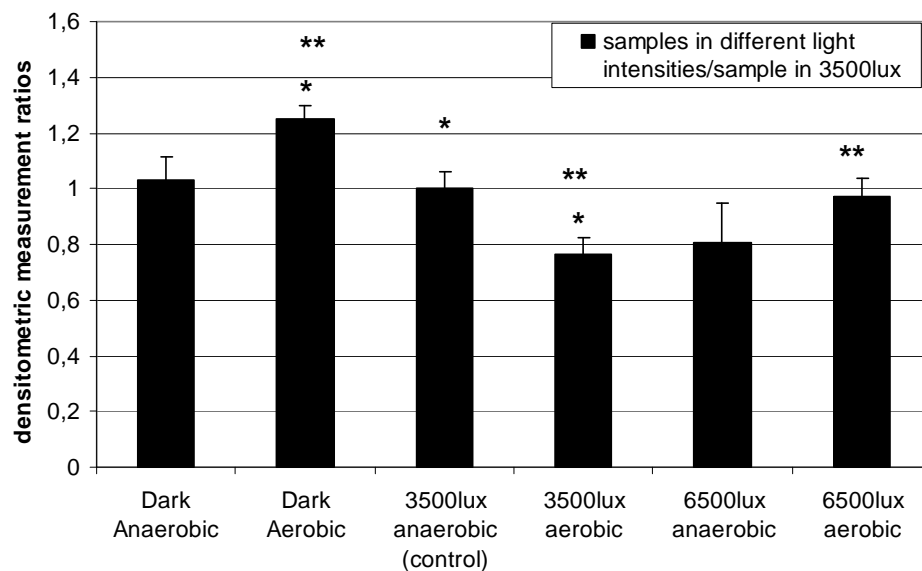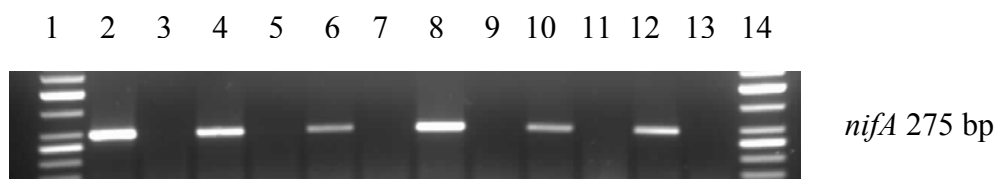     Lane 13: False positive control of sample in lane 12



**Figure 3.34. The expression levels of *prrA* gene in malate-glutamate containing medium at different light intensities under aerobic and anaerobic conditions**

The expression levels of *prrA* gene decreased in aerobic conditions of all light intensities. The highest expression level was observed at 3500 lux under anaerobic conditions. When the expression levels of *nifA* increased, it was observed that the expression level of *prrA* gene decreased. There was a negative correlation between these two genes. It seems as if there was no significant difference between the expression levels of *prrA* at different light intensities.

### 3.2.4.2 The Effect of Different Light Intensities on Expression Levels of Nitrogenase Genes in Acetate-Glutamate Containing Medium

The expression levels of *nifH*, *nifA* and *prrA* genes were investigated in 30mM acetate and 2mM glutamate containing medium at different light intensities under aerobic and anaerobic conditions. In addition to reverse transcriptase PCR, real-time PCR was also performed for *prrA* gene.

### 3.2.4.2.1 The Expression Level of *nifH* gene



**Figure 3.35.  RT-PCR amplification products of *nifH* and 16S rRNA genes in acetate-glutamate containing medium at different light intensities**

*nifH* gene product of;
Lane 2: Dark anaerobic grown bacteria
Lane 3: Dark aerobic grown bacteria
Lane 4: 3500 lux anaerobic grown bacteria
Lane 5: 3500 lux aerobic grown bacteria
Lane 6: 6500 lux anaerobic grown bacteria condition
Lane 7: 6500 lux aerobic grown bacteria

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16S rRNA gene product of;
Lane 9: Dark anaerobic grown bacteria
Lane 10: Dark aerobic grown bacteria
Lane 11: 3500 lux anaerobic grown bacteria
Lane 12: 3500 lux aerobic grown bacteria
Lane 13: 6500 lux anaerobic grown bacteria
Lane 14: 6500 lux aerobic grown bacteria

Lane 1, 8, 15: DNA ladder (50 base pair)

Variation in expression levels of *nifH* gene under different light intensities in acetate-glutamate medium was shown in Figure 3.36. Expression levels were compared considering the expression level at 3500lux anaerobic conditions as a control medium. There was no significant difference between the expressions of *nifH* in dark and in the light intensity of 3500lux under both aerobic and anaerobic conditions. However the expression decreased under 6500 lux both aerobic and anaerobic conditions. The hydrogen production at 3500lux was 2 folds higher than the production at 6500 lux under anaerobic conditions (Table 3.3). Higher light intensity inhibited both the expression of *nifH* gene and hydrogen production in acetate containing medium. The highest expression level was observed in dark aerobic conditions like in the case of malate-glutamate containing medium. This can be explained by dark respiration which may induce the synthesis of nitrogenase by supplying the required energy. The lowest expression of *nifH* was seen at 6500lux aerobic condition (2 folds lower than control condition) which was repressing condition because of high light intensity and oxygen. There was a significant difference between the expression levels at 6500 lux aerobic conditions and 3500 lux anaerobic conditions with $p<0.02$
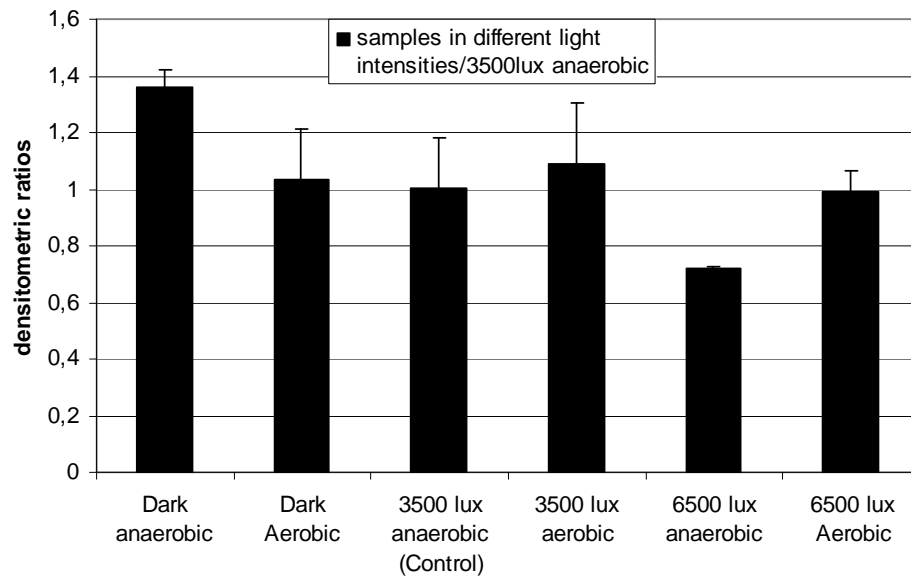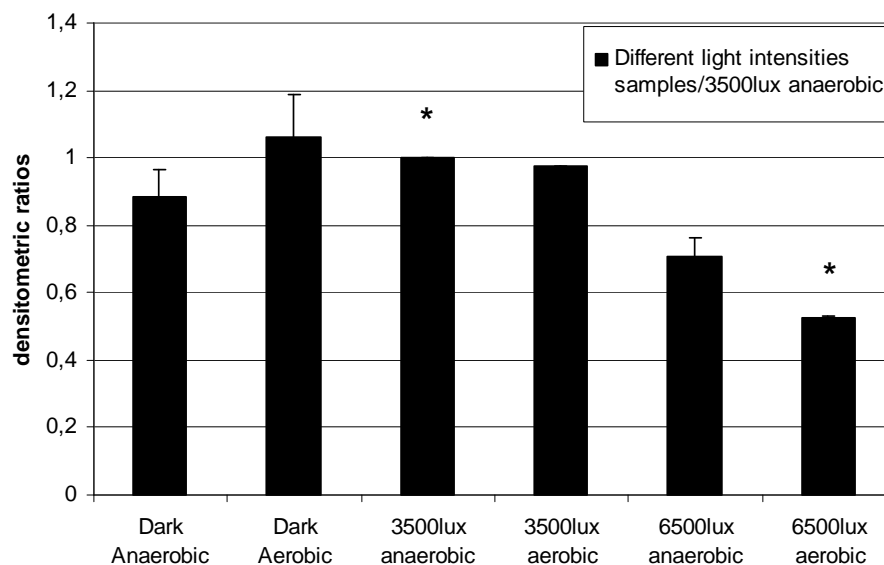
**Figure 3.36. The expression levels of *nifH* gene in acetate-glutamate containing medium at different light intensities under aerobic and anaerobic conditions.** (* represents significant difference between groups $p<0.05$)

### 3.2.4.2.2. The Expression Level of *nifA* gene



*nifA* 275 bp
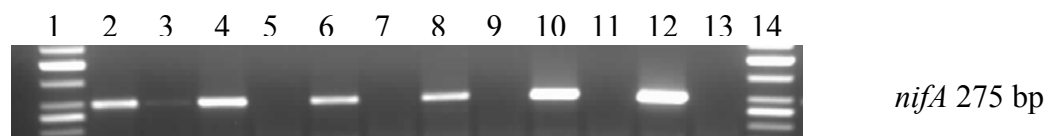
**Figure 3.37. RT-PCR amplification products of *nifA* gene in acetate-glutamate containing medium at different light intensities**

Lane 1, 14: DNA ladder (50 base pair)
Lane 2: *nifA* gene product of dark anaerobic grown bacteria
     Lane 3: False positive control of sample in lane 2
Lane4: *nifA* gene product of dark aerobic grown bacteria
     Lane 5: False positive control of sample in lane 4
Lane 6: *nifA* gene product of 3500 lux anaerobic grown bacteria
     Lane 7: False positive control of sample in lane 6
Lane 8: *nifA* gene product of 3500 lux aerobic grown bacteria
     Lane 9: False positive control of sample in lane 8
Lane 10: *nifA* gene product of 6500 lux anaerobic grown bacteria
     Lane 11: False positive control of sample in lane 10
Lane 12: *nifA* gene product of 6500 lux aerobic grown bacteria
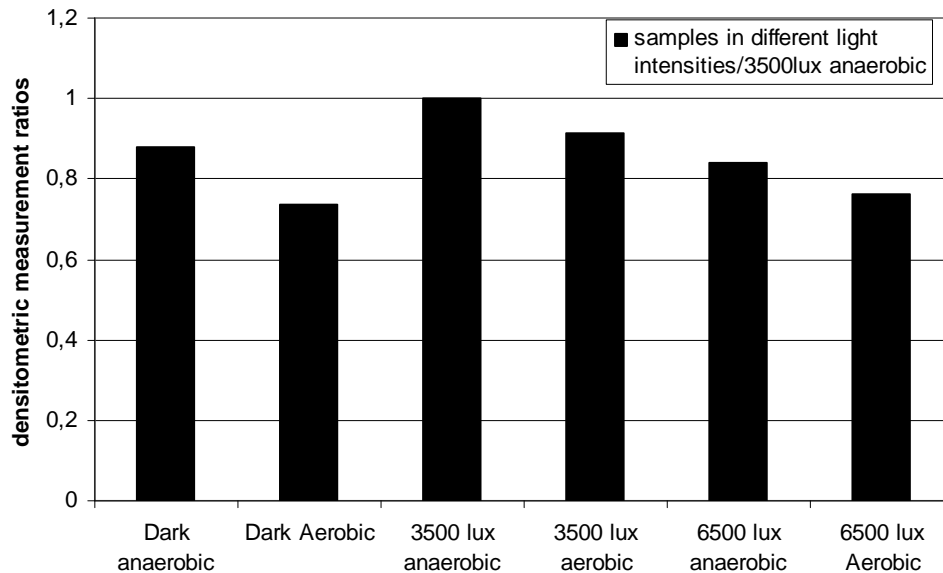     Lane 13: False positive control of sample in lane 12

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**Figure 3.38.  The expression levels of *nifA* gene in acetate-glutamate containing medium at different light intensities under aerobic and anaerobic conditions**

The change in expression levels of n*ifA* gene was shown in Figure 3.38 schematically. When Figure 3.38 was evaluated, it seems like that under light conditions the expression of *nifA* was controlled by negative autoregulation because as the expression of its target gene *nifH* increased, the expression of *nifA* decreased or visa versa. *nifA* was expressed in high levels at dark which may be due to different regulatory mechanisms at dark and light. Oxygen seems not to have effect on the expression of *nifA* gene. The expression of *nifA* in *H. seropedicae* is regulated by fixed nitrogen (4–5-fold reduction by $NH_4Cl$) but not by oxygen (Souza et al., 2000). This may not be said for *R.sphaeroides* however, the concentration of oxygen may be the factor that determines the regulation of *nifA*. *nifA* may be regulated by oxygen when  concentration of oxygen was above a certain value.  There was no significant difference among the groups.

### 3.2.4.2.3. The Expression Level of *prrA* gene
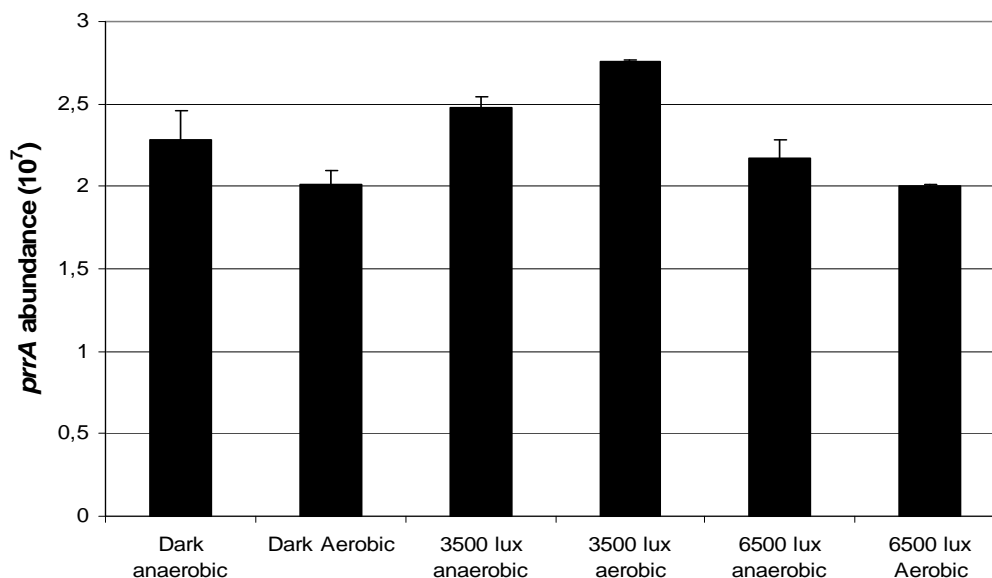


**Figure 3.39. The expression levels of *prrA* gene in 30mM acetate-2mM glutamate containing medium (real-time PCR results)**

According to the real-time PCR results, the expression of *prrA* gene was highest at light intensity of 3500 lux and aerobic conditions. As seen in previous expression data, *prrA* expression was suppressed in the conditions where the expression of target gene (*nifA*) was induced. Figure 3.38 and Figure 3.39 show obviously the reverse relation between the expression levels of *prrA* and *nifA* gene. There was not a significant difference between the groups.

### 3.2.4.3. The Effect of Different Light Intensities on Expression Levels of Nitrogenase Genes in Malate-NH$_4$Cl Containing Medium

The effect of different light intensity on expression levels of *nifH*, *nifA* and *prrA* was investigated in 15mM malate and 1mM NH$_4$Cl containing medium under aerobic

and anaerobic conditions. In addition to reverse transcriptase-PCR, Reverse transcriptase real- time PCR was also performed for *prrA* gene.

### 3.2.4.3.1. The Expression of *nifH* gene



**Figure 3.40. RT-PCR amplification products of *nifH* and 16S rRNA genes in malate-NH₄Cl containing medium at different light intensities**

Lane 1, 8, 15: DNA ladder (50 base pair)

*nifH* gene product of;
Lane 2: Dark anaerobic grown bacteria
Lane 3: Dark aerobic grown bacteria ondition
Lane 4: 3500 lux anaerobic grown bacteria
Lane 5: 3500 lux aerobic grown bacteria
Lane 6: 6500 lux anaerobic grown bacteria
Lane 7: 6500 lux aerobic grown bacteria

16S rRNA gene product of;
Lane 9: Dark anaerobic grown bacteria
Lane 10: Dark aerobic grown bacteria
Lane 11: 3500 lux anaerobic grown bacteria
Lane 12: 3500 lux aerobic grown bacteria
Lane 13: 6500 lux anaerobic grown bacteria
Lane 14: 6500 lux aerobic grown bacteria

**Figure 3.41. The expression levels of *nifH* gene in 15mM malate-1mM NH₄Cl containing medium at different light intensities**

Expression levels were compared considering the expression level at 3500lux anaerobic conditions as a control medium. Approximately the same level of *nifH* gene expression was observed at dark aerobic and anaerobic conditions. It can be suggested that the expression of *nifH* gene was not lost when exposed to dark for 36 hours. *nifH* gene transcribed at dark however nitrogenase was not active. There was no significant change in expression levels among different conditions. However expression level decreased 1.6 fold at 6500 lux aerobic condition when compared with the expression level at 3500 lux anaerobic conditions.

### 3.2.4.3.2. The Expression of *nifA* gene



1mM NH₄Cl
*nif*A 275 bp
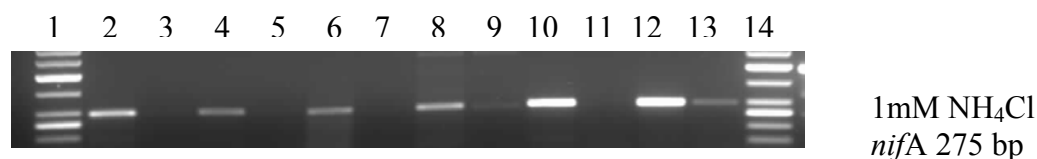
**Figure 3.42. RT-PCR amplification products of *nifA* gene in malate-NH₄Cl containing medium at different light intensities**

Lane 1, 14: DNA ladder (50 base pair)
Lane 2: *nifA* gene product of dark anaerobic grown bacteria
     Lane 3: False positive control of sample in lane 2
Lane4: *nifA* gene product of dark aerobic grown bacteria
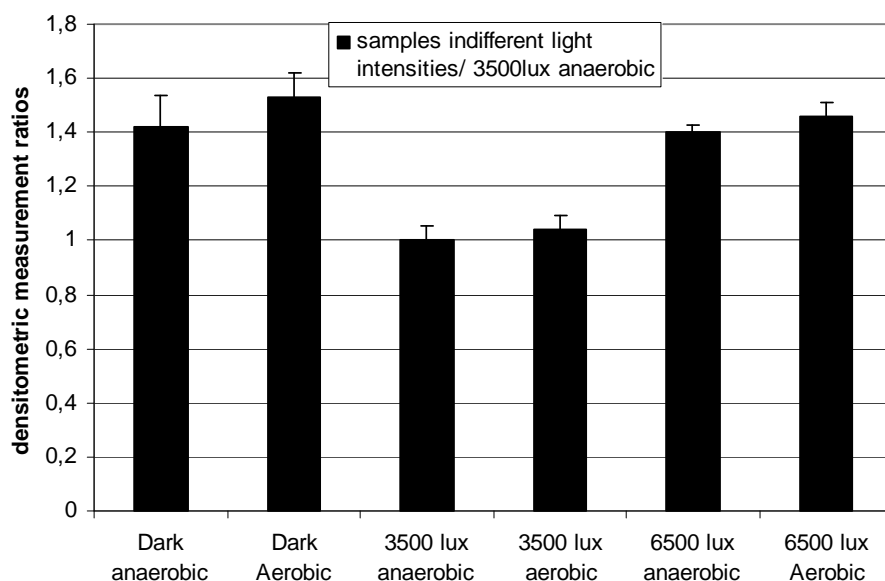     Lane 5: False positive control of sample in lane 4
Lane 6: *nifA* gene product of 3500 lux anaerobic grown bacteria
     Lane 7: False positive control of sample in lane 6
Lane 8: *nifA* gene product of 3500 lux aerobic grown bacteria
     Lane 9: False positive control of sample in lane 8
Lane 10: *nifA* gene product of 6500 lux anaerobic grown bacteria
     Lane 11: False positive control of sample in lane 10
Lane 12: *nifA* gene product of 6500 lux aerobic grown bacteria
     Lane 13: False positive control of sample in lane 12

*nifA* gene expression inceased with the increase in light intensity except the dark anaerobic condition. The expression levels of *nifA* gene increased 1.7 fold at 6500 lux aerobic condition when compared with the expression level at 3500 lux anaerobic condition (control). This increase can be suggested the negative autoregulation of NifA because *nifH* gene under the same conditions have the lowest level of expression. As target gene expression decreases the expression of *nifA* gene can increase.
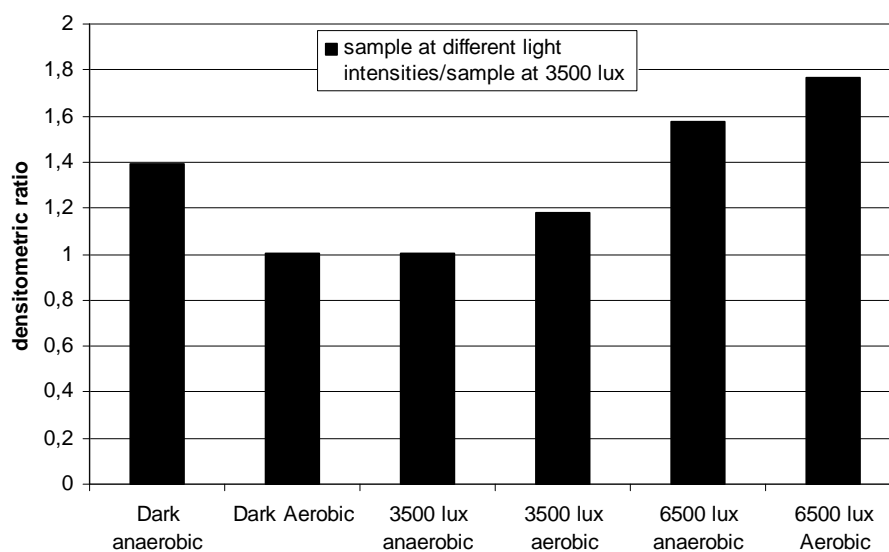
**Figure 3.43. The expression levels of *nifA* gene in 15mM malate-1mM NH₄Cl containing medium at different light intensities**
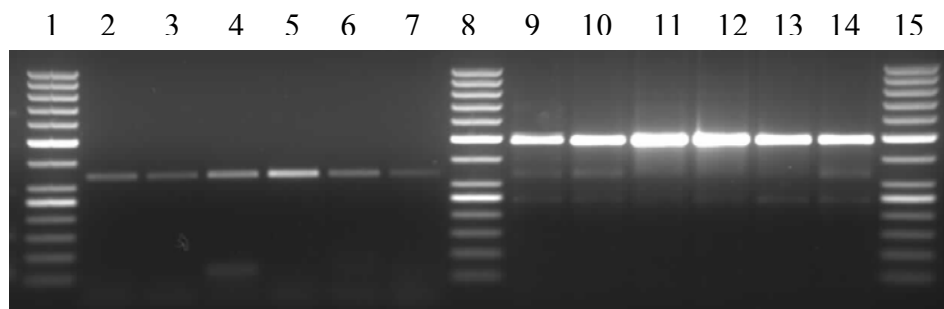
### 3.2.4.3.3. The Expression of *prrA* Gene



prrA 150 bp
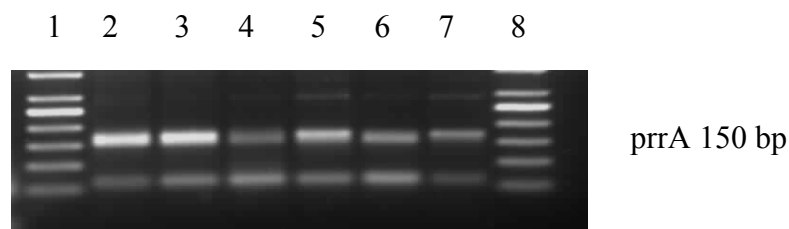
**Figure 3.44. RT-PCR amplification products of *prrA* gene in malate-NH₄Cl containing medium at different light intensities**

Lane1, 8: DNA ladder (50 base pair)

*prrA* gene product of;
Lane 2: Dark anaerobic grown bacteria
Lane 3: Dark aerobic grown bacteria
Lane 4: 3500 lux anaerobic grown bacteria
Lane 5: 3500 lux aerobic grown bacteria
Lane 6: 6500 lux anaerobic grown bacteria
Lane 7: 6500 lux aerobic grown bacteria

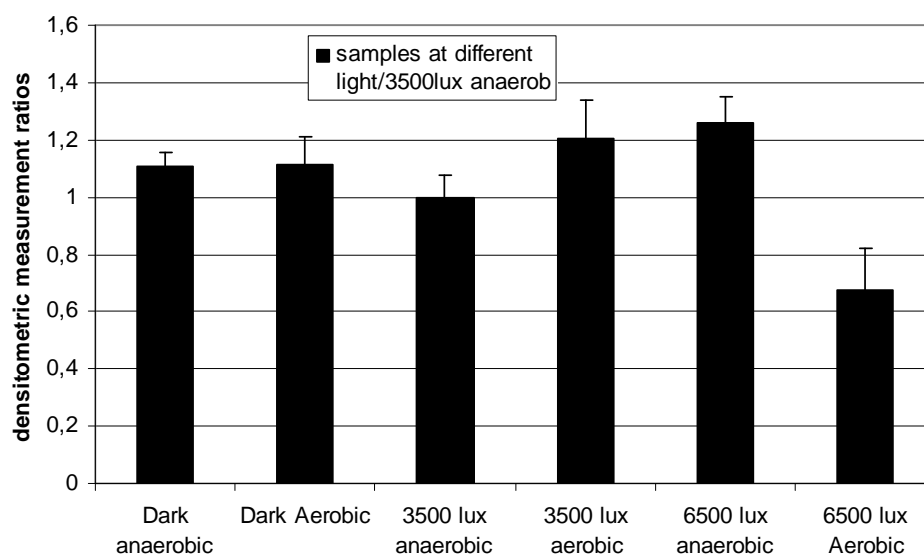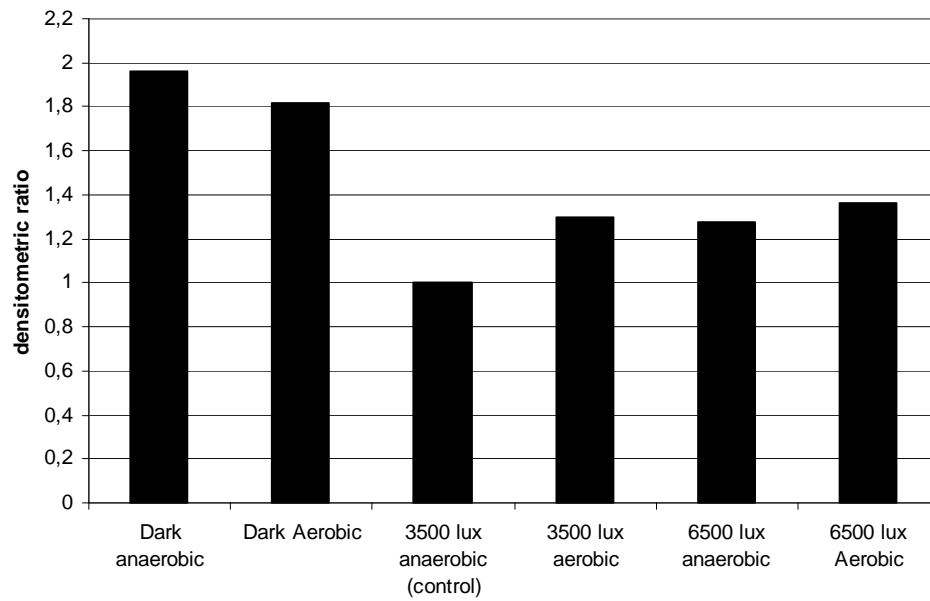**Figure 3.45. The expression levels of *prrA* gene in 15mM malate-1mM NH₄Cl containing medium at different light intensities**
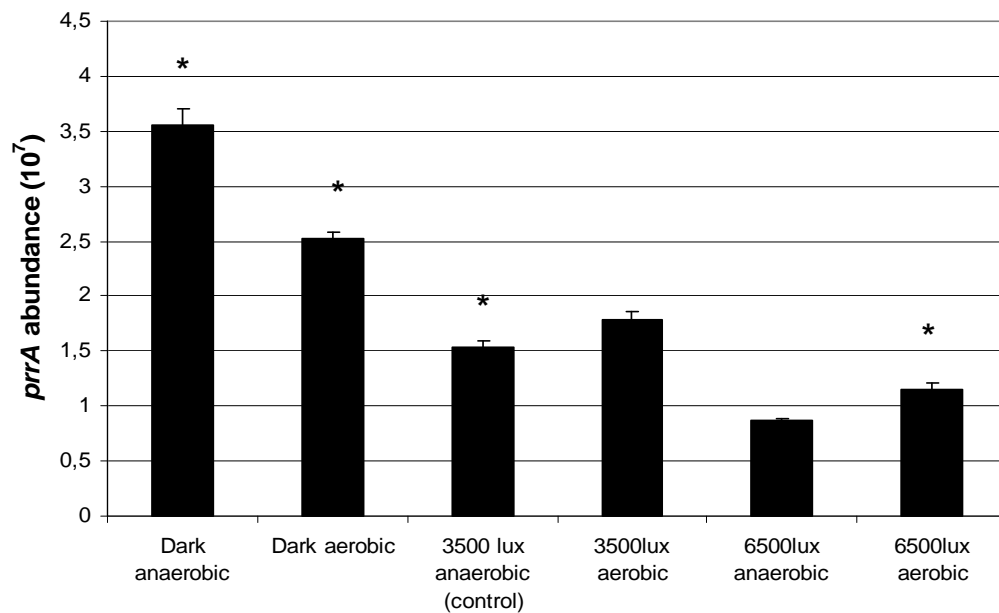


**Figure 3.46. The expression levels of *prrA* gene in 15mM malate-1mM NH₄Cl containing medium at different light intensities (Real-time PCR results)**

The expression level of *prrA* was found to be highest at dark and lowest at 3500 lux anaerobic conditions in reverse-transcriptase PCR. Again the highest expression was observed at dark anaerobic conditions in real-time PCR. However the lowest expression was at 6500 lux anaerobic condition in this case. Real-time PCR data is more reliable because the risk of error is lower because of its precision. There is a significant difference between the control conditions and dark anaerobic, dark aerobic, 6500 lux aerobic conditions with a p-value of 0.05, 0.007 and 0.046, respectively. The lower expression level at 6500 lux can be explained by negative correlation between the expression levels of *prrA* and *nifA* gene. As seen in Figure 3.43 *nifA* expression was higher at 6500 lux. However the expression levels of *prrA* were lower at 6500 lux under aerobic and anaerobic conditions.

# CHAPTER 4

# CONCLUSION

- 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 1mM $NH_4Cl$ containing medium was 2.4 folds lower than in 2mM glutamate containing medium. There was no hydrogen production when ammonium chloride concentration above 2mM.

- 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 5mM and 10mM ammonium chloride containing medium. *prrA* gene was expressed at higher levels with increasing concentrations of $NH_4Cl$.

- There was no significant difference of the growth of bacteria in different concentrations of ammonium chloride containing media under aerobic conditions. Total biomass in 2mM glutamate- 15mM malate containing medium reached 2.7 folds higher values than the total biomass in 2mM $NH_4Cl$ containing media since glutamate can also be used as carbon source.

- Hydrogen production was also observed in aerobic conditions of 1mM, 2mM $NH_4Cl$ and 2mM 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 *nifH* decreased as the concentration of ammonium chloride increased in aerobic conditions and the expression was lost at 5mM

and 10mM $NH_4Cl$. There was expression of *nifA* gene under anaerobic conditions however; it was lost in 5mM and 10mM $NH_4Cl$ containing media under aerobic conditions.

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

- Although no hydrogen production was observed at dark conditions, the expressions of *nifH*, *nifA* and *prrA* genes still exist.

- Bacterial growth was highest at 6500 lux aerobic conditions in all types of media (malate-$NH_4Cl$, malate- glutamate and acetate-glutamate).

- The highest hydrogen production was obtained at 3500 lux anaerobic conditions in acetate-glutamate and malate-ammonium chloride containing media. High light intensity seems to inhibit the hydrogen production in these two media.

- The expression levels of *nifH* gene were not found to be directly proportional to the yield of hydrogen produced. Although the highest hydrogen production was obtained at 3500 lux anaerobic conditions in acetate-glutamate and malate-$NH_4Cl$ containing media, the expressions of *nifH* gene were not highest in the same conditions. Either the translation of mRNA or the nitrogenase enzyme activities may be changing.

- There was no significant difference in expression levels of *nifA* gene at different light intensities in aerobic or anaerobic conditions.

- Expression of *prrA* gene was decreased as the expression level of *nifA* gene increased. This negative correlation requires further investigations.

# REFERENCES

Alber, B. E., Spanheimer, R., Ebenau-Jehle C., Fuchs, G. (2006). **"**Study of an alternate glyoxylate cycle for acetate assimilation by *Rhodobacter sphaeroides"*.Molecular Microbiology **61**(2): 297–309

Asada Y., Miyake J. (1999). "Photobiological Hydrogen production". Journal of Bioscience and Bioengineering **88** (1):1-6

Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., Struhl, K. (Editors) (1995). Short protocols in molecular biology. John Wiley&sons, Inc.

Barbosa, M. J., Rocha, J. M.S., Tramper, J., Wijffels, R. H. (2001). "Acetate as a carbon source for hydrogen production by photosynthetic bacteria". Journal of Biotechnology **85**: 25-33

Basak N., Das D. (2007). "The prospect of purple non-sulfur (PNS) photosynthetic bacteria for hydrogen production: the present state of the art". World J Microbiol Biotechnol **23**:31-42

Bauer, E., Kaspar, T., Fischer, H. M., Hennecke, H. (1998). "Expression of the *fixR-nifA* operon in *Bradyrhizobium japonicum* depends on a new response regulator, RegR". J. Bacteriol. **180**: 3853-3863

Bauer, C.E., Elsen, S., Bird, T.H. (1999). "Mechanism for Redox Control of Gene Expression". Annu. Rev. Microbiol. **53**: 495–523

Biohydrogen, (2008). Hyvolution Extension-Update Annex 1, http://www.biohydrogen.nl/downloadattachment/24778/15387/Annex%20I%20Do W290605update131006TV.doc, February 10, 2008.

Buhrke, T., Lenz O., Krauss N., and Friedrich B. (2005). "Oxygen Tolerance of the $H_2$-sensing [NiFe] Hydrogenase from *Ralstonia eutropha* H16 Is Based on Limited Access of Oxygen to the Active Site". J. Biol. Chem **280** (25): 23791-23796

Burris, R. H. (1991). "Nitrogenases". The Journal of Biological Chemistry **266**(15): 9339-9342

Comolli, J. C., Carl, A.J., Hall, C., Donohue, T. (2002). "Transcriptional activation of the Rhodobacter sphaeroides cytochrome c2 gene p2 promoter by the response regulator PrrA". Journal of bacteriology **184** (2): 390-399

Das D., Veziroglu T. N. (2001). "Hydrogen production by biological processes: a survey of literature". Int J Hydrogen Energy **26**:13–28

Department of Microbiology and Molecular Genetics at the University of Texas Health Science Center, Houston, (2007), *Rhodobacter sphaeroides* genome Project, http://mmg.uth.tmc.edu/sphaeroides/, December 23, 2007.

Dixon, R., Kahn, D. (2004). "Genetic regulation of biological nitrogen fixation". Nature Reviews Microbiology **2** (8): 621-631

Drepper, T., Gross, S., Yakunin, A. F., Hallenbeck, P.C., Masepohl, B., Klip, W. (2003). Role of GlnB and GlnK in ammonium control of both nitrogenase systems in the phototrophic bacterium *Rhodobacter capsulatus*". Microbiology **149**: 2203-2212

Dubbs, J., Tabita, F. R. (2004). "Regulators of nonsulfur purple phototrophic bacteria and the interactive control of CO2 assimilation, nitrogen fixation, hydrogen metabolism and energy generation". FEMs Microbiology Reviews **28**: 353-376

Elsen, S., Dischert, W., Colbeau, A., Bauer, C. E. (2000). "Expression of Uptake Hydrogenase and Molybdenum Nitrogenase in *Rhodobacter capsulatus* Is Coregulated by the RegB-RegA Two-Component Regulatory System". Journal of bacteriology **182** (10): 2831-2837

Eraso, J. M., Kaplan, S. (1996). "Complex Regulatory Activities Associated with the Histidine Kinase PrrB in Expression of Photosynthesis Genes in *Rhodobacter sphaeroides* 2.4.1". Journal of bacteriology **178** (24): 7037-7046

99

Eraso , J. M., Kaplan, S. (2000). "From redox flow to gene regulation: Role of the PrrC protein of *Rhodobacter sphaeroides* 2.4.1". Biochemistry **39**: 2052-2062


Eroglu, İ., Aslan, K., Gündüz, U., Yücel, M., Türker, L. (1999). "Substrate consumption rates for hydrogen production by *Rhodobacter sphaeroides* in a column photobioreactor". Journal of Biotecnology **70**: 103-113


Fang, H. H. P., Liu, H., Zhang, T. (2005). "Phototrophic hydrogen production from acetate and butyrate in wastewater". International Journal of Hydrogen Energy **30**: 785-793


Filatova, L. V., Berg, I. A., Krasil'nikova, E. N., Tsygankov, A. A., Laurinavichene, T. V., Ivanovsky, R. N. (2005). "A study of the mechanism of acetate assimilation in purple nonsulfur bacteria lacking the glyoxylate shunt: acetate assimilation in *Rhodobacter sphaeroides*".  Microbiology **74**: 265-269


Franchi, E., Tosi, C., Scolla, G., Penna, G. D., Rodriguez, F., and Pedroni, P. M. (2004). "Metabolically Engineered Rhodobacter sphaeroides RV strains for Improved Biohydrogen Photoproduction Combined with Disposal of Food Wastes". Marine Biotechnology **6**: 552-565


Goldberg, I., Nadler, V., Hochman, A. (1987).  "Mechanism of Nitrogenase Switch-Off by Oxygen". Journal of Bacteriology **169**: 874-879


Hallenbeck, P. C., Benemann, J. R. (2002). "Biological hydrogen production; Fundamentals and limiting processes". International Journal of Hydrogen Energy **27**: 1185-1193


Happ, H.N., Braatsch S., Broschek, V., Osterloh, L., Klug, G. (2005). "Light-dependent regulation of photosynthesis genes in *Rhodobacter sphaeroides* 2.4.1 is coordinately controlled by photosynthetic electron transport via the PrrBA two-component system and the photoreceptor AppA". Molecular microbiology **58**(3): 903-914


Halbleib, C. M., Ludden, P. W. (2000). "Regulation of Biological Nitrogen Fixation". Journal of nutrition **132** (12): 1081-1084

Hillmer, P., Gest, H. (1977). "H$_2$ metabolism in the photosynthetic bacterium *Rhodopseudomonas capsulata*: production and utilization of H$_2$ by resting cultures". Journal of Bacteriology **129**:732–739

Hochman, A, Burris, RH (1981) "Effect of oxygen on acetylene reduction by photosynthetic bacteria". Journal of Bacteriology **147**: 492–499

Holt, J. G., Stanley, J. T., Bryant, M. P., Pfenning, M. P. (Editors) (1984). Bergey's Manual of Systematic Bacteriology, Vol. 3, Williams and Wilkins, Baltimore

Hübner, P., Willison, J.C., Vignais, P.M., Bickle T. A. (1991). "Expression of regulatory nif genes in Rhodobacter capsulatus". Journal of bacteriology **173** (9): 2993-2999.

Hydrogen and Fuel Cells Canada, (2008). About Hydrogen, http://www.h2fcc.ca/cfm/index.cfm?It=904&Id=3, January 15, 2008

Igarashi, R. Y., Seefeldt, L. C. (2003). "Nitrogen Fixation: The Mechanism of the Mo-Dependent Nitrogenase". Critical Reviews in Biochemistry and Molecular Biology**, 38:** 351–384

Ivanovsky, R N., Krasilnikova, E.N., Berg I. A. (1997). "A proposed citramalate cycle for acetate assimilation in the purple non-sulfur bacterium *Rhodospirillum rubrum*". FEMS Microbiology Letters **153**: 399-404

Kern, M., Kamp, P. B., Paschen, A., Masepohl, B., Klip, W. (1998). "Evidence for a Regulatory Link of Nitrogen Fixation and Photosynthesis in *Rhodobacter capsulatus* via HvrA". Journal of Bacteriology **180**: 1965-1969

Kiley, P.J., Kaplan, S. (1988). "Molecular Genetics of Photosynthetic Membrane Biosynthesis in Rhodobacter sphaeroides". Microbiological reviews **52** (1): 50-69

Koku, H., Eroglu, İ., Gündüz, U., Yücel, M., Türker, L. (2002). "Aspects of the metabolism of hydrogen production by *Rhodobacter sphaeroides*". International Journal of Hydrogen Energy **27**: 1315 – 1329

Koku, H. (2001). "Hydrogen metabolism and factors affecting  hydrogen production in *Rhodobacter sphaeroides*". Msc. Thesis, Middle East Technical University

Kovacs, K. L., Bagyinka, C. (1990). "Structural properties, Functional States and Physiological Roles of Hydrogenase in Photosynthetic Bacteria". FEMS Microbiology Reviews **87**: 407-412

Madigan, M., Cox, S.S., and Stegeman, R.A. 1984. "Nitrogen fixation and nitrogenase activities in members of the family *Rhodospirillaceae*". J. Bacteriol. **157**: 73–78.

Madigan,M.T., Martinko, J.M., Parker, J. Brock Biology of Microorganisms, 10th edition, Prentice Hall, Inc, 2003

Maner, K., Oelze, J. (1999). "Control by light of whole-cell nitrogenase activity and of nitrogenase and bacteriochlorophyll a formation in Rhodobacter capsulatus strain B10S". Archives of Microbiology **171**: 243-248

Merrick, M. J. and Edwards, R. A. (1995). "Nitrogen control in bacteria". Microbiol.Revs., **59**, 604-622.

Merrick, M. J. (2004). "Genetics and Regulation of Nitrogen Fixation in Free-Living Bacteria" (Klipp W., Masepohl B., Gallon J.R. and Newton W.E., eds.), pp. 197-223, Kluwer Academic Publishers, Netherlands.

Meyer, J., Kelley, B.C., Vignais, P.M. (1978). "Nitrogen fixation and hydrogen metabolism in photosynthetic bacteria". Biochimie **60**:245-260

Miyake, J., Miyake, M., Asada, Y. (1999). " Biotechnological hydrogen production: research for efficient light energy conversion". Journal of biotechnology **701**: 89-101

Nath, K., Kumar, A., Das, D. (2005). "Hydrogen production by *Rhodobacter sphaeroides* strain O.U.001 using spent media of *Enterobacter cloacae* strain DM11". Appl Microbiol Biotechnol **68**: 533–541

Oelze J., Klein, G. (1996). "Control of nitrogen fixation by oxygen in purple nonsulfur bacteria". Archives of Microbiology **165**: 219-225

O'Gara, J. P., Eraso, J. M., Kaplan S. (1998). "A Redox-Responsive Pathway for Aerobic Regulation of Photosynthesis Gene Expression in *Rhodobacter sphaeroides* 2.4.1". Journal of Bacteriology **180** (16): 4044-4050

Paschen, A., Drepper, T., Masepohl, B., Klip, W. (2001). "*Rhodobacter capsulatus nif*A mutants mediating *nif* gene expression in the presence of ammonium". FEMS Microbiology Letters **200**: 207-213

Pawlowski, A., Riedel, K. U., Klip, W., Dreiskemper, P., Gross, S., Bierhoff, H., Drepper, T., Masepohl, B. (2003). "Yeast two-hybrid studies on interaction of proteins involved in regulation of nitrogen fixation in the phototrophic bacterium *Rhodobacter capsulatus*". Journal of Bacteriology **185** (17): 5240-5247

Qian, Y. and Tabita, F. R. (1996). "A global signal transduction system regulates aerobic and anaerobic $CO_2$ fixation in *Rhodobacter sphaeroides*". J Bacteriol, **178**, 12-18.

Rey, F. E., oda, Y., Harwood, C. S. (2006). "Regulation of Uptake Hydrogenase and Effects of Hydrogen Utilization on Gene Expression in *Rhodopseudomonas palustris*". Journal of Bacteriology **188** (17): 6143-6152

Rey, F. E., Heiniger, E. K., Harwood, C. S. (2007). "Redirection of metabolism for biological hydrogen production". Applied and environmental microbiology **73** (5): 1665-1671

Riis, T., Hagen, E. F. (2005). Hydrogen Production - Gaps and Priorities IEA. Hydrogen Implementing Agreement (HIA). pp: 1-11

Ribbe, M., Gadkari, D., and Meyer, O. (1997). $N_2$ fixation by *Streptomyces thermoautotrophicus* involves a molybdenum-dinitrogenase and a manganese-superoxide oxidoreductase that couple $N_2$ reduction to the oxidation of superoxide produced from $O_2$ by a molybdenum-CO dehydrogenase. *J Biol Chem* **272**: 26627-26633.

Roh, J. H., Smith, W. E., Kaplan, S. (2004). "Effects of oxygen and light intensity on transcriptome expression in *Rhodobacter sphaeroides* 2.4.1". the Journal of Biological chemistry **279** (10): 9146-9155

Ryalls, J.Z., Gomelsky, M., Erosa, J.M., Yeliseev, A., O'Gara, J., Kaplan S. (1998). "Control of photosystem formation in *Rhodobacter sphaeroides*". Journal of bacteriology **180** (11): 2801-2809

Sasikala, K, Ramana, ChV, Rao, PR. (1991). "Environmental regulation for optimal biomass yields and photo production of hydrogen by *Rhodobacter sphaeroides* O.U.001". Int J Hydrogen Energy **16**:597–601

Siemann, S., Schneider, K., Dröttboom, M., and Müller, A. (2002). "The Fe-only nitrogenase and Mo nitrogenase from *Rhodobacter capsulatus*. A comparative study on the redox properties of the metal clusters present in the dinitrogenase components". Eur J. Biochem **269**, 1650–1661.

Souza EM, Pedrosa F.o., Drummond M., Rigo L.U., Yates M.G. (1999). "Control of Herbaspirillum seropedicae NifA activity by ammonium ions and oxygen". Journal of bacteriology **181**: 681-684

Souza, E. M., Pedrosa, F. O., Rigo, L. U., Machado H. B., and. Yates, M. G. (2000). "Expression of the *nifA* gene of *Herbaspirillum seropedicae*: role of the NtrC and NifA binding sites and of the -24/-12 promoter element". Microbiology, **146**, 1407-1418

Tichi, M.A., Tabita, F.R. (2001). "Interactive Control of *Rhodobacter capsulatus* Redox-Balancing Systems during Phototrophic Metabolism". Journal of bacteriology **183**(21): 6344-6354

Tremblay, P.L ., Drepper, T., Masepohl, B., Hallenbeck, P. C. (2007). "Membrane Sequestration of PII Proteins and Nitrogenase Regulation in the Photosynthetic Bacterium *Rhodobacter capsulatus*". Journal of Bacteriology **189** (16):5850-5859

Uyar, B., Eroglu, İ., Yücel, M., Gündüz, U., Türker, L., (2007). "Effect of light intensity, wave length and illumination protocol on hydrogen production in photobioreactors". International Journal of Hydrogen Energy **32**: 4670-4677

Van Niel, C. B. (1944). "The culture, general physiology, morphology, and classification of the nonsulfur purple and brown bacteria". Bacteriol. Rev. **8:**1-118.

Vignais, P.M., Colbeau, A., Willison, J.C., Jouanneau, Y. (1985). "Hydrogenase, nitrogenase and hydrogen metabolism in photosynthetic bacteria". Adv. Microbial Phys. **26**:154-234

Vignais, P. M., Magnin, JP., Willison J. C. (2006). "Increasing Biohydrogen production by metabolic engineering". International journal of hydrogen energy **31**: 1478-1483

Waligórska, M., Seifert K., Szymańska K., and Łaniecki M. (2006). "Optimization of activation conditions of *Rhodobacter sphaeroides* in hydrogen generation process". Journal of Applied Microbiology **101**: 775–784

Wikipedia The Free Encyclopdia (2007). Hydrogen, http://en.wikipedia.org/wiki/Hydrogen#Properties, December 21, 2007

Yakunin, A.F., Hallenbeck, P.C. (1998). "Short-term regulation of nitrogenase activity by $NH_4^+$ in *Rhodobacter capsulatus*: Multiple in vivo nitrogenase responses to $NH_4^+$ addition". Journal of bacteriology **180** (23): 6392-6395

Yakunin, A. F., Hallenbeck, P. C. (2000). "Regulation of nitrogenase activity in *Rhodobacter capsulatus* under dark microoxic conditions". Archives of Microbiology **173:** 366-372

Yakunin, A. F., Fedorov, A. S., Laurinavichene T. V., Glaser, V. M., Egorov, N. S., Tsygankov, A. A., Zinchenko, V. V., Hallenbeck P. C. (2001). **"**Regulation of nitrogenase in the photosynthetic bacterium *Rhodobacter sphaeroides* containing *draTG* and *nifHDK* genes from *Rhodobacter capsulatus"*. Can. J. Microbiol. **47**(3): 206–212

# APPENDIX A

# LIST OF CHEMICALS AND SUPPLIERS

**Table A.1. The chemicals and suppliers**

| | | | |
|---|---|---|---|
| **KH$_2$PO$_4$** | MERCK | **Glutamic acid** | SIGMA |
| **NH$_4$Cl** | MERCK | **DL-Malic Acid** | SIGMA |
| **CaCl$_2$.2H$_2$O** | MERCK | **Thiamin** | SIGMA |
| **Biotin** | MERCK | **Tri-Reagent** | SIGMA |
| **Acetic acid** | MERCK | **Dimethylsulfoxide (DMSO)** | SIGMA |
| **H$_3$BO$_3$** | MERCK | **Phenol solution** | ALDRICH |
| **HCl** | MERCK | **CuCl$_2$.2H$_2$O** | ALDRICH |
| **Niacin** | APPLICHEM | **DNA ladder (GeneRuler 50 bp)** | FERMENTAS |
| **MnCl$_2$. 4H$_2$O** | APPLICHEM | **dNTP set** | FERMENTAS |
| **ZnCl$_2$** | APPLICHEM | **6x loading dye** | FERMENTAS |
| **Agarose Low EEO** | APPLICHEM | **Taq DNA polymerase** | FERMENTAS |
| **CoCl$_2$.6H$_2$O** | APPLICHEM | **M-MuLV Reverse transcriptase** | FERMENTAS |
| **Ni Cl$_2$.6H$_2$O** | APPLICHEM | **Fe-citrate** | MERCK |
| **Diethylpyrocarbonate** | APPLICHEM | **C$_6$H$_5$Na$_3$O$_7$.5.5H$_2$O** | APPLICHEM |
| **NaCl** | APPLICHEM | **Bacteriological agar** | APPLICHEM |
| **MgSO$_4$.7H2O** | APPLICHEM | **NaOH** | Riedel-de Haën |
| **Tris-HCl** | APPLICHEM | **Ethanol absolute** | APPLICHEM |
| **Tris-base** | APPLICHEM | **Isopropyl alcohol** | AMBRESCO |
| **EDTA** | APPLICHEM | **NaMoO$_4$.2H$_2$O** | MERCK |

# APPENDIX B

# SOLUTIONS AND BUFFERS

## B.1. Composition of Culture Media

**Table B.1. Composition of the culture media**

| Ingredients | Concentration (g/l) |
|---|---|
| $KH_2PO_4$ | 0.5 |
| $MgSO_4. 7H_2O$ | 0.2 |
| NaCl | 0.4 |
| Sodium glutamate (2mM) | 0.37 |
| $CaCl_2. 2H_2O$ | 0.05 |
| L-malic acid (15mM) | 2.0 |
| Vitamin solution | 1ml |
| Trace element solution | 1 ml |
| Fe-citrate solution | 1ml |

## B.2. Trace Element Solution

The composition of 1000ml trace element solution is given in Table A.2. The solution is autoclaved for sterilization after all ingredients are ingredients are dissolved in 1000ml water.

**Table B.2. The composition of trace element solution**

| Ingredients | Concentration (mg/l) |
|---|---|
| $ZnCl_2$ | 70 |
| $MnCl_2.4H_2O$ | 100 |
| $H_3BO_3$ | 60 |
| $CoCl_2.2H_2O$ | 200 |
| $CuCl_2.2H_2O$ | 20 |
| $NiCl.6H_2O$ | 20 |
| $Na_2Mo_4.2H_2O$ | 40 |
| HCl | 1 ml/l |

## B.3. Vitamin Solution

Vitamin solution is sterilized by filtering after all ingredients are dissolved in 1000 ml water.

**Table B.3 The composition of vitamin solution**.

| Vitamins | Concentration (mg/l) |
|---|---|
| Thiamine | 500 |
| Niacin | 500 |
| Biotin | 15 |

## B.4. Fe-Citrate Solution

0.5 g of Fe (III). Citrate.Hydrate was dissolved in 100ml water and autoclaved for sterilization.

## B.5. Solutions and Buffers

**TAE**

40 mM Tris base (Buffer grade)

1mM EDTA disodium dihydrate

Glacial acetic acid

**Protoplasting buffer**

15 mM Tris-HCl

0.45 M Sucrose

8 mM EDTA

pH is adjusted to 8.0

**Gram-negative Lysing Buffer**

10mM Tris-HCl

10 mM NaCl

10 mM sodium citrate

1.5 % (w/v) SDS

pH is adjusted to 8.0

**Saturated NaCl**

40g NaCl in DEPC-treated water

**Ethidium Bromide**

10mg ethidium bromide is dissolved in 1 ml water.

# APPENDIX C

# FORMULAS

- Substrate Conversion Efficiency is calculated by the equation below:

$$\text{Substrate conversion efficiency} = \frac{n_{actual}}{n_{theoretical}} \cdot 100 \qquad (C.1)$$

$n_{actual}$: number of moles of hydrogen that is actually produced

$n_{theoritical}$: number of moles hydrogen that would be produced if all of the substrate is converted to hydrogen through the stoichiometric equation

Stoichiometry of malate and acetate consumption for hydrogen production:

$$C_4H_6O_5 + 3H_2O \longrightarrow 6H_2 + 4CO_2 \qquad (C.2)$$

$$C_2H_4O_2 + 2H_2O \longrightarrow 4H_2 + 2CO_2 \qquad (C.3)$$
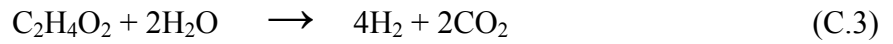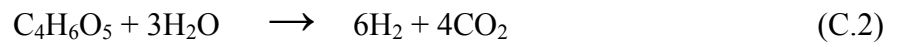
- The average gas production was calculated by the formula below:

$$r_{gas,\ avg} = \frac{(V_2 - V_1)}{(t_2 - t_1) \cdot V_{culture}} \qquad (C.\ 4)$$

The volume of hydrogen gas produced per volume of culture versus time graph was plotted and the rate of average gas was calculated from the slope of that graph by omitting the lag time for hydrogen production.

- Yield of hydrogen was calculated by the formula below:

$$\text{Weight of hydrogen produced/weight of substrate} \qquad (C.5)$$

Weight of hydrogen produced was calculated by taking atmospheric pressure as 680 mmHg at Ankara, and temperature as 30°C. Therefore 1mol $H_2$ gas was defined as 25.47 l ($H_2$ was assumed as ideal gas). Organic acids (malic acid and acetic acid) were taken as substrate. Therefore the weights of malic acid and acetic acid were calculated.
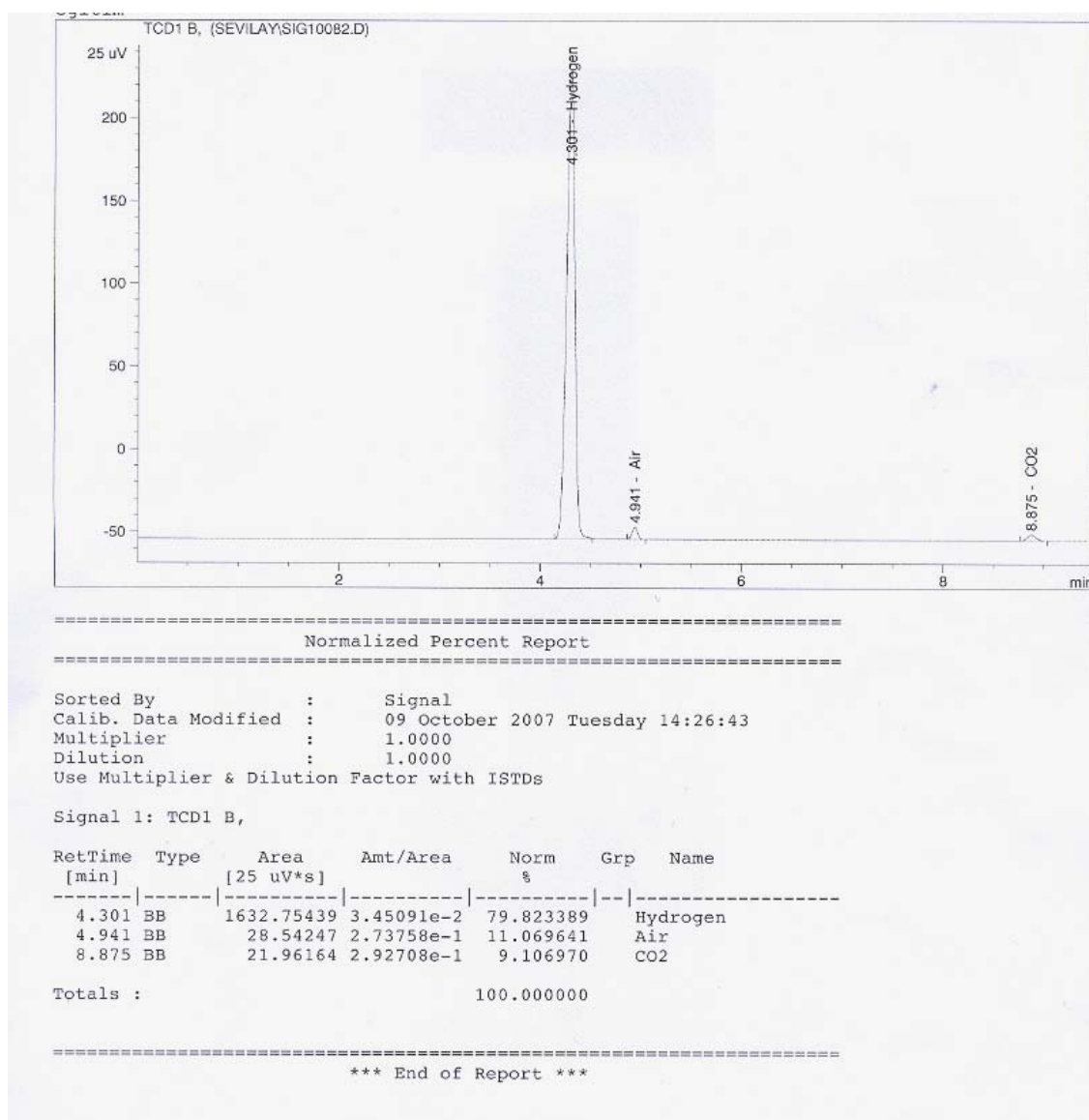
- Light conversion efficiency was calculated according to the equation below:

$$\eta = [(33.61 \cdot \rho_{H2} \cdot V_{H2}) / (I \cdot A \cdot t)] \cdot 100 \qquad (C.6)$$

where $V_{H2}$ is the volume of hydrogen produced in liter, $\rho_{H2}$ is the density of the produced hydrogen gas in g/l, I is the light intensity in W/m², A is irradiated area in m² and t is the duration of hydrogen production in hours.

# APPENDIX D

# SAMPLE GC ANALYSIS



```
==================================================================
                      Normalized Percent Report
==================================================================

Sorted By              :      Signal
Calib. Data Modified   :      09 October 2007 Tuesday 14:26:43
Multiplier             :      1.0000
Dilution               :      1.0000
Use Multiplier & Dilution Factor with ISTDs

Signal 1: TCD1 B,

RetTime Type     Area      Amt/Area     Norm     Grp   Name
 [min]         [25 uV*s]                  %
-------|------|----------|----------|----------|--|-------------------
  4.301 BB    1632.75439 3.45091e-2  79.823389       Hydrogen
  4.941 BB      28.54247 2.73758e-1  11.069641       Air
  8.875 BB      21.96164 2.92708e-1   9.106970       CO2

Totals :                            100.000000


==================================================================
                       *** End of Report ***
```

# APPENDIX E

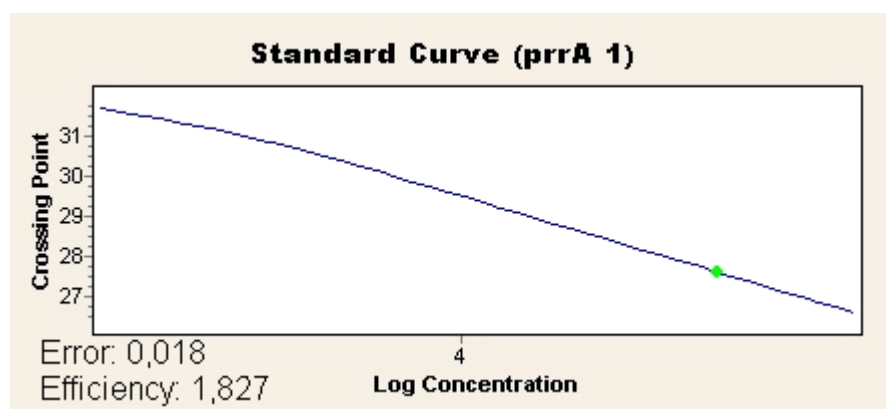# STANDARD CURVE AND AMPLIFACTION CURVES OF
# REAL-TIME PCR



**Figure E.1. Standard curve of *prrA* gene for real-time experiments**
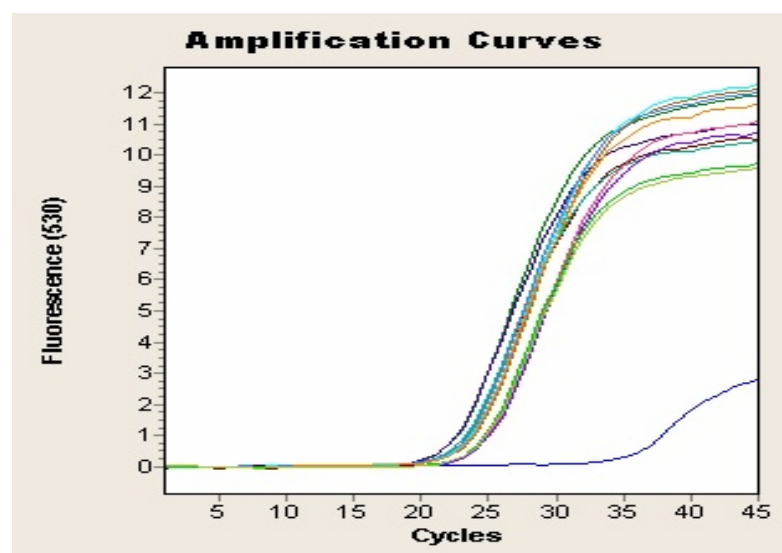


**Figure E.2. Amplification curve of *prrA* gene products from 1mM NH₄Cl and 15mM malate containing medium at different light intensities**
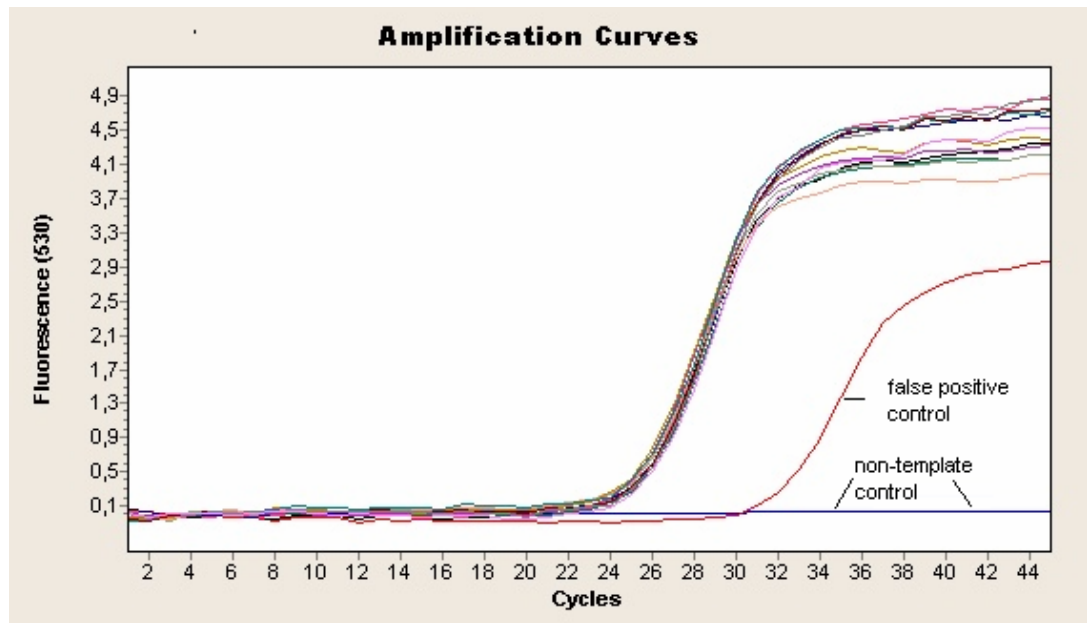
**Figure E.3. Amplification curve of *prrA* gene product from 2mM glutamate and 30mM acetate containing medium at different light intensities**
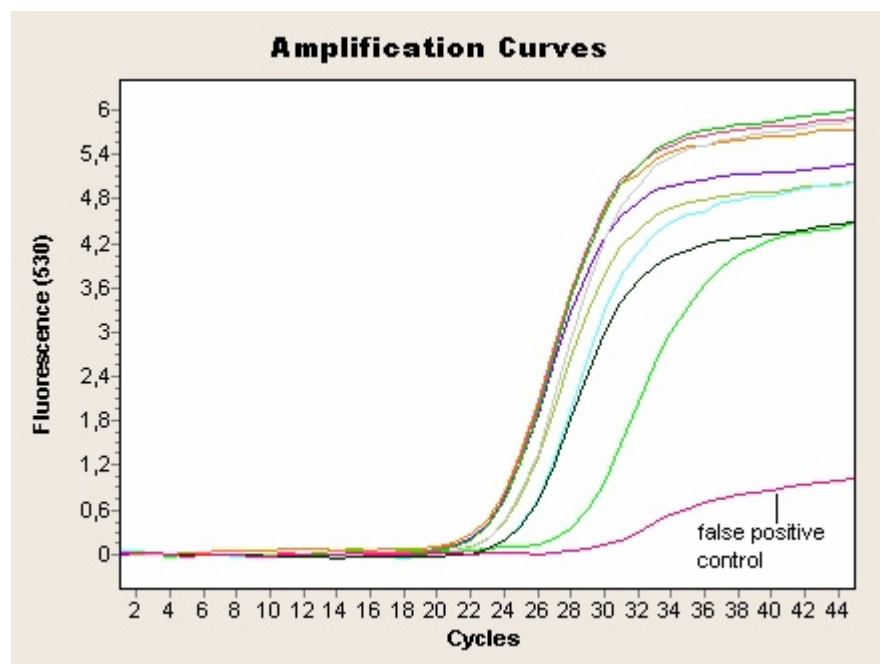


**Figure E.4. Amplification curve of *prrA* gene product from different concentrations of ammonium chloride containing medium under aerobic condition**