IMPROVEMENT OF BIOHYDROGEN PRODUCTION BY
GENETIC MANIPULATIONS IN *RHODOBACTER SPHAEROIDES* O.U.001

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**IMPROVEMENT OF BIOHYDROGEN PRODUCTION BY
GENETIC MANIPULATIONS IN *RHODOBACTER SPHAEROIDES* O.U.001**

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I hereby declare that all information in this document has been obtained and presented in accordance with academic rules and ethical conduct. I also declare that, as required by these rules and conduct, I have fully cited and referenced all material and results that are not original to this work.

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ABSTRACT

IMPROVEMENT OF BIOHYDROGEN PRODUCTION BY GENETIC MANIPULATIONS IN *RHODOBACTER SPHAEROIDES* O.U.001

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Rhodobacter sphaeroides O.U.001 is a purple non-sulphur bacterium producing hydrogen under photoheterotrophic, nitrogen limited conditions. Hydrogen is produced by Mo-nitrogenase but substantial amount of H_2 is reoxidized by a membrane bound uptake hydrogenase. In this study, hydrogen production and the expression of structural nitrogenase genes were investigated by varying molybdenum and iron ion concentrations. These two elements are found in the structure of Mo-nitrogenase and they are important for functioning of the enzyme. The results showed that hydrogen production and *nifD* gene expression increased upon increase in molybdenum concentration. Increasing iron concentration had also positive effect on hydrogen production and *nifK* gene expression.

To improve the hydrogen producing capacity of *R. sphaeroides* O.U.001, *hup*SL genes encoding uptake hydrogenase were disrupted in two different methods. In the first method, *hup* genes were disrupted by gentamicin resistance gene insertion. In the second method, part of the *hup* gene was deleted without using antibiotic resistance gene.

The wild type and the *hup*⁻ mutant cells showed similar growth patterns but substantially more hydrogen was produced by the mutant cells.

The genes coding for hox1 hydrogenase of *Thiocapsa roseopersicina* was aimed to be expressed in *R. sphaeroides* O.U.001 to produce H_2 under nitrogenase repressed and mixotrophic conditions. The hox1 hydrogenase genes of *T. roseopersicina* were cloned and transferred to *R. sphaeroides*. Although the cloning was successful, the expression of hydrogenase was not achieved by using either the native promoter of hox1 hydrogenase or the *crtD* promoter of *T. roseopersicina*.

ÖZ

RHODOBACTER SPHAEROIDES O.U.001 DE BİYOHİDROJEN ÜRETİMİNİN GENETİK YOLLARLA ARTTIRILMASI

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Rhodobacter sphaeroides O.U.001 mor sülfürsüz bir bakteri olup fotoheterotrofik ve kısıtlı azot ortamlarında hidrojen üretebilmektedir. Yabansoy bakteride hidrojen, Mo-nitrojenaz tarafından üretilmekte ancak önemli bir kısmı gerialım hidrojenazı tarafından kullanılmaktadır. Bu çalışmada değişen molibden ve demir derişimlerinin hidrojen üretimine ve Mo-nitrojenaz ı kodlayan genlerin ifadelerine olan etkisi araştırılmıştır. Bu iki element Mo-nitrojenazın yapısında bulunup enzimin fonksiyonu için gereklidir. Sonuçlara göre, hidrojen üretimi ve *nifD* gen ifadesi artan molibden derişimine paralel olarak artmıştır. Artan demir derişimide hidrojen üretimine ve *nifK* gen ifadesine olumlu etki yapmıştır.

R.sphaeroides O.U.001 de hidrojen üretimi verimliliğini arttırmak için *hupSL* genleri iki farklı yöntemle bloke edilmiştir. İlk yöntemde, *hup* genleri, içine gentamisine dirençlilik geni sokularak tahrip edilmiştir. İkinci yöntemde ise, *hup* geninin bir kısmı silinerek enzim tahrip edilmiştir. Mutant ve yaban soy bakteriler benzer üreme profili göstermiş ve mutant bakteriler önemli derecede daha çok hidrojen üretmişlerdir.

Hidrojenin nitrojenaz enziminin baskılandığı koşullardada üretilebilmesi için *Thiocapsa roseopersicina* da bulunan hox1 hidrojenaz genlerinin, *R. sphaeroides* O.U.001 de ifade edilmesi hedeflenmiştir. Bunun için *hox1* genleri klonlanmış ve *R. sphaeroides* O.U.001' e aktarılmıştır. *Hox*1 ve *crtD* promotoru kullanılarak yapılan klonlamalar başarılı olmuş ancak hox1 hidrojenazı hücre içinde oluşmamıştır.

Anahtar Kelimeler: *Rhodobacter sphaeroides*, Biyohidrojen, Gerialım hidrojenazı, Yönlendirilmiş mutagenez, Hox1 hidrojenaz

To The Memory of Zekai Kars

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LIST OF SYMBOLS

anf HDGK	Genes coding for iron only nitrogenase
ATP	Adenosine tri phosphate
DEPC	Diethylpyrocarbonate
Fe	Iron
FADH	Flavine adenine dinucleotide (reduced form)
GC	Gas chromatography
H_2	Hydrogen gas
H_2ase	Hydrogenase
hupL	Gene coding for uptake hydrogenase large subunit
hupS	Uptake hydrogenase small subunit
Mo	Molybdenum
N_2	Nitrogen gas
N_2ase	Nitrogenase
NADH	Nicotinamide adenine dinucleotide (reduced form)
NCBI	National Center of Biotechnology Information
Ni	Nickel
nifDK	Molybdenum nitrogenase component 1
nifH	Molybdenum nitrogenase component 2
PAGE	Poly acrylamide gel electrophoresis
PHB	Polyhydroxybutyrate
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
vnf HDGK	Genes coding for vanadium nitrogenase

CHAPTER 1

INTRODUCTION

1.1 Biological hydrogen production

The world's energy demand is continuously increasing though the fossil fuels being the major energy sources are not being formed at the consumption rates. Therefore, new alternative energy sources are still being investigated. Hydrogen as being one of the future alternative energy carriers can help to discover and improve renewable and sustainable energy systems. The hydrogen as an energy carrier could safely be used in the future due to the following reasons;

1. Hydrogen could be produced from sustainable energy sources such as biomass, solar energy, hydropower, wind power etc.
2. The wide range use of hydrogen will omit the environmental impacts of fossil based fuels due to the emissions.
3. The storage and distribution of hydrogen could be done safely.
4. Hydrogen can be used as a fuel for a wide range of end-use applications such as in the transportation and household uses.
5. Since hydrogen production would be location independent, it would assist in energy diversity and world peace (Elam *et al.*, 2003).

Today, although the hydrogen gas was mostly produced through the non-biological means (chemical ways) such as steam reforming, biological hydrogen production methods are still being investigated and being improved. The chemical methods which rely on fossil fuels should be replaced with biological hydrogen pro-

duction processes which are mostly operated at ambient temperatures and pressures, thus less energy intensive (Das and Veziroğlu, 2001). Biological hydrogen production processes can be classified as follows:

1. Hydrogen production by dark fermentation
2. Photobiological hydrogen production
      2.1 Photoautotrophic hydrogen production
      2.2 Photoheterotrophic hydrogen production
3. Two stage hydrogen production

The biological hydrogen production processes were carried out by microorganisms or their enzymes. Therefore, it was suggested that the overall efficiency of the process fundamentally depends on the amount or activity of these enzymes (Hallenbeck and Benemann, 2002). However, studies showed that, not only the activity and amount of the enzymes but also the other metabolic events such as electron flow to the enzymes determine the overall efficiency of the process (Jeong and Jouanneau, 2000). The simple reaction catalyzed by hydrogen producing enzymes is as follows;

$$2H^+ + 2e^- \leftrightarrow H_2 \tag{1.1}$$

Although the reaction seems simple, hydrogen evolving enzymes show that they contain complex metallo-clusters as active sites and that the active enzyme units are synthesized in a complex processes involving auxiliary enzymes and protein maturation steps. These active sites in hydrogenases unusually interact with CO and CN (Leach and Zamble, 2007). There are four enzymes taking role in hydrogen production processes: nitrogenase, FeFe-hydrogenase, NiFe hydrogenase and metal free hydrogenase all of which will be explained later in this chapter.

In the biohydrogen production processes, the ultimate energy source is the sun. The bacteria can use the biomass, CO_2, and light energy to drive the hydrogen reactions. These processes are examined in the following sections.

1.1.1 Hydrogen production by dark fermentation

Many microorganisms living under anoxic environments can produce hydrogen. These microorganisms can oxidize organic substrates and reduce them to small organic acids while producing enough energy for their cellular metabolism. In more efficient bioprocesses, the feedstocks, or waste materials could also be used. But in that case, mechanical or enzymatic pretreatment are applied to produce hexoses, or pentoses from polysaccharides. They do not need additional energy source such as light as in the case of photobiological hydrogen production. However, since the reduction of organic substrates (hexoses) is not complete up to CO_2, the reduction ends up with the small organic acids such as acetic acid, lactic acid, butyric acid etc (Akkerman *et al.*, 2003). Various kinds of specific controls are necessary to regulate electron flow in the metabolism of strict and facultative anaerobs. One of these regulatory mechanisms is reflected by the ability of many such organisms to dispose excess electrons in the form of molecular hydrogen through the activity of hydrogenase. The reduction of protons to hydrogen provides the bacterium balancing its reducing environment. However, not all types of hydrogenases are hydrogen producing, but there are hydrogenases called uptake hydrogenases (NiFe hydrogenases) convert the hydrogen back to protons and electrons. The electrons are then used to reduce NAD to NADH+H^+ via quinine pool in the membrane. Therefore, these hydrogenases function to reproduce the reducing equivalents which then could be used for cellular purposes (Akkerman *et al.*, 2003).

The theoretical yield per mole of glucose is described in the following reaction:

$$C_6H_{12}O_6 + 4H_2O \longrightarrow 2CH_3COO^- + 2HCO_3 + 4H^+ + 4H_2, \quad \Delta G^0 = -206 \ kJ.mol^{-1} \quad (1.2)$$
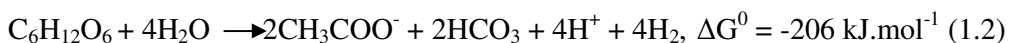
One of the advantages of dark fermentation based hydrogen production is that microorganisms can produce hydrogen constantly through day and night from organic substrates. Moreover, there are hydrogenases which are tolerant to oxygen and are not affected by the high hydrogen pressure (Vincent *et al.*, 2007).

Species of *Clostridia*, rumen bacteria, thermophiles such as *Caldicellulosiruptor* and *Thermotoga* and methanogens are strict hydrogen producing anaerobs. The yield depends on the type of microorganisms and fermantation. In the cultures of *Caldicellulosiruptor saccharolyticus* grown on sucrose at 70 ºC, stoichiometries of 3.3 mole H_2 per mole hexose was obtained which is 83 % of the theoretical value. Maximal hydrogen production rate of 8.4 mmol/L.h was measured. These results show that higher hydrogen yields on hexose can be reached by extreme and hyper-themophiles compared to mesophilic facultative and strict anaerobes (Akkerman *et al.*, 2003). Although the methanogens produce hydrogen gas, they then consume these hydrogen gasses in order to produce methane.   Therefore, the reactor operating conditions should be well adjusted depending on the purpose.

Strict anaerobes as the names imply are very sensitive to oxygen and often do not survive at low oxygen concentrations but facultative anaerobes are resistant to oxygen and these bacteria have the advantage of rapidly consuming oxygen thereby restoring anaerobic conditions immediately in reactors which is suitable for the hydrogen production. But facultative anaerobes could probably serve as a model to find oxygen tolerant hydrogenase. In recent studies, the oxygen insensitive hydrogenases are being investigated and the studies are promising for the future of biohydrogen production (Duche *et al.*, 2005 and Vincent *et al.*, 2007).

1.1.2 Photobiological hydrogen production

Photobiological hydrogen production can occur by photoautotrophic or photoheterotrophic organisms. Microalgae and cyanobacteria are photoautotrophic hydrogen producers because they can use light as the energy source and carbon dioxide as sole carbon source. Photoheterotrophic hydrogen production was performed by nitrogen fixing bacteria since the responsible enzyme is nitrogenase. Both processes are discussed in detail in the following sections.

1.1.2.1 Photoautotrophic hydrogen production

Microalgae and cyanobacteria are able to fix CO_2 to make energy-rich organic compounds $[C_n(H_2O)_n]$ using sunlight during oxygenic photosynthesis. Under anaerobic conditions, these microorganisms can produce hydrogen by photolysis of water using light as the energy source. The catalyst is oxygen sensitive hydrogenase.

$$4H_2O + \text{light energy} => 2O_2 + 4H_2, \Delta G^0 = +1498 \text{ kJ} \quad\quad\quad\quad (1.3)$$
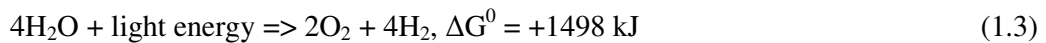
Therefore, the produced hydrogen is mixed with oxygen which is a series problem. Hence the hydrogen gas should be separated from oxygen. This process in which the light energy is directly used for water photolysis is called direct photolysis. Moreover if the oxygen and hydrogen productions were separated in time or place, the process is then called indirect photolysis. In studies done with the green algae *Chlamydomonas reinhardtii*, the phase of photosynthetic O_2 evolution and carbon accumulation to a phase of H_2 production and consumption of metabolites were separated by sulphur (S) deprivation (Melis *et al.*, 2000 and Melis 2002). The heterocystous cyanobacteria are the examples for the separation of the processes by place. Heterocysts are specialized cells in which the nitrogenase is protected from O_2 inhibition, and substrates are provided from the vegetative cells. Despite there are potentials for direct photolysis to be used for hydrogen production, the oxygen sensitivity of hydrogenases and low light conversion efficiency remain as the main limiting factors. And, the indirect photolysis even remains to be studied and improved (Hallenbeck and Benemann, 2002).

1.1.2.2 Photoheterotrophic hydrogen production

The microalgae and plants have two photosystems PSI and PSII but the purple bacteria contain only one photosystem (PS) which is fixed in the intracellular membrane (Akkerman *et al.*, 2003). However, the photosystem of purple bacteria is

not powerful enough to split water and to produce hydrogen. These bacteria under anaerobic conditions are able to use simple organic acids such as acetic acid and the electrons that are liberated from the organic acids are delivered to the electron carriers in the membrane. During the transfer of electrons in the membrane, the protons are pumped out creating proton motive force. In addition to organic acids dihydrogensulfide (H_2S) can also be used as electron donor. The hydrogen formation is catalyzed by nitrogenase which takes up electrons from ferredoxin (Fd). Under ammonia-limited circumstances, these electrons are used by the nitrogenase enzyme to reduce molecular nitrogen into ammonium using extra ATP energy. In the absence of any substrate such as N_2 nitrogenase acts as ATP dependent hydrogenase and all the electrons are utilized for H_2 production with the electrons derived from the ferredoxin (Fd) (Kars *et al*., 2008; Thorneley and Lowe, 1983; Liang and Burris, 1988). For each electron to pass from nitrogenase, two ATP is used. Since two electrons are needed for one H_2, four ATPs are consumed by nitrogenase;

$$2H^+ + 2e^- + 4ATP \longrightarrow H_2 + 4ADP + 4P_i \qquad (1.4)$$

Since oxygen is not produced during the process (anoxygenic photosynthesis), nitrogenase is not inactivated. The hydrogen production property of nitrogenase is an inherent character of the enzyme.

The disadvantage of the nitrogenase based hydrogen production over hydrogenase based hydrogen production is that it requires extra energy in the form of ATP. However, the hydrogenases do not need extra energy. Despite this, capturing energy from the sun and electrons from the feedstocks or waste streams, photoheterotrophic hydrogen production process becomes feasible.

1.1.3 Two stage hydrogen production and HYVOLUTION project

As seen from the figure 1.1, the complete oxidation of glucose to CO_2 is not energetically possible by dark fermentation. If the substrate was completely converted to CO_2, there would not be any energy left for the cellular processes. The

anaerobic fermentation and hydrogen evolution by hydrogenase is energetically favorable but energy requirement of photofermentation is met by light energy (Das and Veziroğlu, 2001).
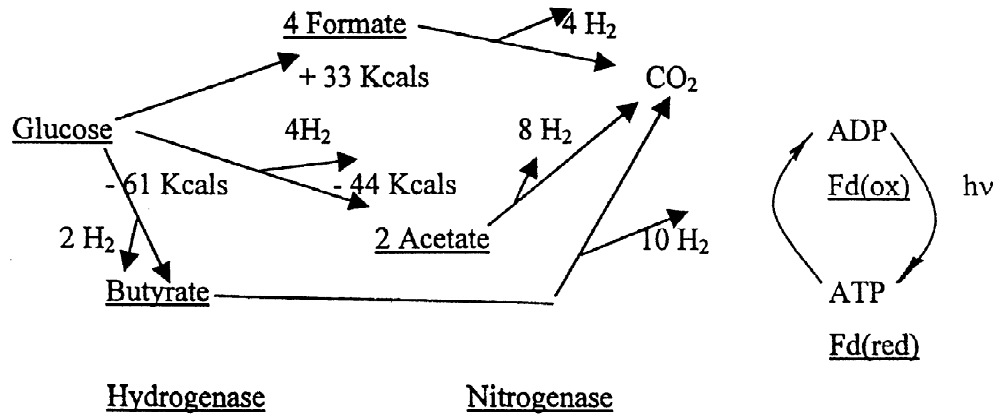
Figure 1.1 The biochemical oxidation of glucose in two stage process (Das and Veziroğlu, 2001).

If the two processes with their free energy change were considered, Eqn. 1.5 and Eqn. 1.6, the coupled system (dark fermentation and photofermentation) run without need for extra energy.

Thermophilic bacteria;

$$C_6H_{12}O_6 + 2H_2O \longrightarrow 2CO_2 + 2CH_3COOH + 4\,H_2, \qquad \Delta G^{o'} = -206 \text{ kJ/mole} \qquad (1.5)$$

Purple, non-sulfur bacteria;

$$CH_3COOH + 2H_2O \longrightarrow 2CO_2 + 4\,H_2, \qquad\qquad \Delta G^{o'} = +104 \text{ kJ/mole} \qquad (1.6)$$
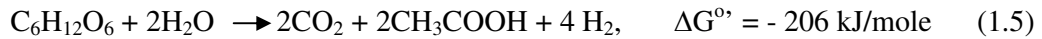
HYVOLUTION is an integrated project, funded by the European Union in the Sixth Framework Programme, Sustainable Energy Systems. The full title of the project is the Non-thermal production of pure hydrogen from biomass. "The aim of HYVOLUTION is to develop a blue-print for decentral hydrogen production process using local biomass. Pretreatment of biomass, thermophilic fermentation, photofermentation, gas upgrading, logistics, system integration and societal integration are addressed" (www.biohydrogen.nl). The Project Coordinator is Wageningen UR, Agrotechnology & Food Innovations (The Netherlands).
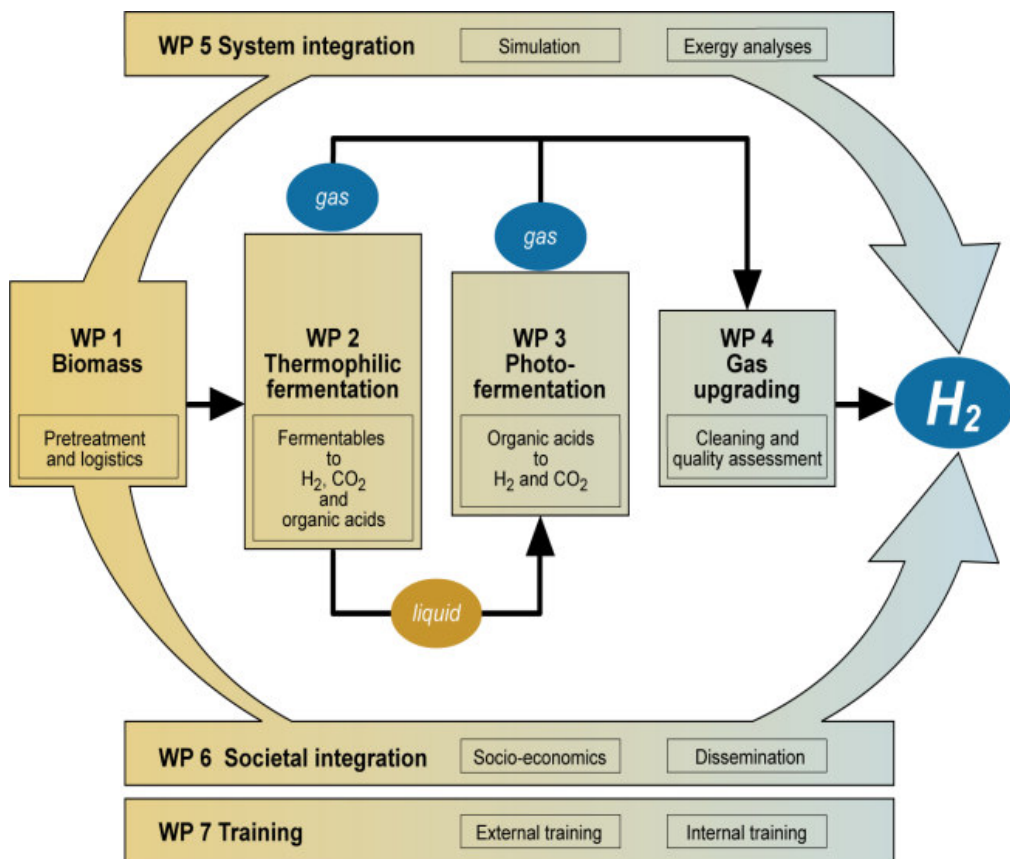
Figure 1.2 The overall scheme representing the HYVOLUTION project.

In this project, the fermentable biomass feedstocks are being used to feed the dark fermentation and the effluent rich in organic acids are then used in photofermentation step. Therefore, this project is an example of two stage process. In the dark fermentation step, an extreme thermophile *Caldicellulosiruptor saccharolyticus* is being used mainly to decompose the pretreated biomass in to organic acids and hydrogen. Then several PNS bacteria are used in photofermentation step. The main PNS bacteria used are *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*. The main organic acids found in dark fermentation effluent are acetate and lactate, therefore, hydrogen production experiments using acetate as a carbon source is of special importance in this study.

1.2 *Rhodobacter sphaeroides*

Rhodobacter sphaeroides previously known as *Rhodopseudomonas sphaeroides* is a gram negative bacterium belonging to the α-3 subgroup of the Proteobacteria (Imhoff *et al*., 1984). Cells have highly variable morphology, especially in complex media. Cells are spherical to ovoid and frequently occur in pairs or as a chain of beads. In young cultures cells are actively motile; they lose their motility with age, especially if the medium becomes alkaline. The size of the individual cells is extremely variable; without capsule they range from 0.7 µm to as much as 4 µm in diameter (van Niel, 1944). As cultures age, they become viscous due to slime layer formation. *R. sphaeroides* 2.4.1 strain was shown to have two circular chromosomes (~0.9 Mbp & ~3 Mbp) and five extra chromosomal plasmids (www.rhodobacter.org; Suwanto and Kaplan, 1989). The photosystem of *R. sphaeroides* contains carotenoids, spheroidene (SE) and spheroidenone (SO) which give the bacterium its color. The presence of oxygen determines the color of the bacterium by arranging its carotenoid types (Kiley and Kaplan, 1988). When the bacteria are cultured aerobically, the SO is the predominant carotenoid in the cells and the color of the culture is pink. When the bacteria are harvested in anaerobic conditions, the SE is the predominant carotenoid in the cells and the color of the culture is yellow/brown (Yeliseev *et al*., 1996; van Niel, 1944). The pH range over

which the bacteria can grow is wide, and extends from at least pH 6 to 8.5. The optimum temperature is relatively low, and lies below 30°C (van Niel, 1944).

1.2.1 Diverse metabolism of *Rhodobacter sphaeroides*

The metabolisms of Purple non-sulfur (PNS) photosynthetic bacteria are quite diverse. They can grow as photoheterotrophs, photoautotrophs or chemoheterotrophs. *Rhodobacter sphaeroides* belonging to a group of anoxygenic PNS bacteria also show a versatile carbon, nitrogen and energy metabolism. Depending on the physiological conditions such as availability of carbon sources, light intensity and oxygen tension; they change their mode of growth. If the CO_2 as carbon source and an electron donor such as molecular hydrogen are available, the bacteria can grow photoautotrophically. The light and anaerobic conditions are needed during photosynthetic mode of growth. Although *R. sphaeroides* can grow photoautotrophically, it grows best photoheterotrophically using organic acids such as malate or succinate as carbon source. PNS bacteria were told as ''non-sulfur'' because they were thought not to use hydrogen sulfide as an electron donor while growing photoautotrophically. However, PNS bacteria can use sulfide as an electron donor but not at high concentrations like sulfur bacteria (Basak and Das, 2007; Brock *et al.*, 2003).

The membrane bioenergetics of *R. sphaeroides* was well studied by Kaplan *et al.* (Kiley and Kaplan, 1988; Lee and Kaplan 1992; Lee and Kaplan 1995; Lee and Kaplan 1996). It was stated that when *R. sphaeroides* is growing chemoheterotrophically, the growth is supported by aerobic respiration and it has a typical gram-negative cell envelope. However, when the anaerobic conditions are set, a series of events takes place in the cell resulting in the differentiation of the cytoplasmic membrane (CM) through a process of invagination into specialized domains which comprise the photosynthetic intracytoplasmic membrane system (ICM). The ICM is physically continuous with the CM but structurally and functionally distinct in that the ICM specifically contains all of the membrane components required for the light reactions of photosynthesis. That is, the ICM

houses the photosynthetic apparatus.

One of the interesting findings about the relationship of three fundamental biological processes; Photosynthesis, biological nitrogen fixation, and carbon dioxide assimilation were well documented by Joshi and Tabita, 1996 and Qian and Tabita, 1996. Previously it was known that the RegA/RegB system controls the ability of *Rhodobacter* to respond to different intensities of light (Sganga and Bauer, 1992; Mosley *et al*., 1994; Eraso and Kaplan, 1994; Eraso and Kaplan, 1995), however, in this study, it was proved that this two-component regulatory system also influence the nitrogen fixation in addition to photosynthesis and carbon dioxide reduction (Figure 1.3). It was shown that mutations in the *regB* gene of *R. sphaeroides* blocked transcription of the *cbb* regulon which contains genes that encode both forms of RubisCO and other enzymes of the CBB cycle. And, inactivation of the *regB* gene of *R. sphaeroides* resulted in the production of active nitrogenase in the normally repressive ammonia media. Similar result was obtained when the CBB route is blocked by mutation in the genes coding for form I and II RubisCO, even in the presence of normally repressive levels of ammonia. The explanation for why the nif system is on even in the presence of normally repressive levels of ammonia is that organisms seek alternative mechanisms to dispense the large amounts of reducing power generated via photosynthesis and the oxidation of organic carbon (such as malate) when the CBB route is blocked. In other words, CO_2 is no longer capable of functioning as the major electron sink when the CBB route is blocked, and the large amount of reducing power is dissipated through nitrogenase action (Joshi and Tabita, 1996; Qian and Tabita, 1996). However, nitrogenase derepressive effect of mutations in *cbb* and *reg* operons might result from the direct effect of these mutations on the *nif* operon. That is, the mutations can positively influence the *nif* operon so that the nitrogenase gene expression occurs continuously without being affected from normally repressive levels of ammonia.
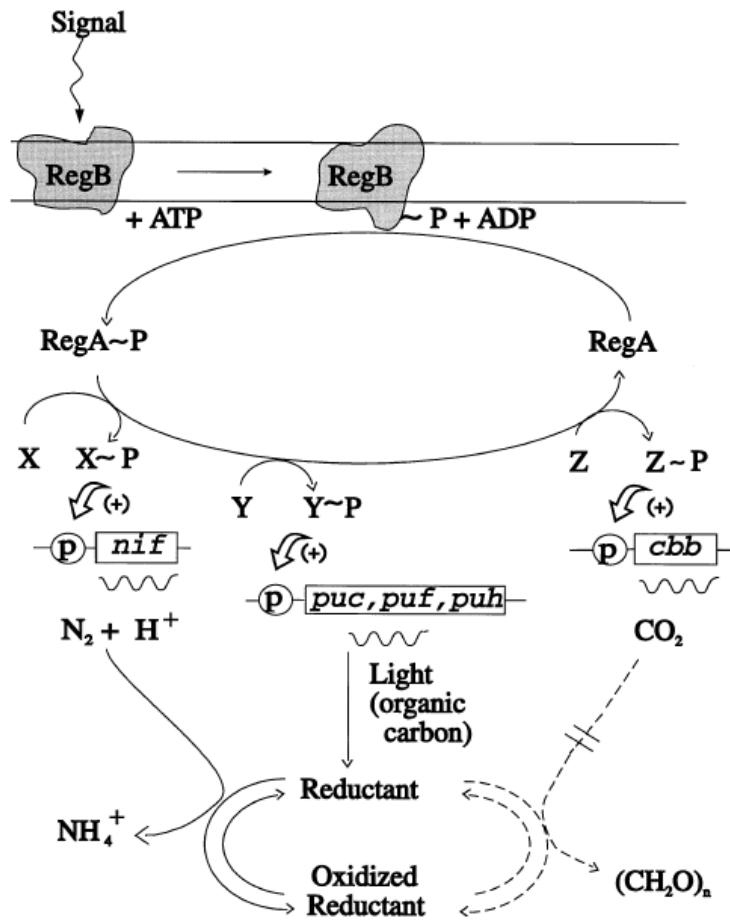
Figure 1.3 The regulation of three processes: photosynthesis, CO_2 assimilation, and N_2 fixation in *R. sphaeroides*. When the CBB pathway is blocked in the RubisCO deletion mutants, excess reducing power is directed to the nitrogenase system (Joshi and Tabita, 1996).

1.2.2 Genetics of *Rhodobacter sphaeroides*

The genomic composition of *Rhodobacter sphaeroides* 2.4.1 is well studied. The whole genome of this bacterium was completely sequenced and annotated (Mackenzie *et al*., 2001 and www. rhodobacter.org). *Rhodobacter sphaeroides* 2.4.1 was initially shown to posses two chromosomes, chromosome I (2973 kb) and chro-

mosome II (911 kb) and five extra chromosomal replicons pRS241a (113.6 kb), pRS241b (104 kb), pRS241c (100 kb), pRS241d (99 kb) , pRS241e (42 kb). The rich genome composition of *Rhodobacter sphaeroides* reflects its metabolic complexity as well.

The gene transfer to *R. sphaeroides* was reported in several ways although the most efficient way is the conjugation or diparental mating. The chemical transformation was reported in only one case by Fornari and Kaplan in 1982. Conjugation is the most widely applied technique of transferring DNA into *R. sphaeroides*. Transfer occurs between the special *E.coli* donor and *R. sphaeroides*. The conjugation simply takes place by mixing donor and recipient on solid surface and then the selection of the recipient by an antibiotic resistance marker. Most engineered plasmids systems used are mobilizable but not self transmissible. The transfer function is either from a helper plasmid or the host *E.coli* S.17-1 (Suwanto and Kaplan, 1992, Kars *et al*., 2008).

Heterologous gene expressions or interspecies gene expressions were also achieved. Mostly, the genes from PNS bacteria were expressed in *E.coli* (Muller *et al*., 1985; McEvan *et al*., 1989; Chen *et al*., 1991 and Hallenbeck and Kaplan, 1987). Moreover, there are studies in which the genes from one PNS bacterium were expressed in other PNS bacteria (Davidson *et al*., 1989).

1.3 The Nitrogenases

The function of nitrogenases is basically to maintain the nitrogen cycle on earth. They convert the molecular nitrogen to ammonia which is then useable for other organisms. Three genetically distinct types of nitrognase systems (nif, vnf, anf) have been proved to exist in nature (Eady, 1991; Eady, 1996). The most widely characterized system is the Mo-containing nitrogenase found in all diazotrophs. All three nitrogenase systems consist of two dissociable component metalloproteins, component 1, dinitrogenase, (MoFe protein, VFe protein, FeFe protein) and component 2, dinitrogenase reductase, (Fe protein). Whereas component 2, which is a dimer of two identical subunits bridged by a single [Fe4S4] cluster, has an identical

structure in all nitrogenase systems, component 1 of the alternative nitrogenases differs from the conventional tetrameric MoFe protein in that it contains, in addition to α and β subunits, a small 14 kDa γ subunit resulting in hexameric structure (α2β2γ2) (Eady, 1991). The Specific activities of VFe, FeFe, and MoFe proteins were compared in Table 1.1. As shown in Table1.1, the FeFe Protein of *R. capsulatus* showed the highest hydrogen evolving activity.

Table 1.1. Comparison of the specific activities of VFe, FeFe, and MoFe proteins (Eady, 1996).

	Specific activity (nmol of product/mg of protein/min)		
	NH_3 formation	H_2 evolution under N_2	H_2 evolution under Ar
	MoFe Proteins		
Klebsiella pneumoniae	990	648	2100
Azotobacter vinelandii	1040	nd	2220
Rhodobacter capsulatus	470	210	1300
	VFe Proteins		
Azotobacter chroococcum (Δ*nifHDK*)	350	928	1348
Azotobacter vinelandii (Δ*nifHDK*)	660	nd	1400
	FeFe Proteins		
Azotobacter vinelandii 3[S]	38	213	253
Azotobacter vinelandii (Δ*nifHDK* W[r]) 3[F]	30	145	203
Azotobacter vinelandii (Δ*nifHDK*; Δ*vnfHDK*)	110	220	350
Rhodobacter capsulatus (Δ*nifHDK*, *modB*)	350	1300	2400

1.3.1 Mo-Nitrogenase

Studies indicate that all diazotrophs have Mo-nitrogenase, which is encoded by *nif* HDK genes (Eady, 1991; Eady, 1996; Burgess and Lowe, 1996). This enzyme consists of two oxygen sensitive metalloproteins, an Fe-protein containing a single [4Fe-4S] center which act as a specific ATP-dependent electron donor to the MoFe protein and encoded by *nif* H gene and MoFe protein encoded by *nif* DK genes as shown in Figure 1.4.
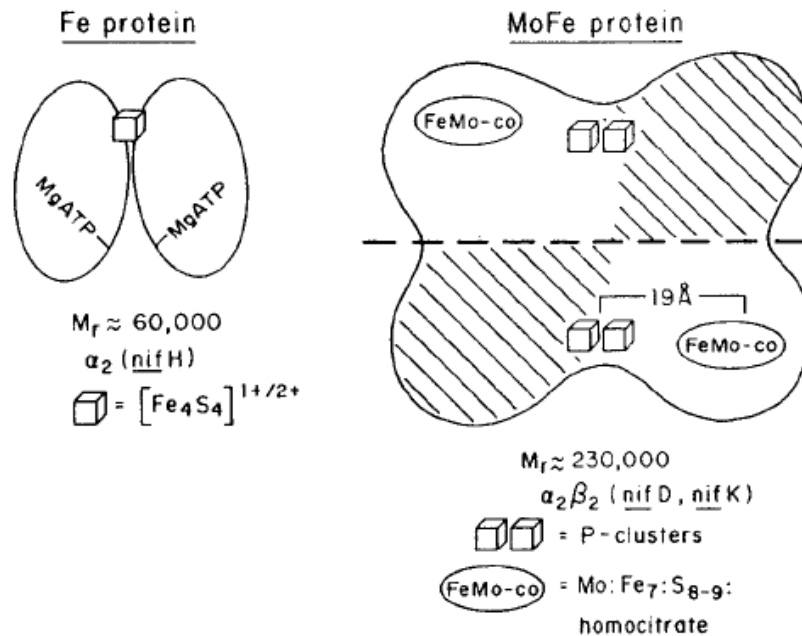
Figure 1.4 Two component proteins of Molybdenum nitrogenase (Burgess and Lowe, 1996).

In addition to transferring electrons to MoFe protein, Fe protein has been proposed to have two or possibly three functions. First, it is required for the initial biosynthesis of FeMo cofactor. Second, it is essential for the incorporation of FeMo

cofactor into FeMo cofactor deficient MoFe protein. Third, it may have a role in the regulation of alternative systems (Burgess and Lowe, 1996).

The MoFe protein has two types of redox centers, two P-clusters consisting of two bridged [4Fe-4S] cubanes, and two Fe and Mo containing cofactor centers (FeMo-co), the probable site binding and reduction of N_2 (Eady, 1996).

The Mo-Nitrogenase catalyses the following reactions;

$$N_2 + 6H^+ + 6e^- + 12ATP \rightarrow 2NH_3 + 12ADP + 12P_i \qquad (1.7)$$

$$2H^+ + 2e^- + 4ATP \rightarrow H_2 + 4ADP + 4P_i \qquad (1.8)$$

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + 16ADP + 16P_i + H_2 \qquad (1.9)$$

In the molecular nitrogen atmosphere, the nitrogenase catalyzes the formation of ammonia from molecular nitrogen, and, at least 25% of the electron throughput is used to reduce protons to molecular hydrogen, which is released from the cells by photosynthetic bacteria under anoxic conditions. Thus, the nitrogenase, and its inherent hydrogenase activity, acts as a safety valve for the dissipation of excess reducing equivalents and in the absence of any substrates such as N_2, nitrogenase acts as ATP dependent hydrogenase and all the electrons are utilized for H_2 production increasing the efficiency of the process (Liang and Burris, 1988; Thorneley and Lowe, 1983; Simpson and Burris, 1984; Joshi and Tabita, 1996; Hillmer and Gest, 1977). However, for the transfer of an electron through nitrogenase, 2 ATPs are consumed. Therefore, for one H_2 to be produced by the nitrogenase, 4 ATPs are needed as shown in the Eqn. 1.8. Nitrogen fixation reaction competes with the hydrogen production, therefore, in the hydrogen production experiments; there should not be considerable amount of free nitrogen. However, since the hydrogen production is an inherent character of nitrogenase enzyme, the hydrogen evolution occurs even under 50 atmosphere of N_2 (Liang and Burris, 1988).

The structure of the Mo-Nitrogenase is given in Figure 1.5.
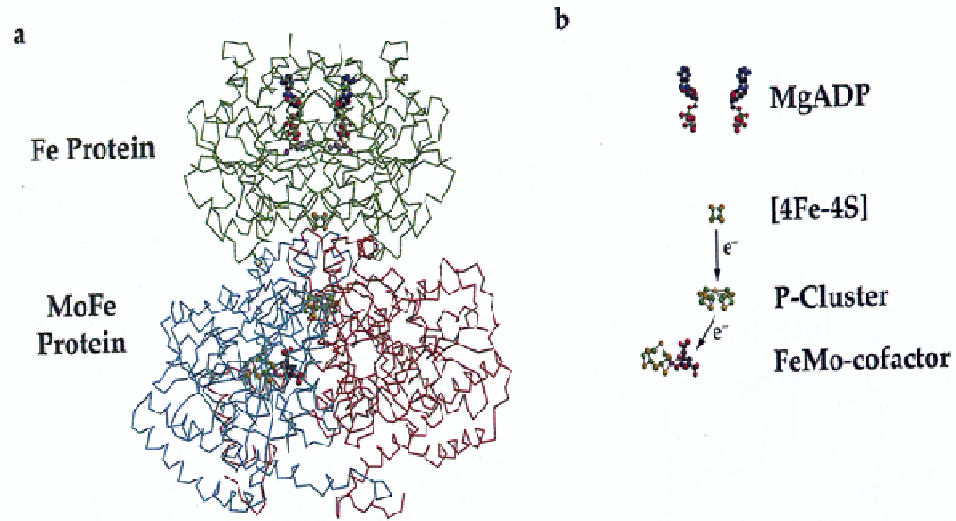
Figure 1.5 The structure of Mo-Nitogenase (Christiansen *et al.*, 2001).

1.3.2 V-Nitrogenase

The second type of nitrogenase is the V-nitrogenase which contains vanadium in the catalytic center of the enzyme in place of molybdenum. The V-nitrogenase was purified from *Azotobacter chroococcum* and *Azotobacter vinelandii* (Eady, 1996). Due to the homology between the genes coding for these enzymes, the structure of the enzymes are similar (Eady, 1995). The enzyme is composed of two main subunits, Fe protein and VFe protein, similar to the Mo-nitrogenase. Although the Fe proteins of the alternative nitrogenase systems have not been extensively characterized when compared with the Fe proteins of Mo-nitrogenases, the Fe proteins of V-nitrogenase were isolated from *A. vinelandii* and *A. chroococcum* and these proteins were shown to be dimeric with Mr ~ 63 kDa and contain 4 Fe and 4 S^{2-} atoms per dimer (Eady, 1996). The VFe proteins differ from the MoFe proteins in

that they have an additional δ subunit forming hexameric structure ($\alpha2\beta2\delta2$) of Mr ~ 250 000 Da. The enzyme is encoded by *vnfHDGK* genes which form a transcriptional unit (additional vnfG, δ subunit) (Joerger *et al.*, 1989; Eady, 1996).

1.3.3 Fe only Nitrogenase

The presence of third nitrogenase system was demonstrated in 1988 by Chisnell *et al.* when *Azotobacter vinelandii* was grown under Mo and V limitation. The presence of alternative nitrogenase was shown and characterized in *R. capsulatus* by Schneider *et al.* (1991). The enzyme is composed of two main subunits, Fe protein and FeFe protein, similar to the Mo-nitrogenase. The Fe protein has not been well characterized but it is dimeric with Mr ~ 64 kDa and contains 4 Fe and 4 S^{2-} atoms per dimer. The FeFe protein has a $\alpha2\beta2\delta2$ subunit structure of 268 kDa and Mr values of 61, 58, and 15 kDa for α, β and δ subunits respectively similar to the V-nitrogenase (Eady, 1996). The enzyme is encoded by *anfHDGK* (additional anfG, γ subunit).

1.4 The Hydrogenases

The other key enzyme in the H_2 metabolism is hydrogenase. The enzyme catalyzes the simplest chemical reaction:

$$2H^+ + 2e^- \longleftrightarrow H_2 \tag{1.10}$$

In *R. sphaeroides* the nitrogenase is mainly responsible for hydrogen production but depending on the Km values of hydrogenases for H_2 and the preferences of the bacteria, hydrogenases can also produce hydrogen as stated earlier (Van Haaster *et al.*, 2005). The reaction catalyzed by the hydrogenases is reversible, and its direction depends on the redox potential of the components such that in the presence of H_2 and an electron acceptor, they act as H_2 uptake enzyme; in the presence of an electron donor, they may use the protons from water as electron ac-

ceptors and release H_2 (Vignais and Billoud, 2007). Hydrogenases are first described in 1931 by Stephenson and Stickland and they are widespread in prokaryotic and lower eukaryotic biological systems. The first classification of these enzymes was based on the identity of specific electron donors and acceptors, namely, NAD, cytochromes, coenzyme F420, or ferredoxins. However, recently three major groups of hydrogenases are distinguished according to their metal atoms at their active site: one Ni and one Fe atom (in [NiFe]-hydrogenases), two Fe atoms (in [FeFe]-hydrogenases), no Fe-S cluster and no Ni (Hmd enzyme, metal free hydrogenase or [Fe]-Hydrogenases) (Vignais and Billoud, 2007). The Hmd enzyme was a different type of hydrogenase and discovered in some methanogens functioning as H_2-forming methylenetetrahydromethanopterin dehydrogenase (Hmd). The Hmd enzymes are now known to contain a mononuclear iron center that has not yet been completely defined, however, significant progress has been made in understanding the assembly of the [NiFe] and [FeFe] hydrogenase active sites and accessory proteins (Kovacs *et al*., 2002; Vignais *et al*., 2004; Leach and Zamble, 2007; Vignais and Billoud, 2007; Böck *et al*., 2006)

1.4.1 [NiFe]-Hydrogenases

[NiFe]-hydrogenases are said to be the most numerous and best studied class of hydrogenases of Bacteria (Vignais and Billoud, 2007). The core enzyme consists of the large subunit (α-subunit) of ca. 60 kDa hosting the bimetallic active site and the small subunit (β-subunit) of ca. 30 kDa hosting the Fe-S clusters forming an αβ heterodimer. The small subunit transfers electrons via [Fe-S] clusters, while the large subunit contains the unique heterobinuclear [NiFe] metallocenter, which is the catalytic site. Crystal structure of [NiFe] hydrogenase of *Desulfovibrio gigas* is shown in Figure 1.6 as an example.
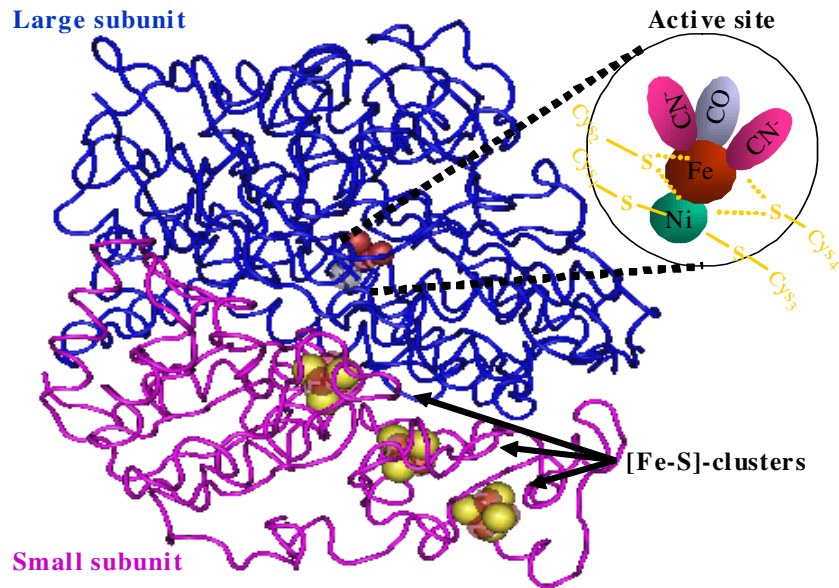
Figure 1.6 Crystal structure of [NiFe] hydrogenase of *Desulfovibrio gigas* (Frey, 2002).

There are many subgroups of [NiFe] hydrogenases depending on the complete sequence analyses of the small and large subunits. Membrane bound uptake hydrogenases (i.e; hupSL and hynSL), hydrogen sensors (i.e; hupUV), NADP-reducing (i.e; HydDA), bidirectional NADP/NAD-reducing (i.e; hoxYH) and energy converting membrane associated H_2 evolving hydrogenase (Vignais and Billoud, 2007; Kovacs *et al.*, 2002; Vignais *et al.*, 2004; Vignais *et al.*, 2001). The membrane-bound respiratory hydrogenases perform the oxidation of hydrogen which then the reduction of quinone occurs. In anaerobic respiration the final electron acceptors are NO^{3-}, $SO4^{2-}$, fumarate, or CO_2 while in aerobic respiration it is the O_2. After the electron transport, the protons are passed across the membrane which recovers the energy in the form of a protonmotive force. Therefore, the membrane bound uptake hydrogenases help to maintain the cellular energy balance.

The purple sulphur phototrophic bacterium, *Thiocapsa roseopersicina* BBS contains a nitrogenase and several [NiFe] hydrogenases. In addition to the regulatory

and accessory proteins, there are mainly four types of hydrogenases in *Thiocapsa roseopersicina* BBS namely Hyn, Hup, Hox1 and Hox2. HupSL and HynSL are the membrane bound [NiFe] hydrogenases found in *T. roseopersicina*. HynSL hydrogenase is more stable, more resistant to heat and oxygen, than HupSL (Kovacs *et al.*, 2002; Vignais *et al.*, 2001; Kovacs *et al.*, 2006). Recently, a third, cytoplasmic NAD$^+$ reducing NiFe hydrogenase was identified and genes were sequenced (*hoxEFUYH*) (Rákhely *et al.*, 2004). The five genes (*hoxEFUYH*) were localized on a single transcript and the gene products showed the highest similarity to the corresponding subunits of the cyanobacterial bidirectional soluble hydrogenases. This hydrogenase is a bi-directional type hydrogenase and it can produce H_2 under the conditions where nitrogenase is repressed such as in the presence of ammonium. Therefore, the Hox enzyme creates a novel approach for biohydrogen production (Kovacs *et al.*, 2006).

1.4.2 [FeFe]-Hydrogenases

The [FeFe]-hydrogenases are generally monomeric and consist of the catalytic subunit only unlike [NiFe]-hydrogenases which are composed of at least two subunits. The [FeFe]-hydrogenases are generally known to produce H_2 unlike the [NiFe]-hydrogenases, and found in anaerobic prokaryotes such as *clostridia* and sulfate reducers. The [FeFe]-hydrogenases are the only type of hydrogenase to have been found in eukaryotes in some anaerobic eukaryotes such as in anaerobic fungi and in some green algae (Vignais and Billoud, 2007). *R. rubrum*, a PNS bacterium, contains Fe-only hydrogenase (HydC) which can produce hydrogen. In the structure of HydC, an H-cluster domain, an active site of H_2-evolving Fe-only hydrogenase, which includes two iron metals and one 4Fe–4S cluster are present (Gorrel and Uffen, 1977; Albracht *et al.*, 2006; Kim *et al.*, 2008).

1.4.3 Fe only Hydrogenases (Hmd Hydrogenase)

The first Hmd hydrogenase was discovered in *Methanothermobacter*

marburgensis and it is the most extensively studied one (Zirngibl *et al.*, 1992). It has been observed in many methanogenic species and catalyzes the conversion of CO_2 with H_2 to methane (Thauer, 1998). Hmd hydrogenase has two identical subunits (38 kDa), encoded by a monocistronic gene but does not contain iron sulfur cluster. It was initially thought to be purely organic catalyst yet it contains two irons per homodimer and its activity depends on an iron-containing cofactor (Vignais and Billoud, 2007).

1.5 The Physiological effectors of Mo-Nitrogenase

Due to the slow turnover time making diazotrophs synthesize large amount of nitrogenase and the consumption of a considerable amount of ATP as shown in Eqn. 1.9, the regulation of the nitrogenase genes at different levels (transcriptional, posttranscriptional and posttranslational levels) in response to environmental stimuli is quite stringent to save energy and time (Dixon and Kahn, 2004). Reversible posttranslational modification of nitrogenase, also known as nitrogenase switch-off/switch-on, has been observed in *Rhodobacter sphaeroides*, *Rhodobacter capsulatus*, *Rhodospirillum rubrum* and *Azospirillum brasilense* which are alpha-proteobacteria. The main extrinsic factors affecting the nitrogenase enzyme activity are oxygen in the environment, the presence and the amount of fixed nitrogen (NH_4), light and availability, the presence of molecular nitrogen and amount of metal ions (iron and molybdenum) which are present in the structure of Mo-nitrogenase. In addition, the redox status of the cells as an intrinsic factor influences the activity nitrogenase.

Mo-nitrogenase is sensitive to oxygen which causes an irreversible damage to the enzyme. Dinitrogenase reductases (Fe proteins) were known to be more sensitive to inactivation by O_2 than dinitrogenases (Mo-Fe proteins). Oxygen also causes a reversible inhibition (switch-off) of nitrogenase activity similar to the ammonium effect. Therefore the nitrogen fixation and hydrogen production are possible only under anaerobic conditions in anoxygenic photosynthetic bacteria (Goldberg *et al.*, 1987).

The levels of nitrogenase control in response to ammonium are presented in Figure 1.7 in *R. capsulatus*. When the fixed nitrogen sources such as ammonium is present in the medium, the nitrogenase enzyme activity is reversibly inactivated in a way that an ADP-ribose group from NAD^+ is attached to an arginine residue in one subunit of the homodimeric NifH protein resulting in NifH inactivation (switch-off). This process is catalysed by dinitrogenase reductase ADP-ribosyltransferase (*DraT*). However, this inactivation is reversible such that when the added ammonium is exhausted by cellular metabolism, the ADP-ribose group is removed by dinitrogenase reductase activating glycohydrolase (*DraG*) leading to NifH activation (switch-on) (Huergo *et al.*, 2006; Drepper *et al.*, 2003). However, it was shown that, the ammonium regulation of the molybdenum nitrogenase are completely circumvented in the *glnB–glnK* double mutant resulting in the synthesis of active molybdenum nitrogenase even in the presence of repressive concentrations of ammonium in *R. capsulatus* (Drepper *et al.*, 2003). In the case of *R. sphaeroides*, although the switch-off response was seen when the ammonium was present, neither *draTG* genes nor the ADP-ribosylation of dinitrogenase reductase were not detected (Yoch *et al.*, 1988; Yakunin *et al.*, 2001). NtrBC is another two component system which senses the fixed nitrogen status of the cell and accordingly regulates the *nif* operon. If the Gln/α K-Glutarate ratio is low, the *nif* genes are positively activated by NtrBC system (Zinchenko et al, 1997).

Light availability is a very important parameter for the hydrogen production and the activity of nitrogenase enzyme. It was found that light strongly stimulates nitrogenase activity of cells and therefore the hydrogen production activity is higher (Jouanneau *et al.*, 1985). The photophosphorylation capacity is also slightly greater in cells grown under high light intensity than in cells grown under low light intensities (Steinborn and Oelze, 1989). Therefore, the high ATP production rate under well illuminated conditions might result in higher hydrogen production activity

Another factor which affects the activity of nitrogenase complex is the presence of molecular nitrogen (N_2) in the environment. In the presence of molecular nitrogen, the nitrogenase enzyme fixes the nitrogen which is favored and, therefore, a lot of energy and electrons are used in this process. In the case of the presence of mo-
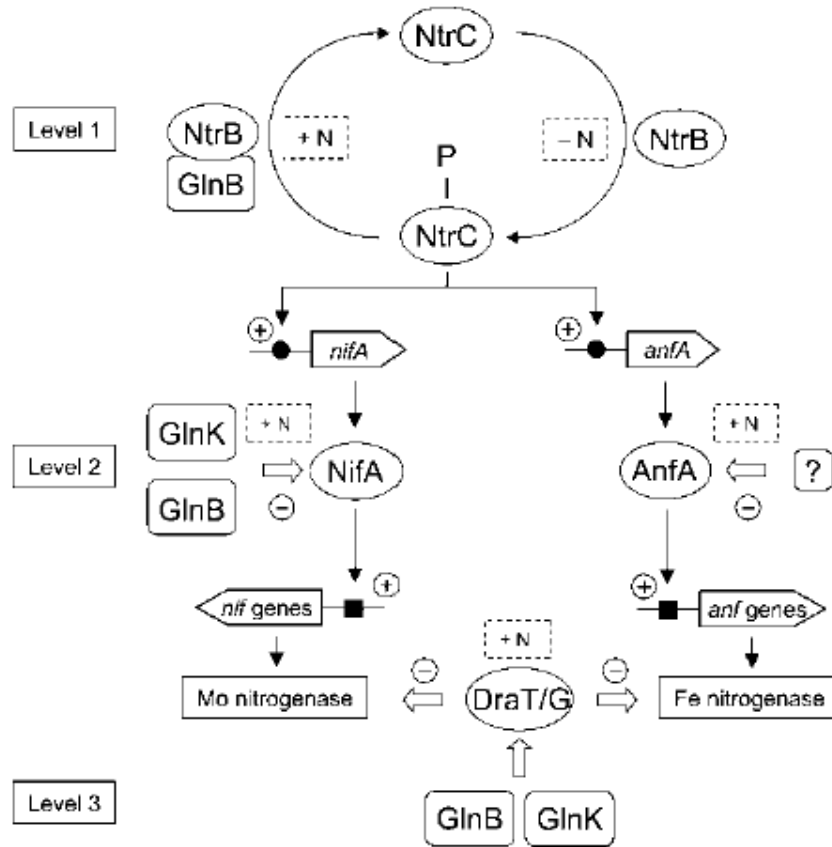
Figure 1.7 The levels of nitrogenase control in response to the availability of ammonium (Drepper *et al.*, 2003).

lecular nitrogen, about 75 % of the electrons are allocated for the nitrogen fixation and remaining 25 % of the electrons are used for H_2 evolving (Rey *et al.*, 2007; Burris, 1991). Therefore, an efficient hydrogen production process should be performed without molecular nitrogen.

Availability of certain metal ions such as molybdenum and iron which are found in the structure of Mo-nitrogenase is also critical. In *Azotobacter vinelandii*, *nifHDK* transcription was repressed under Mo-deprived conditions (Jacobson *et al.*, 1986). Moreover, the presence of Mo was shown to repress alternative nitrogenases in *Rhdobacter capsulatus* (Schneider *et al.*, 1991).

The charge or redox status of the cells is a very important parameter in terms of biohydrogen production since the nitrogenase needs electrons to reduce protons to hydrogen. In the photosynthetic bacterium *Rhodobacter sphaeroides* and *Rhodobacter capsulatus*, a putative membrane-bound complex encoded by the *rnfABCDGEH* operon is thought to be dedicated to electron transport to nitrogenase (Jeong and Jouanneau, 2000). It was shown that overexpression of *rnf* operon in *R. capsulatus* enhanced *in vivo* nitrogenase activity. This result suggests the rate limiting step in the nitrogenase activity to be the electron flow to nitrogenase.

1.6 The physiological effectors of uptake hydrogenase

The main physiological effectors of uptake hydrogenase are oxygen, hydrogen and availability of metal ions (iron and nickel). Certain transcriptional analysis of hydrogenases have been done to investigate the effects of oxygen, hydrogen and nickel on the expression level of [NiFe] hydrogenases (Axelson *et al.*, 2002; Boison *et al.*, 2000). According to these studies, the increased level of both hydrogen and nickel increased the activity of uptake hydrogenase. In addition, reduced level of oxygen has a positive effect on the uptake hydrogenase activity. The presence of the substrate molecule of hydrogenases, H_2, triggers the expression of some hydrogenases through hydrogen sensing regulatory hydrogenase (HupUV/HoxBC) and a two-component system (HupT/HoxJ and HupR/HoxA) which was examined in detail in *R. capsulatus* (Dischert *et al.*, 1999).

1.7 Improvements in biohydrogen production: Towards a super H_2 producer

The hydrogen evolution in purple non sulphur bacteria is carried out by nitrogenases whose primary duty is to fix molecular nitrogen not to produce hydrogen. Therefore, the efficiency of photobiological hydrogen production by these bacteria such as *R. sphaeroides* is inherently limited. In addition to the inherent characteristics of nitrogenase, the total hydrogen production is constrained due to several reasons such as consumption of hydrogen by uptake hydrogenase, limited e-

lectron flow to the nitrogenase due to the production of poly-3-hydroxybutyrate (PHB) or CO_2 fixation, inhibition by presence of fixed nitrogen sources such as ammonium, light requirement of the nitrogenase etc.

The uptake hydrogenase decreases the efficiency of H_2 production by catalyzing the conversion of molecular hydrogen to electrons and protons (Vignais *et al.*, 1985). However, the hydrogen respiration let the cells recapture the electrons from molecular hydrogen and deliver it to the electron carriers in the membrane while making proton motive force which is then used to produce ATP. It was reported that the inactivation of uptake hydrogenase resulted in increase in total hydrogen production in several microorganisms (Kars *et al.*, 2008; Franchi *et al.*, 2004; Öztürk *et al.*, 2006; Kim *et al.*, 2006). Therefore, uptake hydrogenase has been the target in many studies to enhance the hydrogen production efficiency.

It is known that the hydrogen production under nitrogen fixing conditions is inefficienct process due to the fact that 75 % of the reducing power is consumed by nitrogen fixing and only the 25 % of the reductant is used for H_2 evolving (Federico *et al.*, 2007; Burris, 1991). In a study done by Jeong and Jouanneau in 2000, the enhanced electron flow to nitrogenase increased the activity of the enzyme. These results suggested that the electron flow to nitrogenase is one of the bottlenecks of the hydrogen production process. Moreover, since the PHB production and CO_2 fixation share the reducing equivalents with the nitrogenases, these pathways were also targeted for elimination (Kim *et al.*, 2006; Franchi *et al.*, 2004; Joshi and Tabita, 1996; Qian and Tabita, 1996).

The photobiological hydrogen production by PNS bacteria such as *R. sphaeroides* is limited by the presence of ammonia. Therefore, strains which are insensitive to repressive concentrations of ammonia were developed. In a study done by Federico *et al.* in 2007, constitutive hydrogen production in the presence of repressive concentrations of ammonia by *Rhodopseudomonas palustris* having mutations in the four different sites in the NifA transcriptional regulator was reported. The mutations made the bacteria escape from the repressive effect of ammonia. In addition, the post-translational inhibition of nifA activity is completely abolished in a *glnB–glnK* double mutant of *R. capsulatus* (Drepper *et al.*, 2003).

The efficiency of photobiological hydrogen production by PNS bacteria is highly dependent on the intensity of the light required for the energy generation. However, continuous hydrogen production under light and dark conditions would certainly improve the efficiency of biological hydrogen production process. In a study done by Kim *et al.*, 2008, constitutive hydrogen evolution under both photoheterotrophic and dark fermentative conditions by recombinant *R. sphaeroides* was reported. The H_2 production by the recombinant *R. sphaeroides* under both conditions appears to be mediated by the concerted actions of FHL and Fe-only hydrogenase of *R. rubrum* and nitrogenase of its own.

1.9 The scope of the work

In this study, the effect of various concentrations of molybdenum on the expression level of the *nifK* gene and of iron on the expression level of *nifD* and *hupS* genes were investigated. The hydrogen production was also evaluated under the same growth conditions. The gene expression analyses were performed with respect to time to understand the relations among the gene expression level, biohydrogen production and the growth phases.

Then, the purple non-sulfur bacterium *R. sphaeroides* O.U.001 was manipulated such that the uptake hydrogenase was inactivated in two different methods. In the first method, the uptake hydrogenase was inhibited by gentamicin resistance gene insertion into the *hup* genes. In the second method, the central portion of the *hup* genes was deleted without inserting any antibiotic resistance genes. Total hydrogen production, the rate of H_2 production and substrate conversion efficiency were investigated in wild and *hup*$^-$ mutant *R. sphaeroides*.

In another study, a [NiFe] hydrogenase (hox1) which is present in *T. roseopersicina* and capable to catalyze hydrogen evolution under mixotrophic conditions even in the presence of ammonia was put into *R. sphaeroides.* The expression of the genes and hox1 dependent hydrogen production were investigated.

Finally, the approximate amount of Mo-nitrogenase in *R. sphaeroides* O.U.001 was investigated by SDS-PAGE followed by densitometric analysis.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial strains and plasmids

Bacterial strains and plasmids used in this study are listed together with their relevant characteristics in Table 2.1. *R. sphaeroides* O.U. 001 was the bacterium that was used for hydrogen production and expression analysis. Moreover, the mutation and other genetic manipulations were done on this strain using the *E .coli* strains and certain plasmids as general cloning and genetic manipulation tools. *E.coli* XL1 Blue was used as a general host for cloning and blue-white screening. *E. coli* S17-1(λpir) was used as a special strain to deliver plasmids to *R. sphaeroides* via conjugation.

Table 2.1. The plasmids and bacterial strains used in this work.

Strains	Characteristics/Genotype	Reference
E.coli		
XL1 Blue	Δ(*mcrA*)183, Δ(*mcrCB-hsdSMR-mrr*) 173, *endA1*, *supE44*, *thi-1*, *recA1, gyrA96, relA1 lac* [F' *proAB lacIqZ*ΔM15 Tn10 (Tet^r)]	Stratagene
S17-1(λpir)	294 (*recA pro res mod*) Tp^r, Sm^r (pRP4-2-Tc::Mu-Km::Tn7), λ pir	Herrero *et al.*, 1990
R. sphaeroides		
O.U 001	wild	DSM 5864
GK1	*hup*$^-$, Gm^r	This work
ZK1	*hup*$^-$, without antibiotic resistance gene	This work
T.roseopersicina		
BBS	wild	Bogorov *et al.*, 1974
Plasmids		
pK18*mobsacB*	Km^r, *sacB, RP4 oriT, ColE1 ori*	Schafer *et al.*, 1994
pBluescript SK(+)	Amp^r	Stratagene
p34-SGm	Gm^r gene source	Dennis *et al.*, 1998
pGhup 1	3 kb *hup* gene fragment cloned into pBluescript SK (+)	This work

Table 2.1. (continued).

Strains	Characteristics/Genotype	Reference
pGhup1Gm	Gm^r gene inserted into pGhup1	This work
pGhup2Gm	Gm^r gene with *hup* gene cloned into pK18*mobsacB*	This work
pBBR1MCS4	Amp^r, expression vector	Kovach *et al.*, 1995
pTCB4/2	The *hoxEFUYHW* operon	Rákhely *et al.*, 2004
pGhox1a	The *hoxEFUYHW* operon cloned into pBBR1MCS4	This work
pGhup3	916 bp partial *hupS* gene cloned into pBluescript SK (+)	This work
pGhup4	844 bp partial *hupL* gene cloned into pGhup3	This work
pGhup5	1778 bp deleted *hupSL* gene cloned into pK18*mobsacB* vector	This work
pMHE2crt	*crtD* promoter	Fodor *et al.*, 2004
pGhox2	1.2 kb partial *hoxEF* gene cloned into pMHE2crt	This work
pGhox3	4781 bp *Stu*I and *Hind*III cut pGhox1a fragment cloned into pGhox2	This work
pGhox4	Km^r gene inserted into pGhox3	This work

2.2 Growth media and culture conditions

2.2.1 General growth media for *R. sphaeroides* and *E. coli* strains

Rhodobacter sphaeroides O.U.001 is generally grown on Biebl and Pfenning minimal medium (Biebl and Pfennig, 1981) in which the malate and glutamate (15mM/2mM) were used as carbon and nitrogen sources. Vitamin (Thiamin, Niacin and Biotin), iron and trace element solutions were added according to the procedure (Appendix B). The vitamin solution was filter sterilized (as they are prone to degradation under high temperature) and added to the autoclaved media which was cooled to 30 ºC. The solid media for the growth of *R. sphaeroides* was prepared by the addition of agar (1.5 %) before autoclave and pouring onto Petri dishes. After the media was solidified, the bacteria was spread or streaked on these plates. If needed, the antibiotics were added at the following concentrations (μg mL-1): gentamicin (Gm), 50; kanamycin (Km), 25; ampicillin (Amp), 100; streptomycin (Sm), 25.

The *E.coli* strains were grown on Luria Broth (LB) media at 37 ºC at constant shaking with antibiotics supplemented in the following concentrations (μg mL-1): ampicillin 100; kanamycin 25; streptomycin (Sm) 25; chloramphenicol (Cm) 25 and tetracycline (Tet) 10 (Appendix C). The 1.5 % agar was used as a solidifying agent when solid media were needed.

2.2.2 Growth media and culture conditions for aerobic and anaerobic growth of *R. sphaeroides*

For the aerobic growth of *R. sphaeroides* in liquid cultures, the penicillin bottles were not filled with medium totally but enough space was left on top as oxygen source and it was not necessary to shake them. The solid media, agar plates, were incubated at 30 ºC without light. The reddish colonies which were 2-3 mm in diameter normally appear on plates after 3-4 days incubation. The agar plates were sealed with parafilm in order not to let them dry after 3-4 days incubation. The aerobically grown *R. sphaeroides* cultures were reddish in color whereas anaerobic-

ally grown *R. sphaeroides* cultures were brown-yellow in color.

(A) (B) (C)
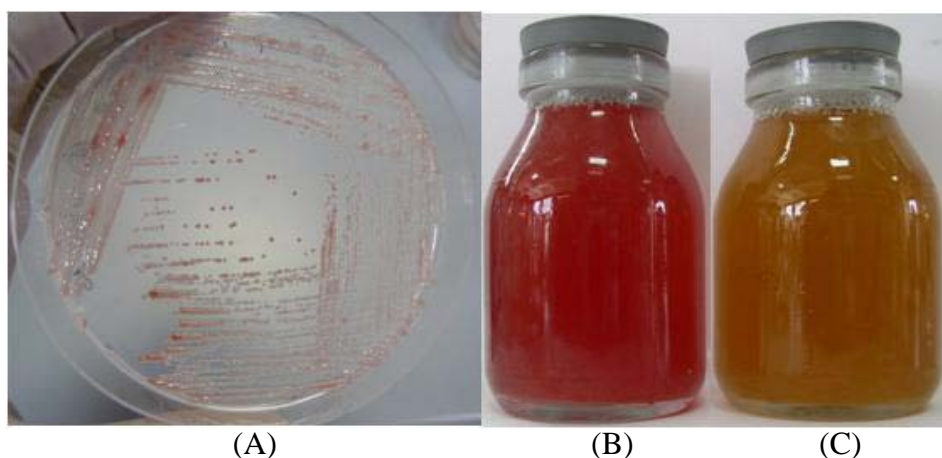
Figure 2.1 The aerobically grown *R. sphaeroides* on plates (A), in liquid culture (B) and anaerobically grown cells in liquid culture (C).

The anaerobic condition was provided by flushing the media with argon for a few minutes, or by filling the bottles completely. However, it is best to flush the media to remove all traces of oxygen which inhibits the nitrogenase activity. Anaerobic environment is of special importance when nitrogen fixing, photosynthetic or hydrogen producing conditions are needed. It was stated that a number of *bch*, *crt* and *puc* genes were suppressed under aerobic conditions by a repressor protein PpsR in *R. sphaeroides* (Moskvin *et al.*, 2005). In the cases when the anaerobic growth on plates was needed, anaerobic jars were used to provide anaerobic environment which was created by flushing the jar by argon gas.

2.2.3 The media for H$_2$ production, pH and OD measurements

In hydrogen production, pH and OD measurement experiments, malate (15 mM) and acetate (30 mM) were used as carbon sources. As a nitrogen source 2 mM of glutamate was used. The other components were not changed except that the buf-

fer concentration was increased to six fold (3 g/l, KH_2PO_4) in acetate containing media due to high pH rise.

2.2.4 The media for the hydrogen production and transcriptional analyses at different metal ion (iron and molybdenum) concentrations

R. sphaeroides O.U.001 was grown in Biebl and Pfenning minimal medium in which D, L-malate and L-glutamate (15 mM/2mM) were used as carbon and nitrogen sources. Except for the concentrations of the metal ions, iron and molybdenum, all other contents of the media kept the same. Since, in this study, the metal ion (iron and molybdenum) effect on the growth, hydrogen production and expression of *nif* and *hup* genes was investigated, the bottles and the other glassware were washed with 30% (v/v) nitric acid and then with ultrapure water several times to remove traces of metal ions. Furthermore, possible contaminants of molybdenum and iron accompanied with the inoculums (10%) were eliminated with the following treatments. The fresh cultures were first centrifuged and the pellets were dissolved in media with no molybdenum or iron. After several passages in their media, the final inoculations were done. The medium named as control or standard (1X) contained 0.165 µM of sodium molybdate ($Na_2MoO_4 \cdot 2H_2O$) as a molybdenum source and 0.1 mM of iron citrate ($Fe(C_6H_5O_7) \cdot H_2O$) as an iron source. The molybdate is the mostly used and stable form of molybdenum and transported into the cells by a specific transport proteins encoded by *mop* genes (Grunden and Shanmugam, 1997). Although the iron is supplied as ferric citrate, citrate is not taken up by the cells and it does not affect the growth of *R. sphaeroides* (Moody and Dailey, 1984). The media denoted as 1/10X, 10X and 100X indicate how many times the medium was concentrated or diluted in terms of that metal content. The Table 2.2 shows the relative concentrations of molybdenum and iron in the growth media. At different time intervals, the absorbance at 660 nm, pH and hydrogen gas evolved were measured.

Table 2.2. The metal ion composition in the media used for hydrogen production and transcriptional analyses.

Run name	[Na$_2$MoO$_4$2H$_2$O]	[Fe(III)citrate.hydrate]
1. Control	0.165μM	0.1mM
2. (No Mo)	0	0.1mM
3. (Mo 1/10X)	0.0165μM	0.1mM
4. (Mo 10X)	1.65μM	0.1mM
5. (Mo 100X)	16.5μM	0.1mM
6. (No Fe)	0.165μM	0
7. (Fe 1/10X)	0.165μM	0.01mM
8. (Fe 10X)	0.165μM	1mM
9. (Fe 100X)	0.165μM	10mM

2.3 Hydrogen production in photobioreactor

2.3.1 Hydrogen production under nitrogenase derepressed conditions

The hydrogen production with wild and mutant bacteria was performed in Biebl and Pfenning minimal medium with malate or acetate as carbon source and glutamate as nitrogen source using 62 ml photobioreactors. Since the media are devoid of any ammonia, the nitrogenase enzyme is fully active and act as ATP dependent hydrogenase in these conditions. The cultures were incubated at 30 ºC under irradiance of 940 μE/m^2/s which was provided by 100 watt tungsten lamp from a distance of 30-40 cm. Hydrogen gas evolved was measured by a construct develop-

ed by Uyar *et al.* (2007) (Figure 2.2). In this setup, the hydrogen gas was collected in the water filled tubes through empty cables spanning from cultivation bottles to gas collection tubes. The purity of gas collected was analyzed by gas chromatography. The rate and yield of H_2 production were calculated as shown in Appendix D.

Figure 2.2 The hydrogen production setup.

2.3.2 *In vitro* hydrogen evolution measurement under nitrogenase repressed conditions

In order to check the hox1 hydrogenase dependent hydrogen production, *in vitro* hydrogen production measurements were done under nitrogenase repressed conditions (in the presence of ammonia, rich medium). For this purpose, 20 ml of *R.sphaeroides* culture was centrifuged at 12 000 x g for 10 minutes and the pellet was dissolved in 2 ml of 20 mM phosphate buffer (pH=7). The cell suspension was transferred to a vial together with 60 μl of 20 mM BV (final concentration was 0.5

mM). After flushing the vial with N_2 gas for 10 minutes to anaerobize it, 0.5 ml of anaerobized dithionate (0.1 g/ml) was added with syringe. The mixture was incubated at 30 ℃ for 1 h and 200 μl of gas phase was taken from the vial and analyzed in GC to check the presence of hydrogen produced by hox1 hydrogenase. In the reactions shown below, DT serves as e^- donor (in excess amount) and reduces BV which then acts as an e^- donor to Hox1 hydrogenase (Rákhely *et al.*, 2004).

$$DT + BV^+ \longrightarrow BV_{reduced} \tag{2.1}$$

$$H^+ + BV_{reduced} \xrightarrow{\text{Hox1 H}_2\text{ase}} H_2 + BV_{oxidized} \tag{2.2}$$
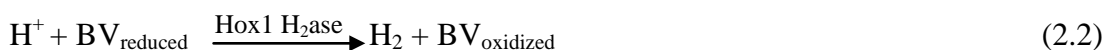
2.4 Analyses and sampling

Small aliquots from the bioreactors were taken in time intervals for pH and cell density analyses. The cell density analysis was performed by measuring the relative turbidity of the cultures using spectrophotometer (Shimadzu UV-1208, Japan). The absorbance of the cells was measured at 660 nm in 1 cm width glass cuvettes.

The pH of the cultures was initially set to 6.8-7, and was not controlled during the cultivation. At different time intervals the small aliquots from the bioreactors were taken and analyzed by pH meter (WTW series inoLab pH/Cond 720, Germany).

The gas composition of the samples was analyzed by gas chromatography with thermal conductivity detector (Agilent Technologies 6890N, USA). Argon was used as a carrier gas at a flow rate of 1.4 ml/min and HP19091F-413 column was used. The temperature sets for oven, injector and detector were 150 ℃, 50 ℃ and 250 ℃ respectively. A sample gas chromatogram was given in Appendix E.

2.5 Molecular genetic techniques

2.5.1 Genomic DNA isolation

Genomic DNA isolation was performed according to the procedure defined by Murray, M. G. and Thompson W. F. in "Short Protocols In Molecular Biology" edited by Ausubel *et al.* (1996). According to the protocol, 1.5 ml of culture having OD_{660}~1 was centrifuged at 13 000 rpm for 2 minutes. The pellet was resuspended in 567 μl TE buffer by repeated pipetting. 30 μl of 10 % SDS and 3 μl of 20 mg/ml proteinase K were added and mixed. The mixture was incubated at 37 ℃ for 1 hour. 100 μl of 5 M NaCl was added and mixed thoroughly. 80 μl of CTAB/NaCl solution was added, mixed and incubated at 65 ℃ for 10 minutes. Equal volume (780 μl) of chloroform/isoamyl alcohol was added, mixed, and centrifuged at 13 000 rpm for 5 minutes. The supernatant was transferred to a fresh tube and equal volume of phenol/chloroform/isoamyl alcohol was added, mixed, and centrifuged at 13 000 rpm for 5 minutes. 0.6X volume of cold isopropanol was added to a fresh tube and then the supernatant was poured onto it and mixed gently until the DNA precipitates. After a brief centrifugation, the gDNA pellet was obtained at the bottom of the tube. Then, 1 ml of 70 % ethanol was added and centrifuged again for 5 minutes at 13 000 rpm. The supernatant was discarded, and the pellet was dried. The pellet was then dissolved in 40 μl of sterile water at 65 ℃ for 1 hour. All the centrifugation processes were done using microcentrifuge. The solutions used here are given in Appendix C.

2.5.2 Plasmid DNA isolation

The plasmid isolation was performed according to the protocol developed by Birnboim and Doly (1979). According to the protocol developed by Birnboim and Doly (1979), 1.5 ml of overnight grown *E.coli* was centrifuged at 13 000 rpm at 4 °C and all the supernatant was discarded. 100 μl of solution I was added and the pellet was dissolved completely by vortexing or by pipetting. 3 μl of RNase A (100mg/ml)

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was added and the tube was vortexed for 3-5 seconds and incubated at room temperature for 5-10 minutes. After that 200 μl freshly prepared solution II was added and the mixture was mixed by inversion for 5 times, and incubated for 3 minutes on ice. Then, 150 μl of ice cold solution III was added and the tube was quickly mixed by inversion for 10 seconds and incubated on ice for 5 minutes. After centrifugation for 10 minutes at 13 000 rpm at 4 °C, approximately 400 μl supernatant was transferred to a new sterile tube. 200 μl phenol (Tris saturated, pH:8) and 200 μl chloroform:IAA (24:1 v/v) were added to the tube and it was mixed by vortexing for 5-10 seconds and the tube was centrifuged for 5 minutes at 13 000 rpm at 4 °C. Approximately 350 μl supernatant (the upper water part) was taken into a new sterile tube and mixed with 2 volumes of pure ethanol or 1X volume of isopropanol. The mixture was vortexed for 5 seconds and incubated at RT for 3 minutes. Then, it was centrifuged for 15 minutes at 13 000 rpm at 4 °C. The supernatant was poured without discarding the pellet and 1 ml 70 % ethanol was put gently onto the pellet and the tube was re-centrifuged for 3-5 minutes at 13 000 rpm at 4 °C. The supernatant was taken out and the pellet was dried in sterile cabinet or near flame. The pellet was dissolved in 20 μl sterile water. All the centrifugation processes were done using microcentrifuge.

If higher amount of plasmid DNA was needed, midi scale plasmid isolation protocol was performed using 10 ml of culture. In this protocol, only the volume of the solutions were changed such that 200 μl solution I, 400 μl solution II and 300 μl solution III were used. The generally used plasmids were shown in Appendix F.

2.5.3 Restriction enzyme digestion

The restriction enzyme digestion was generally performed in a total volume of 20 μl. Several μg of plasmid DNA can be cut in a single tube (i.e. 5 μg of plasmid DNA). After putting plasmid DNA into a tube, 2 μl of 10X related enzyme buffer and 20 units of enzyme were added to the reaction tube. The volume was completed to 20 μl by sterile water and the mixture was incubated at the temperature suitable for

the enzyme for 2-3 hours. 1-2 μl of sample was run on agarose gel to check if the reaction was completed. If DNA was completely digested then the reaction was stopped by heat inactivation. If the cut DNA was planned to be purified, heat inactivation was not necessary. If the reaction was not completed, it was incubated more. If overnight incubation was planned, less amount of enzyme was used.

Moreover, in certain conditions, the plasmid DNA or gDNA were digested with more than one enzyme at the same time in the same reaction tube. In this case, an online tool named as "DoubleDigest™" was used (www.fermentas.com). The tool selects the optimum buffer and temperature for the restriction enzymes used. The used restriction endonucleases with their relevant characteristics were listed in Appendix G.

2.5.4 Blunting sticky end of cut plasmid DNA

If digested DNA yielded sticky ends with 3' or 5' protruding ends, it was blunted using polymerase enzyme such as T4 DNA polymerase (Fermentas) or Klenow fragment (Fermentas) to be able to use the vector in blunt end ligation. If the digested DNA had 5' protruding end which was wanted to be kept same, Klenow fragment was preferred due to its higher fill-in activity. After digestion with sticky end producing restriction enzyme, 2 μl of 2 mM of dNTP was added to the same reaction tube together with 1 μl of T4 DNA Polymerase and the mixture was incubated for 10 minutes at RT. If the Klenow fragment was used, the reaction tube was incubated at 37 °C. Since phosphatases such as CIAP can accept nucleotides as substrate, this reaction mixture was cleaned to remove nucleotides before phosphatase treatment with CIAP.

2.5.5 Phosphorylation of PCR product

The PCR products were phosphorylated with kinase enzyme such as T4 Polynucleotide kinase (PNK) (Fermentas) to be used in ligation. Before phosphorylation, the PCR product was concentrated by salt precipitation or by ex-

tracting from the gel if there were non-speficic products. The PCR product wasdissolved in 8 µl of water and mixed with 10 µl of 10 mM of rATP and 2 µl of 10X polynucleotide kinase buffer A for forward reaction (Fermentas). 1.8 µl of T4 PNK (10 U/ µl) was added and the mixture was incubated for 30 minutes at 37 °C. After cleaning the solution by either phenol:chloroform extraction followed by precipitation or solution cleaning kits, PCR product can be used in ligation reaction.

2.5.6 Dephosphorylation of cut plasmid DNA

Calf intestinal alkaline phosphatase (CIAP) (Fermentas) was used to dephosphorylate the cut vector to prevent self ligation of the vector during ligation reaction. Since most of the restriction enzyme buffer was compatible with this kind of DNA modifying enzyme, the reaction was done with the same buffer which was used for the digestion of the DNA. 8 µl of 10X same restriction digestion buffer and 64 µl of water were added to the tube where DNA digestion was performed. 8 µl of (1U/µl) CIAP was added and the reaction was continued at 55 °C for at least 1 hour. Since CIAP was a stable enzyme, it was removed from the reaction by phenol:chloroform treatment or with other reaction solution cleaning kits. If phenol:chloroform treatment was applied, first the reaction volume was increased by adding water up to 100 or 200 µl.

2.5.7 Purification and concentration of DNA

In order to clean the enzymatic reaction from enzymes, dNTPs, buffers, salts and to concentrate DNA, either salt precipitation or reaction cleaning kits was used. If salt precipitation was preferred, 100 µl of PCR product or reaction mixture was mixed with 10 µl (1/10X volume) of 3 M sodium acetate (pH: 5.2) making final concentration 0.3M and 3 volumes of pure ethanol was added. After mixing the tube, it was incubated at -80 °C for 15 minutes or at -20 °C for 30 minutes and centrifuged at 13 000 rpm for 15 minutes at 4°C. The supernatant was carefully discarded with-

out disturbing the pellet, 1 ml of 70 % ethanol added and the tube was recentrifuged at 13 000 rpm for 3-5 minutes at 4 °C. The pellet was dried and dissolved in appropriate volume of sterile water. The centrifugation processes were done using microcentrifuge.

2.5.8 Extraction of DNA from agarose gel

In order to obtain digested DNA fragments from agarose gel, QIAquick Gel Extraction Kit (cat. #: 28704) was used. This kit was also used to cleanup enzymatic reactions. According to the protocol (QIAquick^R Spin Handbook, July 2002), the gel containing the DNA fragments was excised from the agarose gel and put into pre-weighed tube. Three volumes of buffer QC was added to 1 volume of gel (100 mg~100 µl) and incubated at 50 °C for 10 min. until the gel was completely melted. One volume of isopropanol was added to tube and mixed. The solution was loaded into QIAquick column and centrifuged at 13 000 rpm. Then 750 µl of buffer PE was added to the tube and centrifuged for 1 min. In order to remove traces of ethanol, the tube is re-centrifuged for 1 min. In order to elute DNA, 30 µl of sterile water was put onto membrane and incubated 1 min. at RT. Then the tube is centrifuged at 13 000 rpm for 1 min. to obtain DNA. For more concentrated DNA, 30 µl of water was used during elution. The centrifugation processes were done using microcentrifuge.

2.5.9 Ligation of insert to plasmid DNA

In general, molar ratio of insert to plasmid was selected as 3:1 when sticky end cloning was performed. In the case of blunt end cloning, 1:1 ratio was used. The molar ratio was converted to mass ratio by the following formula: ng of vector x (size of insert in kb/size of vector in kb) x (molar ratio of insert/vector) = ng of insert (Promega usage information). The DNA concentration by agarose gel electrophoresis with size marker was estimated by comparing the brightness of the band of interest relative to the nearest marker band. The vector, insert, 2 µl of 10X ligation buffer, 4 µl of PEG 4000 or PEG 8000 (50 % w/v), water, and T4 DNA ligase (5-10 units)

were mixed in a total volume of 20 μl and incubated at 22 °C for 1-2 hours after which 1-1.5 μl of ligation mixture was transformed to *E.coli* and the remaining of mixture was incubated o/n at 22 °C. In general, o/n ligation was preferred for the sake of simplicity.

2.5.10 RNA isolation

Rapid RNA isolation protocol was performed according to the protocol defined in "Short Protocols in Molecular Biology" edited by Ausubel *et al.* (1996). According to the protocol, 10 ml of bacteria culture was centrifuged at 12 000 x g at 4 °C for 10 minutes. The pellet was resuspended in 10 ml of protoplasting buffer, and 80 μl of 50 mg/ml lysozyme was added before incubation on ice for 15 minutes. Protoplasts were centrifuged at 5 900 x g for 5 minutes at 4 °C. The pellet was resuspended in 0.5 ml of gram negative lysing buffer and 15 μl of DEPC was added. The solution was mixed, transferred to centrifuge tube and incubated at 37 °C for 5 minutes. Then the solution was chilled on ice. 250 μl of saturated NaCl was added, mixed, and incubated on ice for 10 minutes. The mixture was centrifuged at 13 000 rpm for 10 minutes at room temperature and the supernatant was removed into two clean centrifuge tubes. 1 ml 100% cold ethanol was added and precipitated overnight at - 20 °C. The tubes were centrifuged at 13 000 rpm for 15 minutes at 4 °C. The pellet was rinsed with 500 μl, 70 % ethanol and centrifuged again at 13 000 rpm for 10 minutes at 4 °C. The pellet was dried and dissolved in 30 μl DEPC treated water.

In addition to commercially available kits, RNA isolation was also successfully performed using Tri reagent™ (Sigma, Product No. T9424). According to the protocol, 100 mg of cell pellet was obtained after several centrifugation at 13 000 rpm for 2-3 minutes. 1ml of Tri reagent™ was added to the pellet and dissolved by pipetting. 200 μl of chloroform was added, shaked for 15 seconds and left at RT for 15 minutes. The mixture was centrifuged at 13 000 rpm for 15 minutes at 4 °C and supernatant was transferred to a clean tube and 1-3 times phenol:chloroform (1:1) extraction was performed until the elimination of white particles between the

phases. The upper water phase was transferred to clean tube and 0.5 ml of isopropanol was added per ml of Tri reagent™ used. The solution was mixed and incubated at RT for 5-10 minutes. After centrifugation at 13 000 rpm for 12 minutes at 4 °C, the RNA pellet was obtained and then washed with 75 % ethanol by centrifuging for 5 minutes at 13 000 rpm at 4 °C. The pellet was dried and dissolved in 30 μl DEPC treated water. The ratio of OD_{260}/OD_{280} >1.8 shows a good quality of RNA.

2.5.11 Primer and probe design

The primers used in PCR and cDNA synthesis were generally designed using Primer 3 (v.0.4.0) online tool developed by Rozen and Skaletsky (Rozen and Skaletsky, 2000). Moreover, in the expression analyses of *hupS* and *nifD* gene, tag extended RT primers were used (Cobley *et al.*, 2002) in order to eliminate the non-specific binding of primers to the gDNA. In this approach, a high GC content tag (5-AGACCGTGTGGG-3) was attached to the transcript sequence. The following RT primer was used in the cDNA synthesis of *hupS* transcript, 5-AGACCGTGTGGGATCTTGTAGAGGCAGTAGC-3. In this primer, the underlined sequence was the GC rich tag, and the remaining was the sequence complementary to the transcript of *hupS* gene. Then, in the PCR reaction the following primer was used as one of the primers, AGACCGTGTGGGATCTTGTAG. The underlined sequence was only complementary to the tag sequence in the cDNA not to the gDNA, therefore, false positive result was eliminated in PCR. The same strategy was followed to design tagged primer for the expression analysis of *nifD* gene. The primers used in expression analyses with their properties were given in Table 2.3. The all primers were listed with their relevant characteristics in Appendix I.

Table 2.3. The primers used in the RT-PCR

	Primer pair for PCR (5'→3')	Primer for RT (5'→3')	Position
*nif*D	<u>agaccgtgtgggg</u>catctgac	<u>agaccgtgtggggcatctgac</u>ggaacgggatg*	2277013-2277032
	cgtcgacgcctaccatgacc		2277229-2277248
*nif*K	gaggtgcgagcgatagtagg	random hexamer	2276476-2276495
	gatctgttcaaggaacccga		2276729-2276748
*hup*S	ccgtgcacaaggtcatcctc	<u>agaccgtgtggg</u>atcttgtagaggcagtagc	2232113-2232132
	<u>agaccgtgtggg</u>atcttgtag		2232353-2232371
16S rRNA	cagctcgtgtcgtgagatgt	random hexamer	34683-34702
	tagcacgtgtgtagcccaac		34833-34852

* The underlined sequence represents the tag.

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2.5.12 Polymerase chain reaction (PCR)

The PCR reaction was performed either in 25 or 50 µl reaction volumes. The reaction ingredients were as follows;

Ingredients	Volume (µl)	Volume (µl)
Buffer (10X *Taq* buffer)	2.5	5
MgCl$_2$ (25 mM)	1.5	3
dNTP mix (10 mM each)	0.5	1
Forward Primer, (10 pmol/µl)	1	2
Reverse Primers (10 pmol/µl)	1	2
Template DNA (10 pg-1 ng/µl)	0.5	1
Enzyme (*Taq* DNA polymerase, 5u/µl)	0.5	1
Water	Completed to 25 µl	Completed to50 µl

In addition to *Taq* DNA polymerase (Fermentas), *Pfu* DNA polymerase (Fermentas), Phusion DNA polymerase (Finnzyme), DyNAzyme DNA polymerase (Finnzyme), *Pwo* DNA polymerase (Roche) were depending on the purpose. In the case different enzyme was used, the buffer and MgCl$_2$ content were changed while the others were kept same.

The cycling parameters were as follows;

Cycle name	Temperature (°C)	Time	Cycle number
Initial Denaturation	94	1-5 min.	1
Denaturation	94	10-60 sec	30-35
Annealing	T_m-5	10-60 sec.	30-35
Extension	72	30 sec.-5 min.	30-35
Final Extension	72	5	1

The time for denaturation, annealing and extension was adjusted depending on the type of polymerase used in PCR.

2.5.13 Colony polymerase chain reaction (cPCR)

This type of PCR was performed to screen and confirm the single recombinants, or to find out the correct clone. For his purpose, 100 μL of sterile water was put into a sterile 1.5 ml microfuge tube and the colony grown on agar plates was put into the tube and dissolved by pipetting or by vortexing. After dissolving the single colony in water, the suspension was incubated at 95 º C for 10 minutes. During incubation, the suspension was vortexed several times to fasten the cell burst. Then, the cells were centrifuged at 13 000 rpm for 3 minutes to pellet the cell debris and the supernatant containing the gDNA was transferred to another sterile centrifuge tube. PCR was performed using this sample as template. In some cases the colony itself was directly put into the PCR tube though it was not as efficient as previous one.

2.5.14 Sequence analyses

The sequence analyses were performed to ensure that the PCR product was our expected one and to prove the *hup* mutant double recombinants. The region to be sequenced was amplified using specific primers for that region and then loaded onto agarose gel. The specific region on agarose gel was cut and eluted. Then the purified PCR product and a primer were sent to sequencing done by a company (İontek, İstanbul).

2.5.15 Complementary DNA (cDNA) synthesis and reverse transcription-polymerase chain reaction (RT-PCR)

The complementary-DNA synthesis was performed as follows; 0.1-5 μg of genomic DNA free total RNA was put in a sterile tube. 0.2 μg of random hexamer or

15-20 pmol sequence specific primer was added and it was completed to 11 µl with nuclease free water. The mixture was incubated at 70 °C for 5 minutes and then chilled on ice. 4 µl of 5X reaction buffer, 2 µl of 10 mM dNTP were added and the mixture was completed to 19 µl with nuclease free water. The mixture was incubated at 37 °C for 5 minutes. If random primer was used, incubation was done at 25 °C for 5 minutes instead. 40 units of M-MuLV reverse transcriptase (Fermentas) were added. The reaction mixture containing sequence-specific primer was incubated at 37 °C for 1 hour. If random hexamer was used, mixture was incubated at 25 °C for 10 minutes first and then at 37 °C for 1 hour. The reaction was stopped by incubating the mixture at 70 °C for 10 minutes before chilling on ice for a few minutes.

3-5 µl of cDNA was used in the PCR to look at the transcript level of the gene of interest. The cycling conditions were same as mentioned in section 2.5.12.

2.5.16 Southern blotting and hybridization

2.5.16.1 Probe design and preparation for Southern hybridization

For the Southern hybridization, DIG DNA Labeling and Detection Kit (Roche) was used. The experiment is based on the digoxigenin (DIG) labeled probes and hybridization of the probes to the denatured DNA on the membrane. Digoxigenin is a steroid hapten attached to the dUTP (Fig. 2.3).

The DIG-labeled probes for the Southern hybridization was prepared according to the manufacturer's instructions (Roche DIG DNA Labeling and Detection Kit, Instruction Manual, Version October 2004) as follows: A 10 ng-3µg DNA (purified PCR product or DNA fragment) was put into a tube (the volume must be 15 µl, if it was not, it was completed by sterile H_2O). The DNA was then denatured by boiling at 95-100 ºC for 10 min. and rapidly chilled on ice for 1-3 minutes. Then, 2 µl of hexanucleotide mix, 2 µl of dNTP labeling mix and 1 µl of Klenow enzyme were added to the reaction tube. The final reaction volume was 20 µl. The solution was mixed, centrifuged briefly and incubated at 37 ºC for overnight

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(16-20 hours). Longer incubation time increases the efficiency of the labeling. Therefore it is advised to incubate up to 20 hours. The reaction was stopped by adding 2 µl of 0.2M EDTA (pH 8.0) and/or heating to 65 ℃ for 10 minutes. Theoretically, the length of the resulting DIG-labeled DNA fragments range from 200 to 1000 bp.
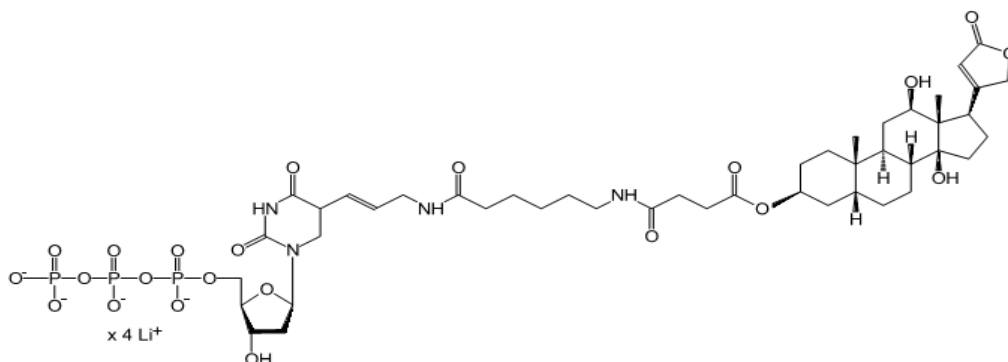
Figure 2.3 DIG-11-dUTP.

2.5.16.2 Southern blotting of fragmented gDNA to membrane

After the probes were synthesized, gDNA was cut with a frequent cutter restriction enzyme and loaded onto 0.7 % agarose gel prepared by using 0.5X TBE buffer (pH:8.3). The agarose gel electrophoresis and Southern transfer were performed by using 0.5X TBE buffer too. The thickness of the agarose gel was smaller than 0.6 mm (or equal to 0.6 mm) to make an efficient Southern transfer. However, the thinner gels did not accommodate all cut gDNA, therefore 0.6 mm agarose gels were preferred. The Southern transfer was performed using Bio-Rad Gel Transfer System (Germany) at 13 volts for 4-5 hours at 4 cm electrode distance (high electric field). The DNA fragments were blotted to positively charged nylon membrane (Roche).

2.5.16.3 Southern hybridization and detection

The optimal hybridization temperature (T_{hyb}) for DNA:DNA hybrids in any buffer was calculated according to the following formula given in Roche instruction manual; $Tm = 16.6 \log [Mol\ Na^+] + 0.41\ (\%\ G + C) + 81.5\ T_{hyb} = T_m - 25\ °C$ Where T_m is the melting point of probe-target hybrid (in the absence of formamide) and T_{hyb} is the optimal temperature for hybridization of probe to target. This calculation was performed if there was a 100 % homology between the target and probe which was the case here. Theoretically if the homology is smaller, then the hybridization temperature is lowered. The detection was performed by using Anti-Dig-Alkaline Phosphatase conjugate (Roche).

2.5.17 Gene delivery methods

2.5.17.1 CaCl₂ mediated transformation of *E. coli* and *R. sphaeroides*

The CaCl₂ mediated chemical transformation was used as a general way of gene transfer to *E.coli* strains. Either a sample of ligation reaction or a bare vector was successfully transferred to *E.coli* strains. First, the competent *E.coli* was prepared according to the protocol described by Sambrook *et al.* (1989). According to the protocol, a single colony was inoculated into LB broth and grown at 37 °C with shaking o/n. Then, a new LB broth was inoculated with freshly grown cells and the bacteria were grown until the OD_{660}=0.5-0.8. The culture was centrifuged at low speed such as 4000 rpm at 4 °C for 10 min using microcentrifuge. Then the pellet was dissolved in 2 ml of ice-cold 0.1 M CaCl₂ for each 10 ml of bacteria centrifuged. The cells were re-centrifuged and dissolved in 1.2 ml of ice-cold 0.1 M CaCl₂ containing % 25 glycerol for each 10 ml culture centrifuged. Since the competent cells were stored at -80 °C, they were harvested with glycerol a cryoprotectant. The small aliquots were stored in -80 °C for later use.

The transformation was performed as follows; 100 µl of aliquot was mixed with 1-0 µl of ligation reaction or 1-10 ng of DNA and incubated on ice for 30 min.

Then a heat shock was applied to the cells by incubating them at 42 °C for 90 sec exactly. Then the cells were put on ice for 5 min. After the incubation, 900 µl of LB or SOC medium was added and the bacteria were incubated at 37 °C for one hour. Then small aliquots were spread on agar plates with a selection marker.

Transformation of *R. sphaeroides* has been only reported by Fornari and Kaplan (1982). To prepare the competent *R. sphaeroides*, 20 ml of *R. sphaeroides* culture with a density of 8 x 10^8 cells/ml was centrifuged at 7 000 rpm for 5 min. at 4 °C. The pellet was dissolved in 10 ml of ice-cold 0.5 M Tris-Cl (pH 7.2). After re-centrifugation, the cell pellet was suspended in ice-cold 0.1 M Tris-Cl (pH 7.2), 0.2 M $CaCl_2$. Plasmid DNA was added to 0.2 ml of competent cells together with 40 % PEG in 0.1 M Tris-Cl (pH 7.2). The suspension was mixed and left on ice for 10 min. A 2 min heat shock at 35 °C was applied followed by the addition of 1 ml of fresh medium and the tubes were incubated at 35 °C for 20 min. Three milliliters of medium were added and the tubes were further incubated at 35 °C for over 6.5 hours to allow phenotypic expression before spreading onto selective plates. However, this method was found to be less efficient than conjugation.

2.5.17.2 Electroporation of *E. coli* and *R. sphaeroides*

Although the efficiency of transformation by electroporation was said to be higher compared to that of chemical transformation, the chemical transformation of *E.coli* was straight forward and worked efficiently. And, the conjugation was the most effective one in the case of *R. sphaeroides*. The preparation of electrocompetent cell was very crucial in terms of the efficiency. According to the protocol provided by Manufacturer (Bio-Rad), to prepare electrocompetent *E.coli*, 500 ml of L-broth was inoculated with 1/100 volume of a fresh overnight *E. coli* culture and the cells were grown at 37 °C by shaking at 300 rpm to an OD_{600} of circa 0.6. The cells were chilled on ice for ~20 minutes. All the subsequent steps were performed at the temperature as close to 0 °C as possible. The cells were centrifuged at 4 000 x g for 15 minutes at 4 °C. The pellet was re-suspended in 500 ml of ice-cold 10 % glycerol and then the cells were centrifuged at 4 000 x g for 15 minutes at 4 °C. The superna-

tant was discarded and the pellet was re-suspended in 250 ml of ice-cold 10 % glycerol. After centrifugation at 4 000 x g for 15 minutes at 4 °C, the supernatant was carefully poured off and the pellet was re-suspended in ~20 ml of ice-cold 10 % glycerol. The suspension was transferred to a 30 ml sterile tube and centrifuged at 4 000 x g for 15 minutes at 4 °C. The cell pellet was dissolved in a final volume of 1–2 ml of ice-cold 10 % glycerol. The cell concentration should be about 1–3 x 10^{10} cells/ml. The cells were aliquoted in 100 µL samples and frozen by liquid nitrogen and stored at -80 °C. The cells could be stable for several months under these conditions.

According to the manufacturer's instructions (Bio-Rad), the electroporation of *E.coli* cells were performed as follows; the *E. coli* cells were thawed on ice and for each sample to be electroporated, a 1.5 ml microfuge tube and a 0.2 cm electroporation cuvette were placed on ice. In a cold 1.5 ml microfuge tube, 40 µl of the cell suspension was mixed with 1 to 2 µl of ligation mixture or bare plasmid and the mixture was incubated on ice for about 1 minute. The mixture was transferred to a cold electroporation cuvette which was then tapped to push the suspension to the bottom. After the pulse was applied once, 1 ml of SOC medium was put into the cuvette as soon as possible (the shorter the time of SOC addition, the higher the efficiency of transformation, delaying this transfer by even 1 minute causes a 3-fold drop in transformation). The cell suspension was transferred to a bigger tube and incubated at 37 °C for 1 hour by shaking at 225 rpm. After 1 hour certain volumes were plated on selective medium which contains antibiotic, IPTG and X-Gal for blue white screening. The applied voltage should be 1.8 kV if 0.1 cm cuvette was used or 2.5 kV if 0.2 cm cuvette was used. The time constant should be around 5 miliseconds.

For the preparation of electrocompetent *R. sphaeroides*, 500 ml of freshly grown cells (OD_{660}=0.5-07, aerobically grown cells formed better pellet than anaerobically grown cells and usually aerobically grown cells were used for competent cell preparation) were incubated on ice for 15 minutes and then centrifuged for 10 minutes at 10 000 x g at 4 °C. The pellet was formed well when the tubes were balanced before centrifugation. The pellet was dissolved in 500 ml

sterile ice-cold distilled water and centrifuged again at the same conditions. After discarding the supernatant, the pellet was dissolved in 250 ml of sterile ice-cold dH2O. After another centrifugation, the pellet was dissolved in 125 ml sterile ice-cold dH_2O. The cells were centrifuged again and the pellet was dissolved in 15 ml of sterile ice-cold 10 % glycerol. After the final centrifugation, the pellet was suspended in ice-cold 10 % glycerol such that the concentration of the cell suspension will be 10^{10} cells/ml. Then, the cells were aliquoted in 90 μl samples and stored at -80 °C for several months (Donohue and Kaplan, 1991).

The electroporation protocol was quite different for *R. sphaeroides* than *E.coli*. The competent cells were thawed on ice and 40 μl of competent cells were placed in a chilled 1.5 ml microfuge tube together with 1-2 μl prechilled ligation mixture. The cells and the DNA were mixed and incubated on ice for 1 minute and then transferred to a prechilled 0.2 cm electroporation cuvette. For one sample, 4 pulses were applied and between each pulse 1 minute incubation on ice was done. The time constant should be around 8.5 miliseconds (A resistance of 400 ohms using 0.2 cm cuvettes with 2.5 kV and 25 μF settings was used to generate pulses). If the time constant was low (too conductive sample), the cells were diluted or the kilovolt setting was lowered. After the final pulse, 1 ml of SOC medium was added as soon as possible and the cells were incubated at 32 °C for 6 hours (two doublings time) by shaking. The samples were spread on selective media.

2.5.17.3 Diparental mating (Conjugation)

For the delivery of plasmids and constructs into *R. sphaeroides*, diparental mating was applied. The construct was first transferred into *E.coli* S17-1(λpir) strain which provides the transfer function by *tra* genes on the chromosome. The *mob* region also required for the transfer was provided by the construct (vector) and this type of constructs can be delivered into *R. sphaeroides* by conjugation from *E.coli* S17-1(λpir) cells. The freshly grown *E.coli* S17-1(λpir) cells containing the construct were grown until mid-log phase of growth ($OD_{600} \sim 0.5$) but the inoculum must be also fresh. Therefore it is better to re-inoculate the culture into a fresh LB media with

1:10 proportion to ensure the log phase. The *R. sphaeroides* culture was also freshly grown until log phase (OD_{600}~0.5-0.7) in minimal medium under aerobic conditions. About 40 ml of recipient cells were mixed with 4 ml of donor cells (1:10 ratio). Before mixing, the donor culture was centrifuged at 5000 rpm then the pellet was dissolved in 4 ml cold minimal medium to remove the antibiotic. The mixture was centrifuged at 10 000 x g for 10 minutes and the pellet was dissolved in 200-300 μl of minimal medium and this mixture was spotted onto a 0.45 μm pore size nitrocellulose filter that was previously placed on LB plate. The mating pairs were incubated at 32º C for 6 hours. After that the filter was placed onto a new plate and washed with 1 ml of minimal medium and then this cell suspension was put into a tube. From 50 to 300 μL of this mixture was spread onto selective B&P minimal media. Since *E.coli* S17-1(λpir) strain was proline auxotroph, they were not able to propagate in minimal media. Therefore, they were eliminated during passages in minimal media. Moreover, since *R. sphaeroides* cells were resistant to tellurite up to 200 μg/ml, it could also be included in the selective media though it seemed not necessary. If only the bare vector was going to be transferred to *R. sphaeroides*, dilutions were needed before spreading to plates. The colonies should appear on selective media within 2-3 days (Donohue and Kaplan, 1991). The gene transfer by conjugation is explained schematically in Figure 2.4.
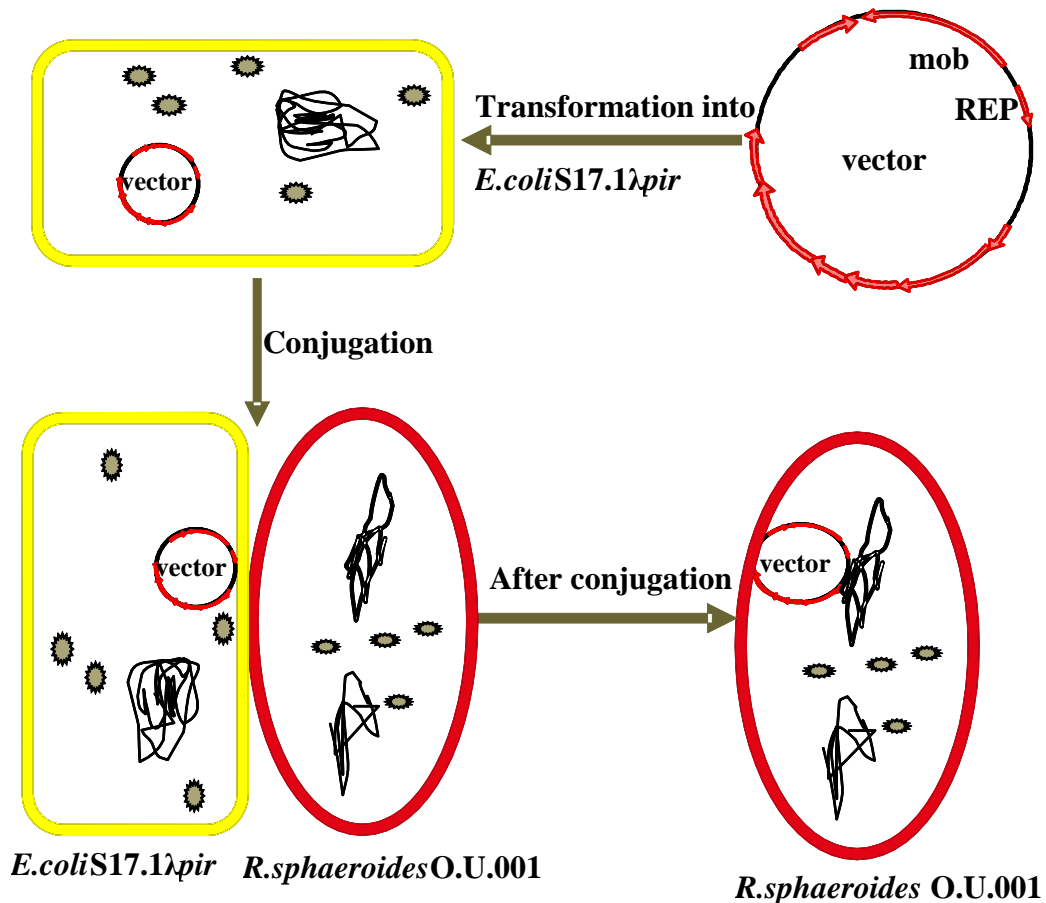
Figure 2.4 The transfer of the vector from *E.coli* S17-1(λpir) to *R sphaeroides* by conjugation.

2.6 Biochemical Techniques

2.6.1 Quantification of nitrogenase protein in *Rhodobacter sphaeroides*

Previously, it was stated that the nitrogenase comprises about 20 % of all cellular protein in the nitrogen fixing cell although it was not clearly demonstrated and proved. If that was the case, it constitutes a big burden for the cells to turnover that much protein. For this reason, in order to have a relative idea about the amount of enzyme in cells under H_2 producing and nitrogenase repressed conditions; the total cellular proteins of *R. sphaeroides* were analyzed by SDS-PAGE.

2.6.1.1 Harvesting and disrupting *Rhodobacter sphaeroides* for SDS-PAGE

R. sphaeroides was cultivated in three different ways (aerobic, anaerobic and anaerobic with 10 mM NH_4Cl instead of Glutamate) to compare the relative amount of nitrogenase enzyme in the cells. The ammonium was expected to repress nitrogenase enzyme. The freshly grown cells were collected after centrifugation at 10 000 x g for 10 min. and dissolved in Tris-Cl (pH 8). They were freeze-thawed five times in liquid nitrogen and centrifuged at 14 000 x g for 30 min. The supernatant was transferred to clean tubes to be used in Bradford assay.

2.6.1.2 Protein concentration determination by Bradford assay

Total protein of the cells was measured by Bradford protein assay (Bradford, 1976). The calibration curve was drawn using protein standards. Then, the concentrations of three unknown samples were calculated from this standard curve to be used in SDS-PAGE. The preparation of Bradford reagent was explained in Appendix C.

2.6.1.3 Polyacrylamide gel electrophoresis (SDS-PAGE)

The protein samples (5.5 µg each) were run on polyacrylamide gel consisting of 12.5 % separating part and 5 % stacking part. The samples were run at 20 mA at stacking gel and 40 mA at separating gel. The gel was stained with Coomasie Brillant Blue G-250. The composition of gel together with the buffers and solutions were given in Appendix C. After staining of the gel, the densitometric analyses of the corresponding nitrogenase protein subunits (nifH, nifD and nifK) were done by Scion Image Analyzer (Scion Corporation, USA).

2.6.2 Uptake hydrogenase activity assay

The uptake hydrogenase assay was performed using an artificial electron

acceptor, benzyl viologen with the molecular formula $C_{24}H_{22}Cl_2N_2$ (1,1′-Dibenzyl-4,4′-bipyridinium dichloride, BV) (Goodman and Hoffman, 1983). After the conversion of molecular hydrogen to protons and electrons by uptake hydrogenase, BV accepts the electrons and reduced to colored form and this color formation is monitored by spectrophotometric measurements during certain time intervals. 1.5 ml of anaerobically grown cells was centrifuged and dissolved in 1 ml of 20 mM phosphate buffer (pH=7.0). 1 ml of phosphate buffer was added together with 100 μl of 40 mM BV. The mixture was first flushed with nitrogen gas to make anaerobic environment and then with hydrogen gas used as a substrate of uptake hydrogenase for 5 minutes. This mixture was prepared in anaerobic cuvette and the kinetics of the reaction was measured by spectrophotometer at the temperature at which the bacteria grow or at 60 ℃. The color change due to reduction of BV was recorded for certain time at the wavelength of 600 nm.

2.6.3 Nitrogenase activity assay

Wild type and *hup* mutant *R. sphaeroides* were grown under hydrogen producing conditions and their nitrogenase activities were measured by acetylene reduction assay (Schneider *et al.*, 1991). 5 ml of anaerobically grown wild type and mutant cells were placed in 10 ml XTT bottles and anaerobized by N_2 flushing. 15 % of the remaining volume of the bottle was replaced by acetylene gas by injecting it with Hamilton Syringe a few times. After incubation at 30 ℃ for 2 hours under the light, 50-100 μl gas sample was taken and analyzed by GC. The analysis was done using PLOT Q column with TCD detector at 40 ℃ oven temperature in Agilent 6890N gas chromatography (U.S.A).

2.7 Software and online tools

Primer design online tool: Primer 3 (v.0.4.0) ((Rozen and Skaletsky, 2000)
DNA double digestion online tool: DoubleDigest™ (Fermentas)
Densitometric gel analyses: Scion Imaging Software (Scion Corporation)

CHAPTER 3

RESULTS AND DISCUSSIONS

3.1 The effect of molybdenum and iron on hydrogen production and expression of *nifD*, *nifK* and *hupS* genes in *R. sphaeroides* O.U.001

It was stated that due to the slow turnover time making diazotrophs synthesize large amount of nitrogenase and the consumption of a considerable amount of ATP, the regulation of the nitrogenase genes at the transcriptional level in response to environmental stimuli is quite stringent (Dixon and Kahn, 2004). These environmental effectors which also significantly determine the overall hydrogen production capacity could be listed as light, oxygen, ammonia, temperature, metal ions such as molybdenum and iron. Considerable amount of studies has been conducted to see the influence of various parameters like light/dark cycle (Jouanneau *et al.*, 1985), oxygen (Oelze and Klein, 1996; Axelsson and Lindblad, 2002), hydrogen (Axelsson and Lindblad, 2002), pH (Tsygankov, 1997), nitrogen sources (Arp and Zumft, 1983; Jouanneau *et al.*, 1985), and metal ions (Axelsson and Lindblad, 2002, Tsygankov, 1997, Jacobson *et al.*, 1986) on hydrogen production by bacteria. According to the results, light strongly stimulates nitrogenase synthesis (Jouanneau *et al.*, 1985) but oxygen and ammonia inhibit nitrogenase (Dixon and Kahn, 2004). Availability of certain metal ions such as molybdenum and iron which are found in the structure of Mo-nitrogenase also alters the hydrogen production efficiency significantly. As an example, in *Azotobacter vinelandii*, *nifHDK* transcription was repressed under Mo-deprived conditions (Jacobson *et al.*, 1986). Moreover, the presence of Mo was shown to repress alternative nitrogenases in *R. capsulatus* (Schneider *et al.*, 1991). Yang and Shen (2006), in their studies, showed

that the hydrogen production was increased with increasing iron concentrations (the highest hydrogen production was obtained at 150 mg/l $FeSO_4$ iron concentration) in an anaerobic bio-hydrogen production process from soluble starch.

In this part of the study, the effect of various concentrations of sodium molybdate on the expression level of the *nifK* gene and of ferric citrate on the expression level of *nifD* and *hupS* genes were investigated. Although the gene transcript level does not reflect the exact amount of the protein, it gives us valuable information about the amount of the protein. The gene expression analyses were performed with respect to time to understand the relations among the gene expression level, biohydrogen production and the growth phases. The hydrogen production was also measured under the same growth conditions to show the effect directly.

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

Figure 3.1 illustrates the pH and absorbance changes during the growth of *R. sphaeroides* O.U. 001 in different media (Table 2.2). Although the initial pH of the media was buffered to 7, it increased upto 9.0 in No Mo and 1/10X Mo media (Figure 3.1A). On the other hand, in 1X Mo, 10X Mo and 100X Mo media, there was no significant rise of the pH, which remained below 8.0. If we refer to the hydrogen production curve (Figure 3.2), the rise in the pH started at t=24 h corresponding to the beginning of the hydrogen production in 1X Mo, 10X Mo and 100X Mo media. This observation might suggest that the lack of molybdenum in No Mo medium and its deficiency in 1/10X Mo medium led to decrease in hydrogen production and thus the reducing equivalents were used for the formation of products such as polyhydroxybutyrate (PHB), which might lead to the increase in pH. Similar results were also recorded by Uyar B., in his PhD thesis such that an increase in pH was always seen after the growth of bacteria (Uyar B., PhD Thesis, Middle East Technical University, 2008 Ankara). However, then, a drop in pH was observed. This fluctuation might be due to first consumption of organic acids such as malate or

acetate which results in pH rise then the production of protons from carbon sources which lead to drop in pH.

In Figure 3.1B, it can be seen that molybdenum insufficiency did not interfere with growth. On the contrary, the bacteria in the absence of Mo and in 1/10X Mo media reached relatively higher absorbance values, which were 1.74 and 1.90 respectively. Yet, the other three cultures reached relatively lower absorbance values, which were below 1.5. Based on these observations, it could be concluded that the bacteria that did not spend their energy for hydrogen production reached relatively higher cell masses and densities.

(A)

(B)
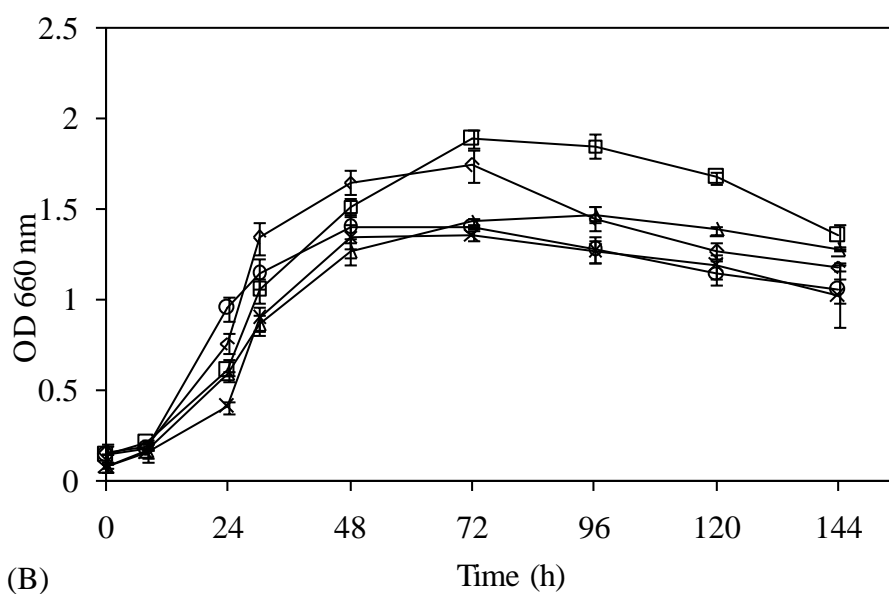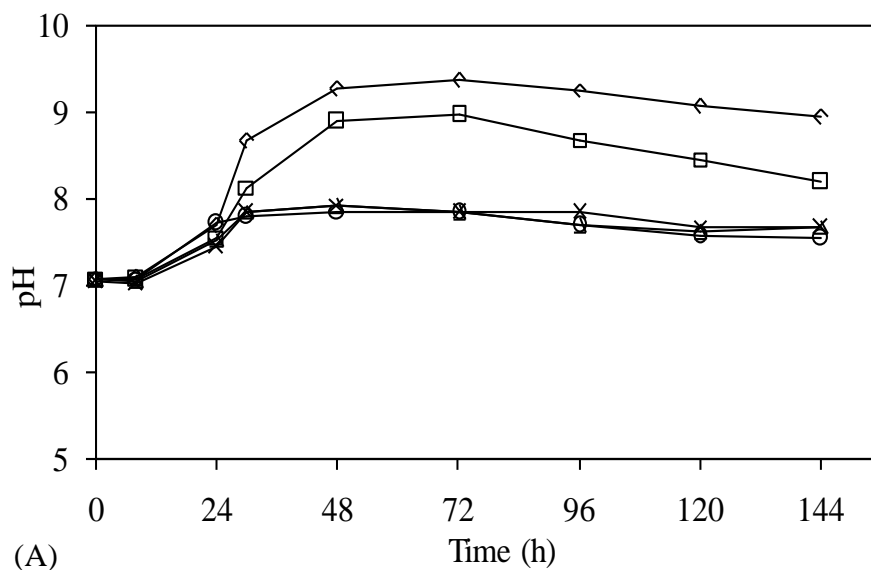
Figure 3.1 The pH (A) and absorbance (B) changes during growth of *R. sphaeroides* O.U.001 in media with different molybdenum concentrations. The initial pH of the cultures was buffered to 7 and was not controlled during incubation. Each value in the pH and growth curve is the mean of three measurements with ± standard deviation. (◊) No Mo; (□) 1/10X Mo; (Δ) 1X Mo; (×) 10X Mo; (o) 100X Mo.

Figure 3.2 points out the influence of different concentrations molybdenum on hydrogen production by *R. sphaeroides* O.U. 001. There was almost no hydrogen production in No Mo medium and little hydrogen production in 1/10X Mo medium; however, there was an increase in total hydrogen production accompanied with elevated molybdenum concentrations. The maximal hydrogen accumulation was achieved in 100X Mo medium (0.84 l/l) followed by 10X Mo (0.74 l/l) and 1X Mo (0.64 l/l) media. The results might imply that increase in molybdenum concentration stimulated hydrogen production. Since nitrogenase enzyme accommodates molybdenum in its active center, either the amount of enzyme was increased or the activity of the enzyme was increased by increasing concentrations of molybdenum. The gas chromatography analyses showed that the hydrogen constituted 97-99 % (v/v) of the overall gas evolved.
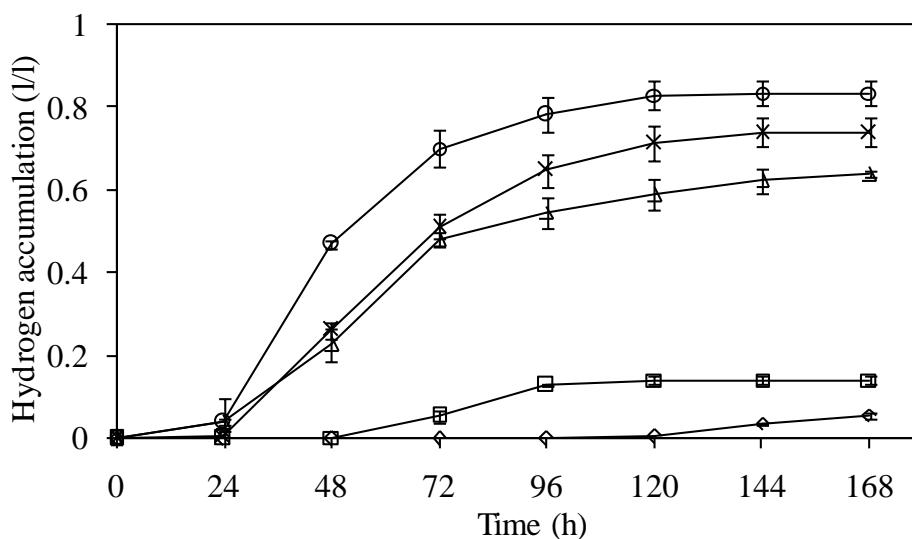
Figure 3.2 Total hydrogen production by *R. sphaeroides* O.U. 001 in media with different molybdenum concentrations. Each value in the curve is the mean of three measurements with ± standard deviation. (◊) No Mo; (□) 1/10X Mo; (Δ) 1X Mo; (×) 10X Mo; (o) 100X Mo.

3.1.2 Time-dependent expression analysis of *nifK* at different molybdenum concentrations

The primers for the *nif*K and 16S rRNA genes were carefully designed and there was no complete nonspecific match as a result of similarity search done using the BLAST at the NCBI web page (www.ncbi.nlm.nih.gov/BLAST/). The primers were also checked by PCR followed by sequence analysis in which gDNA was used as a template to show that the primers amplified the *nifK* exactly (Figure 3.3).
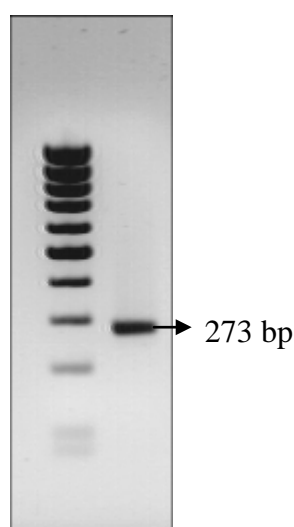
273 bp

Figure 3.3 The *nifK* PCR product was run on agarose gel together with 100 bp DNA ladder. The PCR product gave expected 273 bp band on the gel.

The sequence analysis of the PCR product was done and (Appendix H) and it was proved that the PCR product was exactly the *nifK* gene of *R. sphaeroides*.

False positive results due to the DNA contamination was checked by a direct PCR from each RNA sample and no bands were observed at all. The expression of *nif*K with respect to time was followed during the growth of *R. sphaeroides* O.U.001 in No Mo, 1/10X Mo, 1X Mo and 100X Mo media (Figure 3.4). There was no ex-

pression at t=23 h and very low expression until t=120 h in the cells grown in medium without molybdenum; however, there was some expression in the late stationary phase of the growth (t=120 h). Hydrogen production also started in this phase in No Mo medium, although the amount of hydrogen was not considerable. This lag time might be the period for the cells to collect the traces of molybdenum to express nitrogenase genes. The expression in the cells grown in 1/10X Mo medium started at t=23 h and reached the highest level at t=41 h corresponding to the late log phase. Moreover, a small amount of expression was seen at t=65 and 120 h. The expression level of *nif*K in the cells grown in 1X Mo and 100X Mo media was almost the same in all phases of the growth. The expression started at t=23 h and reached the highest level in the late log phase (t=41 h). Then, it decreased towards the end of the stationary phase of growth.

In general, *nif*K expression was maximal in the late log phase of the bacterial growth (almost the beginning of the stationary phase) in 1/10X Mo, 1X Mo and 100X Mo media while it decreased towards the end of the stationary phase of growth. Colbeau *et al.* (1980) and Krahn *et al.* (1996) found similar results which demonstrated that hydrogenase and nitrogenase activities were maximal in this phase of growth in *R capsulatus*. Although there was more hydrogen accumulation in 100X Mo medium than in 1X Mo and 1/10X Mo media, the gene expression levels were not different significantly. This might result from higher amount of properly assembled Mo-nitrogenase in 100X Mo medium due to higher Mo concentration.

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

In this study, four types of media (No Fe, 1/10X Fe, 1X Fe, and 10X Fe) were prepared to see the effect of varying concentrations of iron on pH, growth, biohydrogen production and *nif*D expression. pH changes during the growth of *R. sphaeroides* O.U.001 at various iron concentrations are illustrated in Figure 3.5A. The pH values of 1/10X Fe, 1X Fe and 10X Fe media were all similar (~7.0) and did not change considerably. However, the pH of No Fe medium started to rise after t=46
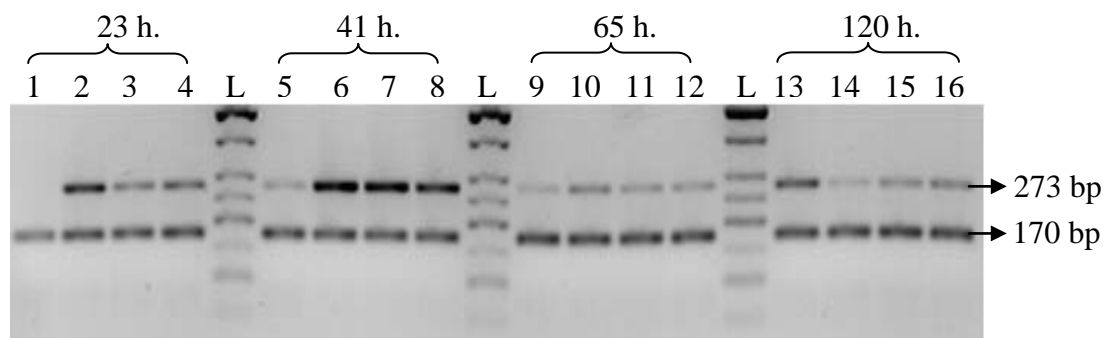
Figure 3.4 Agarose gel of RT-PCR products of *nif*K in *R. sphaeroides* O.U.001 cultured in media with various concentrations of molybdenum ([Na$_2$MoO$_4$.2H$_2$O]: without molybdenum (No Mo); 0.0165 μM (1/10X Mo); 0.165 μM (1X Mo); and 16.5 μM (100X Mo)). Products of RT-PCR were run on a 1.5 % agarose gel at 90V for 1h together with a 100 bp DNA ladder (L). The analyses were performed at t=23, 41, 65 and 120 h. Lane 1-4, 5-8, 9-12 and 13-16 represent the results obtained from the cells grown in No Mo, 1/10X Mo, 1X Mo and 100X Mo media respectively. In each lane, the *nif*K product was normalized using the internal control (16S rRNA), which was expressed uniformly throughout the growth phase.

h and became alkaline (~9.0). The rise in pH in media with reduced hydrogen production due to metal ion deficiency and the beginning of the hydrogen production were observed almost at the same time (t=48 h). Therefore, this might imply that evolved hydrogen or the accumulation of some acidic compounds might keep the pH from rising in media with hydrogen production. There was a growth delay in No Fe and 1/10X Fe media indicating the vitality of iron for cellular functions (Figure 3.5B). The fresh cultures in 1X Fe and 10X Fe media immediately entered the log phase yet this phase was only seen after t=24 h in No Fe and 1/10X Fe media. The observed colors of the iron starved cultures were also different from that of the others showing the interference of iron starvation with other cellular processes. The maximal absorbance value for the iron starved culture was 0.8 but the other three media reached optical densities circa 1.0. Moreover, it was also possible for the iron

starved cells to get traces of iron during the sampling with syringe and therefore grew better than expected.

The highest total amount of hydrogen production was observed in 1X Fe medium (1.14 l/l). In addition, 1.02 l/l and 0.98 l/l total hydrogen accumulation was achieved in 1/10X Fe and 10X Fe media (Figure 3.6). However, no hydrogen production was recorded in No Fe medium showing that iron is necessary for hydrogen evolution. As explained in detail in the introduction part, the iron is not only found in the structure of nitrogenase, but also found in the structure of electron carriers like ferrodoxin. Therefore, the availability of iron strictly influences the hydrogen production performance of the cells. The highest iron concentration (1 mM) caused a decrease in hydrogen production which might be due to a toxic effect or due to intense color of the medium because of iron which might have limited the light penetration. Therefore, the optimum iron concentration was found to be 0.1 mM. The GC analyses of the collected gas showed that the fraction of the hydrogen was between 98-99 % (v/v) in the overall gas.
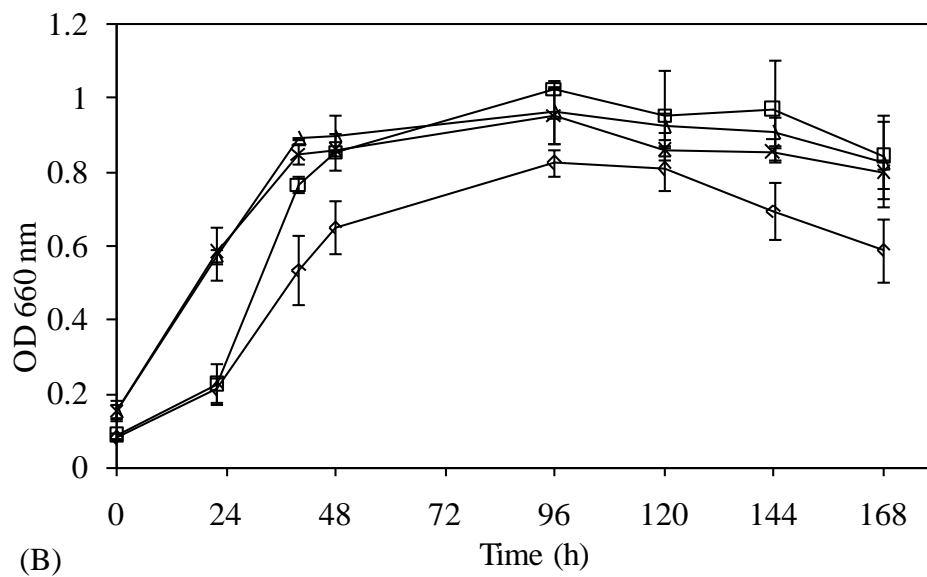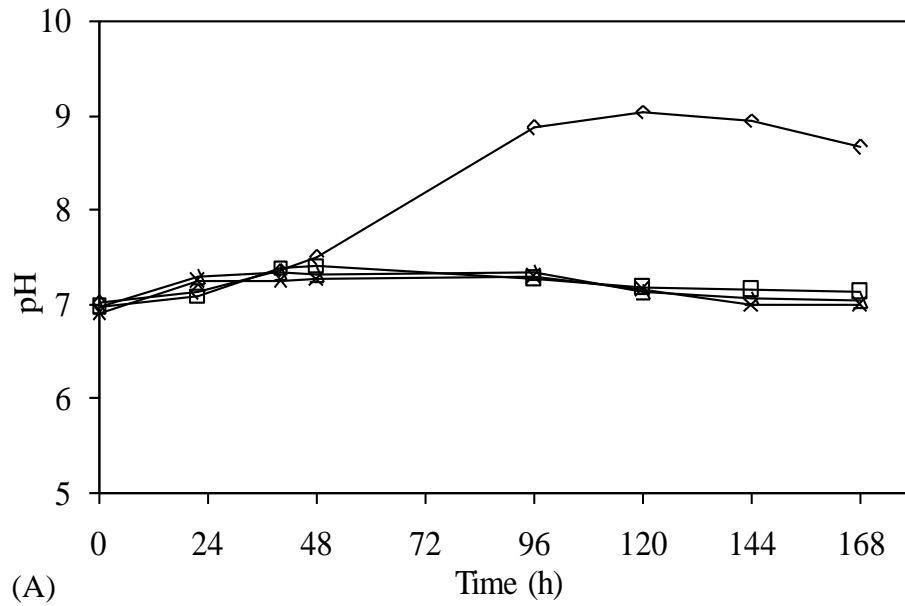
Figure 3.5 The pH (A) and absorbance (B) changes during growth of *R. sphaeroides* O.U.001 in media with different iron concentrations. The initial pH of the cultures was buffered to 7 and was not controlled during incubation. Each value in the pH and growth curve is the mean of three measurements with ± standard deviation. (◊) No Fe; (□) 1/10X Fe; (Δ) 1X Fe; (×) 10X Fe.
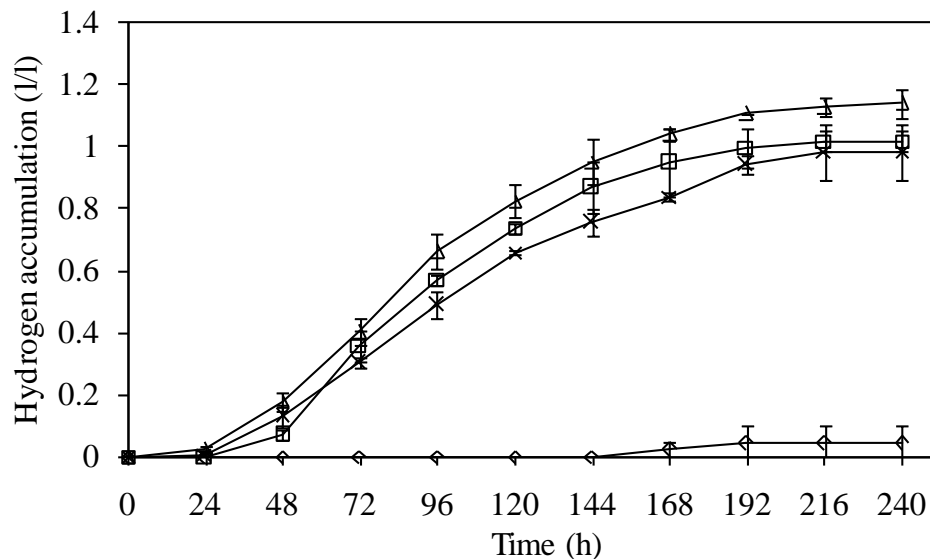
Figure 3.6 Total hydrogen production by *R. sphaeroides* O.U. 001 in media with different iron concentrations. Each value in the curve is the mean of three measurements with ± standard deviation. (◊) No Fe; (□) 1/10X Fe; (Δ) 1X Fe; (×) 10X Fe.

3.1.4 Time-dependent expression analysis of the *nifD* gene at different iron concentrations

The primers for the *nif*D gene were carefully designed and there was no complete nonspecific match as a result of similarity search done using the BLAST at the NCBI web page (www.ncbi.nlm.nih.gov/BLAST/). The primers were also checked by PCR followed by sequence analysis in which gDNA was used as a template to show that the primers amplified the *nifD* exactly (Figure 3.7). The sequence analysis of the PCR product was done (Appendix H) and it was proved that the PCR product was exactly the *nifD* gene of *R. sphaeroides*.
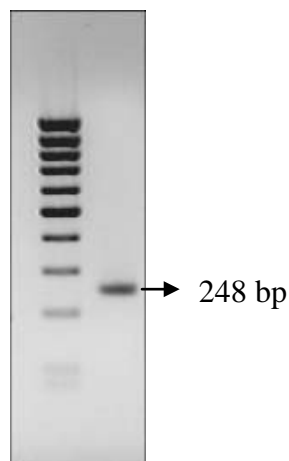
Figure 3.7 The *nifD* PCR product was run on agarose gel together with 100 bp DNA ladder. The PCR product gave expected 248 bp band on the gel.

The stringent PCR conditions due to using carefully designed tagged primers did not allow false positive results as confirmed by PCR using RNA as a template. The expression analyses were done at t=16, 24, 44, 66, 118 and 191 h and the results were illustrated in Figure 3.8. The *nifD* expression analysis in the cells grown in No Fe medium was started at t=44 h and there was still no expression. Although a considerable amount of expression was detected at t=66 and 118 h in the cells grown in No Fe medium, there was no hydrogen production at all. This could be due to the fact that although the genes were transcribed, incorporation of sufficient amount of iron and correct assembly of fully active nitrogenase could not take place. There was a considerable amount of *nifD* expression in the cells grown in 1/10X Fe at t=16 and 24 h. Moreover, the expression level increased at t=44 and 66 h where the highest expression level was obtained. At t=118 and 191 h, the *nifD* expression in the cells decreased considerably. The expression in the cells grown in 1X Fe medium has already started at t=16 h and continued at the same level at t=24 h. Furthermore, a significant amount of expression was observed at t=44, 66, and 118 h and then the expression declined at t=191 h. Considering the overall expression analysis of *nifD* gene, it was seen that maximal expressions were obtained in the cells grown in 1X Fe medium throughout the growth phases. This result is in agreement with the hydrogen

production experiment in that the maximal hydrogen production was achieved in 1X Fe medium implying that the optimum iron concentration for the hydrogen production is 0.1 mM. The expression of the *nifD* gene in the cells grown in 10X Fe medium also started at t=16 and it increased until t=66 h where the highest expression was observed. However the amount of expression lessened towards the end of stationary phase. This result pointed out that further addition of iron did not increase the expression amount but rather it caused a reduction in the gene expression. In general, the maximal expressions were observed around the beginning of the stationary phase. Also, maximal rates of hydrogen production were seen in the period from the beginning of the stationary phase to the mid stationary phase.
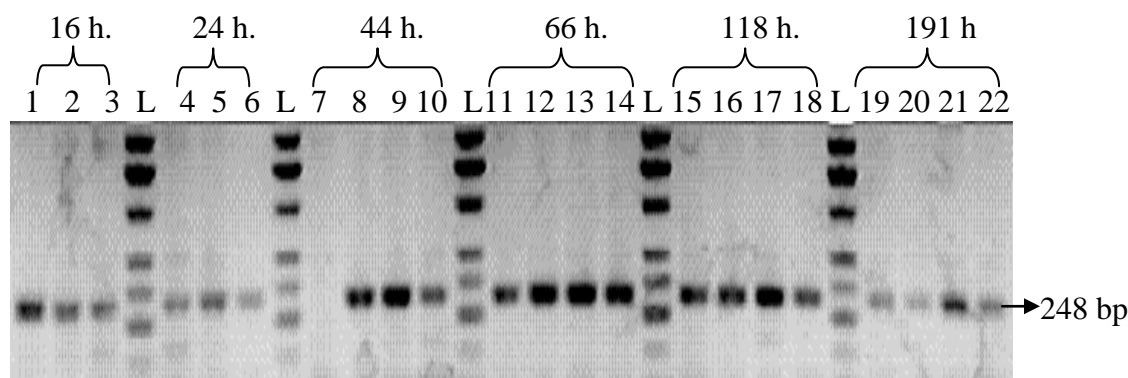
Figure 3.8 Agarose gel of RT-PCR products of *nif*D in *R. sphaeroides* O.U.001 cultured in media with various concentrations of iron ([Fe(III) citrate.hydrate]: without iron (No Fe); 0.01 mM (1/10X Fe); 0.1 mM (1X Fe) and 1 mM (10X Fe)). Products of RT-PCR were run on a 1.5 % agarose gel at 90V for 1h together with a 100 bp DNA ladder (L). The analyses were performed at t=16, 24, 44, 66, 118 and 191 h. Lane 1-3, 4-6 represent the results obtained from the cells grown in 1/10X Fe, 1X Fe and 10X Fe media. Lane 7-10, 11-14, 15-18 and 19-22 represent the results obtained from the cells grown in No Fe, 1/10X Fe, 1X Fe and 10X Fe media respectively.

3.1.5 Time-dependent expression analysis of the *hupS* gene at different iron concentrations

Membrane bound uptake hydrogenase is known to oxidize molecular hydrogen thereby decreasing the total hydrogen production. In addition to the Mo-nitrogenase gene expressions, the expression of *hup*S with respect to time was also monitored and the result was given in Figure 3.9. The tagged primer strategy was also applied for *hup*S expression analysis and there were no false positive results. It was observed that *hup*S was not expressed considerably until t=23 h in the iron starved cells. Although there was some expression at t=23 h in the cells, it disappeared in the following analyses. This transient gene expression might have occurred as opposed to the physiological stress due to iron deficiency. Moreover, the presence of the transcript may not always reflect the presence of active protein in the cell if we consider the role of cofactors in the activity of enzymes. There was a very small amount of gene expression in the cells grown in 1/10X Fe medium at t=16 h but the expression increased at t=23 h. And, a significant amount of *hup*S expression was seen until t=112 h where the amount of expression decreased to a certain level. Moreover, the expression in the cells grown in 1X Fe and 10X Fe media was quite pronounced throughout the growth phases. Since there was still sufficient amount of hydrogen, which could be used by the uptake hydrogenase, it was expected to see *hup*S expression until t=112 h (Figure 3.6 and Figure 3.9).
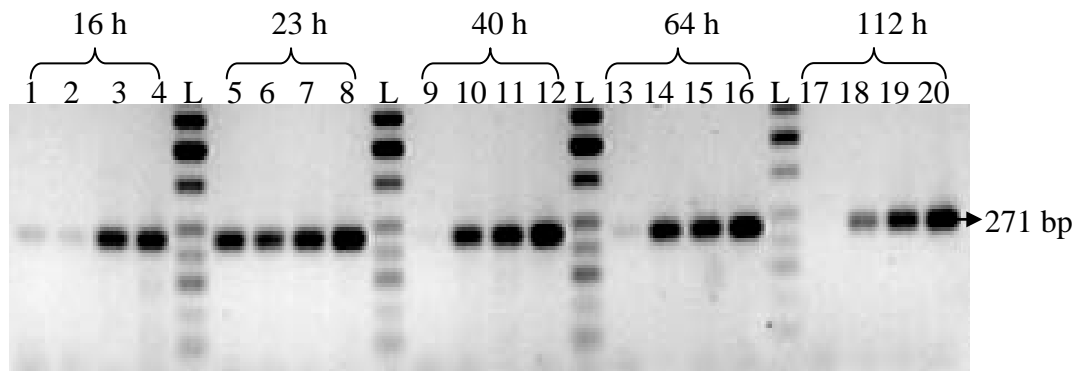
Figure 3.9 Agarose gel of RT-PCR products of *hup*S at different iron concentrations ([Fe(III) citrate.hydrate]: without iron (No Fe); 0.01 mM (1/10X Fe); 0.1 mM (1X Fe) and 1 mM (10X Fe)). Products of RT-PCR were run on a 1.5 % agarose gel at 90V for 1h together with a 100 bp DNA ladder (L). The analyses were performed at t=16, 23, 40, 64 and 112 h. Lane 1-4, 5-8, 9-12, 13-16 and 17-20 represent the results obtained from the cells grown in No Fe, 1/10X Fe, 1X Fe and 10X Fe media respectively.

3.2 Inactivation of uptake hydrogenase by antibiotic resistance gene insertion into *hup* genes

In *R. sphaeroides*, several metabolic pathways take role in the production and consumption of hydrogen as shown schematically in Figure 3.10. The total hydrogen production is limited due to several metabolic events occurring in the cells such as the production of poly-3-hydroxybutyrate (PHB) or consumption of hydrogen by uptake hydrogenase. The electrons flow from the last electron donor ferrodoxin to nitrogenase and together with ATP produced by ATPase and protons, the H_2 evolution occurs. The membrane-bound uptake hydrogenase decreases the efficiency of H_2 production by catalyzing the conversion of molecular hydrogen to electrons and protons (Vignais *et al.*, 1985). It was reported that the inactivation of uptake hydrogenase resulted in total increase in hydrogen production (Franchi *et al.*, 2004;

Öztürk *et al.*, 2006; Kim *et al.*, 2006). In this part of the study, the purple non-sulfur bacterium *R. sphaeroides* O.U.001 was manipulated such that the uptake hydrogenase was inactivated. Total hydrogen production, the rate of H_2 production and substrate conversion efficiency were improved in modified *hup⁻ R. sphaeroides* O.U.001.
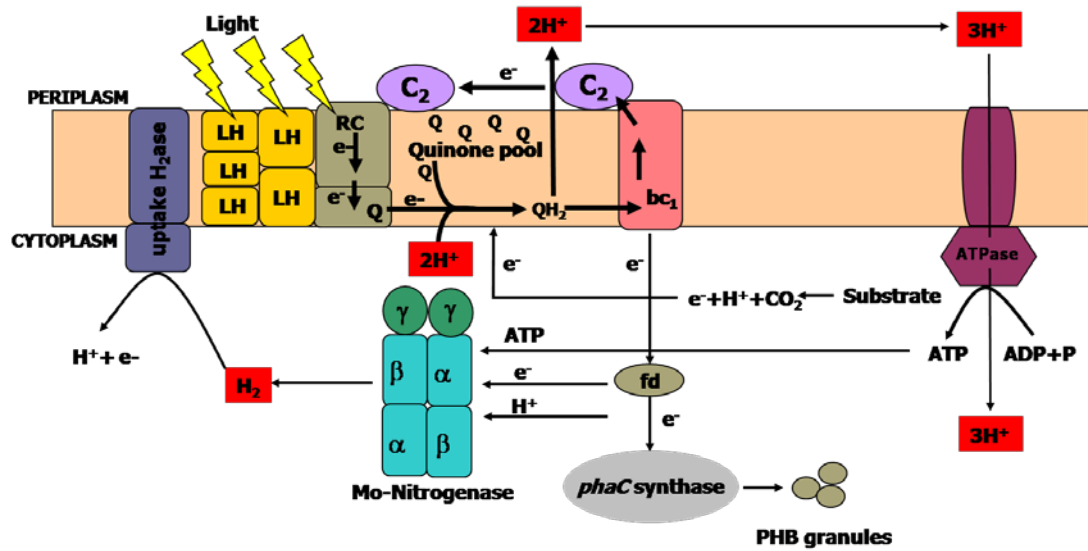
Figure 3.10 The schematic view of H_2 related pathways in *R. sphaeroides*.

3.2.1 The construction of suicide vector for insertional inactivation of *hup* genes

The strategy applied for the mutation of *hup* gene was insertional inactivation of the *hupS* gene after homologous recombination events. First, the gDNA of wild type *R. sphaeroides* was isolated as shown in Figure 3.11A and then the 3082 bp long *hup* gene fragment was amplified using this DNA as a template in PCR (Figure 3.11B).
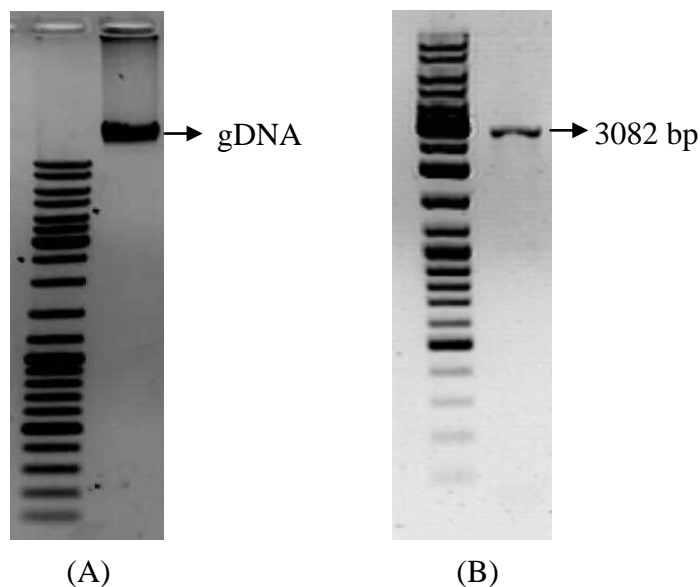
Figure 3.11 The gDNA (A) was isolated from wild type *R. sphaeroides* to be used as a template in PCR in which a 3082 bp *hupSL* gene was amplified (B).

Pfu DNA polymerase (Fermentas) and the following primer pairs; hupSL3 (left): 5'-TAACGGATTTCACCCCTTCC-3', hupSL4 (right): 5'-GAATGGCGAG-CAGTTTCTTC-3' were used. The PCR program was as follows: 2 min. at 95 ° C for pre-denaturation, 30 cycles of amplification step (1 min. at 95 ° C, 1 min. at 50 ° C and 7 min. at 72 ° C) followed by a final extension of 5 min. at 72 ° C. The PCR fragment was phosphorylated and cloned into *Eco*RV cut pBluescript SK (+) plasmid. The resulting construct (pGhup1, Figure 3.12A) was analyzed by restriction enzyme digestion with *Stu*I and sequence of the insert was confirmed by sequence analysis (Appendix H). When pGhup1 was cut with *Stu*I, the expected 6036 bp single fragment was obtained as shown in Figure 3.12B. In addition to T7 and T3 general primers the following primers were used for the sequencing (5'→3'); RSHUSQ1: 5'-ATGCGCTGGCTGTAGAACAT-3', RSHUSQ 2: 5'-GATCTCGATCCCCGACA-AT-3', RSHUSQ3: 5'-CAGGTTCAGCTGCATCATGT-3', RSHUSQ4: 5'-GCAA-TCTCGAGGAAGTGCAT-3'. The sequence analyses both confirmed the construct and gave the exact sequence of *hupSL* gene which could be used for further studies.

(A)   (B)
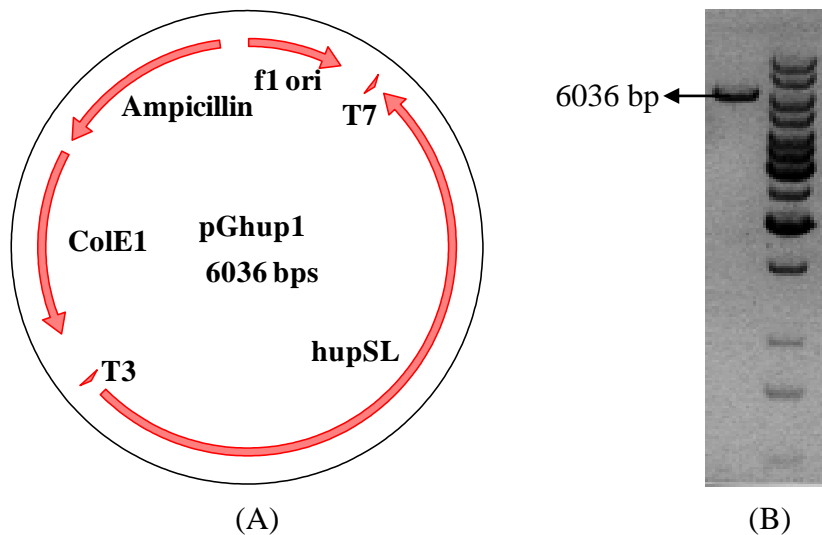
Figure 3.12 The whole vector with its properties was illustrated (A). The construct pGhup1 was digested with *StuI* and gave expected 6036 bp single band confirming the correct ligation of *hupSL* PCR product into pBtSK plasmid (B).

After the confirmation of pGhup1, an antibiotic resistance cassette was prepared to insert it into the *hup* gene. As a source of antibiotic resistance gene, gentamicin was selected. The sensitivity of the wild type *R. sphaeroides* was investigated by inoculating the bacteria into several B&P media in which there were different concentrations of gentamicin ranging from 1 µg/ml to 50 µg/ml. *R. sphaeroides* did not grow even in the medium containing 2 µg/ml gentamicin. Therefore, gentamicin was used as 25 µg/ml as a working concentration in the experiments. The Gm^r gene was excised from the p34SGm plasmid by digestion with *KpnI* and cloned into *StuI* digested pGhup1 making pGhup1Gm construct (Figure 3.13A). The pGhup1Gm vector was double digested with *SmaI* and *SalI* to confirm the integration of Gm fragment as shown in Figure 3.13B.

(A)   (B)
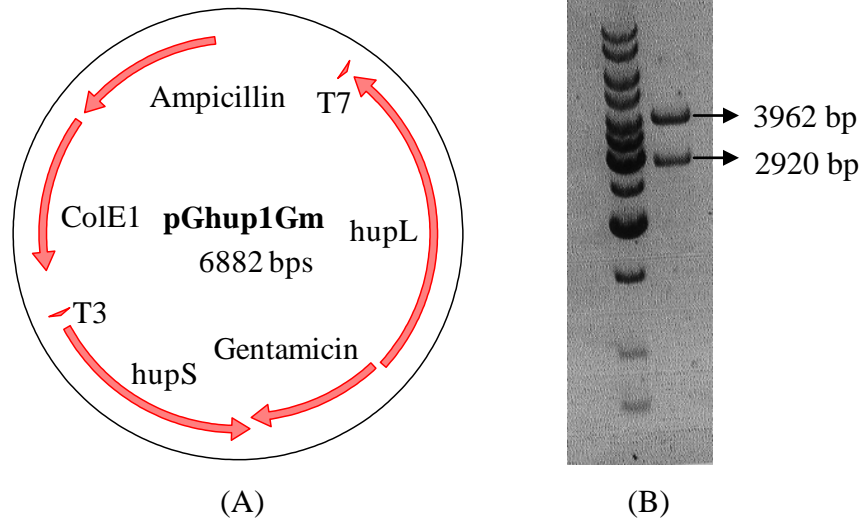
Figure 3.13 The whole vector with its properties was illustrated (A). The construct pGhup1Gm was double digested with *Sma*I and *Sal*I and the digestion gave expected 3962 and 2920 bp bands confirming the correct integration of Gm^r gene into pGhup1 (B).

For the efficient site directed mutagenesis, the size of the gene fragments flanking the Gm^r gene should be equal and between 500 bp-1 kb. Since the resulting construct (pGhup1Gm) did not suffice this requirement, a new fragment was obtained by PCR using this construct as a template. The new fragment was obtained using the *Pfu* DNA polymerase and the following primer pairs; RSHU1F: 5'-ATGAAATACTGCTCGCTCAC-3',RSHU2R: 5'CATGCACTTCCTCGAGATT G-3'. The same PCR conditions as above were applied as the expected product sizes are very close to each other. This new fragment was around 2.8 kb containing 1 kb *hup* gene fragments flanking 0.8 kb Gm^r gene. The PCR product was phosphorylated and blunt end cloned into *Sma*I cut, dephosphorylated pK18*mobsac*B vector. The resulting vector pGhup2Gm was digested with restriction enzymes to confirm the construct as shown in Figure 3.14A. The properties of the construct were given in the Figure 3.14B and Figure 3.14C.
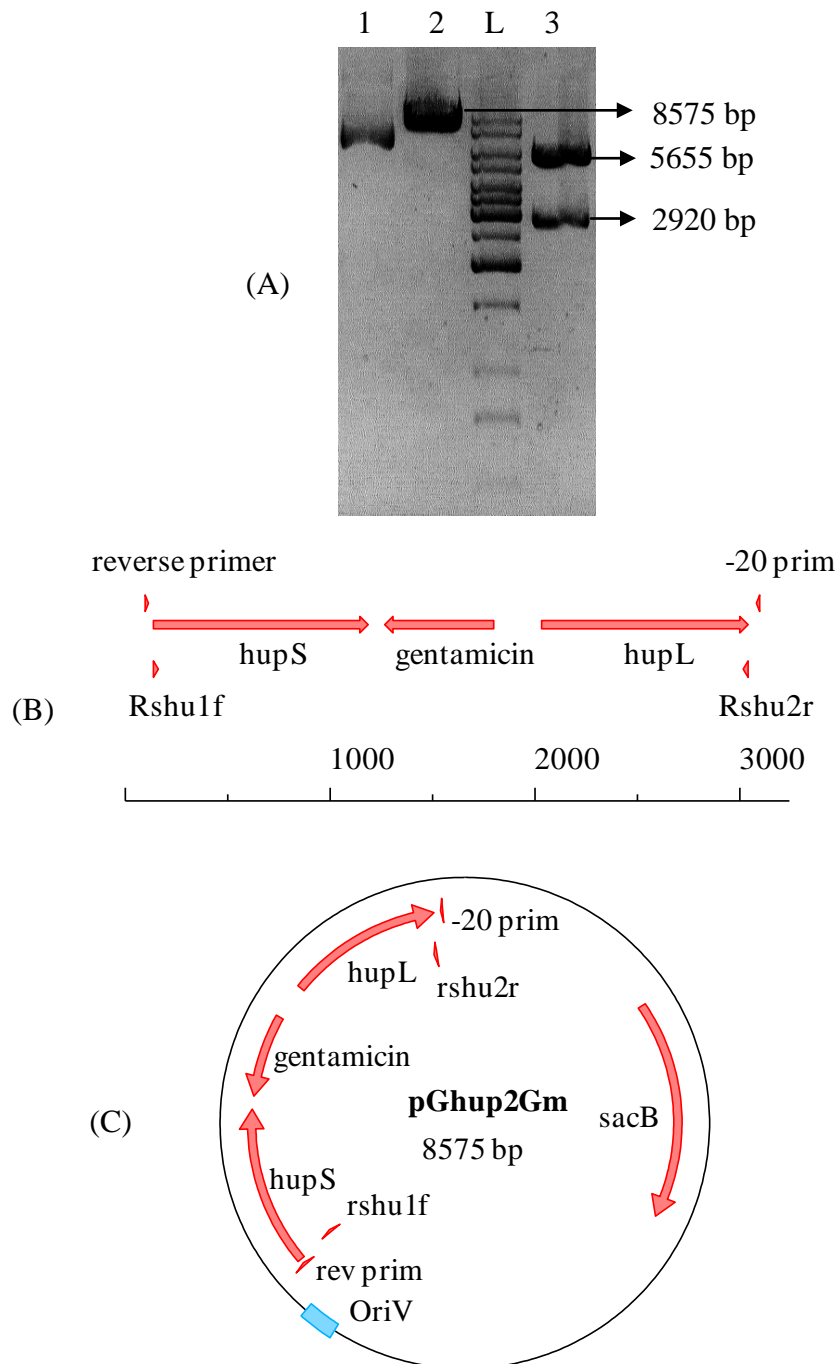
Figure 3.14 In (A), the uncut pGhup2Gm was loaded in agarose gel (Lane1) together with *Xba*I cut (Lane2) and *Xba*I/*EcoR*I double digested pGhup2Gm (Lane3). The digestions resulted in expected products confirming the construct. Lane L represents the DNA marker. In (B) and (C), the properties of pGhup2Gm were shown.

3.2.2 The transfer of suicide vector into *R. sphaeroides* and selection of the double recombinants

The suicide vector was first transferred to *E. coli* S17.1λpir to be delivered to *R. sphaeroides* by diparental mating. The *E. coli* cells containing suicide vector were mixed with wild type *R. sphaeroides* cells and let them to conjugate. Then, a sample of aliquot was spread onto a selective plate containing 50 µg/ml gentamicin (Gm) and 25 µg/ml kanamycin (Km). The single recombinants appeared on selective plates as shown in Figure 3.15.
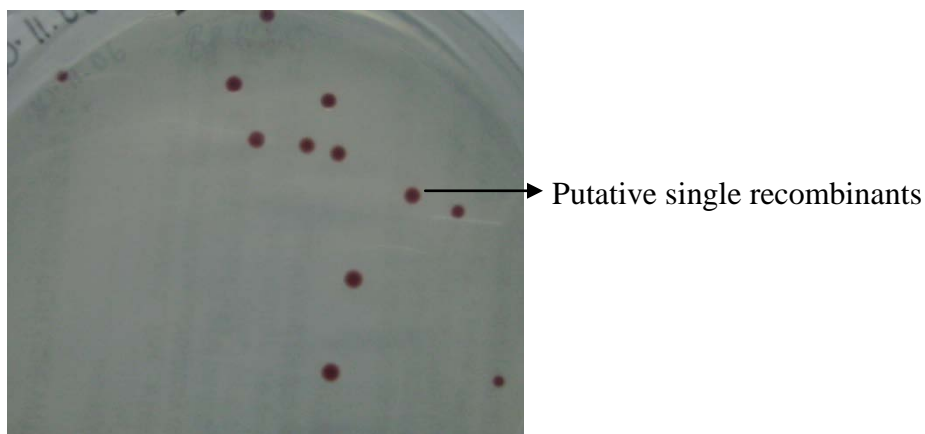
Figure 3.15 The putative single recombinants appeared on selective plates.

After finding single recombinants, a single colony which has grown in medium with Gm and Km was inoculated into non-selective liquid media and passaged two times. Then, certain volumes from this were spread onto minimal medium plates in the presence of gentamicin and 10 % sucrose allowing the simple selection for the double recombinants. The single recombinants carrying the *sacB* gene in the integrated vectorial DNA were expected to be eliminated while the double recombinants were expected to survive. The explanation for the double recombinant selection is as follows; the *sacB* gene encodes levansucrase which cata-

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lyzes both hydrolysis of sucrose to glucose and fructose and polymerization of fructosyl groups to form levan which is a branched fructose polymer of high molecular weight and thought to be toxic to the cells. Therefore, the single recombinants carrying s*acB* gene were eliminated and the double recombinants with only the gentamicin resistant gene survived (Figure 3.16) (Schafer *et al.*, 1994).
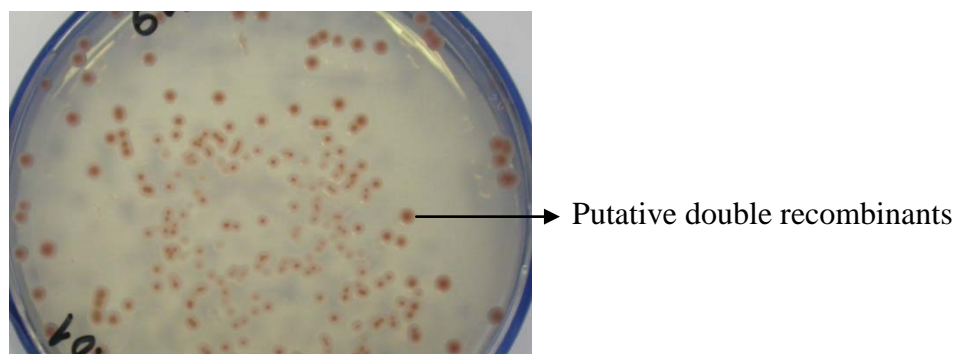
Figure 3.16 The putative double recombinants grown on gentamicin and 10 % sucrose containing plate.

3.2.3 The confirmation of *hup* mutant *R. sphaeroides* by genetic and biochemical tests

The *hup* mutant *R. sphaeroides* was analyzed by genetic and biochemical tests to confirm the correct integration of Gm resistant gene into *hup* genes.

3.2.3.1 PCR and sequence analyses

The first genetic test was performed by doing PCR in which the presence or absence of vectorial part (*sacB* gene) was checked. The primers (pK18forward and pK18reverse, Appendix H) were selected on the suicide vector and the absence of any vectorial part was confirmed by PCR using these primers. The double recombinant mutants were expected to discard any vectorial part and therefore, there

should not be any PCR product when the gDNA of double recombinant was used as a template as shown in Figure 3.17. The pK18*mobsacB* and pGhu2Gm vectors were used as positive control to show that the primers and the PCR conditions were working. In order to show that the amplified product was exactly the *sacB* gene region of the pK18*mobSacB*, the PCR product was sequenced and shown that it corresponds to *SacB* gene region of the suicide vector pGhu2Gm**.**

In addition to showing the absence of any vectorial part in the double recombinants' gDNA, the presence of Gm resistant gene was demonstrated by PCR in which the Gm left and Gm right primers (Appendix H) were used. The gDNA of double recombinant mutants were used as a template and both of the mutants gave the expected 471 bp product (Figure 3.17B). This result proved that the gentamicin resistant gene was successfully inserted into the *hup* gene after double recombination event. In order to show that the amplified product was exactly the gentamicin resistant gene region, the PCR product was sequenced and shown that it corresponds to gentamicin resistant gene of the suicide vector pGhu2Gm**.**
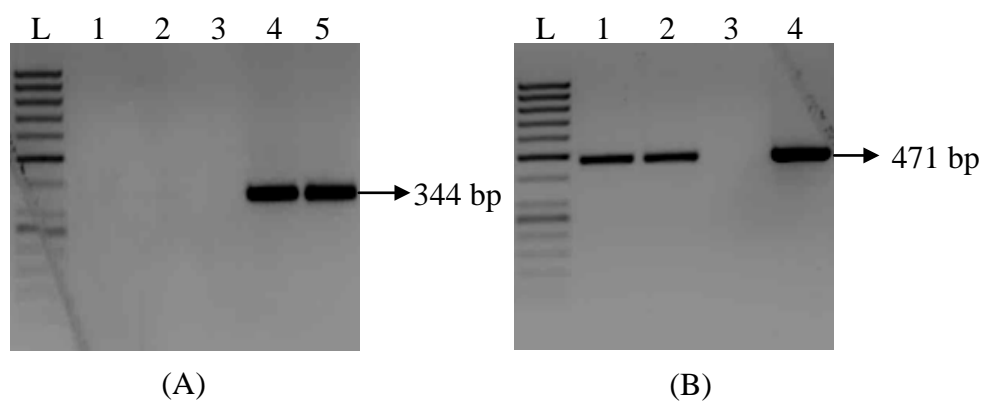
Figure 3.17 The absence of vectorial part (*sacB* gene) was checked by PCR in which *sacB* specific primers were used using the gDNA of double recombinants (A1, A2), water (A3), pK18*mobSacB* (A4) and pGhu2Gm (A5) as templates. While, water was used as non template control (NTC), pK18*mobSacB* and pGhu2Gm vectors were used as positive control. The presence of gentamicin resistant gene was also demons-

Figure 3.17 (continued) trated by PCR using the gDNA of double recombinants (B1 and B2), water (B3), and pGhu2Gm as templates. Water was used as a NTC, and pGhu2Gm was used as a positive control in the PCR (B4). 1 kb DNA ladder was run together with the PCR products in lane L.

The PCR and subsequent sequence analyses proved that after the second homologous recombination event, the vectorial part was discarded out from the gDNA while the gentamicin resistant gene was left in the *hup* gene. These results showed that the correct double recombinants were successfully obtained.

3.2.3.2 Uptake hydrogenase assay

In the next confirmation, the uptake hydrogenase assay was performed to show that the uptake hydrogenase was completely inactivated in these double recombinant mutants. Uptake hydrogenase assay is an effective and simple assay to test the presence of an active uptake hydrogenase developed by Goodman and Hoffman, 1983. The colorimetric test was based on the ability of benzyl viologen to take up electrons resulted from the activity of uptake hydrogenase and turning into its reduced form which is a purple in color. The substrate of uptake hydrogenase was put into the reaction vessel by flushing the reaction solution with hydrogen gas. Therefore the presence of a colored compound indicates the actively existing uptake hydrogenase. The mutants did not give any uptake hydrogenase activity, while wild type cells showed enzyme activity by giving purple color (Figure 3.18). The assay proved that the gene for the uptake hydrogenase was successfully destroyed.

3.2.3.3 Southern blotting and hybridization

As a last confirmation, the presence of gentamicin resistant cassette inserted into the *hup* genes was detected by Southern blot and hybridization experiments. The probe was prepared by using PCR product of gentamicin resistance gene using the
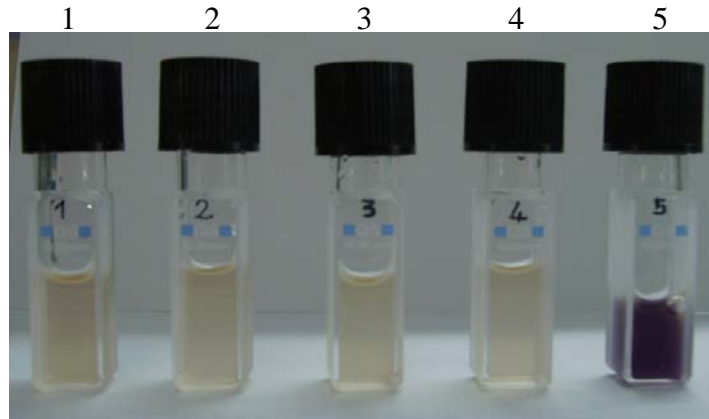
Figure 3.18 The uptake hydrogenase activity assay using Benzyl viologen dye. 1,3: mutants with H_2, 2,4: mutants without H_2, 5: wild type cells with H_2.

primers Gm left (5' GCAGTCGCCCTAAAACAAAG-3') and Gm right (5'-AGTGCATCACTTCTTCCCGTA-3'). The PCR program was as follows: 5 min. at 94 ºC for pre-denaturation, 30 cycles of amplification step (30 sec. at 94 ºC, 30 sec. at 55 ºC and 1 min. at 72 ºC) followed by a final extension of 5 min. at 72 ºC. After the probe was prepared using the PCR product, the gDNA of *hup* mutant and wild type cells were digested with *Hinc*II and *Sma*I and loaded onto 0.7 % agarose gel prepared by using 0.5X TBE buffer (pH:8.3) (Figure 3.19).

The agarose gel electrophoresis and Southern transfer were performed by using 0.5X TBE buffer too. The thickness of the agarose gel was 0.6 mm to make an efficient Southern transfer. The Southern transfer was performed using Bio-Rad Gel Transfer System (Germany) at 13 Volts for 4-5 hours at 4 cm electrode distance (high electric field). The DNA fragments were blotted to positively charged nylon membrane (Roche). After blotting, the hybridization of labeled probes to the DNA fragments was done at 68 ºC for overnight. The detection was performed by using Anti-Dig-Alkaline Phosphatase conjugate. After detection, it was observed that the gentamicin probe hybridized only to the digested gDNA of *hup* mutant *R. sphaeroides*. Therefore, it could be concluded that the gentamicin resistant gene was correctly inserted into the *hup* genes in the genome of *R. sphaeroides*.

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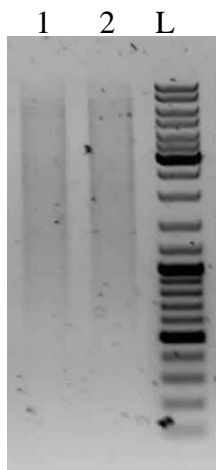
Figure 3.19 The *Hinc*II *and Sma*I digested gDNA of *hup* mutant (1) and wild (2) type *R. sphaeroides* were run on 0.7 % agarose gel prepared by using 0.5X TBE buffer (pH:8.3). Lane L is the Molecular Weight Marker (Fermentas).
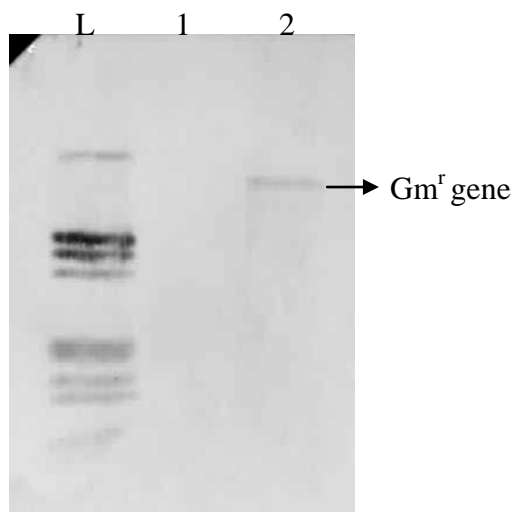
Figure 3.20 The Southern transfer and detection of gentamicin resistance gene in the *Hinc*II *and Sma*I digested gDNA of wild (Lane 1) and *hup* mutants (Lane 2) type *R. sphaeroides*. Lane L is the Dig-labeled Molecular Weight Marker III (Roche).

3.2.4 The growth and hydrogen production profiles of *hup* mutant *R. sphaeroides* in malate/glutamate (15/2 mM) media

The pH changes during the growth of mutant and wild type cells were monitored and illustrated in Figure 3.21A. The initial pH was buffered to 7.0 and was not controlled during the growth. There was no considerable difference in the pH values of mutant and wild type cells and the pH values varied between 7.3 and 7.8.

The growth of mutant and wild type *R. sphaeroides* O.U 001 was monitored by measuring the absorbance at 660 nm at certain time intervals. The wild type cells reached relatively higher absorbance values (OD660=1.90) compared to *hup* mutant strain (OD660=1.71) (Figure 3.21B). Moreover, the total cell dry weight measurements showed that the wild type cells reached higher cell masses (1.26 g/L) while mutant cells reached lower cell masses (1.07 g/L). According to the previous studies (Kars *et al.*, 2006), the cells which could not produce hydrogen obtained higher cell masses than the cells which produced considerable amount of hydrogen. Therefore, the results were in consistent with the previous ones. This phenomenon could be attributed to the total amount of energy and electron spent either to hydrogen production or cell materials which meant that more energy and electrons were directed towards the hydrogen production in *hup* mutant cells while less amount of energy and electrons were consumed for hydrogen production in wild type cells.

(A)

pH / Time (h)

(B)

OD 660 nm / Time (h)
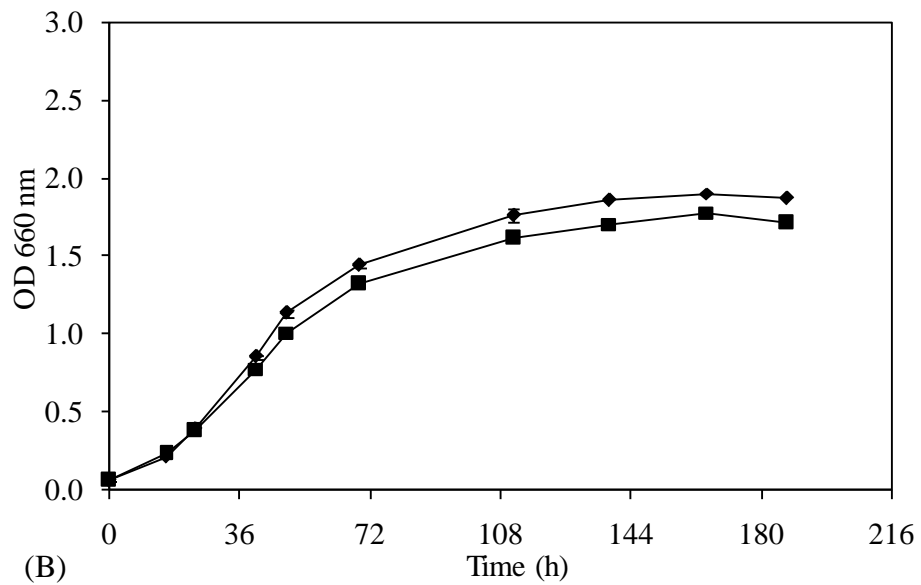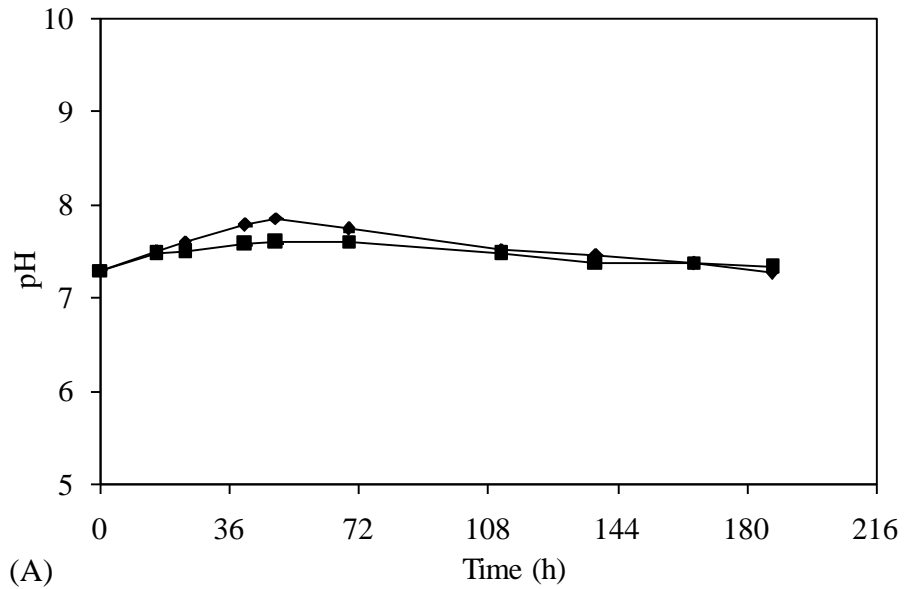
Figure 3.21 The pH (A) and absorbance (B) changes during the growth of wild type and mutant strain of *R. sphaeroides* O.U 001 were monitored with respect to time. Each value in the curves is the mean of three replications with ± standard deviation. (■) *hup* mutant; (♦) wild type *R. sphaeroides* O.U 001.

The total hydrogen photoproduction by *hup* mutant and wild type *R. sphaeroides* O.U. 001 was investigated under the nitrogenase derepressed conditions in 60 ml bioreactors. Previously, it was shown that the inactivation of uptake hydrogenase resulted in increase in total hydrogen production in the cells producing hydrogen by nitrogenases (Franchi *et al.*, 2004; Öztürk *et al.*, 2006; Kim *et al.*, 2006). Similarly, significantly higher amount of hydrogen was accumulated in *hup* mutant *R. sphaeroides* O.U. 001 when compared to wild type cells (Figure 3.22). The total hydrogen accumulation in mutant cells was 20 % higher than that in wild type cells (m=2.85 l H_2/l culture, w=2.36 l H_2/l culture). According to the gas chromatography (GC) analysis, the hydrogen constituted 96–99 % (v/v) of the overall gas. The average gas production rate of mutant cells was 9.2 ml/l/h while that of wild type cells was 6.9 which were calculated by dividing the total volume of gas produced by the volume of the culture and by the duration of gas production.

Another parameter for comparative analysis of hydrogen production is the substrate conversion efficiency which is calculated as the ratio of the actual amount of produced hydrogen to the theoretical amount. The substrate conversion efficiency of mutant cells was 80 % while that of wild type cells was 66.4 %. A substrate conversion efficiency of 35-57 % for the malate was reported in the literature for *R. sphaeroides* (Koku *et al.*, 2002). The yield for mutant cells was calculated as 0.072 g H_2/g malate while the yield for wild type cells was 0.060 g H_2/g malate. Hence, an efficient substrate conversion to hydrogen was achieved in *hup* mutant *R. sphaeroides* O.U.001. The high substrate conversion efficiency demonstrated that more energy and reducing equivalents were directed towards the nitrogenase enzyme and therefore more hydrogen accumulation was achieved.
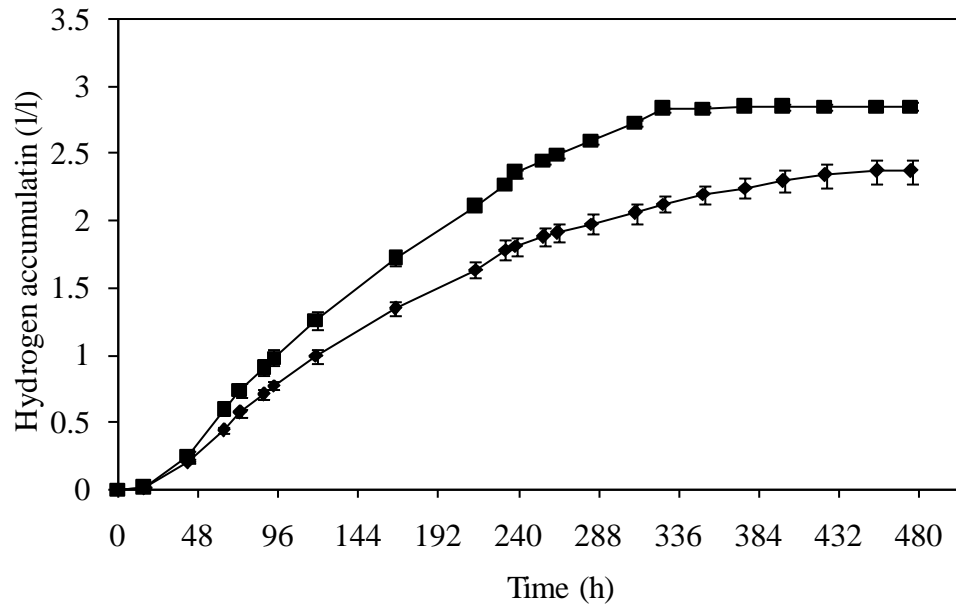
Figure 3.22 Total hydrogen production by wild type (♦) and *hup* mutant (■) *R. sphaeroides* O.U 001 was monitored with respect to time. Each value is the mean of three replications with ± standard deviation.

3.2.5 The growth and hydrogen production profiles of *hup* mutant *R. sphaeroides* in acetetate/glutamate (30/2 mM) media

In the coupled system in which the photobiological hydrogen production proceeds the anaerobic dark fermentation, the low molecular weight organic acids are produced as a result of dark fermentation and used in the photofermentation step. Therefore, the coupled systems increase the efficiency of hydrogen production and facilitate an effective feedstock usage. Acetate is one of the main components of the effluent of anaerobic fermentation. Moreover, in the HYVOLUTION project mentioned in introduction (section 1.1.3), the photofermentation step focuses on the usage of acetate as carbon source. Hence, in these studies the acetate was compared to malate which was used as reference compound since efficient hydrogen production was achieved using malate. The acetate was used in 30 mM concentration which was found as optimum concentration by Uyar B., in his PhD studies (Uyar B.,

PhD Thesis, Middle East Technical University, 2008 Ankara). 2 mM Glutamate was used as nitrogen source. The pH changes and growth of the mutant and wild type *R. sphaeroides* in acetate containing medium was monitored and given in Figure 3.23. The pH of the media was first buffered to pH 7 and was not controlled during the cultivation. The pH of the medium in which the wild type cells were grown rose up to pH 8.8 while the pH of the media where mutant cells were grown increased up to pH 8.4 (Figure 3.23A). This result was consistent with the previous studies (growth and H_2 production with malate) such that the cells which produced less amount of H_2 reached relatively higher pH values. These results suggest that there may be an effect of H_2 production on the pH values of the media. Regarding the growth of cells in acetate containing media, both wild and mutant cells reached much higher cell masses compared to the cells grown in malate containing media. Moreover, if the hydrogen production profiles of the cells grown in acetate and malate were considered, the cells grown in malate produced significantly more hydrogen than the cells grown in acetate. These findings may imply that the cells directed much of its energy towards producing cell masses in the acetate media. The mutant cells reached relatively small absorbance values compared to wild type cells (wild $OD_{660nm}=3.70$; mutant $OD_{660nm}=3.34$). This could be attributed to the relatively high hydrogen production capacity of mutant cells. Since they produce more hydrogen, they spent more energy to produce hydrogen and less energy to make cell mass. Another finding during the growth of bacteria in acetate containing media was that there occurred a lot of PHB granules when acetate was used instead of malate.

(A)
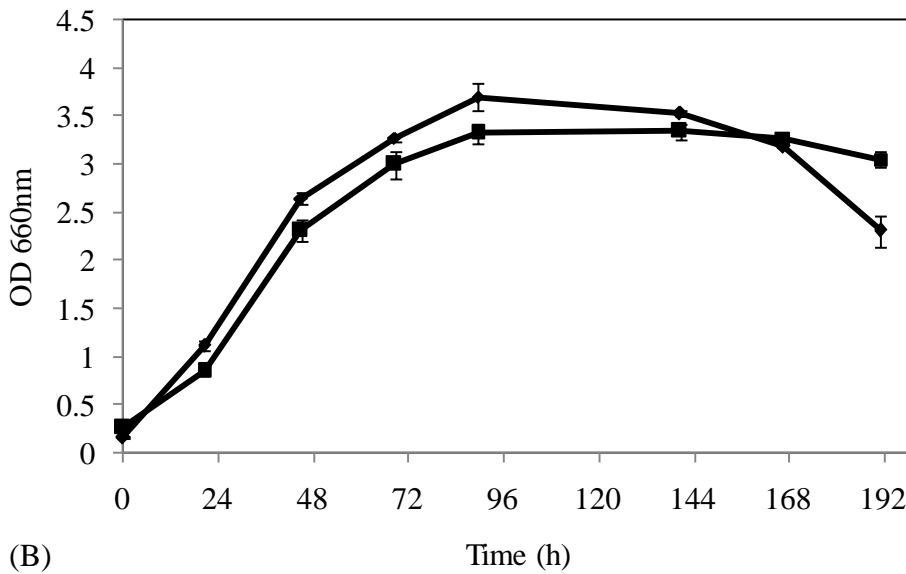
(B)

Figure 3.23 The pH (A) and absorbance (B) changes during the growth of wild type (♦) and *hup* mutant strain (■) of *R. sphaeroides* O.U 001 in acetate media. Each value is the mean of three replications with ± standard deviation.

In Figure 3.24, the hydrogen production performance of mutant and wild type cells in acetate/glutamate (30mM/2mM) medium was illustrated. As it is seen from the figure, the mutant cells

produced 0.31 l H_2/l culture whereas wild type cell produced 0.25 l H_2/l culture. Moreover, the hydrogen production yield of mutant cells in acetate medium was 0.014 g H_2/g acetate and that of wild type cells was 0.011 g H_2/g acetate. While the mutant cells had a substrate conversion efficiency of 6.9 %, the wild type cells had a substrate conversion efficiency of 5.8 %. If the hydrogen production efficiencies of the cells in malate and acetate media were compared, both wild and mutant cells produced significantly less amount of hydrogen in acetate media than the cells grown in malate containing media. Similar results were found by Uyar B., in his PhD studies such that in many experiments only very few of them resulted in hydrogen production and the amount of hydrogen was not significant (Uyar B., PhD Thesis, Middle East Technical University, 2008 Ankara). However, there are studies in which the hydrogen production was achieved by photosynthetic bacteria (Barbosa *et al.*, 2001).

The inability of *R sphaeroides* to produce hydrogen may come from the acetate assimilation pathway in this bacterium. In a study done by Birgit *et al.* in 2006, it was stated that the *R sphaeroides* has an alternative glyoxylate cycle for acetate utilization since two main enzymes (isocitrate lyase and malate synthase) and the genes for them are absent in *R sphaeroides*. According to the proposed pathway, acetoacetyl-CoA is a common intermediate for both polyhydroxybutyrate synthesis and acetate assimilation pathways. This means that the initial steps of both pathways are the same, or in other words, simultaneous PHB synthesis is required for acetate assimilation. These results suggest that the PHB synthesis and hydrogen production by nitrogenase compete with each other for reducing equvalents. In the proposed acetate assimilation pathway, it was stated that acetoacetyl-CoA reductase is an NADPH dependent enzyme and therefore requires NADPH. Probably, there are more NADH or FADH requiring enzymes showing that the electrons are allocated for PHB synthesis rather than hydrogen production. This proposal also explains why there were a lot of PHB granules when the cells were grown in the acetate containing media. Probably, the acetate as a substrate in the environment acted as a driving force for the PHB synthesis as the initial steps were the same. The higher pH rise in acetate media may also resulted from the higher PHB synthesis.
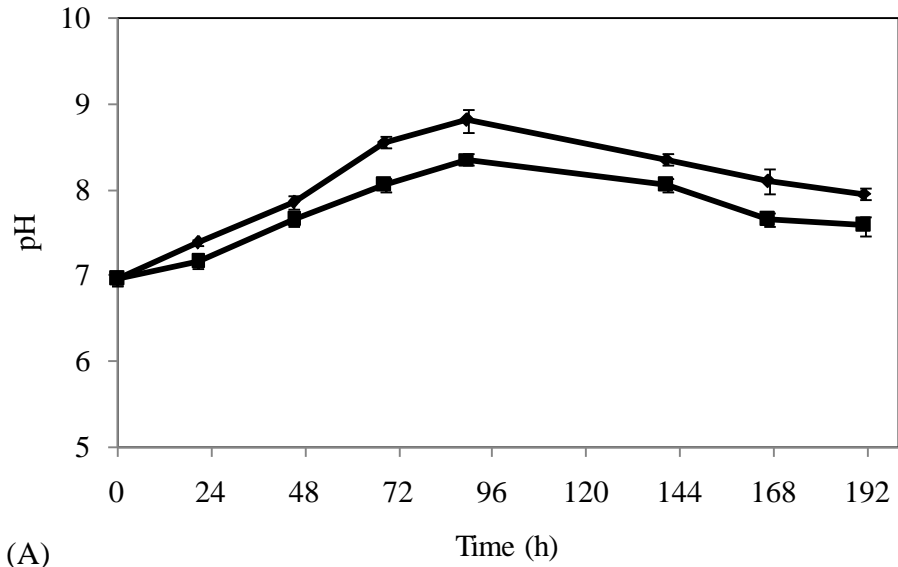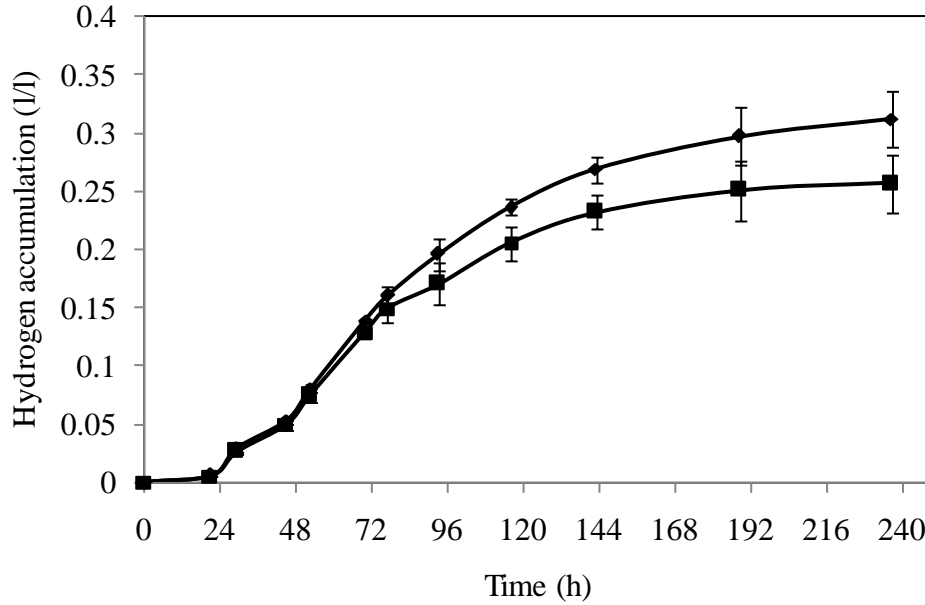
Figure 3.24 Total hydrogen production by wild type (■) and *hup* mutant (♦) *R. sphaeroides* O.U 001 in acetate media. Each value in the curves is the mean of three replications with ± standard deviation.

3.3 Inactivation of uptake hydrogenase by deleting a part of *hup* genes

Since *hup* mutation resulted in an increase in hydrogen production, there is a possibility to use this mutant in large scale application (outdoor experiments). However, as the mutant bacteria are carrying gentamicin resistance gene, there is the possibility for the gentamicin resistant gene to be transferred to other microorganisms found in the nature. Therefore, they may not be favored to be used in industrial applications. Taking these facts into account, in this study, the uptake hydrogenase was aimed to be inactivated by deleting a part of *hup* gene without inserting any antibiotic resistant gene. The significance of the study is that, since there is no addition of extra gene from outside, the industrial application of this bacterium for hydrogen production is safer.

3.3.1 The construction of suicide vector for the deletion of *hup* genes

First, a partial *hup*S gene was amplified by PCR using the hupSL3 and hupSL5 primers (Appendix H) giving a 916 bp long PCR product. The partial *hup* gene was blunt end cloned into *EcoRV* cut pBtSK(+) yielding pGhup3 vector. The pGhup3 vector was linearized by cutting with *Hinc*II to verify. The expected single band (3877 bp) was obtained as shown in Figure 3.25.
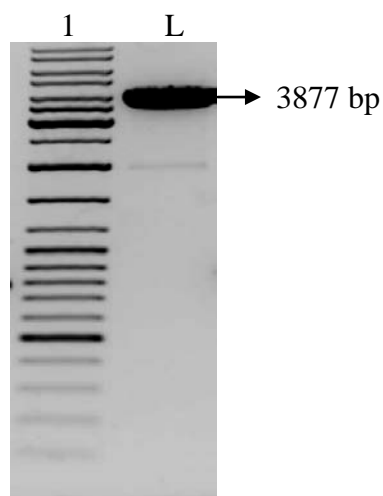
Figure 3.25 The pGhup3 vector was linearized by cutting with *Hinc*II to see if it was the correct construct. The expected 3877 bp band (Lane 1) was seen on gel. The digested vector was run together with DNA ladder (L).

Then, a fragment of *hupL* gene (844 bp) was PCR amplified using hupSL4 and Rshusq4 primers (Appendix H) and blunt end cloned into *Sma*I cut pGhup3 yielding pGhup4. The direction of the *hupS* and *hupL* genes on the pGhup4 construct was determined by doing several PCR using pGhup4 as a template. The result showed that the *hupS* and *hupL* genes on the pGhup4 construct was correctly placed as shown in Figure 3.26.
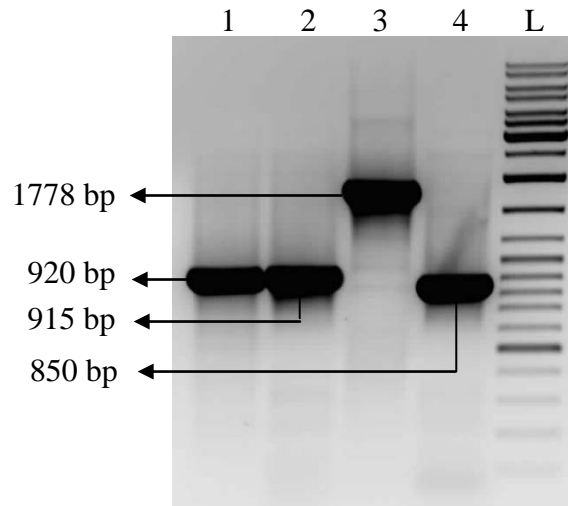
Figure 3.26 The direction of the *hupS* and *hupL* genes on the pGhup4 construct was determined by doing several PCR using pGhup4 as a template. The primer sets used were as follows; Lane1: hupSL3-hupSL5, Lane2: T3-Rshusq4, Lane3: hupSL3-hupSL4 Lane:4: hupSL4-Rshusq4, L:DNA ladder.

The pGhup4 construct was digested with *Kpn*I and *Xba*I for further confirmation. As seen from the Figure 3.27, the digestion of pGhu4 with *Kpn*I and *Xba*I gave the expected two bands on the gel.

The 1778 bp long deleted *hupSL* gene was PCR amplified using *pfu* polymerase using hupSL3 and hupSL4 primers. The pGhup4 vector was used as a template in the PCR. The PCR product was blunt end cloned into *SmaI* cut pK18*mobsacB* vector yielding the suicide vector pGhup5. The direction of genes and the location of primers are shown in Figure 3.27A and B.
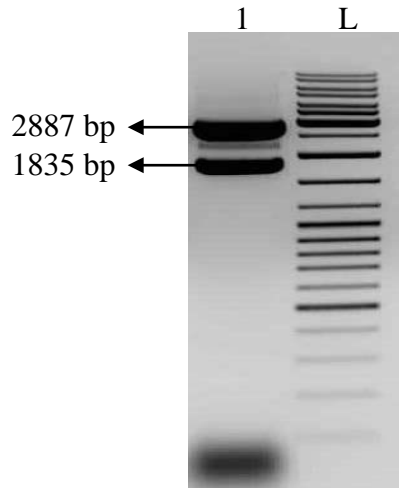
Figure 3.27 The *Kpn*I and *Xba*I digested products of pGhup4 were run on the gel (Lane 1) together with DNA ladder (L).
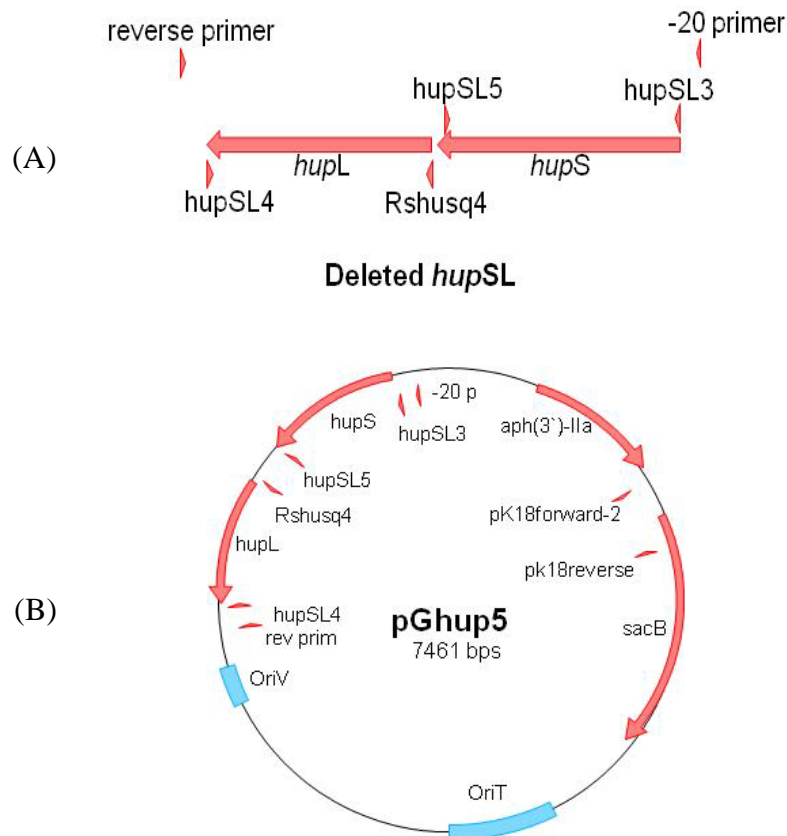
Figure 3.28 The orientation of deleted *hup* genes and the location of primers on

Figure 3.28 (continued) pGhup5 were shown (A) and the schematic view of suicide vector pGhup5 was also given (B).

After construction of the suicide vector, it was confirmed by PCR using three different primer sets to obtain three different PCR products (Figure 3.29). The correct construct was delivered to *R. sphaeroides* by to obtain *hup* mutant bacterium.
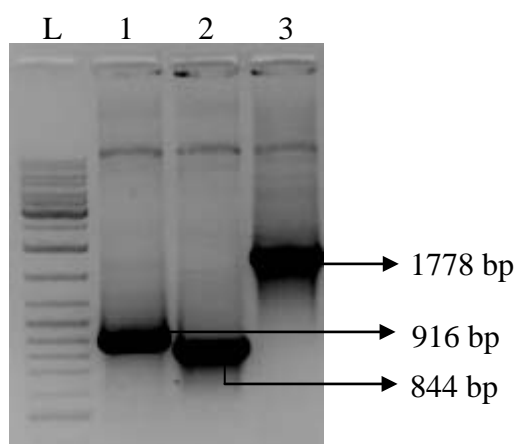
Figure 3.29 The suicide vector pGhup5 was used as a template to obtain three different PCR products using three different primer sets; Lane 1:hupSL3-hupSL5, Lane 2: hupSL4-Rshusq4, Lane 3: hupSL3-hupSL4, Lane L:DNA ladder.

3.3.2 The transfer of suicide vector into *R. sphaeroides* and selection of the double recombinants

The suicide vector pGhup5 was first transferred to *E. coli* S17.1λpir to be delivered to *R. sphaeroides* by diparental mating. The *E. coli* cells containing suicide vector were mixed with wild type *R. sphaeroides* cells and let them to conjugate as explained in section 2.5.17.3. Then, a sample of aliquot was spread onto a selective plate containing 25 µg/ml of kanamycin. The single recombinants appeared on selective plate as shown in Figure 3.30.
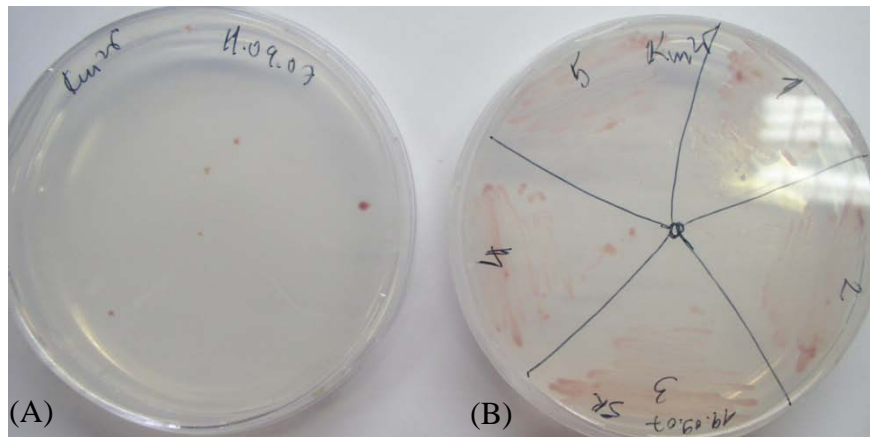
Figure 3.30 The putative single recombinants (A) appearing on selective plate were re-streaked on another kanamycin containing plate (B).

After finding single recombinants, a single colony was inoculated into non-selective liquid media and passaged two times to let the second homologous recombination occur. After several dilutions (up to 10^{-8}), certain volumes were spread onto minimal medium plates in the presence of 10 % sucrose allowing the simple selection for the double recombinants. The single recombinants carrying the *sacB* gene in the integrated vectorial DNA were expected to be eliminated while the double recombinants were expected to survive in this medium (Figure 3.31).

The putative double recombinants were then replica plated on both Km containing and non-selective plates. As seen from the Figure 3.32, the mutants were Km sensitive implying that the single recombinants were successfully converted to double recombinants. There is always a probability for single recombinants to convert back to wild type cells as well, but these cells were further checked by uptake hydrogenase assay, PCR and subsequent sequence analysis and Southern blot.
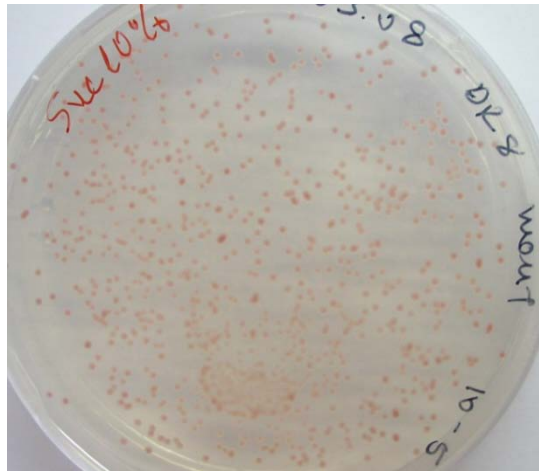
Figure 3.31 The putative double recombinants grown on 10 % sucrose containing plate.

(A)   (B)
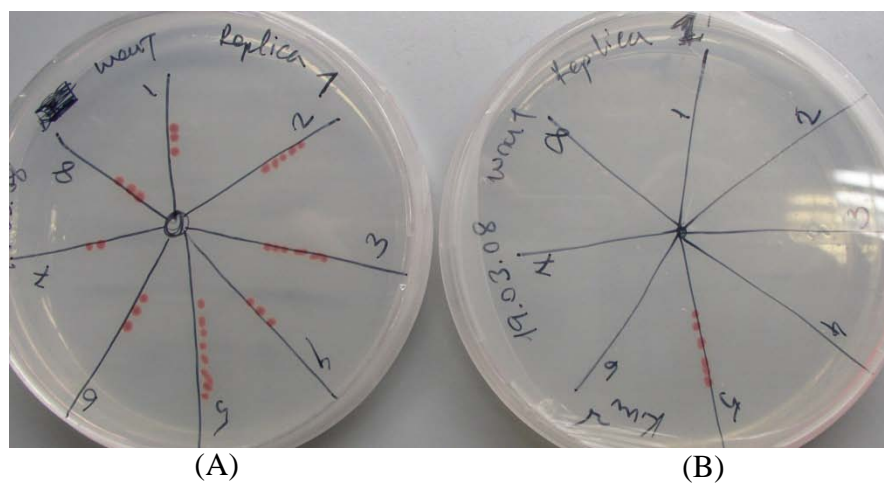
Figure 3.32 The putative double recombinants were replica plated on both Km containing and non-selective plates.

3.3.3 The confirmation of *hup* deleted mutant of *R. sphaeroides* by genetic and biochemical tests

3.3.3.1 PCR and sequence analyses

The confirmation of the genotype of *hup* cell was done by sequence analysis of the deleted *hup* gene after PCR. This confirmation method is simple but straight forward. The PCR was done using the hupSL8 and hupSL9 primers (Appendix H) using the gDNA of mutant cells as a template. The PCR program was as follows: 5 min. at 94 ℃ for pre-denaturation, 30 cycles of amplification step (30 sec. at 94 ℃, 30 sec. at 55 ℃ and 30 sec. at 72 ℃) followed by a final extension of 5 min. at 72 ℃. The 276 bp PCR product shown in Figure 3.33 was sent to sequence analysis.
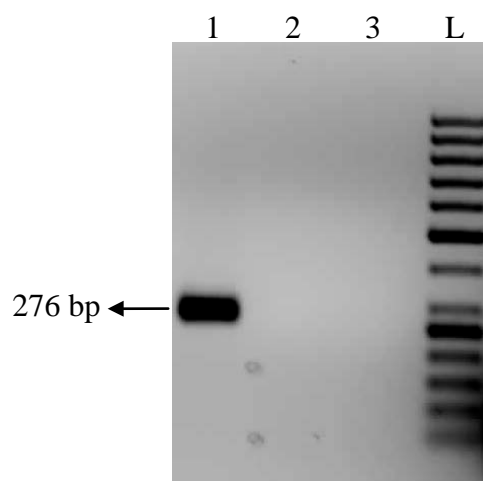
Figure 3.33 The PCR products were obtained by using gDNA of mutant cells (Lane 1), gDNA of wild type cells (Lane 2). Non template control was also loaded (Lane 3) into the gel together with DNA ladder (L).

According to the sequence analysis (Appendix H), the PCR product was exactly the deleted *hup* gene. Therefore, it could be concluded that the central fragment of the *hupSL* gene in the gDNA of mutant cells was successfully deleted.

3.3.3.2 Uptake hydrogenase assay

The uptake hydrogenase assay was performed using an artificial electron acceptor, benzyl viologen (BV). The assay was performed to show that the uptake hydrogenase enzyme was completely inactivated in the *hup* deleted *R. sphaeroides*. The Figure 3.34 indicates that the mutants did not give any uptake hydrogenase activity, while wild type cells showed enzyme activity by giving purple color.

Figure 3.34 The uptake hydrogenase activity assay using mutant and wild type *R.sphaeroides* using benzyl viologen dye. 1: mutant cells with H_2, 2: mutant cells without H_2, 3: wild type cells with H_2, 4: wild type cells without H_2.

According to the results obtained from uptake hydrogenase assay, it can be concluded that the uptake hydrogenase enzyme was correctly inactivated in the *hup* deleted mutant cells.

3.3.3.3 Southern blotting and hybridization

For the Southern hybridization, DIG DNA Labeling and Detection Kit (Roche) was used. The DIG-labeled probes for the Southern hybridization is prepar-

ed according to the manufacturer's instructions (Roche DIG DNA Labeling and Detection Kit, Instruction Manual, Version October 2004). The probe was prepared from the PCR product which was obtained using the primers RshupSL6 and RshupSL7 using pGhup5 as a template. The PCR program was as follows: 5 min. at 94 ℃ for pre-denaturation, 30 cycles of amplification step (30 sec. at 94 ℃, 30 sec. at 55 ℃ and 90 sec. at 72 ℃) followed by a final extension of 5 min. at 72 ℃. The probes were synthesized freshly before the hybridization experiment. The probe used here was expected to hybridize to the gDNA of the mutant cells but not to that of wild type cells by giving a 946 bp band on the membrane because the probe corresponds to the joining part of deleted *hupSL* gene. The location of the probe was shown in the gDNA of the mutant cells in Figure 3.35.
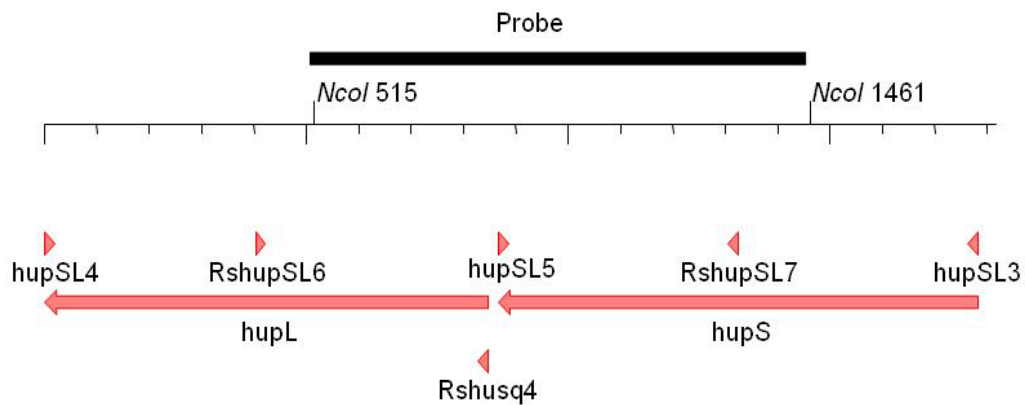
Figure 3.35 The location of the probe used to detect deleted *hup* gene.

After the probes were prepared, the gDNAs of *hup* mutant and wild type cells were digested with *Nco*I and *Sma*I and loaded onto 0.7 % agarose gel prepared by using 0.5X TBE buffer (pH:8.3). The agarose gel electrophoresis and Southern transfer were performed by using 0.5X TBE buffer too. The thickness of the agarose gel was equal to 0.6 mm to make an efficient Southern transfer. The Southern transfer was performed using Bio-Rad Gel Transfer System (Germany) at 13 Volts for 4-5 hours at 4 cm electrode distance (high electric field). The DNA fragments

were blotted to positively charged nylon membrane (Roche). After blotting, the hybridization of labeled probes to the DNA fragments was carried out at 75 °C for overnight. The detection was performed by using Anti-Dig-Alkaline Phosphatase conjugate. As shown in Figure 3.36, the probe hybridized as expected confirming the *hup* deletion.
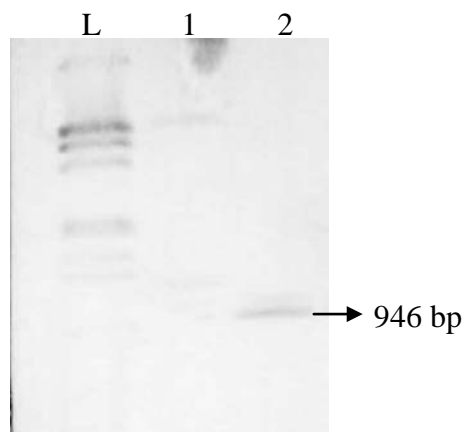
Figure 3.36 The Southern transfer and detection of *hup* genes in the *NcoI* and *Sma*I digested gDNA of wild type (Lane 1) and *hup* mutant (Lane 2) *R. sphaeroides*. Lane L is the Dig-labeled Molecular Weight Marker III (Roche).

3.3.4 The growth and hydrogen production profiles of *hup* deleted mutant of *R. sphaeroides* in malate/glutamate (15/2 mM) media

The aim of this study was to test the growth and hydrogen production performance of *hup⁻* and wild type *R. sphaeroides* in malate/glutamate medium. The mutant and wild type bacteria were grown in Biebl & Pfenning growth medium (15/2 mM, Malate/Glutamate). The pH values of the media were first set to pH 7 before inoculation and were not controlled during cultivation. However, the pH value of both cultures did not change significantly and followed the same pattern (Figure 3.37A). It increased to 7.4 and then declined to circa 7.2. The growth of the mutant

and wild type *R. sphaeroides* was monitored by measuring the turbidity of the culture at 660 nm (Figure 3.37B). The wild type cells and mutant cells showed very similar growth patterns. While the maximal OD value of the wild type cells was 1.65 that of mutant cells was 1.62.

(A)

(B)
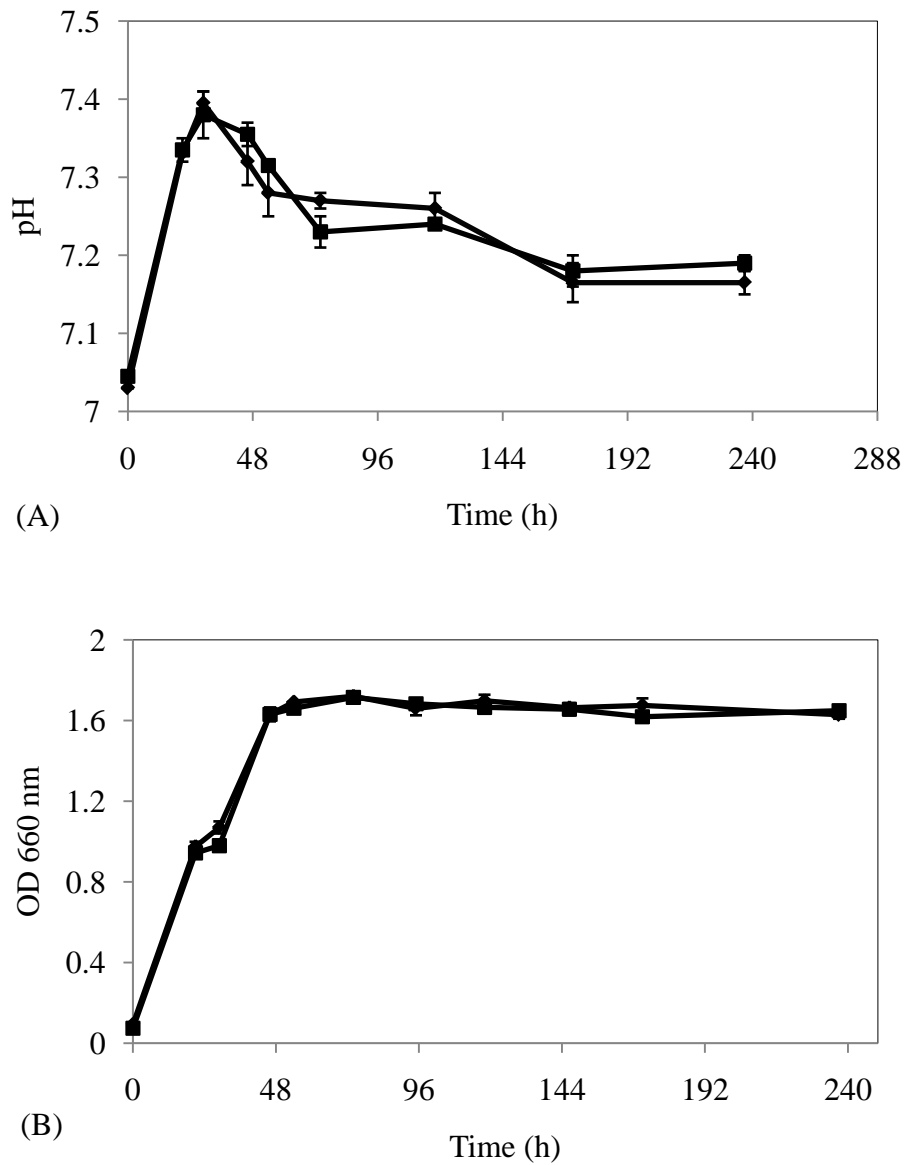
Figure 3.37 The pH (A) and absorbance (B) changes during the growth of wild type (■) and mutant (♦) strain of *R. sphaeroides* O.U 001.

In Figure 3.38, the hydrogen production performance of mutant and wild type cells was illustrated. As it is seen from the figure, the mutant cells produced significantly more hydrogen than the wild type cells. The mutant cells produced 2.42 l H_2/l culture whereas wild type cell produced 1.97 l H_2/l culture. Moreover, the hydrogen production yield and substrate conversion efficiency of mutant cells were 0.064 g H_2/g malate and 71.5 % while that of wild type cells were 0.052 g H_2/g malate and 58 %.
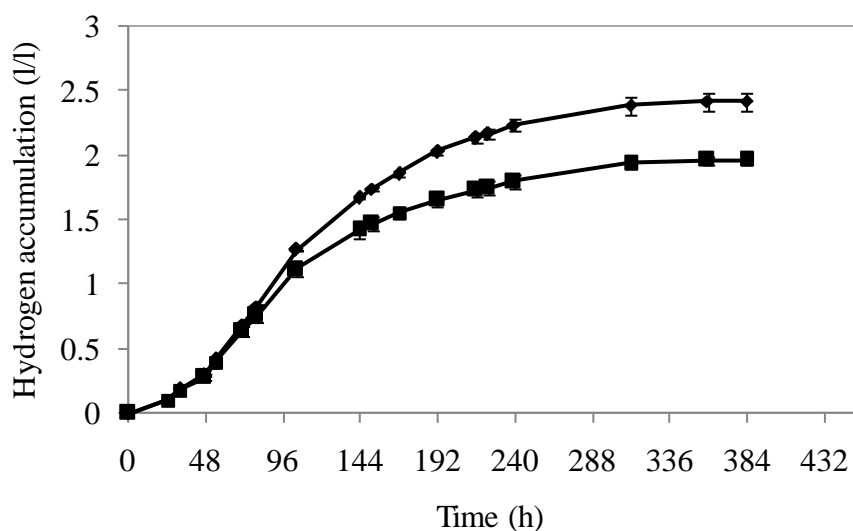
Figure 3.38 Total hydrogen production by wild type (■) and *hup⁻* (♦) *R. sphaeroides* O.U 001. Each value is the mean of three replications with ± standard deviation.

These results suggested that the *hup* mutation did not interfere with the growth of *R. sphaeroides* and resulted in an increase in hydrogen production. Therefore, the mutants could be safely used in industrial scale.

3.3.5 The growth and hydrogen production profiles of *hup* deleted mutant of *R. sphaeroides* in acetate/glutamate (30/2 mM) media

In these studies, the growth and hydrogen production performance of wild type and *hup* deleted *R. sphaeroides* were evaluated. According to the results obtained so far, the pH of the culture where bacteria were grown using acetate as a carbon source increased significantly compared to the pH of the culture in which the malate was used. Similarly, the pH of the medium in which the wild type cells were grown reached pH 9.8 while pH of the medium in which the mutant cells were grown reached pH 9.1 (Figure 3.39A). The products formed during acetate utilization might cause this pronounced rise in pH. In the case of cell growth, the bacteria grown in acetate containing media reached relatively higher cell masses compared to the cells grown in malate containing media. While the maximal OD value of the wild type cells was 3.4, that of mutant cells was 3.0 (Figure 3.39B).

In Figure 3.40, the hydrogen production performance of mutant and wild type cells in acetate containing media was illustrated. As it is seen from the figure, the mutant cells produced significantly more hydrogen than the wild type cells. The mutant cells produced 0.25 l H_2/l culture whereas wild type cell produced 0.21 l H_2/l culture. Moreover, the hydrogen production yield and substrate conversion efficiency of mutant cells were 0.011 g H_2/g acetate and 5.5 % while that of wild type cells were 0.009 g H_2/g acetate and 4.6 %. The possible reasons for this very low hydrogen production yields were explained in section 3.2.5. The results suggested that either the cells could not feed the nitrogenase enzyme energetically which was more probable, or acetate metabolism somehow inhibited the nitrogenase synthesis. Then the question "how this unwanted situation can be solved" arises. There could be several approaches. First, the electrons are redirected to nitrogenase rather than PHB production by knocking out the gene coding for the PHB synthesis enzymes. Second, the media are supplemented with additional carbon or nitrogen sources such as malate, lactate, etc. which trigger the hydrogen production. The problem could be attacked more easily when the complete acetate assimilation pathway of the *R. sphaeroides* was known.
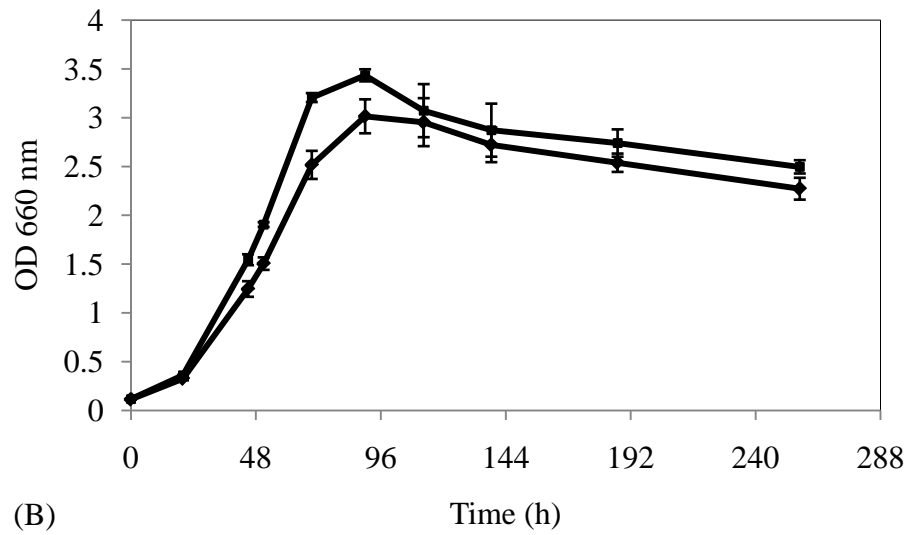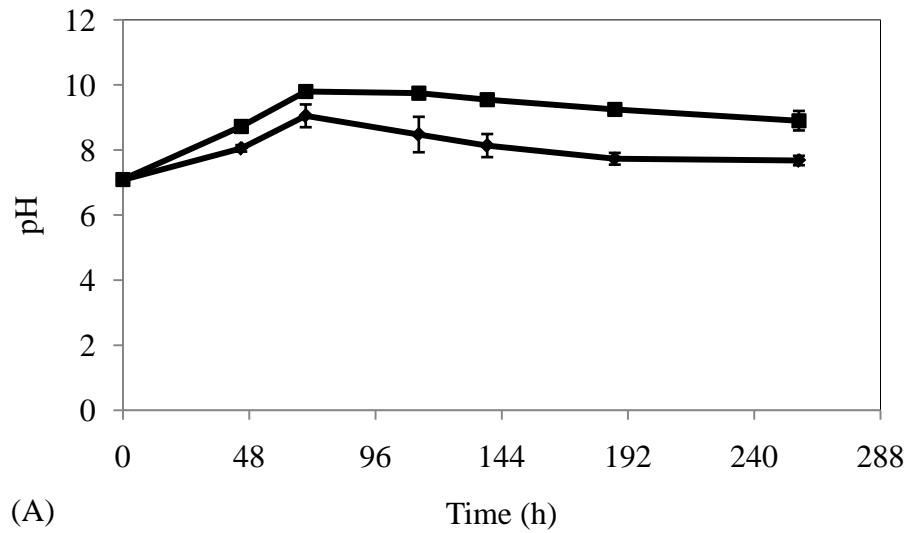
Figure 3.39 The pH (A) and absorbance (B) changes during the growth of wild type
(■) and *hup* deleted mutant strain (♦) of *R. sphaeroides* O.U 001 in acetate media.
Each value is the mean of three replications with ± standard deviation.
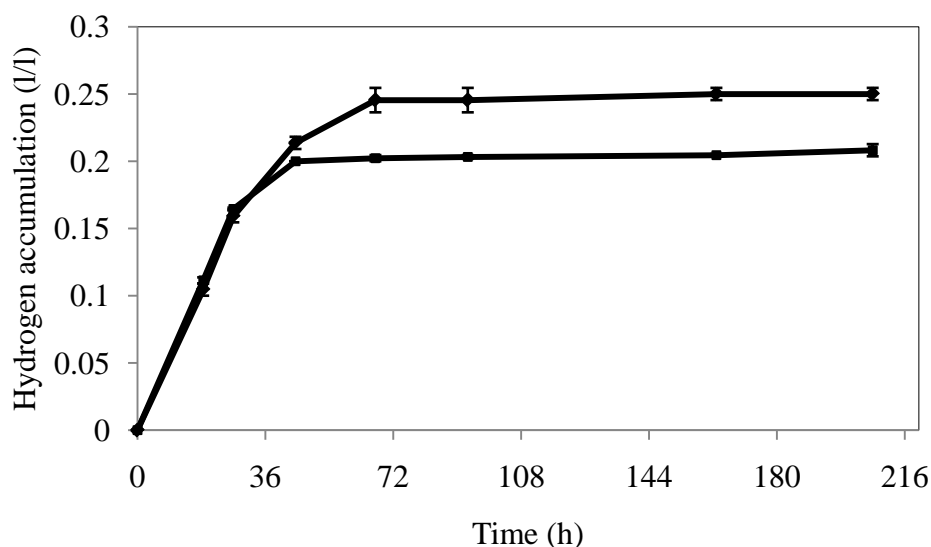
Figure 3.40 Total hydrogen production by wild type (■) and *hup* mutant (♦) *R. sphaeroides* O.U 001 in acetate media. Each value is the mean of three replications with ± standard deviation.

3.4 The heterologous expression of *hox1* hydrogenase gene with its own promoter

In industrial applications, the biohydrogen production by the nitrogenase is also limited by the availability of various nitrogen sources which might repress the process. Hence, in these studies, a NiFe hydrogenase capable to catalyze hydrogen production was planned to integrate into the metabolic processes of *R. sphaeroides*. If the expression of both nitrogenase and hox1 hydrogenase was achieved, the hydrogen gas would be produced under mixotrophic conditions in the presence of ammonia without need for reduced sulfur compounds.

3.4.1 The construction of wide range of expression vector

The functional operon of the bidirectional hydrogenase (Hox1 hydrogenase) from *Thiocapsa roseopersicina* was cloned into a broad host range expression vector

and delivered to *R. sphaeroiodes* O.U.001. The whole *hox* operon (*hoxEFUYHW*) was obtained from pTCB4/2 plasmid by digestion with *BstX*I enzyme. And after polishing with T4 DNA polymerase, it was cloned into *Hinc*II and *Sma*I double digested pBBR1MCS4Amp vector (Figure 3.41).
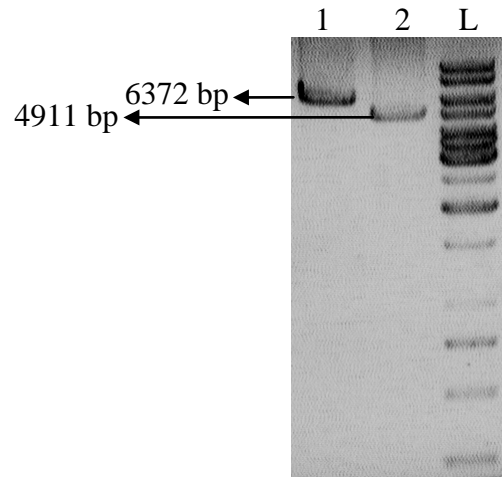
Figure 3.41 The *hox1* operon (Lane 1) obtained from pTCB4/2 plasmid and *Hinc*II and *Sma*I double digested pBBR1MCS4Amp vector (Lane 2) before ligation.

One of the constructs (pGhox1a, Figure 3.42) containing the *hox1* operon in the orientation of the ampicillin resistance gene in the vector was chosen and transferred into *R. sphaeroides* by conjugation. The transformed *R.sphaeroides* was selected on ampicillin containing minimal media.
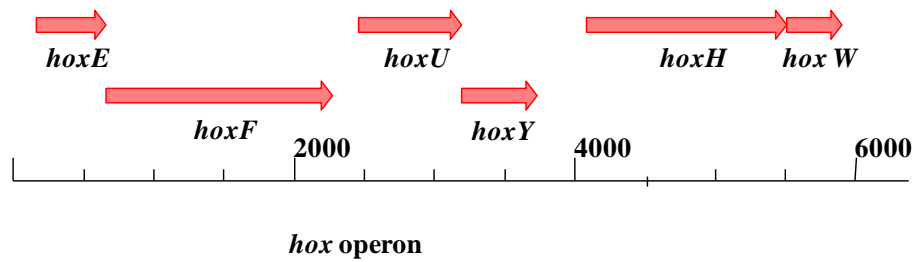
(A)
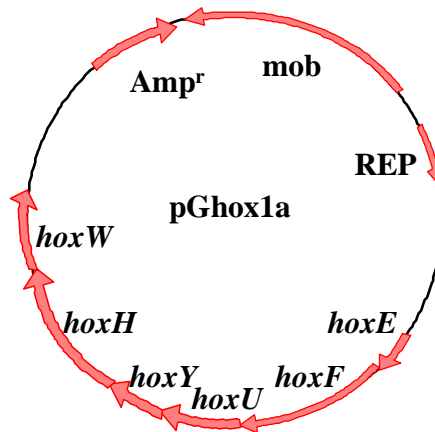
(B)

Figure 3.42 The whole *hox1* operon (A) and the schematic view of the expression vector pGhup5 (B) were illustrated.

In order to ensure the presence of construct in the bacterium, the construct was isolated from the *R. sphaeroides* and digested with *Hind*III and *Nde*I for characterization it (Figure 3.43).
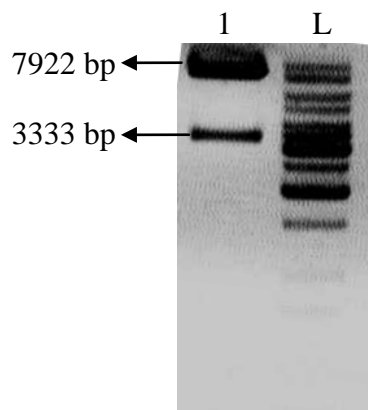
Figure 3.43 The *Hind*III and *Nde*I digested pGhox1a (Lane 1) was loaded into agarose gel with the DNA ladder (Lane L).

3.4.2 The analysis of the expression of *hox1* genes in *R. sphaeroides* by RT-PCR

R. sphaeroides which contained the construct with *hox1* structural genes was grown in the presence of ampicillin (100 μl mL^{-1}) and the total RNA was isolated as shown in Figure 3.44.

The cDNA was synthesized using 10 μl of DNase I treated RNA with specific primer (TCHO 08, Appendix H). 3 μl of cDNA was used in the PCR using specific primers (TCHO 03 and TCHO 04, Appendix H). The PCR program was as follows: 2 min. at 94 ° C for pre-denaturation, 30 cycles of amplification step (30 sec. at 94 ° C, 1 min. at 57 ° C and 30 sec. at 72 ° C) followed by a final extension of 5 min. at 72 ° C. The samples were analyzed on 1 % agarose gel. As it is seen from the Figure 3.45, the *hox1* gene was successfully expressed in *R. sphaeroides*. In order to detect the interference of gDNA in the PCR which leads to false positive results, RT-experiments were also performed. In RT- experiments, reverse transcriptase was not added during cDNA synthesis. Therefore, since there were no products in the RT-tubes, it can be concluded that there was no gDNA contamination and the products came from cDNAs.
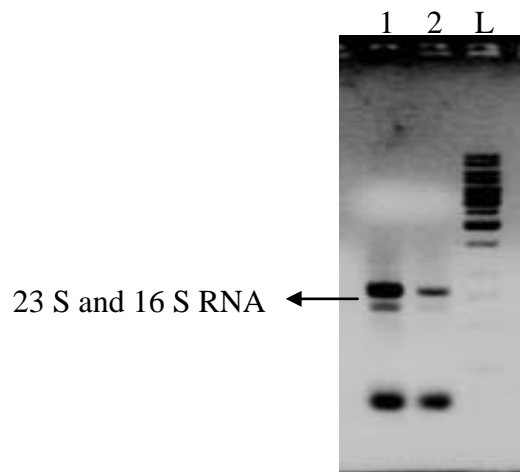
Figure 3.44 Total RNA was isolated from *R.sphaeroides* containing *hox* structural genes (1,2). A 5 μl of aliquot was run on 1 % native agarose gel at 90 V. for 1 hour together with DNA ladder.
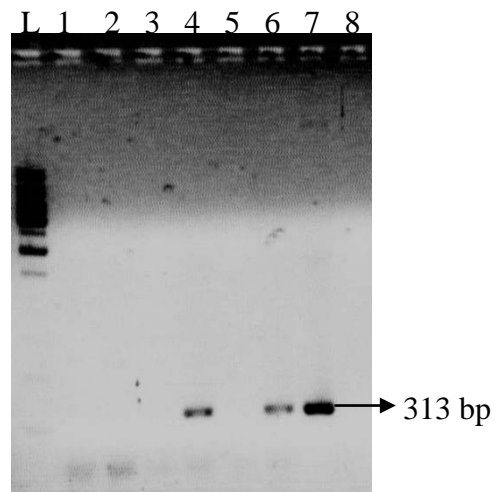
Figure 3.45 The expression analysis of *hox* gene in *R. sphaeroides*, L: DNA ladder, 1: Wild (RT-), 2: Wild (RT+), 3: Strain 1 (RT-), 4: Strain 1 (RT+), 5: Strain 2 (RT-), 6: Strain 2 (RT+), 7: Standart, 8: Non template control (NTC).

3.4.3 *In vitro* hydrogen evolution measurement under nitrogenase repressed conditions

In order to check the hox1 hydrogenase dependent hydrogen production, in vitro hydrogen production measurements were done under ntirogenase repressed conditions (in the presence of ammonia, rich medium) as it is explained in the materials and methods section. However, hydrogen was not detected in GC measurements showing that although the genes were transcribed the functional enzyme was not formed. There could be several reasons for this result. Since the genes were originated from a purple sulfur bacterium, due to the different codon usage or preference of *R. sphaeroides*, the correct active enzyme might not be synthesized. Furthermore, in a very recent article, heterelogous expression of H_2-evolving Fe-only hydrogenase from *R. rubrum* was achieved in *R. sphaeroides* (Kim *et al.*, 2008). But it was stated that the hydrogen-evolving activity by the Fe-only hydrogenase of *R. rubrum* was achieved by introducing its maturation proteins into *R. sphaeroides* as well. This results show the importance of accessory proteins for the function of proteins. Moreover, although the transcription was detected by RT-PCR, the amount of transcription might not be sufficient to synthesize the protein too. The absence of correct posttranscriptional RNA processing or lack of posttranslational modification of the enzyme such as correct folding of the enzyme in *R. sphaeroides* might also be the reasons for this result.

3.5 The heterologous expression of *hox1* hydrogenase gene under the control of *crtD* promoter

3.5.1 The construction of wide range of expression vector

In this study, the structural genes of *hox*1 hydrogenase were in frame cloned to pMHE2crt expression vector which has *crtD* promoter of *T. roseopersicina*. This construct was made in two successive cloning steps. First, 1.2 kb long partial *hox*EF gene was PCR amplified using pGhox1a as a template using Phusion® DNA poly-

merase. The primers were Thihox01 and Thihox02 (Appendix H). The PCR program was as follows: 30 sec. at 98 ºC for pre-denaturation, 30 cycles of amplification step (10 sec. at 98 ºC, 30 sec. at 54 ºC and 90 sec. at 72 ºC) followed by a final extension of 5 min. at 72 ºC. The PCR products were precipitated, gel eluted and then kinase treated. The phosphorylated PCR products were purified before ligation. The pMHE2crt vector was *Nco*I cut and treated with Klenow fragment to make blunt ends. The cut vector was finally treated with CIAP before ligation. The PCR product and vector were ligated to form pGhox2. Three clones were screened by digesting with *StuI* enzyme to find the correct pGhox2. After digestion, one out of three clones was found to be the correct pGhox2 by giving expected fragments as shown in Figure 3.46.
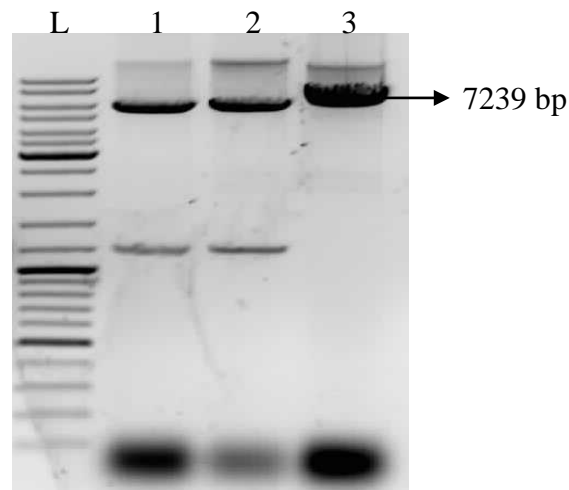
Figure 3.46 Three clones were screened by digesting with *StuI* enzyme to find the correct pGhox2. Lane 1-3 represents the three clones screened. The clone 3 being the correct construct gave the expected band (Lane 3).

The remaining of *hox*1 structural genes were excised from pGhox1a by cutting it with *StuI* and *Hind*III and cloned inframe into *StuI* and *Hind*III cut pGhox2 forming pGhox3 (Figure 3.47).
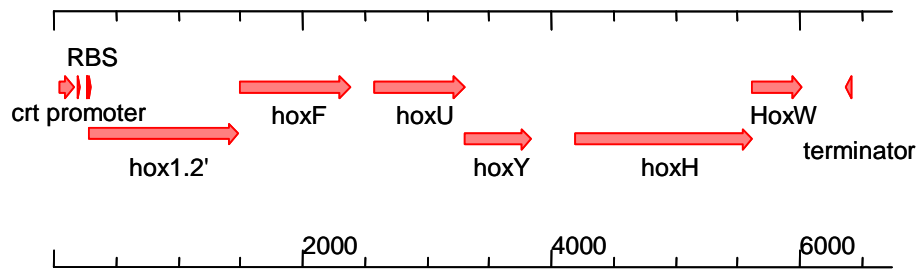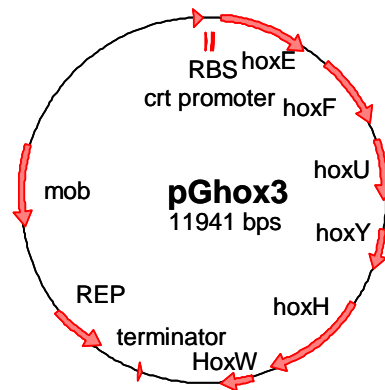
Figure 3.47 The structure of close (A) and open (B) form of pGhox3.

The constructed pGhox3 was double digested with *Nco*I/*Xba*I to see if it is the correct one. As shown in Figure 3.48, double digestion gave the expected bands.

The construct (pGhox3) contained streptomycin resistance gene and therefore it was not possible to deliver it by conjugation using *E. coli* S17 λ.pir which had Sm[r] gene too. Therefore, a kanamycin resistance gene was PCR amplified with *pfu* polymerase using Kmleft and Kmright primers (Appendix H) using pK18*mobsacB* as a template and blunt end cloned into *Hind*III cut pGhox3 making the final expression vector pGhox4 as shown in Figure 3.49. The PCR program to amplify Km[r] gene was as follows: 5 min. at 94 °C for pre-denaturation, 30 cycles of amplification step (1 min. at 94 °C, 1 min. at 55 °C and 3 min. at 72 °C) followed by a final extension of 5 min. at 72 °C.
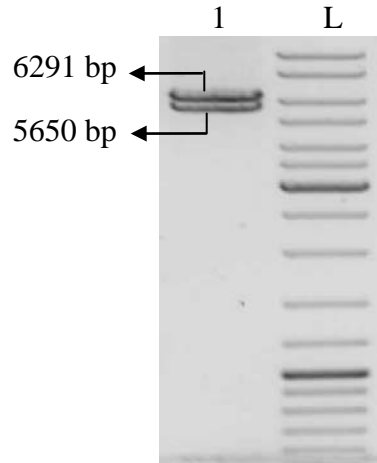
Figure 3.48 The *Nco*I/*Xba*I digested products of pGhox3 (Lane 1) was run on the gel together with DNA ladder (L).
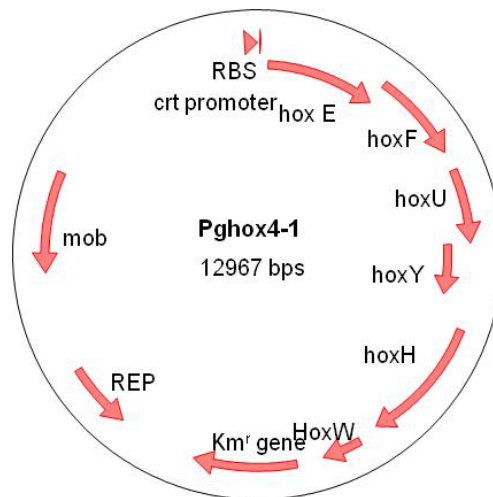
Figure 3.49 The *hox1* expression vector (pGhox4) ready to be delivered to *R. sphaeroides* by conjugation.

After the construction of the final expression vector, it was digested with *EcoRV* to see if it is the correct one. As shown in Figure 3.50, the digestion gave the expected two bands.
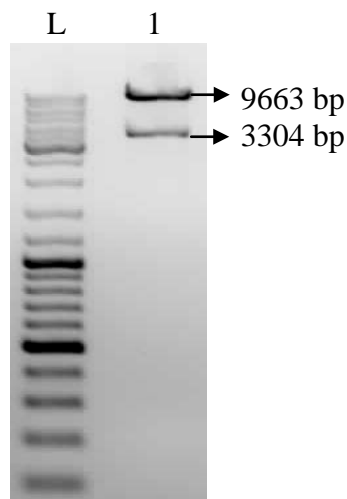
Figure 3.50 The pGhox4 was digested with *EcoRV* and run on the gel together with DNA ladder (L). The digestion gave the expected 9663 and 3304 bp fragments (Lane 1).

Moreover, the direction of the Km^r gene was also checked by restriction enzyme digestion. The pGhox4 expression vector was cut with *NcoI* and the construct which has the transcription direction as shown in Figure 3.49 was expected to give 11874 and 1093 bp fragments after cutting. As seen in Figure 3.51, the correct construct gave the expected bands.

3.5.2 The delivery of expression vector into *R. sphaeroides*

After addition of Km^r gene to the expression vector, the construct was successfully delivered to *R. sphaeroides* by diparental mating as explained before and the expression of the *hox* genes was investigated by RT-PCR.

3.5.3 The analysis of the expression of *hox1* genes in *R. sphaeroides* by RT-PCR

Total RNA isolation was performed using TRİ$^®$-Reagent according to the

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protocol described in Manual (Sigma, Product No. T9424). The extracted total RNA was shown in Figure 3.52
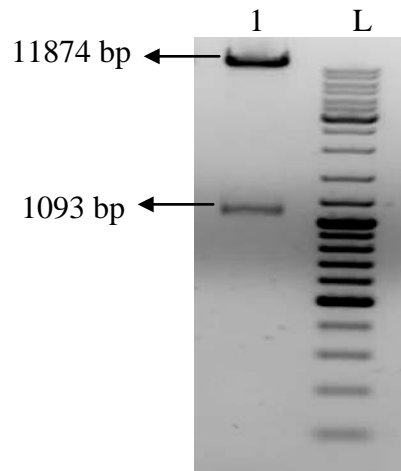
Figure 3.51 The orientation of the Km[r] gene was determined after digestion with *NcoI* enzyme. The digested vector was run together with DNA ladder (L).

.
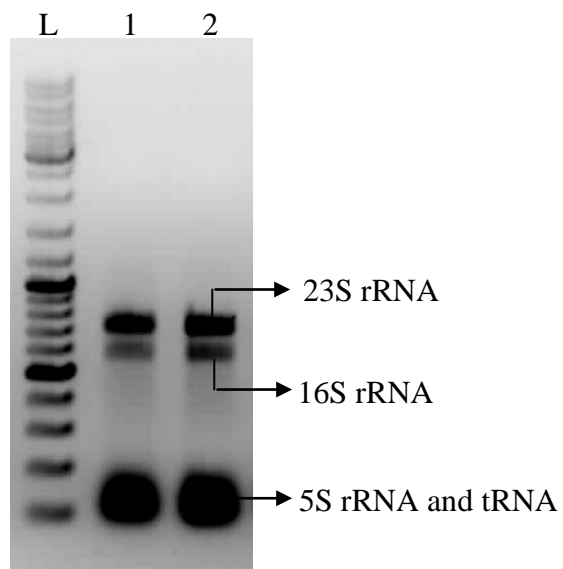
Figure 3.52 Total RNAs were isolated from wild type (Lane 1) and pGhox4 contai-

10 μl of DNase I treated RNA was mixed with 1 μl of specific primer (Tchox2, Appendix H, 10 pmol/μl) and incubated at 70 ° C for 5 minutes and then chilled on ice. After addition of 4 μl of 5X RT buffer and 2 μl of 10 mM dNTP reaction mixture was completed to 19 μl. The mixture was incubated at 37 ºC for 5 minutes. Then, 200 units of Revert aid™ H Minus MMuLV reverse transcriptase (Fermentas) was added and the mixture was incubated at 42 ºC for 1 hour and the reaction was stopped by incubating at 70 ºC for 10 minutes. 3 μl of cDNA was used in the PCR using specific primers (Tchox1 and Tchox2, Appendix H). The PCR program was as follows: 2 min. at 94 ° C for pre-denaturation, 30 cycles of amplification step (30 sec. at 94 ° C, 30 sec. at 55 ° C and 45 sec. at 72 ° C) followed by a final extension of 5 min. at 72 ° C. The samples were analyzed on 1 % agarose gel in Figure 3.53.
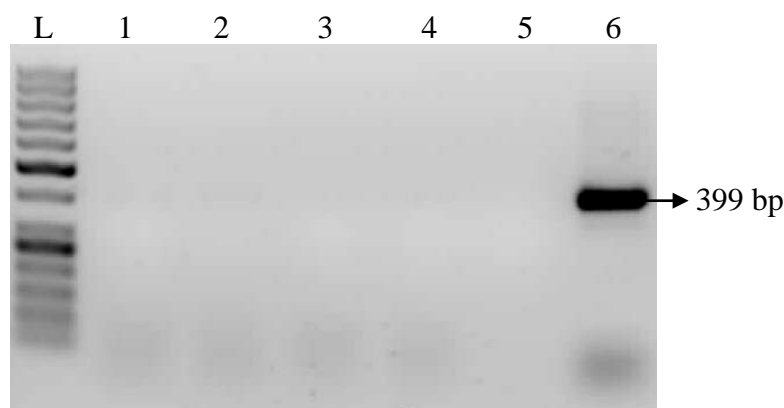
Figure 3.53 The expression analysis of *hox* gene in pGhox4 containing *R. sphaeroides*. L: 50 bp DNA ladder, 1: wild (RT-), 2: wild (RT+), 3: wild with pGhox4 (RT-), 4: wild with pGhox4 (RT+), 5: NTC, 6: positive control.

Total RNA was isolated from both wild and pGhox4 containing *R. sphaeroides* and RT-PCR was performed using both RNA samples to check if the transcription was occurred. As seen from the Figure 3.53, the expression of *hox* gene was not detected. Since the genes were not transcribed, there could be problem with the promoter. Probably, the promoter was not recognized by *R. sphaeroides* transcription machinery. Another reason might be that the promoter alone was not sufficient to drive the transcription. The transcription machinery of might require additional or different transcriptional factors which are probably present in *T. roseopersicina* but absent in *R. sphaeroides*.

3.6 The estimation of relative amount of nitrogenase protein in *R. sphaeroides* O.U.001 by SDS-PAGE

Since hydrogen production was done by nitrogenase in *R. sphaeroides*, its relative amount, turnover time and energy requirement took much attention. One of the arguments was that the high amount of Mo-nitrogenase in *R. sphaeroides* constitutes a big burden for the cells in terms of energy and cell materials. It was argued that under normal nonrepressive conditions, in the absence of ammonia, the nitrogenase enzyme complex is synthesized at extremely high levels to enable the cell to obtain all required nitrogen via the reduction of dinitrogen (Klugkist *et al.*, 1985; Nielsen and Nordlund, 1975; Kranz and Cullen, 1995, Joshi and Tabita 1996). And, at least 25 % of the electron throughput is used to reduce protons to molecular hydrogen (Simpson and Burris, 1984), which is released from the cells by photosynthetic bacteria under anoxic conditions. Therefore, in this study, an approximate amount of Mo-nitrogenase was investigated by SDS-PAGE.

3.6.1 Bradford protein assay and SDS-PAGE

R. sphaeroides was grown in three different physiological conditions to be used in SDS-PAGE. The cells were grown in B&P medium under anaerobic condition (sample 1), in B&P medium under aerobic condition (sample 2) and in nit-

rogenase repressed condition using 10 mM ammonium chloride (sample 3). 10 ml of each culture was collected and freeze-thawed five times and the cell debris was separated by centrifugation. The supernatant was used to calculate the amount of protein by Bradford assay and to load into the gel. The protein standard curve was drawn using BSA (Figure 3.54).
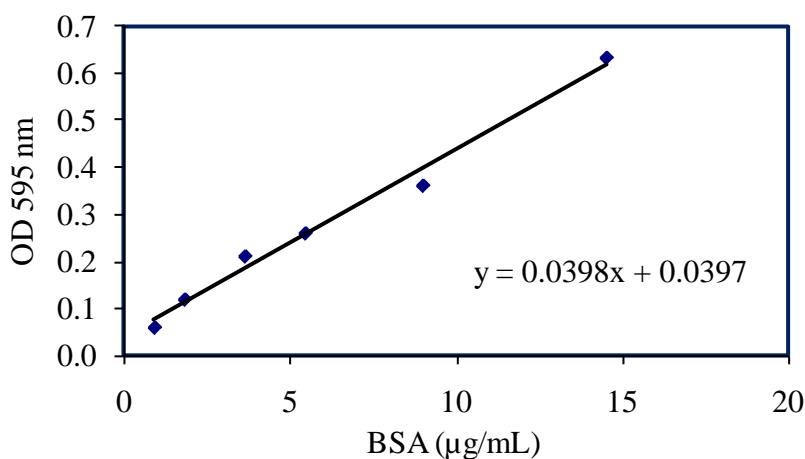
Figure 3.54 Protein standard curve using BSA.

The calculated concentrations of the samples (1, 2 and 3) were 183, 228 and 192 µg/mL respectively. 5.5 µg of each sample was loaded onto the gel and the gel was stained using Comasie Brillant Blue G-250 (Figure 3.55).

The expected sizes of nif H, nif D and nif K subunits are 31.4 kDa, 54.6 kDa and 56 kDa. The gel was used to calculate relative amount of nitrogenase by densitometric analysis. The result showed that the nitrogenase components (H+D+K) constituted less than 8.5 ± 0.5 % of all proteins. This percentage indicates the upper maximal amount of nitrogenase because other proteins most probably overlapped at the same location in the gel. Moreover, there were no significant differences between three samples harvested under different physiological conditions.
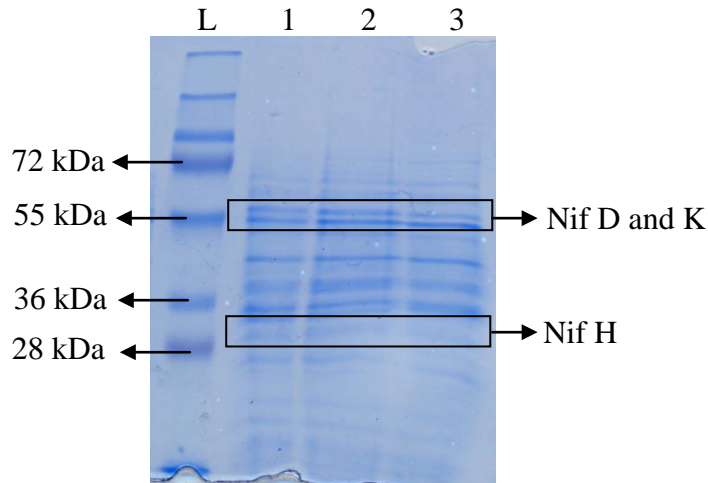
Figure 3.55 The SDS-PAGE of three different total protein samples obtained from *R. sphaeroides* grown under hydrogen producing and non-producing conditions. The total proteins extracted from *R.sphaeroides* grown under anaerobic conditions in B&P medium (1), aerobic conditions in B&P medium (2) and non-nitrogen fixing conditions (3).

3.7 Nitrogenase assay

The hydrogen production in *R. sphaeroides* was mediated by nitrogenase and therefore it is very useful to measure the nitrogenase activity. In order to assess the hydrogen production capacity of the cells without performing hydrogen production experiments, nitrogenase activity measurement by acetylene reduction assay could be performed. In order to set up the nitrogenase activity assay, *R. sphaeroides* cells were grown in nitrogenase derepressed condition and their nitrogenase activity was measured as explained in the section 2.6.5. As seen from the Figure 3.56, the acetylene injected into the reaction vessel was converted to ethylene by the activity of nitrogenase. In the diagram the hydrogen gas produced was also observed as nitrogenase accepts both acetylene and protons as substrates.
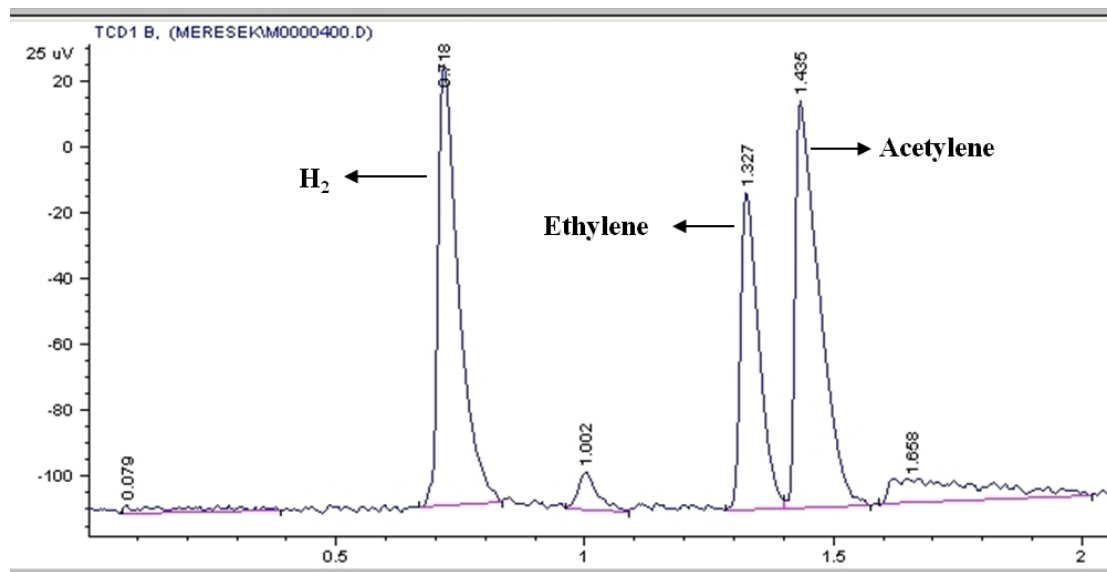
Figure 3.56 The nitrogenase activity of *R. sphaeroides* by acetylene reduction assay.

CHAPTER 4

CONCLUSIONS

The hydrogen production and the expression of Mo-nitrogenase and uptake hydrogenase genes (*nifK*, *nifD* and *hupS*) were investigated in *R. sphaeroides* grown at different concentrations of metals (Mo and Fe). pH and the cell densities were also monitored under the same growth conditions. The cells grown in media without molybdenum and iron reached relatively higher densities since they do not spent energy for hydrogen production but the cells grown in metal ion containing media reached relatively lower absorbance values. Moreover, the pH rised considerably in media without hydrogen production than in the media with hydrogen production. This pH rise could be attributed to the accumulation of more cellular by-products such as PHB in the media without H_2 production. The cells grown in media without molybdenum and iron could not produce considerable amounts of hydrogen and the optimum concentrations of the molybdenum and iron salts were 16.5µM and 0.1 mM, respectively. It could be concluded that molybdenum and iron in the media strongly influences the hydrogen production capacity of the cells, therefore, they should be supplemented in optimum amount to have high hydrogen production.

The gene expression analyses at different molybdenum concentrations showed that *nifK* expression in No Mo medium was strongly reduced but it was expressed significantly in the Mo containing media. The maximal gene expressions were observed in the late logarithmic growth phase of the cells. The *nifD* and *hupS* expression analyses at various concentrations of iron showed that iron deficiency also led to pronounced reduction in the gene expression levels. Similar to the *nifK* expression, the highest *nifD* expression was observed between the very end of log phase and the mid-stationary phase. The *hupS* gene was expressed significantly as soon as the hydrogen and the iron were available in the environment.

In conclusion, if the gene expression analyses and the hydrogen production profiles of the cells were taken into account, it is suggested to keep the cells in the late log phase of the growth to get a high hydrogen production rate with the optimum concentrations of iron and molybdenum. The cells could be kept at their late logarithmic phase of growth in continuous bioreactors by adjusting the parameters such as dilution rate. Or, the cells at this phase of growth could be immobilized onto surfaces and could be used for hydrogen production.

The membrane-bound [NiFe] hydrogenase called also as uptake hydrogenase decreases the efficiency of H_2 production by catalyzing the conversion of molecular hydrogen to electrons and protons (Vignais et al, 1985). Therefore, the genes were targeted to be disrupted in *R. sphaeroides* to enhance the total hydrogen photoproduction. *hup*SL genes coding for uptake hydrogenase were disrupted in two different ways in *R. sphaeroides* O.U.001. In the first method, the *hup* genes were disrupted by gentamicin resistant gene insertion. The wild type and the *hup*$^-$ mutant cells showed similar growth patterns but substantially more hydrogen was produced by the mutant cells in both malate/glutamate (15/2 mM) and acetate/glutamate (30/2 mM) media. The total hydrogen accumulation in mutant cells was 20 % higher than that in wild type cells (2.85 l H_2/l culture and 2.36 l H_2/l culture respectively) in malate/glutamate (15/2 mM) containing medium. The average gas production rate of mutant cells was 9.2 ml/l/h while that of wild type cells was 6 ml/l/h. The substrate conversion efficiency of mutant cells was 80 % while that of wild type cells was 66.4 %. The yield for mutant cells was 0.072 g H_2/g malate while the yield for wild type cells was 0.060 g H_2/g malate. Hence, an efficient substrate conversion to hydrogen was achieved in *hup* mutant *R. sphaeroides* O.U.001 in malate/glutamate medium. The high hydrogen production rate and high substrate conversion efficiency demonstrated that uptake hydrogenase can no longer use the hydrogen produced by nitrogenase and therefore, more hydrogen accumulation was achieved in the *hup* mutant cells.

The hydrogen production performance of mutant and wild type cells was also evaluated in acetate/glutamate (30mM/2mM) medium. The mutant cells produced 0.31 l H_2/l culture whereas wild type cell produced 0.25 l H_2/l culture. Moreover, the

hydrogen production yield of mutant cells in acetate medium was 0.014 g H_2/g acetate and that of wild type cells was 0.011 g H_2/g acetate. While the mutant cells had a substrate conversion efficiency of 6.9 %, the wild type cells had a substrate conversion efficiency of 5.8 %. If the hydrogen production efficiencies of the cells in malate and acetate media were compared, both wild and mutant cells produced significantly less amount of hydrogen in acetate media than the cells grown in malate containing media. The inability of *R sphaeroides* to produce hydrogen may come from the acetate assimilation pathway in this bacterium.

Since *hup* mutation resulted in an increase in hydrogen production, there is a possibility to use this mutant in large scale application (outdoor experiments). However, the mutant bacteria carrying antibiotic resistance gene are not favored for this purpose due to environmental safety issues. Therefore the uptake hydrogenase was inactivated by deleting *hup* gene without inserting antibiotic resistant gene. As expected, the mutant cells produced 2.42 l H_2/l culture whereas wild type cell produced 1.97 l H_2/l culture in malate/glutamate (15/2 mM) medium. Moreover, the hydrogen production yield and substrate conversion efficiency of mutant cells were 0.064 g H_2/g malate and 71.5 % while that of wild type cells were 0.052 g H_2/g malate and 58 %. The hydrogen production performance of mutant and wild type cells was also evaluated in acetate/glutamate (30/2 mM) containing medium. The mutant cells produced significantly more hydrogen than the wild type cells. The mutant cells produced 0.25 l H_2/l culture whereas wild type cell produced 0.21 l H_2/l culture. Moreover, the hydrogen production yield and substrate conversion efficiency of mutant cells were 0.011 g H_2/g acetate and 5.5 % while that of wild type cells were 0.009 g H_2/g acetate and 4.6 %. As a conclusion, *hup* mutation with both methods was successfully achieved and it resulted in increase in the amount of hydrogen evolution significantly.

The biohydrogen production by the nitrogenase is limited by the availability of various nitrogen sources such as ammonia which repress the nitrogenase. Hence, a NiFe hydrogenase (hox1) capable to catalyze hydrogen evolution under mixotrophic conditions in the presence of ammonia without need for reduced sulfur compounds was planned to integrate into the metabolic processes of *R. sphaeroides*. For this pur-

pose, the native promoter of hox1 hydrogenase and the *crtD* promoter were tested for the expression of *hox*1 genes. The expression of hox1 hydrogenase in *R. sphaeroides* was investigated by RT-PCR at the transcription level and by *in vitro* hydrogen production measurements under ntirogenase repressed conditions (in the presence of ammonia, rich medium). However, hydrogen was not detected by GC measurements although the genes were transcribed when using the *hox1* native promoter. It can be concluded from these results that accessory genes are needed to be expressed together with the structural genes to obtain a functional hox1 hydrogenase in *R. sphaeroides*. In addition, when the promoter of *crtD* gene of *T. roseopersicina* was used to drive the expression of *hox1* operon, the transcription was not detected in RT-PCR experiments. It can be concluded that the promoter of *crtD* gene of *T. roseopersicina* was not working in *R. sphaeroides*.

Finally, approximate amount of Mo-nitrogenase was estimated by SDS-PAGE followed by densitometric analysis of the gel. The result showed that the nitrogenase components (H+D+K) constituted much less than 8.5 ± 0.5 % of the total proteins of *R. sphaeroides*. As a conclusion, the amount of nitrogenase in *R. sphaeroides* might not be a big burden for the cells.

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APPENDIX A

LIST OF CHEMICALS AND SUPPLIERS

Acetic acid, Merck

Acrylamide, Sigma

Agar, Applichem

Agarose, Applichem

Ammonium persulfate [APS], Sigma

Ampicillin, Applichem

Biotin, Merck

BSA, [Bovine Serum Albumin], Sigma

$CaCl_2.H_2O$, Merck

Chloroform, Merck

$CoCl_2.6H_2O$, Applichem

$CuCl_2.2H_2O$, Aldrich

DEPC, Applichem

D-L,Malic Acid, Sigma

EDTA, Applichem

Ethanol absolute, Applichem

Fe-citrate, Merck

Gentamicin, Applichem

Glycerol, Sigma

H_3BO_3, Merck

HCl, Merck

Iso-propyl alcohol, Ambresco

Kanamycin, Applichem

KH_2PO_4, Merck

LB Broth (Lennox L Broth Base), Invitrogen

2-Mercaptoethanol, Sigma

$MgSO_4.7H_2O$, Applichem

$MnCl_2.4H_2O$, Applichem

N, N'-Methylene-bis-Acrylamide, Sigma

NaCl, Applichem

$NaMoO_4.2H_2O$, Merck

NH_4Cl, Merck

NaOH, Riedel De Haen

Niacin, Applichem

$NiCl_2.6H2O$, Applichem

Phenol, Sigma

SDS, Applichem

Sodium acetate, Sigma

Sodium Glutamate, Sigma

Spectinomycin, Sigma

Streptomycin, Sigma

Sucrose, Merck

Temed, Sigma

Tetracycline Hydrochloride, Sigma

Thiamine, Sigma

Tri Reagent, Sigma

Tris Base, Applichem

$ZnCl_2$, Applichem

APPENDIX B

GROWTH MEDIA FOR BACTERIA

B.1 Biebl and Pfenning minimal medium

For general purposes, the solid ingredients except for the vitamins, trace elements and ferric citrate were weighed and dissolved in 1 Liter distilled water. The pH of the solution was adjusted to 6.8-7.0 by adding NaOH and sterilized by autoclaving. Vitamin solution, trace element solution and Fe-citrate solution were then added to the medium. If solid media were needed, 1.5 % agar was used.

Table B.1. The composition of *R. sphaeroides* O.U. 001 cultivation media with different DL-Malic Acid to Na Glutamate ratio.

Medium composition (g/l)	DL-Malic acid/Na Glutamate 7.5/10 (mM)	DL-Malic acid/Na Glutamate 15/2 (mM)
KH_2PO_4	0.5	0.5
$MgSO_4.7H_2O$	0.2	0.2
DL-Malic acid	2.0	1.0
Na Glutamate	0.36	1.8
NaCl	0.4	0.4
$CaCl_2.2H_2O$	0.05	0.05
Vitamin Solution	1 ml	1 ml
Trace Element Solution 7	1 ml	1 ml
Fe-Citrate Solution	5 ml	5 ml

B.2 Trace element solution for B&P medium

After the ingredients were weighed and dissolved in 1000 ml of water, the solution was autoclaved for sterilization. However, it is more useful to prepare the solution in a smaller volume in concentrated manner such as 100X trace element solution in 10 ml.

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

Ingredient	mg/l
$ZnCl_2$	70
$MnCl_2.4H_2O$	100
H_3BO_3	60
$CoCl_2.6H_2O$	200
$CuCl_2.2H_2O$	20
$NiCl_2.6H_2O$	20
$Na_2MO_4.2H_2O$	40
HCl (% 25 v/v)	1 ml

B.3 Vitamin solution for B&P medium

After the ingredients were weighed and dissolved in 1000 ml of water, the solution was filter-sterilized. However, it is more useful to prepare the solution in a smaller volume in concentrated manner such as 100X vitamin solution in 10 ml.

Table B.3. The composition of vitamin solution.

Vitamins	mg/l
Thiamine	500
Niacin	500
Biotin	15

B.4 Fe-citrate solution for B&P medium

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

B.5 LB medium for the growth of *E.coli*

For the growth of *E.coli* strains, 20 grams of LB powder was dissolved in distilled water and autoclaved. If solid media were needed, 1.5 % agar was added to the liquid media before autoclaving and poured into petri dishes.

APPENDIX C

SOLUTIONS AND BUFFERS

C.1 Antibiotics

Ampicillin

For 100 mg/ml stock solution;

1 gram ampicillin was dissolved in 10 ml dH$_2$O, filter sterilized, and stored at -20°C. The working concentration was 100 µg/ml.

Gentamicin

For 25 mg/ml stock solution;

250 mg gentamicin was dissolved in 10 ml dH$_2$O, filter sterilized, and stored at -20°C. The working concentration was 25 µg/ml.

Kanamycin

For 25 mg/ml stock solution;

250 mg kanamycin was dissolved in 10 ml dH$_2$O, filter sterilized, and stored at -20°C. The working concentration was 25 µg/ml.

Spectinomycin

For 25 mg/ml stock solution;

250 mg spectinomycin was dissolved in 10 ml dH_2O, filter sterilized, and stored at -20 °C. The working concentration was 25 µg/ml.

Streptomycin

For 25 mg/ml stock solution;

250 mg streptomycin was dissolved in 10 ml dH_2O, filter sterilized, and stored at -20 °C. The working concentration was 25 µg/ml.

Tetracycline

For 5 mg/ml stock solution;

50 mg tetracycline was dissolved in 10 ml 50% (v/v) Ethanol / water, and and stored in dark at -20°C. The working concentration was 5 µg/ml.

C.2 Southern blot solutions

10X TBE

108 grams of Tris base, 55 grams of Boric acid and 40 ml of 0.5 M EDTA in 1 liter dH_2O, pH~8.3

Depurination solution

 250 mM HCl

Denaturation solution

0.5 M NaOH, 1.5 M NaCl

Neutralization solution

0.5 M Tris-HCl (pH 7.5), 1.5 M NaCl

10 % SDS

10 grams of SDS in 100 ml of H_2O

10 % Sarkosyl

10 grams of sarkosyl in 100 ml of H_2O

20X SSC buffer

3 M NaCl, 300 mM sodium citrate, pH 7.0

Low stringency buffer

2x SSC containing 0.1% SDS

High stringency buffer

0.5x SSC containing 0.1% SDS

Hybridization buffer

5X SSC, 0.1 % Sarkosyl, 0.02 % SDS, 1 % Blocking solution

Washing buffer

0.1 M Maleic acid, 0.15 M NaCl;pH 7.5; 0.3% (v/v) Tween 20

Maleic acid buffer

0.1 M Maleic acid, 0.15 M NaCl;adjusted with NaOH (solid) to pH 7.5

Detection buffer

0.1 M Tris-HCl, 0.1 M NaCl, pH 9.5 (20°C)

TE buffer

10 mM Tris-HCl, 1 mM EDTA, pH 8.0

C.3 SDS-PAGE solutions

40 % Gel solution

29.2 grams of acrylamide, 0.8 gram bisacrylamide in 75 ml of H_2O

Stacking buffer

6 grams of Tris base in 100 ml of H_2O, pH adjusted to 6.8

Separating buffer

18.15 grams of Tris base in 100 ml of H_2O, pH adjusted to 8.8

10X running buffer

0.25 M Tris, 1.92 M Glycine in 0.5 liter H_2O

Coomasie Brillant Blue G-250

58.82 ml of phosphoric acid (85 %), 50 grams of ammonium sulfate, 0.6 gram of Coomasie Brillant Blue G-250 in 0.5 liter H_2O

Ammonium persulfate (APS) (10%)

0.l gram of APS in l ml of dH_2O

C.4 General solutions and buffers

TAE Buffer (50X)

242 grams of Tris Base, 57.1 ml of acetic acid (Glacial), 100 ml of EDTA. $2H_2O$ (0.5 M, pH:8), in 1 liter dH_2O

TE Buffer

10 mM tris, 1 mM EDTA, pH adjusted to 8.0

Protoplasting buffer

15 mM Tris-HCl, 0.45 M Sucrose, 8 mM EDTA, pH adjusted to 8.0

Gram negative lysing buffer

10 mM Tris-HCl, 10 mM NaCl, 10 mM sodium citrate, 1.5 % (w/v) SDS, pH adjusted to 8.0

Saturated NaCl

40 grams of NaCl was dissolved in 100 mL DEPC treated water.

CTAB/NaCl solution

4.1 grams of NaCl was dissolved in 80 ml H_2O and 10 grams of CTAB was slowly added while heating and stirring. If necassary, it was heated to 65 ºC to dissolve. Final volume was adjusted to 100 ml.

5X Bradford reagent

500 mg of Commasie Brillant Blue G-250, 250 ml of 95% EtOH and 500 ml of 85% (w/v) phosphoric acid were dissolved in 1 liter of water. The solution should be filtered through coarse filter and kept at 2-8 ºC.

APPENDIX D

FORMULAS AND CALCULATIONS

The calculations were performed under operating conditions

Avarage hydrogen production rate

Avarage hydrogen production rate = $(\Delta V)(liter)/(\Delta t)(hour)$

Where;

ΔV: Final volume of accumulated hydrogen gas

Δt: The time in which final volume of hydrogen gas collected (ΔV)

Yield

Hydrogen production yield: gram H_2 / gram substrate

Assumption: All the substrate was consumed

Substrate conversion efficiency (SCE)

$SCE = (100 \times P)/(6 \times V \times M_0)$

Where;

P: moles of total accumulated H_2

V: volume of culture in liter

M_0: the initial concentration of substrate

Assumption: All the substrate was consumed

SAMPLE GC ANALYSIS OUTPUT


```
RUN#     11              JAN  1, 1901   00:32:23

AREA%
        RT        AREA TYPE   WIDTH        AREA%
       .587     .628194   PB    .126     99.56796
      1.129        941    VB    .094       .05754
      2.733       6125 N PV     .244       .37456

TOTAL AREA=1635260
MUL FACTOR=1.0000E+00
```
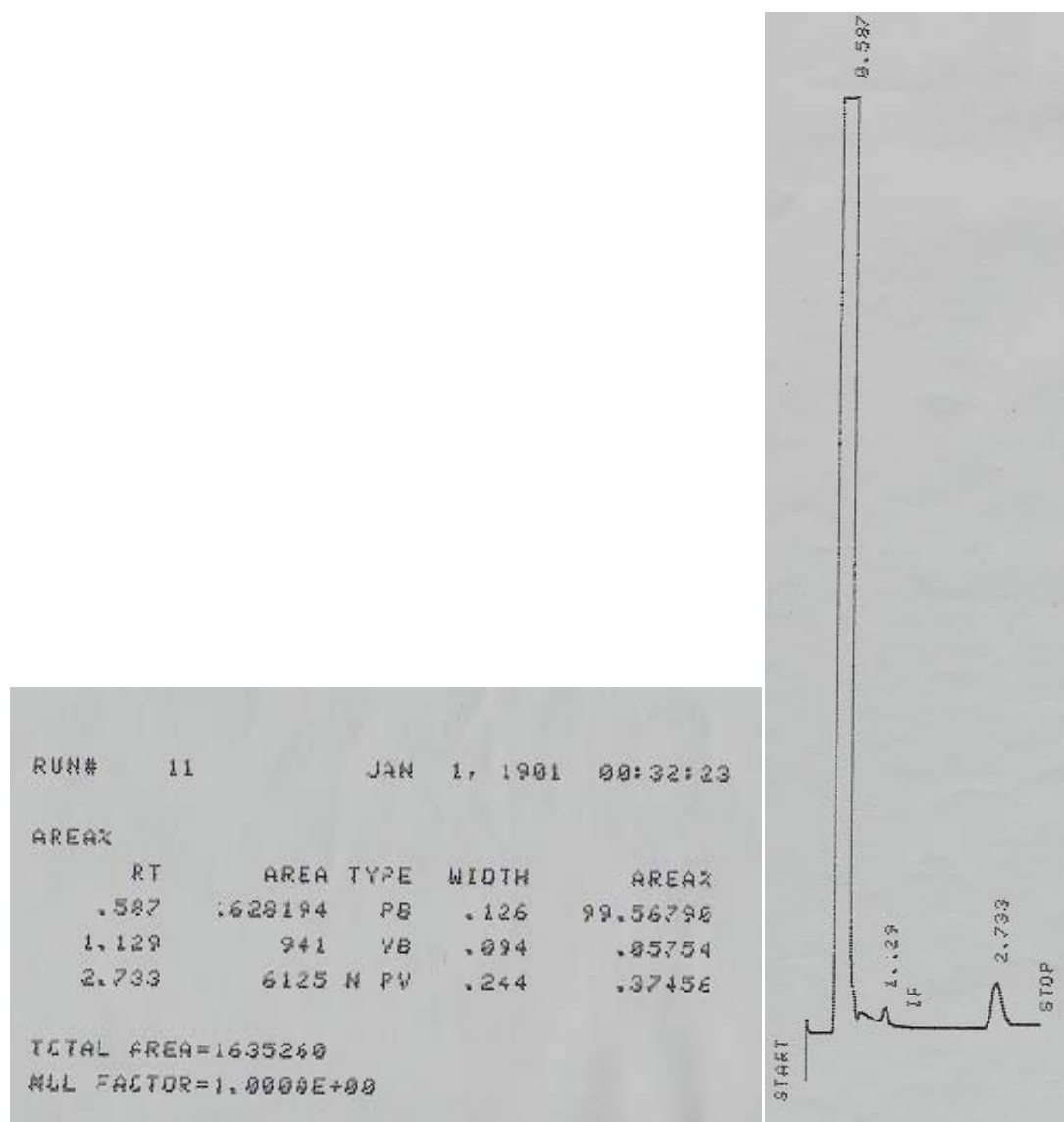
Figure E.1 A sample GC output.

MAP OF PLASMIDS
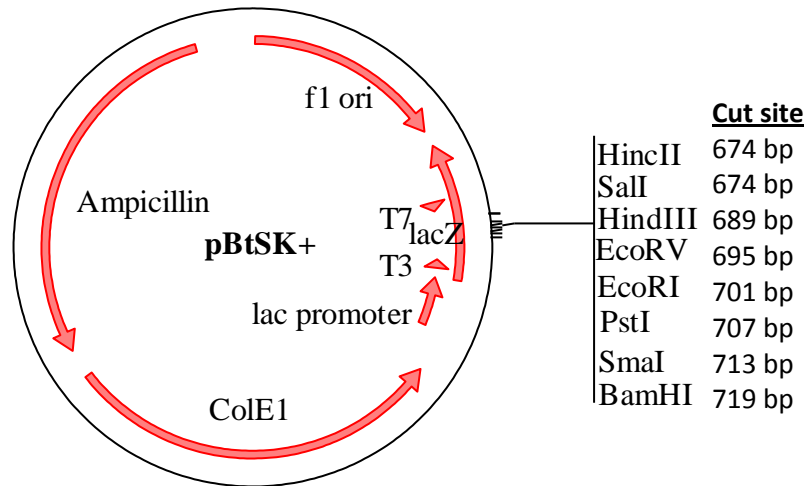
Figure F.1 The pBtSK+ plasmid and its common unique restriction sites.

pTCB4-2 (11927 bps)
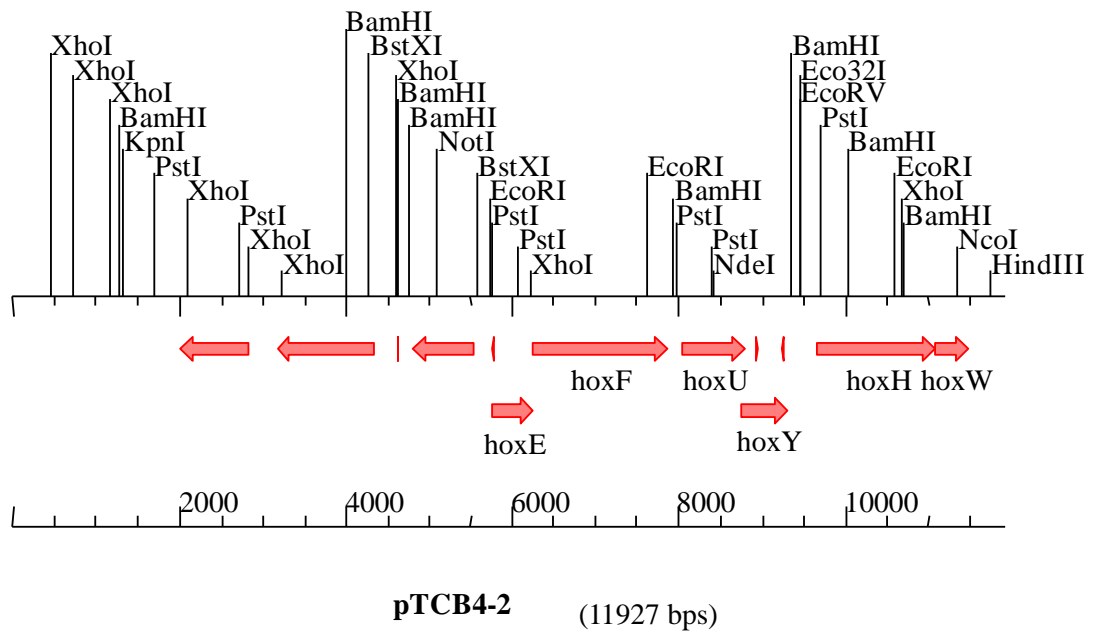
Figure F.2 The open form of pTCB4/2 plasmid and its restriction sites.
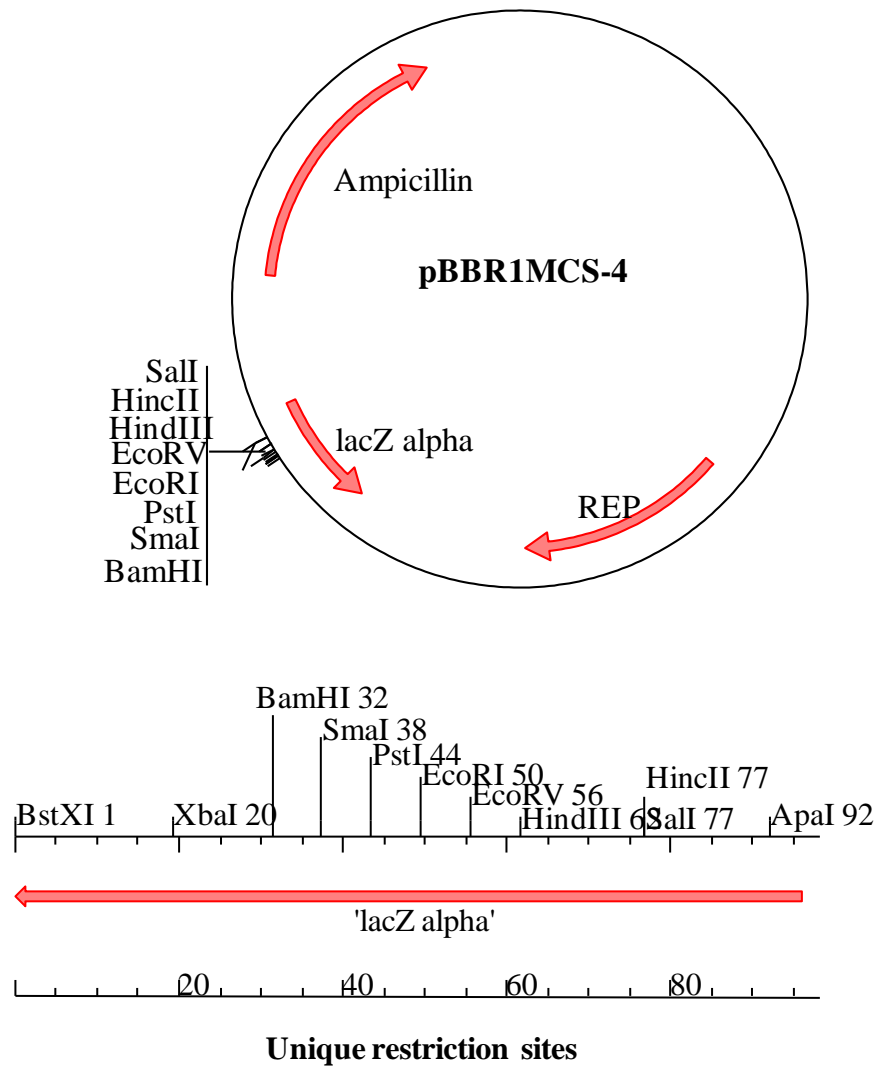
Figure F.3 The pBBR1MCS4 plasmid and its unique restriction sites.
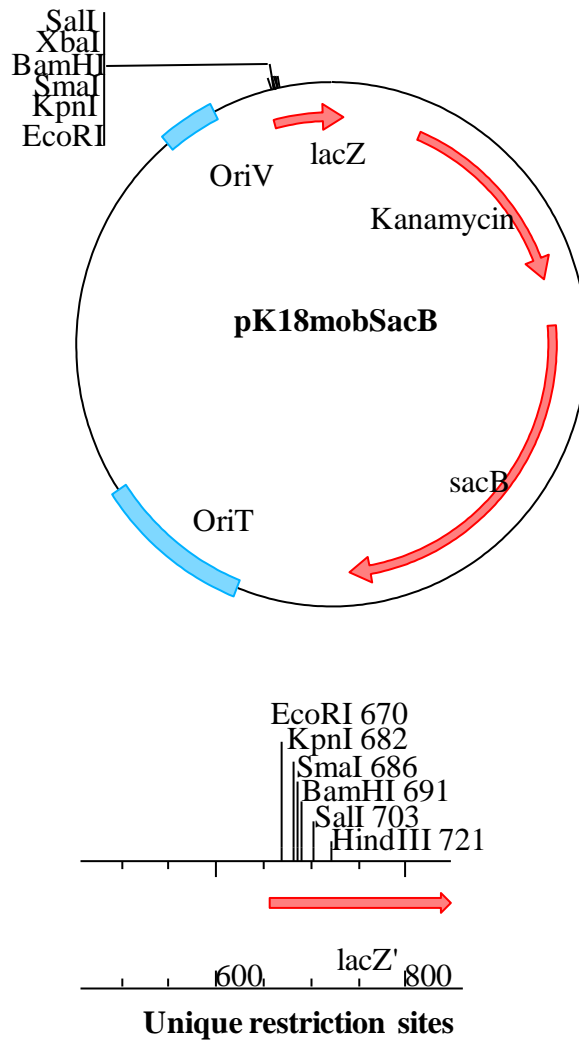
Figure F.4 The pK18*mobSacB* plasmid and its unique restriction sites.
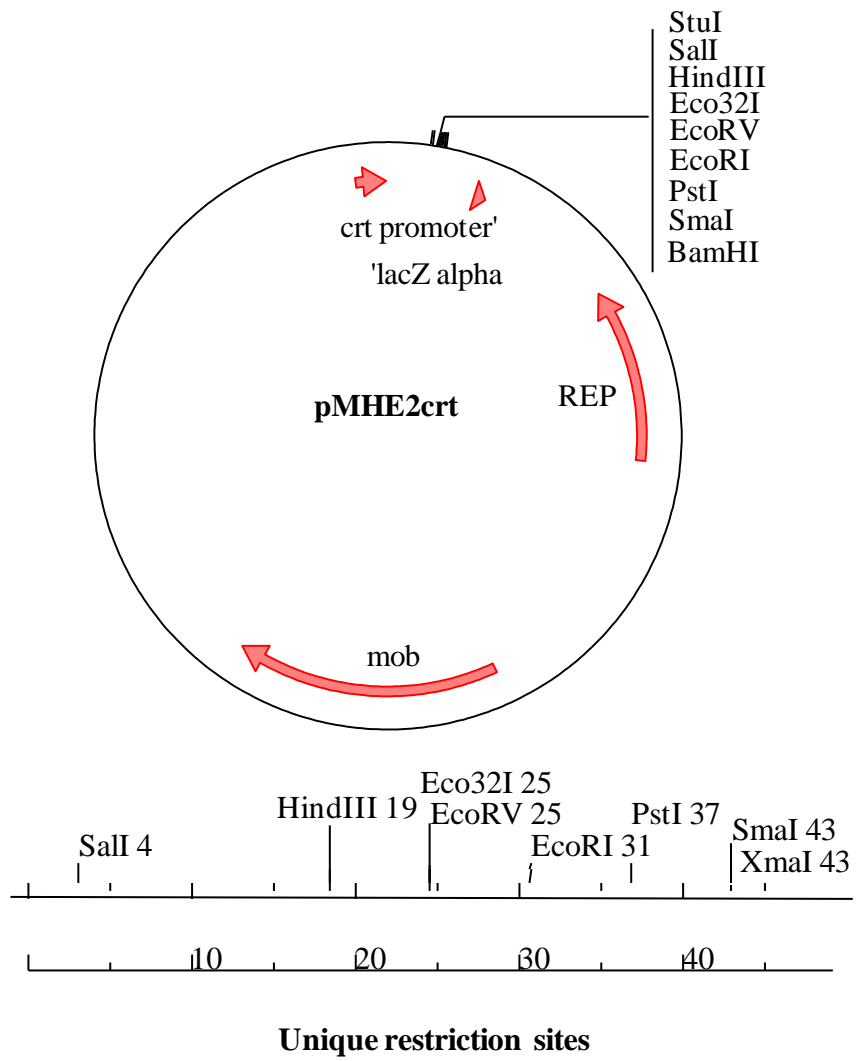
Figure F.5 The pMHE2crt plasmid and its unique restriction sites.
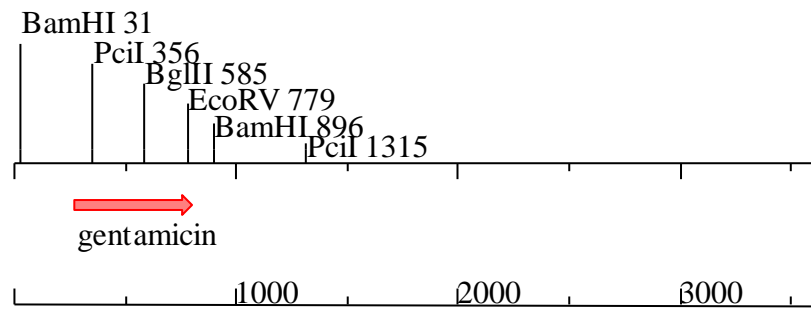
Figure F.6 The open form of p34-SGm plasmid and its unique restriction sites.

APPENDIX G

RESTRICTION ENDONUCLEASES AND DNA/RNA MODIFYING ENZYMES

G.1 Restriction endonucleases

*Bam*HI (MBI Fermentas, cat. # ER0051)

*Stu*I, (Eco147I) (MBI Fermentas, cat. # ER0421)

*Eco*RI (MBI Fermentas cat. # ER0271)

*Eco*RV (Eco32I) (MBI Fermentas, cat. # ER0301)

*Nco*I (MBI Fermentas, cat. # ER0571)

*Hinc*II (HindII) (MBI Fermentas, cat. # ER0491)

*Sac*I (MBI Fermentas, cat. # ER1131)

*Sma*I (MBI Fermentas, cat. # ER0661)

*Hind*III (MBI Fermentas cat. # ER0501)

*Kpn*I (MBI Fermentas cat. # ER0021)

*Xba*I (MBI Fermentas cat. # ER0681)

G.2 Polymerases

Pfu DNA polymerase (MBI Fermentas, cat. # EP0501)

Taq DNA polymerase (MBI Fermentas, cat. # EP0401)

Phusion DNa polymerase (NEB, cat. # F-530G)

DyNAzyme Ext DNA polymerase (NEB, cat. # F-505L)

Pwo DNA polymerase (Roche Applied Science, cat. # 11644947001)

Klenow Fragment (MBI Fermentas, cat. #EP0051)

T4 DNA polymerase (MBI Fermentas, cat. #EP0061)

G.3 Reverse Transcriptase

M-MuLV Reverse transcriptase (MBI Fermentas, cat. #EP0351)

G.4 Ligases

T4 DNA Ligase (MBI Fermentas, cat. #EL0014)

G.5 Phosphatases

Calf Intestine Alkaline Phosphatase (CIAP) (MBI Fermentas, cat. #EF0341)

G.6 Kinases

T4 Poly Nucleotide Kinase (MBI Fermentas, cat. #EK0031)

G.7 DNAse and RNase

DNaseI (MBI Fermentas, cat. #EN0521)
RNAse H (MBI Fermentas, cat. #EN0201)

APPENDIX H

PRIMERS AND SEQUENCES

The sequence analyses of PCR products;

H.1 The partial fragments of *nifHDK* genes used in transcriptional analyses

nif H

CGAGCNGNGCGAGATGATGGCGCTCTACGCCGCCAACAACATCGCCAAG
GAGCATCCTGCAAATACGCCAACTCGNGGCGGCGTCCGTCTCGGCGGCCT
GATCTTGCAACGAGCGCAAGACCGACCGCGAACTGGAACTGGCCGAGGC
GCTGGCCGCCAAGCTCGGCTGCAAGATGATCCACTTCGTCCCGCGCGACA
ATATCGTGCAGCACGCCGAACTCCGCCGCGAGACGGTCTCCCAGTATAN
NNNNN

nif D

CNAGAGGTNTGCCGCCTATCCGACAGGACAAGAAGAAACGCGCCAAGCA
CCTNNGCGTCNCCGAGACGGTTGCCGATGCCGAGCCCGGCATCCAGTCG
AAATGCGACACCGTGAAGTCGAACATCAAGTCGGTTCCCGGCGTGATGA
CCATCCGCGGCTGCGCCTACGCCGGCTCGAAAGGCGTGGTCTGGGGTCCG
GTCAAGGACATGCTGCACATCAGANNN

nif K

AACACGCTAGCTGGAGAAGAAGCGCGCCACCTACGANGAACGCGACCCC

GGNCCGAGAAGGTCGAGGAAGTCGCCGACTGGACGAAGTCCTGGGACTA
CCGCGAGAAGAACCTCGCCCGCTCCTGCGTCACGATCAACCCGGCCAAG
GCCTGCCAGCCGCTCGGCGCCGTCTTCGCCGCCGCGGGCTATGACAGCAC
CATGAGCTTCGTGCACGGCTCGCAGGGATGCGTGGCCTACTATCGTCGCC
AC

H.2 The *hupSL* gene of *R. sphaeroides* O.U.001

TGAATGGCGAGCAGTTTCTTCATGGTTTTCTCCTTCAGCGGACCTTGACGT
GGTGAGTTCCTGGCCGTCCGGCGACAGGACATGGGTGGAGCAGGCGAGG
CAGGGGTCGAACGAGTGCAGGGTGCGCAGGATCTCGACCGGCTCCTCGG
GACGCTCCATCTTGGTGTCGAGCAGGCTCGCCTCGAAGGCGCCGATGTTG
CCGGCCGCGTCGCGCGGGCTGCCGTTCCAGGTGGTGGGCACCACGCACT
GATAGTTCTCGATCCGGCCGTCCTTGATGCGGATCCAGTGGCCGAGCGCG
CCGCGCGGGGCCTCGGTCATGCCGACGCCCTTGGCCTCCTTCGGCCAGGT
GCGCGGCTCCCACTTCTCGACATTGGCGGTGCTGCTGTCGCCGTTCTTCAC
GTTGGTGATGAGCTTGTCGAAGAAATGCTTCTGCAGGCGGCAGCAATATT
CCGCCTCGAGCGCGCGGGCGGCCGTGCGGCCCAGCGTCGAGAACAGTGC
CGAGACCGGCAGGTCCATGGTGCGCAAGAGACCCTCGACCTGGTTCTTG
ATGTCCTCGTGGCCCTTGGCGTAGCCCACGATGTAGCGGGCGAGCGGGCC
CCACCTCCATCGCATGCCCCTTCCAGCGCGGCGCCTTGATCCAGGAATAT
TTCGCCGCCTCGTCGAGCTCGAGGATGTTCGTCCGCGTGCCCTTGGCATT
GGGGCCGAGTTTGTAGCGCGGCTTGGTCACGCCGTTCCCAGGGGTGCAGC
CCGCGCCCCGGCTCGCCATAGGCATACCAGGAGTGATCGACGAATTCCTG
CACCTGCTCGGGGTCGCGCGGATCGACGTCATGCACTTCCTCGAGATTGC
CGTTGATGATCGCCCCCCGCGGCAGGTGGAGCTGTTCGGGCGAGAAAT
CGTTCGGATGCTCGGGGATGTCGCCATAGGCCATCACCGACTTCGACGAG
AGCCCGCCGCCATAGAGCCAGTTGCGGTAGAAGCCGCCGATGGCCACCA
CGTCGGGCAGATAGACGTTGTTGGTGAATTGGATGCACTGGTCGATGATC
GAGGAGACGAGGTTCAGACGCTCCATGTTGATCGCGCCGACCGCGCCCA
CGCCGTCGATGTTGATCGGGCAGGGCACGCCCCCCACCAGCCAGTTCGG

ATGCGGGTTCTTGCCGCCGAAGATCGTGTGGACCTTCACGATCTCCTTCT
GCAGGTCGAGCGCCTCGAGATAGTGGGTCGTGGCCATCAGGTCCGCTTCG
GGCGGCAGCAGATAGGCCGGATTGTCCCAGTAGCCGTTCTTGAACAGGC
CGAGCTGCCCCGATTCCACGAACTTCTTCAGCCGGTTCTGCACGTCGCGG
AAATAGCCCGGCGACGAGAGCGGGTGCGAAGGCGAGACCTTCTGCTGCA
GCTCGGACGTGGCCTTCGGATCGGCGCGCAGCGCATTGACCGGGTTCACC
CAGTCCAGCGCATGCAGATGGTAGAAATGGACGATGTGGTCGTGGATCT
GCAGGTTCAGCTGCATCATGTTGCGGATCGAGTTCGCATTGTCGGGGATC
GAGATCCCCAGCGCATCCTCGACGGCGCGGACGGAGGTCAGCGCATGGG
TGCCGGTGCAGACCCCGCAGATCCGCTCGGTGAAGGCCCAGGCGTCGCG
CGGATCGCGGCCCTTGAGGATGACCTCGAGGCCGCGCCACATCGTCCCCG
TCGAGACGGCATTGCGGATGATGCCCTGATCGTCAACGTTCACTTCGCAG
CGCATGTGACCCTCGATCCGGGTCACCGGGTCCACCACGATGCGGCGGCC
GGTCTTGTCCAGGTTGAAACCGTTCGGTGTCGCGACCATGGCTCAGGCCT
CCGTCTTTTCGTCGGTCTTCGCGGCATTCGCCGCCTGCGATTTCTTCTGCG
CGCTCTTGAGGGCGGTGGCCGCGATATGGGCCGCCACGCCCGCGCCGAG
CGCGCCCACGGCCGTGAGGCCGATCGTGTCGGCATTGGCCTCGACGCCG
AACTGCTTGATGGTGGTCAGCCGGTCGTAGAAGGACCCCTGATCCCAGA
AGCCGTCTTCCGAGCAGCCGATGCAGCCGTGGCCGGACTGGATCGGGAA
GCTCACGCCCTCGTTCCAGCGCACGGTCGAGCAGGCGTTGTAGGTGGTCG
GGCCCTTGCAGCCCATCTTGTAGAGGCAGTAGCCCTTCTTGGCGTAGTCG
TCGTCCCAGGCCTCGACGAACTGGCCCGCGTCGAAATGCGGGCGGCGGT
AGCATTTGTCGTGGATGCGCTGGCTGTAGAACATCGCCGGGCGGNCCTGA
NGGTCNANCTCGGGCANCCGGTCGAAGGGTAGCATGTAGGTGATNACGC
CGGTCATGACCTCGGCGATGGGCGGGCAGCCCGGCACCTTGACGATCGG
CTTGTCGAGGATGACCTTGTGCACGGGCGTGGCCCGCGTCGGGTTGGGCG
CGGCCGCCTGCACGCAGCCGTAGGAGGCGCAGGCCCCCCAGCTGATGAT
GGCCTTGGCGTGCTCGGCCGCCATCTTCAGCTGCTCGACGAAGGGCTTGC
CCCCGATGATGCAGTACATCCCGTCCTCGTTCAGCGGCGGGTTGCCCTCG
ACGGCAAGGATATAGTTGCCCTTGTATTTCTCGATCGTGTCCATCAGCGC
GGCTTCGGCCTGATGGCCCGCCGCCGCCATCAGCGTGTCGTCATAGTCGA

GCGAGATCATCGACAGGACGACGTCCTTGGCCAGCGGGTGCGCCGCGCG
GATGAAGCTCTCCGAGCAGCAGGTGCATTCGAGCCCATGGACCCAAATC
ACCGGTGTGCGCGGCTTCGTCTCCATCGCATGCGCGATCTTCGGCACGAA
NGAAGGGCCGAGCCCCAGCGCCGCCGCGGTGAGCGAGCAGTATTTCATG
AAGCTGCGCCGGGTGATCCCCTGGCGGCGCATCACATCGTAGAANGTTTC
GATCTGGGGGCAACGCATTCCTTCCCTCGCTGGCGGCTTTCCCNGGCCGC
TGGGCCTCTGCGGCTTGTGCCCTTGGGGCTCCGCCGGCTTNCGTATGCCA
AGNAATGCCGGTGAAAGCTCGGGGCCNCNCNAAAAAAAATGCGCGGG

H.3 The partial fragment of gentamicin resistance gene in the *hup* mutant *R. sphaeroides* GK1 genomic DNA

AAAGTAGCATTGGGCTCTTCGCCATGTAGGCTCGGCCCTGACCAGTCAAT
CCATGCGGGCTGCTCTTGATCTTTTCGGTCGTGAGTTCGGAGACGTAGCC
ACCTACTCCCAACATCAGCCGGACTCCGATTACCTCGGGAACTTGCTCCG
TAGTAAGACATTCATCGCGCTTGCTGCCTTCGACCAAGAAGCGGTTGTTG
GCGCTCTCGCGGCTTACGTTCTGCCCAAGTTTGAGCAGCCGCGTAGTGAG
ATCTATATCTATGATCTCGCAGTCTCCGGCGAGCACCGGAGGCAGGGC
ATTGCCACCGCGCTCATCAATCTCCTCAAGCATGAGGCCAACGCGCTTGG
TGCTTATGTGATCTACGTGCAAGCAGATTACGGTGACGATCCCGCAGTGG
CTCTCTATACAAAGTTGGGCATACGGGAAGAAAGAATGCACTAAA

H.4 The deleted *hup* gene region in the *hup* mutant *R. sphaeroides* ZK1 gDNA

NTTAAAGGCGCGCATCAGGGGCGCCGCGCCCGGCTCGCCATAGGCATAC
CAGGAGTGATCGACGAATTCCTGCACCTGCTCGGGGTCGCGCGGATCGA
CGTCATGCACTTCCTCGAGATTGCGGGCTGCAGGAATTCGATCTTCTTGG
CGTAGTCGTCGTCCCAGGCCTCGACGAACTGGCCCGCGTCGAAATGCGG
GCGGCGGTAGCATTTGTCGTGGATGCGCTGGCTGTAGAACATCGCCGGG
A

Table H.1. The complete list of primers used.

Primer name	Sequence
NIF H LEFT	5'-GCGCAGGAAATCTACATCGT-3'
NIF H RIGHT	5'-ATACTGGATGACCGTCTCGC-3'
NIF D LEFT	5'-CGAGACCAACATGAAGCTGA-3'
NIF D RIGHT	5'-CTGATGTGCAGCATGTCCTT-3'
NIF K LEFT	5'-GATCTGTTCAAGGAACCCGA-3'
NIF K RIGHT	5'-GAGGTGCGAGCGATAGTAGG-3'
16S RRNA LEFT	5'-CAGCTCGTGTCGTGAGATGT-3'
16S RRNA RİGHT	5'-TAGCACGTGTGTAGCCCAAC-3'
HUPSL 3	5'-TAACGGATTTCACCCCTTCC-3'
HUPSL 4	5'-GAATGGCGAGCAGTTTCTTC-3'
RSHUSQ 1	5'-ATGCGCTGGCTGTAGAACAT-3'
RSHUSQ 2	5'-GATCTCGATCCCCGACAAT-3'
RSHUSQ 3	5'-CAGGTTCAGCTGCATCATGT-3'
RSHUSQ 4	5'-GCAATCTCGAGGAAGTGCAT-3'
RSHU1F	5'-ATGAAAT ACTGCTCGCTCAC-3'
RSHU2R	5'-CATGCACTTCCTCGAGATTG-3'
REVERSE PRIMER	5'-GGAAACAGCTATGACCATG-3'
-20 PRIMER	5'-GTAAAACGACGGCCAGT-3'
RSHUP 01	5'-GCCTGTTCAAG AACGGCTAC-3'
TCHO 03	5'-GCTCAGCACGTACCAGATTG-3'
TCHO 04	5'-CATCCTGGAAGGCTCGATCA-3'
TCHO 08	5'-TGATGGTCTGGCCGATCTGG-3'
HUPSL 5	5'-CTTCTTGGCGTAGTCGTCGT-3'
GM LEFT	5'-GCAGTCGCCCTAAAACAAAG-3'
GM RIGHT	5'-AGTGCATCACTTCTTCCCGTA-3'
PK18 REVERESE	5'-TGTGGACGAATCGAAGTCAG-3'
PK18 FORWARD	5'-GACTCTGGGGTTCGAAATGA-3'

Table H.1. (continued).

Primer name	Sequence
PK18 FORWARD 2	5'-TACTTTGGCGTCACCCCTTA-3'
KM LEFT	5'-AAAAAGCTTGCAAGCGAACCGGAATTG-3'
KM RIGHT	5'-AAAAAGCTTAAGGGGTGACGCCAAAGTAT-3'
HOX1.2 FORWARD	5'-AGTCTGCAGCAAGCCAAG-3'
THIHOX 02	5'-ATGTCTATGTCCGCGCCGAG-3'
RSHUPSL 6	5'-TGGTGATGAGCTTGTCGAAG-3'
RSHUPSL 7	5'-TGATGGACACGATCGAGAAA-3'
TCHOX 1	5'-GACCTTTGCTCGGTTGG-3'
TCHOX 2	5'-GCCTTGGGCATCCTTGAG-3'
RSHUPSL 8	5'-ATTGGGGCCGAGTTCGTAG-3'
RSHUPSL 9	5'-CCCGGCGATGTTCTACAG-3'

APPENDIX I

EQUIPMENTS USED IN THIS STUDY

Steril cabinet, Nuve MN120

Thermal cycler, Techne, Apollo ATC401

37 ℃ shaker-incubator, Heidolph Unimax1010

30 ℃ Incubator, Nuve EN500

Hybridization oven, Hybaid Midi Dual14

Power supplies, BioRad

Heating and cooling dry bloc, Biosan CH-100

Autoclave, Hırayama

pH meter, InoLab wtw 720

Micropipettes, Eppendorf®, Nichiryo

Automatic Pipette Controller, Accu-jet® pro

Microcentrifuge, Thermo IEC

Analytical centrifuges, Hettich Universal 16R

+4 ℃ Refrigerators, Arçelik

-20 ℃ deepfreeze, Vestel

-85 ℃ deepfreeze, Sanyo MDF 192

Spectrophotometer, Shimadzu UV1208

UV Transilluminator, Hoefer

Electrophoresis Systems for Nucleic acids, BioRad

Electrophoresis Systems for Proteins, Biorad Trans Blot Cell

Gel image analyzer, Vilber Lourmat

Vortex, Nuve

Gas chromatography, Agilent 6890N

PERSONAL INFORMATION

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EDUCATION

Degree	Institution	Year of Graduation
PhD	METU Biotechnology	2008
MS	METU Biotechnology	2004
BS	METU Department of Biology Education (Major) and Department of Biology (Double Major)	2002
High School	Gazi High School	1996

WORK EXPERIENCE

Year	Place	Enrollment
2001 (Three week)	TÜBİTAK-Gen Mühendisligi Biyoteknoloji Arastırma Enstitüsü	Internship
2004 (Three weeks)	Biological Research Center, Szeged, Hungary	Research Scientist
2005-2006 (11 months)	International Training Course, Hungarian Academy of Sciences, Biological Research Center, Szeged, Hungary	Research Scientist

FOREIGN LANGUAGES

Advanced English

PUBLICATIONS

Papers published in international journals

1. Kars G, Gündüz U, Rakhely G, Yücel M, Eroğlu İ, Kovacs K, "Improved Hydrogen Production by Hydrogenase Deficient Mutant Strain of *Rhodobacter sphaeroides* O.U.001", International Journal of Hydrogen energy, 33 (12) (2008) 3056-3060.

2. Kars G., Gündüz U., Yücel M., Türker L., Eroğlu İ., "Hydrogen production and transcriptional analysis of *nifD*, *nifK* and *hupS* genes in *Rhodobacter sphaeroides* O.U.001 grown in media with different concentrations of molybdenum and iron", International Journal of Hydrogen energy, 31 (2006) 1536-1544.

Full papers published in international conferences

1. Kars G, Gündüz U, Devrekanlı A, Yücel M, Rakhely G, Kovacs K, Eroğlu İ, "Improved Hydrogen Production by Uptake Hydrogenase Mutant Strain of *Rhodobacter sphaeroides* O.U.001" International Hydrogen Energy Congress and Exhibition (IHEC 2007), 13-15 July 2007, İstanbul, Turkey.

2. Kars G, Gündüz U, Rakhely G, Yücel M, Eroğlu İ, Kovacs K, "Improved Hydrogen Production by Hydrogenase Deficient Mutant Strain of *Rhodobacter sphaeroides* O.U.001" "HYSYDAYS – 2nd World Congress of Young Scientists on Hydrogen Energy Systems, 6-8 June 2007, Turin, Italy, p178.

3. Kars G., Gündüz U., Yücel M., Türker L., Eroğlu İ., "The effect of different concentrations of Mo and Fe on the expression level of *nifHDK* and *hupS* genes in *Rhodobacter sphaeroides* O.U.001", International Hydrogen Energy Congress & Exhibition (IHEC-2005), Istanbul, TURKEY, (2005), article published in the conference CD.

Full papers published in national conferences

1. Kars G., Gündüz U., Yücel M., Türker L., Eroğlu İ., "*Rhodobacter sphaeroides* O.U.001 suşunda farklı molibdenum (Mo) derişimlerinin Mo-nitrojenaz genlerinin ekspresyon düzeylerine olan etkisinin incelenmesi" Biyoenerji-2004 Sempozyumu, 20-22 Ekim, Ege Üniversitesi-İzmir, 2004, Bildiriler kitabı, s. 199-204.

2. Kars G., Gündüz U., Yücel M., Türker L., Eroğlu İ., "*Rhodobacter sphaeroides* U.U.001 suşunda nitrojenaz genleri üzerine genetik çalışmalar", "Biyolojik Yolla Elde Edilen Hidrojen ve Endüstriyel Kullanımı Kursu", TÜBİTAK- Gen Mühendisliği ve Biyoteknoloji Araştırma Enstitüsü, Gebze-KOCAELİ, 16 Nisan 2004, Bildiri CD' si.

3. Kars G., Gündüz U., Rakhely G., Yücel M., Kovacs K., Eroğlu İ., "Genetiği Değiştirilmiş *R. sphaeroides* ile Biyohidrojen Üretimi", 8. Ulusal Kimya Mühendis-

liği Kongresi, 26-29 Ağustos 2008, Malatya.Bildiri CD'si.

Abstracts published in International conferences

1. Kars G., Gündüz U., Yücel M., Türker L., Eroğlu İ., "Genetic characterization of nitrogenase enzyme systems in *R.sphaeroides* O.U.001", COST- 841 Working Group2 Workshop on Active Centers of Hydrogenases, Max-Planck-Institute for Bioinorganic Chemistry, Mulheim -Germany, 27-30 September 2003, Abstracts p.39.

2. Gündüz U., Kars G., Doğrusöz N., Yücel M., Türker L., Eroğlu İ., "Hydrogenase and Nitrogenase Gene Expression in *Rhodobacter sphaeroides* O.U. 001", COST Action 841 Working Group 4 and Working Group 5 Workshops, Biological and Biochemical Diversity of Hydrogen Metabolism, Porto-Portoqual, 1-3 May 2005, Abstracts p. 12.

3. Kars G., Gündüz U., Yücel M., Türker L., Eroğlu İ., "Investigation of nitrogenase (*nifHDK*) gene expression in *Rhodobacter sphaeroides* O.U. 001 grown in different concentrations of iron and molybdenum" Jordan International Chemical Engineering Conference V (JICEC 05), Amman, 12-15 Sep. 2005, Abstracts p.256.

4. Kars G., Gündüz U., Rakhely G., Yücel M., Kovacs L. K. Eroğlu İ., "Genetic Engineering of *Rhodobacter sphaeroides* O.U.001 to improve biohydrogen production" XII International Congress of Bacteriology and Applied Microbiology (IUMS 2008) İstanbul, 5-9 August 2008, pp.89-90.

Abstracts published in national conferences

1. Kars G., Gündüz U., Yücel M., Türker L., Eroğlu İ., "*Rhodobacter sphaeroides* O.U.001 suşunda değişen molibden ve demir derişimlerinin biyohidrojen üretimine ve Mo-nitrojenaz gen ekspresyonuna olan etkisinin incelenmesi" 14. Biyoteknoloji Kongresi, 31 Ağustos-2 Eylül 2005, Eskişehir p.527.

International Courses

1. "1st Course in Technologies and Genomics of Microbial Hydrogen Production", organized by European School of Genetic Medicine, Bologna University Residental Centre-Bertinoro di Romagna, Italy, March 12-16, 2005.