Investigation of molybdenum iron protein expression and activity in *Wolinella succinogenes*

Dissertation
zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultäten
der Georg-August-Universität zu Göttingen

vorgelegt von

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Göttingen 2010

D7

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Tag der mündlichen Prüfung: 19.04.2010

Contents:

I. Zusammenfassung:

Die Fixierung molekularen Stickstoffs (N_2) ist ein essentieller Schritt im Stickstoffkreislauf und wird durch das Enzym Nitrogenase katalysiert. Obwohl N_2 78% der Atmosphäre ausmacht, ist die Bioverfügbarkeit des Elements begrenz, da es erst nach Reduktion zu Ammoniak für Biosynthesen nutzbar ist. Nitrogenase ist das einzige bekannte enzymatische System, welches die stabile Dreifachbindung von N_2 unter Normalbedingungen zu spalten vermag, um Ammoniak zu bilden. In der Industrie wird dies im Haber-Bosch-Prozess, bei dem hohe Temperatur und Druck benötigt werden, realisiert. Obwohl der Haber-Bosch Prozess zurzeit der einzige bekannte Prozess ist, um fixierten Stickstoff verfügbar zu machen, bringt er viele Probleme mit sich, vor allem im Bezug auf Umweltverschmutzung und Energieverbrauch. Deshalb wären effizientere Katalysatoren nach dem Prinzip der Nitrogenase sehr erstrebenswert. Dies wäre auch von großer Bedeutung für die moderne Industrie, da weniger Energie benötigt und die Umwelt geschont würde. Zudem besteht großes Interesse seitens der Wirtschaft, insbesondere der Agrarwirtschaft, wo Pflanzen Stickstoff nur als Nitrat oder in Form von Ammoniumionen aufnehmen, um die Verfügbarkeit von Stickstoff im Boden zu erhöhen und somit mehr Produktivität zu erhalten.

Das zur Fixierung molekularen Stickstoffs befähigte Enzymsystem Nitrogenase besteht aus zwei Proteinen, der Dinitrogenase Reduktase (oder Eisen-Protein) und der Dinitrogenase (oder Molybdän-Eisen-Protein). Mehrere Strukturen der Nitrogenase-Komponenten von verschiedenen Mikroorganismen wurden in den letzten zwei Jahrzehnten gelöst und kürzlich berichteten Einsle *et al*. von einem neuen Modell des aktiven Zentrums, mit einer verbesserten Auflösung von 1,16 Å, welches ein internes, sechsfach koordiniertes leichtes Atom im FeMo-Cofaktor enthält. Dennoch sind mechanistische Details wie die Distickstoff-Dreifachbindung an dem einzigartigen Metall-Zentrum der Nitrogenase gebrochen wird noch nicht verstanden. Die Untersuchung des Nitrogenasesystems in verschiedenen Arten von Bakterien kann daher einen Beitrag zur Aufdeckung dieser rätselhaften Eigenschaften leisten.

Die Synthese der Nitrogenase und die Stickstofffixierung erfordert einen hohen Energieaufwand. Deshalb werden die Gene, die für das Enzym kodieren, nur dann induziert, wenn keine anderen Stickstoffquellen mehr zur Verfügung stehen. Bisher sind nur wenige Organismen dafür bekannt, dass sie *nif* Gene besitzen. Überraschend wurden solche Gene in einem Bakterium aus dem Pansen von Kühen, *Wolinella succinogenes*, gefunden.

Hauptziel dieses Promotionsprojekts war die Nitrogenase von *W. succinogenes*, die in zwei parallelen Strategien bearbeitet wurde. Erstens wurde *Azotobacter vinelandii*, ein ausgiebig untersuchter Organismus, für Aktivitätsmessung und Reinigung des Nitrogenasesystems benutzt, um die Experimente zu kalibrieren. Als Methode zur Aktivitätsmessung wurde der klassische Nitrogenase-Acetylen-Reduktions-Assay, mit Hilfe von Gaschromatographie durchgeführt. Zusätzlich wurden andere analytische Methoden zur Charakterisierung des Enzyms angewendet, wie SDS-PAGE und Massenspektrometrie. Die zweite parallele Arbeitslinie war an *W. succinogenes*, das die *nif* Gene besitzt aber dessen

Stickstofffixierungsfähigkeit noch nicht genau bekannt und die Art der Nitrogenase völlig unbekannt ist. Die anfänglichen Experimente deckten auf, dass *W. succinogenes* in stickstofflimitiertem Medium Nitrogenase zwar exprimiert, aber nicht diazotroph wächst.

Der Nachweis einer Nitrogenaseaktivität war hier noch nicht voll aussagekräftig, da die Expressionsbedingungen sowie die effektive Stickstoffkonzentration im Medium noch nicht voll erfasst wurden. Daher wurden in Anschluss weitere Schritte zur Charakterisierung und Isolation der Nitrogenase durchgeführt. Das Enzym wurde aus Zellextrakten angereichert und per Massenspektrometrie identifiziert.

II. Summary:

Although biological nitrogen fixation has been discovered more than one and a half centuries ago many questions regarding the mechanism of this process are still awaiting answers. While there is an abundance of nitrogen in nature (78% of our atmosphere), it is almost entirely in a form (gaseous nitrogen, N_2) that is not usable by most organisms, making nitrogen the growth-limiting factor for these organisms. In nature, the ability to fix nitrogen is restricted to some prokaryotes that possess the enzyme nitrogenase. It was possible to simulate this important process and achieve industrial nitrogen fixation through the invention of Haber and Bosch. Although this is the sole industrial process available to provide fixed nitrogen, it does bring along many problems with respect to pollution and energy consumption. More efficient catalysts modeled on the working principles of the enzyme nitrogenase would therefore be highly desirable.

Nitrogenase, the machine that is responsible for converting dinitrogen to ammonium, comprises two oxygen-labile protein components, iron protein or dinitrogenase reductase and molybdenum iron protein or dinitrogenase. Over the last two decades, several structures of nitrogenase components from different microorganisms have been solved and lately, Einsle, *et al*. reported a new model of the active site, at an improved resolution of 1.16 Å, which includes an internal hexa-coordinate light atom within the FeMo-cofactor. However, the mechanistic details of how the dinitrogen triple bond is broken at the unique metal center of nitrogenase remain to be elucidated. Therefore, studying the nitrogenase system in different species of bacteria may contribute in uncovering its enigmatic features.

In the course of genome projects, many species of bacteria, which were not formerly known to be able to fix dinitrogen, have been found to possess the entire set of genes required for the production of a functional nitrogenase system. Such bacteria include the epsilon-proteobacterium *Wolinella succinogenes*, an enteric bacterium isolated from rumen fluid of cattle. Initial experiments on *W. succinogenes* showed that it is not able to grow diazotrophically but it does have nitrogenase activity at nitrogen-limiting conditions.

The main task of this work was to investigate the nitrogenase system in *Wolinella succinogenes*. Activity assays were employed in order to establish cultivation conditions that allow for the purification and characterization of nitrogenase components. As a well-studied diazotroph model, *Azotobacter vinelandii* was used as a standard and a control for comparison at each stage of the work.

Acetylene reduction the ethene, the typical assay for nitrogenase activity, was used in combination with gas chromatography. In addition, other analytic methods were be employed, such as SDS-PAGE and mass spectrometry, for unequivocal identification of the enzyme. Detection of nitrogenase activity in *W. succinogenes* is not sufficient to prove its expression because the conditions in which nitrogenase is expressed and the regulation of nitrogen status are completely unknown in this case. Therefore, further biochemical assays, in parallel with the results of *Azotobacter vinelandii*, were applied for identification of the enzyme in *W. succinogenes*.

1. Introduction:

1.1. Nitrogen in nature:

Nitrogen is a basic element for life because it is an essential component of all biopolymers, such as proteins and nucleic acids. It exists in the biosphere in several oxidation states, ranging from N(+V) (in nitrate) to N(-III) (in ammonia). Interconversions of these nitrogen states constitute the global biogeochemical nitrogen cycle (Einsle, 2004).

Elemental nitrogen is a colourless, odourless, tasteless and mostly inert diatomic gas at standard conditions, constituting 78% of Earth's atmosphere, which equals 98% of the entire available nitrogen on the earth (figure 1-1). Our atmosphere is the only relevant inorganic source of nitrogen, which is one of the major constituents of biological systems and is contained in many industrially important compounds such as ammonia, nitric acid, and cyanides.

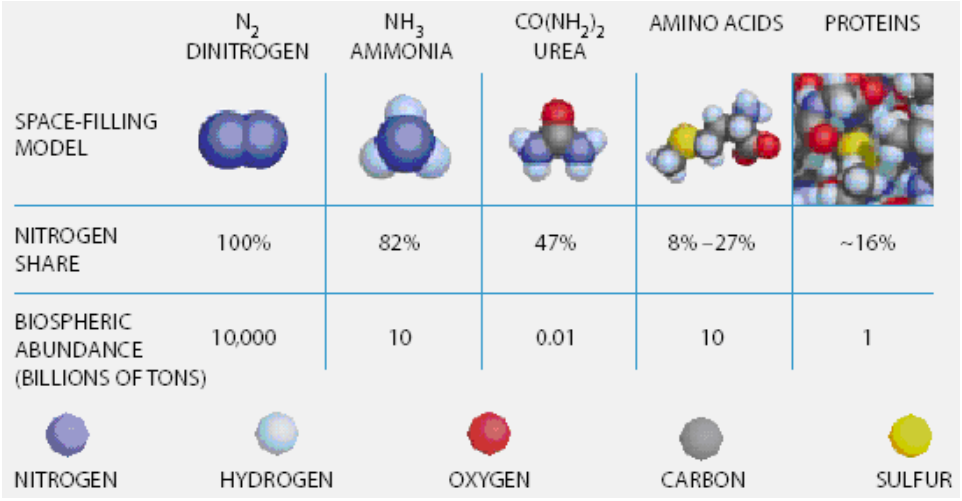

Figure (1-1): Representative of nitrogen and its compounds in nature (Smil, 1997)

Molecular nitrogen (N_2) in the atmosphere is relatively non-reactive due to its high bond energy. In nature, nitrogen is slowly converted biologically into useful compounds by certain bacteria and archaea known as diazotrophs. In contrast, molecular nitrogen is also released into the atmosphere in the process of decay, in dead plant and animal tissues, or through some other bacteria that have the ability to perform the denitrification process.

While there is an abundance of nitrogen in nature, it is almost entirely in a form (gaseous nitrogen, N_2) that is not usable by most organisms. Thus, in the absence of human intervention, there is not enough readily available and easily extractable nitrogen in the environment to sustain human population growth at its present rate. The ability to combine or fix molecular nitrogen is a key feature of modern industrial chemistry, where gaseous nitrogen and hydrogen are converted into ammonia via the Haber-Bosch process, which requires about 500°C and 450 bar of pressure in addition to iron catalyst. Ammonia, in turn, can be used directly as a fertilizer or as a precursor of many other important materials. Although this process is used to produce more than 100 million ton $year^{-1}$ of artificial

fertilizer, which contributes in sustaining about 40% of our planetary population (figure 1-2), it demands roughly 1% of the world's energy (Townsend, 2003). In addition, there are many health and environmental challenges that limit Haber-Bosch process possibilities of increasing nitrogen production in the future (figure 1-3). Therefore, the challenge is now how to increase or at least optimize the production of nitrogen and, at the same time, minimize the negative impacts of this operation on the environment, human health, and consumption of world's energy (Smith, 2002).

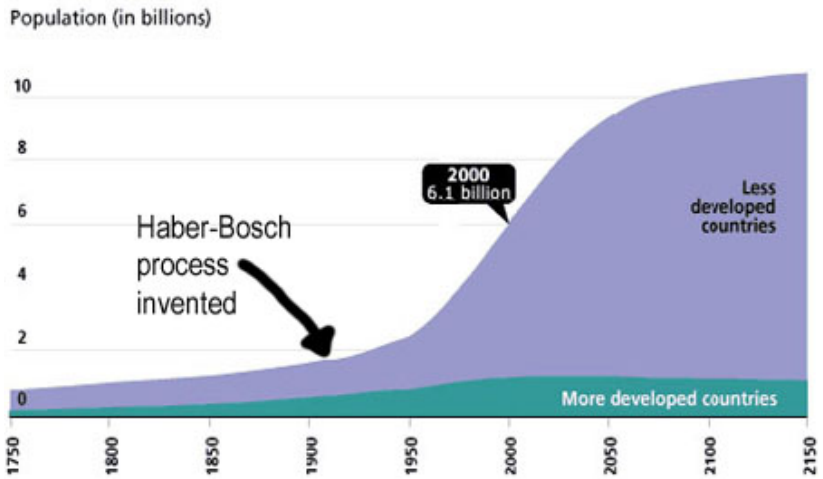

Figure (1-2): Historical estimates of world population (http://www.idsia.ch/~juergen/haberbosch.html)

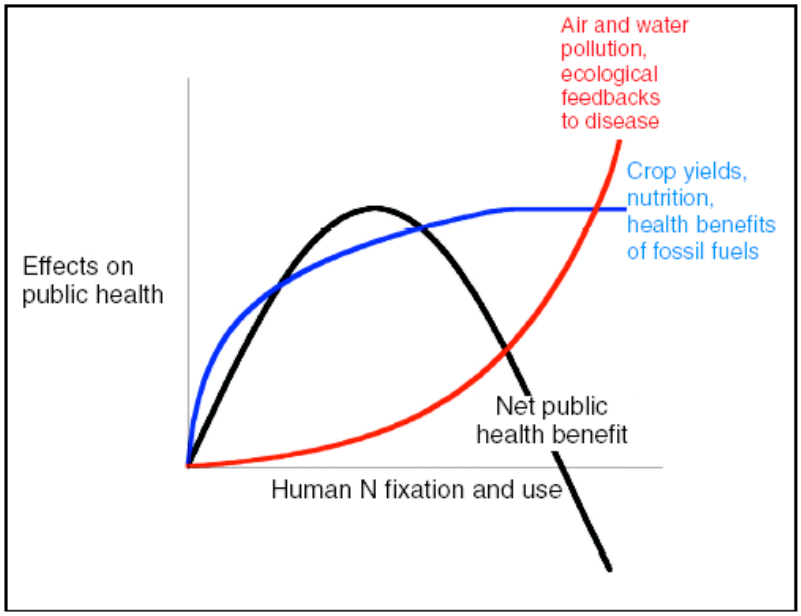

Figure (1-3): Conceptual model of the overall net public health effects of increasing human fixation and use of atmospheric N_2 (Townsend, 2003)

1.2. Nitrogen cycle:

The nitrogen cycle is the biogeochemical cycle that describes the transformations of nitrogen-containing compounds in nature (figure 1-4). Processes of the nitrogen cycle are:

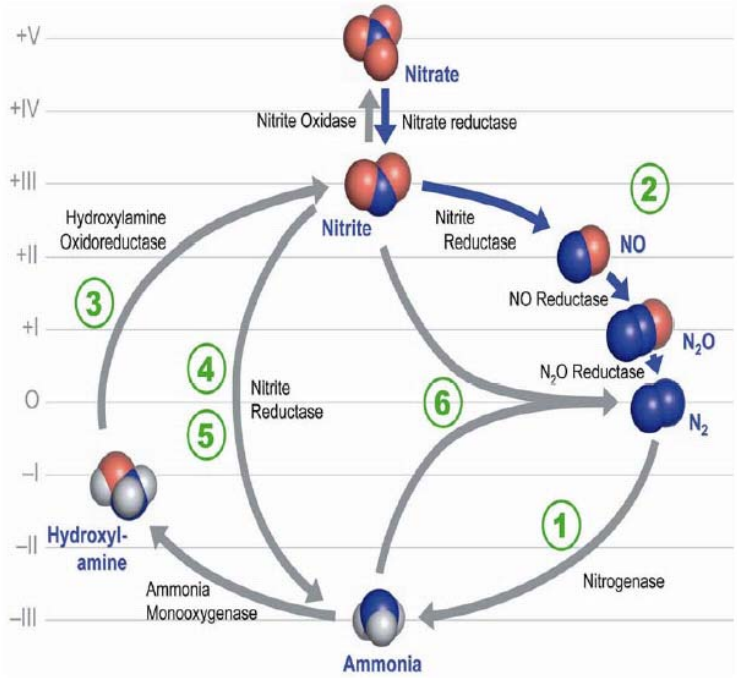

Figure (1-4): Nitrogen cycle; (1) nitrogen fixation, (2) denitrification, (3) nitrification, (4 & 5) Amonification or assimilation, (6) anamox (Einsle, 2004)

1.2.1. Nitrogen fixation:

There are four ways by which atmospheric nitrogen gas (N_2) is converted into more chemically reactive forms:

1. **Biological fixation:** Some symbiotic bacteria (most often associated with leguminous plants) such as *Rhizobium* and *Frankia* or some other free-living bacteria such as *Azotobacter* and *Klebsiella* are able to fix nitrogen and assimilate it as organic nitrogen owing to their unique enzyme system nitrogenase. The stoichiometry of the reaction is usually indicated as:

$$N_2 + 8H^+ + 8e^- + 16MgATP \longrightarrow 2NH_3 + H_2 + 16MgADP + 16P_i$$

2. **Industrial fixation:** In the Haber-Bosch process, dinitrogen (N_2) is converted together with hydrogen gas (H_2) to ammonia (NH_3) in the presence of an iron catalyst under high temperature and pressure conditions.

3. **Combustion of fossil fuels**: Such procedure takes place in automobile engines and thermal power plants, where NO_X compounds are released.

4. **Other processes:** An example is formation of NO from N_2 and O_2 due to UV-radiation and lightning.

1.2.2. Denitrification:

While nitrogen fixation helps in converting nitrogen from the atmosphere into organic compounds, a series of processes called denitrification return an approximately equal amount of nitrogen to the atmosphere contributing in keeping the balance of these

compound.Denitrifying bacteria tend to be anaerobes, or facultative anaerobes, including *Achromobacter*, *Micrococcus, Bacillus,* and *Pseudomonas*. The denitrification process, caused by oxygen-free conditions, converts nitrates via four enzymatic reactions stepwise to nitrite (NO_2^-), nitric oxide (NO), and nitrous oxide (N_2O) to finally yield dinitrogen gas (N_2). All steps within this metabolic pathway are catalyzed by complex multi-site metalloenzymes with unique spectroscopic and structural features (Einsle, 2004). However, fixed nitrogen may circulate many times between organisms and the soil before denitrification returns it to the atmosphere.

1.2.3. Nitrification:

The Russian microbiologist Sergei Winogradsky (1856-1953) discovered this process. Nitrification is a vital part of the nitrogen cycle wherein certain bacteria (nitrifying bacteria) are able to transform nitrogen in the form of ammonium into nitrates, which are available to growing plants, and once again converted there to proteins. Some bacteria such as *Nitrosomonas* and *Nitrosococcus* species perform the primary stage of nitrification, the oxidation of ammonia to nitrite (NO_2^-). Other bacterial species, such as *Nitrobacter*, are responsible for the oxidation of nitrite to nitrate (NO_3^-).

1.2.4. Amonification or assimilation:

Nitrate is reduced to nitrite by nitrate reductase and nitrite is then reduced to ammonia in a six-electron transfer by nitrite reductase (Einsle, 1999 and 2002). The final product, ammonia, is readily incorporated into amino acids.

1.2.5. Anaerobic ammonium oxidation (anammox):

In this biological process, nitrite and ammonium are converted directly into dinitrogen gas under anoxic conditions. It is recognized as the only significant process converting fixed nitrogen to gaseous N_2 (Dasqaard, 2003). It is the balance between nitrogen sources and a sink that controls the global inventory of fixed nitrogen (Thamdrup, 2002).

1.3. Nitrogen fixing organisms:

The successful beginning of the investigation of nitrogen fixation returns to the year 1830, when the French chemist Boussingault proved, through series of successful experiments, that clover and peas increased their nitrogen content upon growing on sand that was virtually free of fixed nitrogen but later he was not able to repeat his experiments successfully. Interpretation of this phenomena was lacking and the main tend was concentrated on plants itself as nitrogen fixer. A half-century later, Hellriegel and Wilfarth reported their truly definitive experiments at a meeting in Berlin 1886. They proved that the source of nitrogen fixation is a white nodule present on roots of some plants. Winogradsky (1894) isolated and characterized, for the first time, the free-living anaerobe *Clostridium pasteurianum* as a bacterium that could use dinitrogen (N_2) as its sole nitrogen source.

Later, a lot of bacteria were isolated and defined as diazotrophs such as *Rhezobium leguminosarum* (1889), a symbiotically living microorganism, and *Azotobacter chroococcum* (1901), a free-living microorganism.

So far, all known nitrogen-fixing organisms (diazotrophs) are prokaryotes and the ability to fix nitrogen is widely, though sporadically, distributed across both the bacterial and archaeal domains (Raymond, 2004). Members are unified only on the basis of their metabolic ability to fix dinitrogen, which relies solely upon the presence of the nitrogenase enzyme system. Although a widespread trait in prokaryotes, nitrogen fixation occurs in only few select genera. Outstanding among them are the symbiotic bacteria rhizobia, bradyrhizobia, cyanobacteria, and the actinomycete *Frankia* that form nodules on the roots of legumes. In addition, there are various free-living nitrogen-fixing prokaryotes in both soil and aquatic habitats. Clostridia and some methanogens fix nitrogen in anaerobic soils and sediments. A common soil bacterium, *Azotobacter*, is a vigorous nitrogen fixer that was isolated in the early 1900, as is *Rhodospirillum,* a purple sulfur bacterium. Even *Klebsiella*, an enteric bacterium closely related to *E. coli*, fixes nitrogen.

Recently, Raymond *et al.* (2004) performed a genomic analysis of *nif* genes encoding the NifH, NifD, NifE, and NifN proteins, and proposed five groups shown in figure (1-5):

I. Typical Mo-Fe nitrogenases predominantly composed of members of the proteobacterial and cyanobacterial phyla.
II. Anaerobic Mo-Fe nitrogenases from predominantly anaerobic organisms including acetogenic bacteria, clostridia and several methanogens.
III. Alternative nitrogenases, including the Mo-independent *anf* and *vnf* genes (except VnfH, which is more similar to NifH rather than AnfH).
IV. Uncharacterized nif homologues detected only in methanogens and some anoxygenic photosynthetic bacteria.
V. Bacteriochlorophyll and chlorophyll biosynthesis genes common to all phototrophs.

Group I consists primarily of Nif sequences from cyanobacteria and proteobacteria, which collectively represent the best-studied nitrogenases and are among the largest *nif* gene operons. The genes comprising theses extensive operons are involved mainly in nitrogenase regulation and assembly. Additionally, these two diverse bacterial groups are intimately associated with O_2 by way of aerobic respiration and oxygenic photosynthesis. Both phyla have intricate but well described spatial and/or temporal mechanisms for keeping nitrogenase and molecular oxygen separate, with responsible genes often encoded within the *nif* operon (Berman-Frank, 2001).

Group II nitrogenases have been well characterized and are very similar in structure and function to their group I homologs (Kim, 1993). The HDKEN operon structure is highly conserved, although group II organisms have smaller operons than their group I counterparts. The taxa from this group are distinct from cyanobacteria and proteobacteria in that they are predominantly obligate anaerobes, including methanogens, clostridia, and sulfate-reducing bacteria. The monophyly of this cluster is supported especially in NifD primary sequence alignments, where sequences from group II organisms all share an approximately 50-residue conserved insertion that is diagnostic for this group of proteins

(Wang, 1988). Protein comparisons between methanogens and clostridia, including and extending beyond nitrogenase, represent several of the most exemplary known cases of horizontal gene transfer (Doolittle, 2000).

The so-called alternative, or Mo-independent, nitrogenases denoted Anf and Vnf fall within a distinct group III clade. This clade is consistently preserved across all different protein alignments with the sole exception of the VnfH proteins, which are phylogenetically indistinguishable from NifH sequences. In fact, the closest relative to VnfH sequences are typically NifH sequences present in the same genome, as found in *A. vinelandii*, indicating relatively recent gene duplication events. The remaining group III sequences support an early paralogous origin for alternative nitrogenases. Hence, modern nitrogenases have likely been refined over hundred millions of years of evolution through a combination of increasing catalytic efficiency and adapting to changing metal availability. Although the Fe-dependent and V-dependent enzymes are less efficient, organisms such as *Rhodopseudomonas palustris* and *Azotobacter vinelandii* maintain these alternative nitrogenases. What remains enigmatic is why all alternative nitrogenases studied so far are found only in organisms that also have Mo-dependent enzymes.

As a whole, groups IV and V include a diverse range of NifH and NifD homologs that are not known to be involved with fixing nitrogen.

Group IV consists of a subset of nitrogenase homologs (Nif-like proteins, herein designated NflH or NflD, depending on homology) that have yet to be characterized. Intriguingly, group IV homologs are found only in methanogens, not all of which are diazotrophs, and in some nitrogen-fixing bacteria, most of which are photosynthetic (Raymond, 2004).

The pigment biosynthesis complexes protochlorophyllide reductase and chlorophyllide reductase, denoted herein as group V nitrogenase homologs, are not only homologous but are functionally analogous to nitrogenase, coupling ATP hydrolysis–driven electron transfer to substrate reduction (Fujita, 2000). As with nitrogenase, electrons flow from a NifH-like ATPase (BchL and BchX) to a NifDK-like putative heterotetramer where the tetrapyrrole is bound (BchNB and BchYZ). These two enzymes catalyze independent reductions on opposing sides of a tetrapyrrole ring that are essential late steps in chlorophyll and bacteriochlorophyll biosynthesis (Nomata, 2006).

Conserved residues in alignments of NifH homologs from all five groups show that 4Fe- 4S cluster-ligating cysteines and the P-loop/ MgATP binding motif are invariant, suggesting that these proteins may function analogously to dinitrogenase reductase. Conversely, NifD homologs are highly diverged from both the nitrogenase subunits and the pigment biosynthesis genes. FeMoco-ligating residues are not conserved among group IV and V proteins, although several—but not all—conserved cysteines involved with P cluster coordination are found in NifD and NifK homologs. This suggests that a less complex FeS cluster, such as a 4Fe- 4S, may be functioning in electron transfer in the Group IV and V proteins.

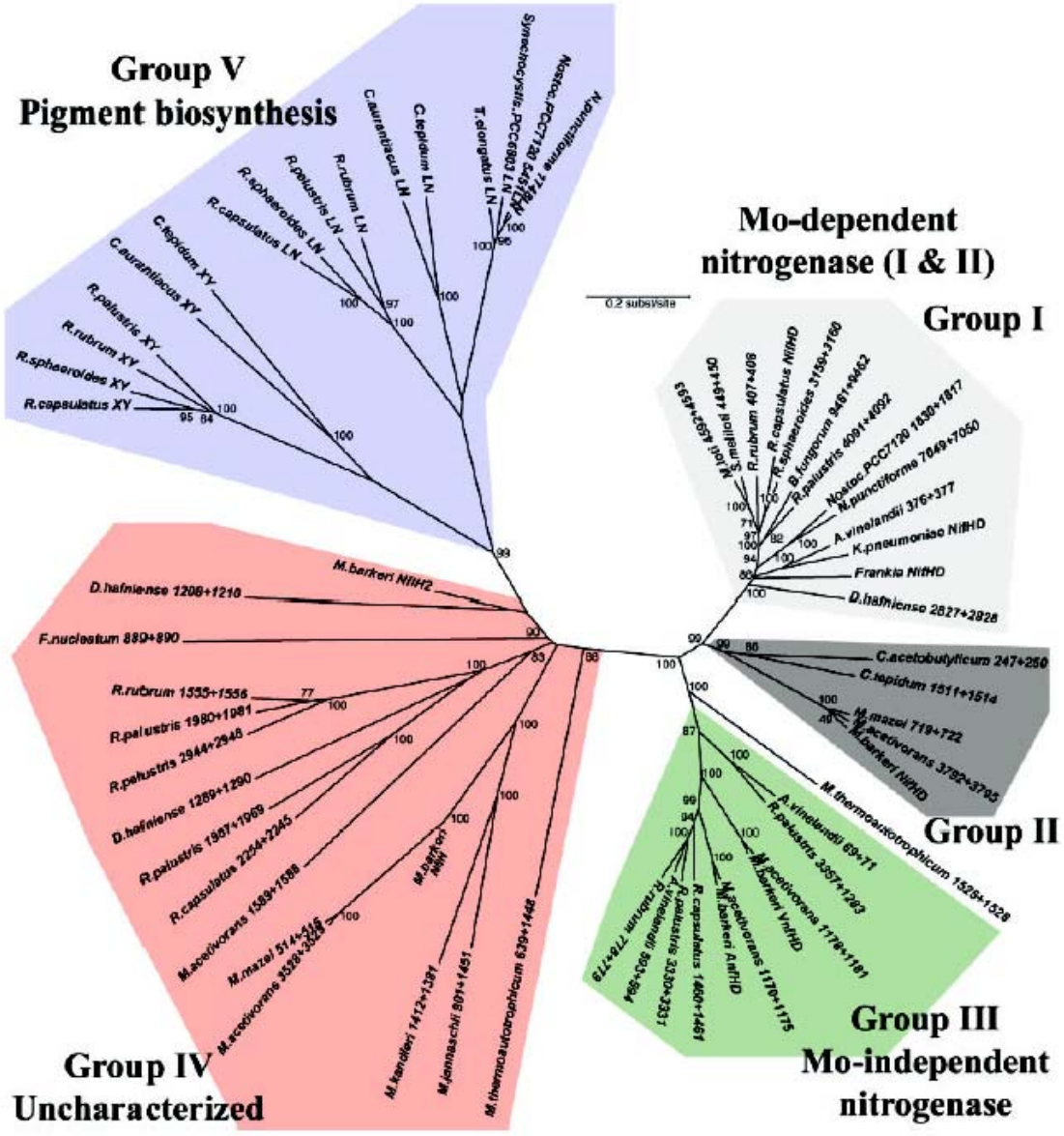

Figure (1-5): Overview of five phylogenetic groups shown on a concatenated phylogenetic tree composed of NifH and NifD homologs found in complete genomes; groups I to III are all functional nitrogenases (Raymond, 2003)

1.3.1. *Azotobacter vinelandii*, nitrogen-fixing model organism:

Azotobacter's cells are large rods, at least 2 microns in diameter. They can live singly, in chains, or in clumps, and may or may not be mobile by flagella. Their resting stage is spent as a thick-walled cyst, which protects the organism from harsh climates. *Azotobacter* is a genus of free-living diazotrophic bacteria belonging to the family *Pseudomonadaceae* of the class Gamma-proteobacteria. It is primarily found in neutral to alkaline soils, in aquatic environments, and on some plants. It has several metabolic capabilities, including atmospheric nitrogen fixation by conversion to ammonia. Azotobacter, interestingly, contains more DNA than most other bacteria, but their genome size is typical of most prokaryotes (e.g. genome of A. vinelandii consists of 5,365,318 bp) (Setubal, 2009). The reason for this above average amount of DNA is not known, but it is possibly because the cells of *Azotobacter* are larger than those of other bacteria. The DNA of *Azotobacter* spp.

displays many similarities, in terms of gene type and recognition factors, to the DNA of *Escherichia coli*.

Azotobacter vinelandii (figure 1-6) was first isolated in 1903 by Lipman from the soil in Vineland, New Jersey, USA, although it is found worldwide. It is a large obligate free-living aerobe that can utilize a large number of different carbon sources such as sugars, alcohols, and salts of organic acids and also synthesize carbon storage molecules such as alginates and poly-β-hydroxybutyric acid.

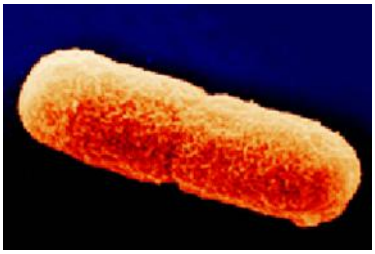

Figure (1-6): *Azotobacter vinelandii* cell

Over the years, *Azotobacter vinelandii* has served as a model for biochemical analyses due to the extraordinary yield and quality of enzymes that can be isolated from this organism. Most recently these studies have been focused on the ability of *A. vinelandii* to fix atmospheric nitrogen using three distinct nitrogenase systems under free-living conditions, a process that occurs in the presence of oxygen levels that typically inactivate the nitrogenase enzyme (Fallik, 1991). The cells uniquely high respiration rate, which is the highest metabolic rate of any organism, allows the normally oxygen-sensitive nitrogenase to experience limited oxygen exposure (Burke, 1930). Besides, *Azotobacter* is also capable of producing a protein which protects the nitrogenase from sudden oxygen-provoked stress. The alternative nitrogenases are encoded by distinct structural genes *vnfHDGK* and *anfHDGK*; the vnfG and anfG genes encoding an extra small subunit not found in molybdenum nitrogenase. However, many of the same ancillary genes e.g. *nifUSVWZ* and *nifM* are used in biosynthesis of all three enzymes. Recently, a new nitrogenase CrFe protein was obtaind from a mutant of *A. vinelandii* grown on Cr-containing medium (Zhigang, 2006).

1.3.2. *Geobacter sulfurreducens*, anaerobic diazotroph:

Geobacter was first isolated in 1987 from fresh water sediments from Potomac River, Washington (Lovley, 1988). The genus *Geobacter* belongs to the order Desulfuromonadales of the class Delta-proteobacteria. It is rod-shaped with pili (figure 1-8) and is known to have unusual electron transfer and environmental restorative capabilities, giving it several industrial applications such as bioremediation (Heitmann & Einsle, 2005, Hoffmann & Einsle, 2009). *Geobacter* spp. are anaerobic respiration bacterial species that were found to be the first ones with the ability to oxidize organic compounds and metals including iron, radioactive metals, and petroleum compounds into environmentally benign carbon dioxide while using iron oxide or other available metals as electron acceptor (Lukat & Einsle, 2008).

Geobacter sulfurreducens, a dissimilatory metal- and sulfur-reducing microorganism, was first isolated from surface sediments of a hydrocarbon-contaminated ditch in Norman, Okla, USA (Caccavo, 1994). It is an obligate anaerobe, nonfermentative, nonmotile, gram-negative rod that can grow in a defined medium with acetate as an electron donor and ferric PP1, ferric oxyhydroxide, ferric citrate, elemental sulfur, Co(III)-EDTA, fumarate, or malate as the sole electron acceptor. The *G. sulfurreducens* genome is a single circular chromosome of 3,814,139 base pairs (Methe, 2003).

Figure (1-7): *Geobacter sulfurreducens* cells

Preliminary studies have suggested that *G. sulfurreducens* might have genes for nitrogen fixation. The ability to fix nitrogen may be required for *Geobacter* to compete successfully in petroleum-contaminated subsurface environments which are carbon rich but contain little fixed nitrogen (Bazylinski, 2000). Further genetic studies showed that *G. sulfurreducens* fixes nitrogen in a manner similar to that of other nitrogen-fixing microorganisms (Coppi, 2001).

1.3.3. *Wolinella succinogenes*, a *nif* gene-containing bacterium:

The genus *Wolinella* belongs to the family Helicobacteraceae (epsilon subclass of the Proteobacteria). There is only one species of the genus, *W. succinogenes*.

Wolinella succinogenes dwells in the reticulum and rumen compartments of cattle's stomach. It was first isolated during the search for pathogens in bovine rumen fluid and considered, depending on morphology and present of cytochromes, a new species of the genus *Vibrio* (Wolin, 1961). Later, the name *Wolinella* was suggested to this bacterium among others and considered as a new genus including anaerobic, asaccharolytic, rod-shaped bacteria with single polar flagella (figure 1-8) and deoxyribonucleic acid guanine-plus-cytosine contents of 42 to 49 mol% (Tanner, 1981). According to 16S rRNA studies, *W. succinogenes* was classified as a member of the genus *Campylobacter* (Paster, 1988 and Vandamme, 1991) and more recently, 23S rRNA studies revealed that it is more related to the genus *Helicobacter*, which belongs to the Campylobacterales order of Epsilon-proteobacteria class (Dewhirst, 2005).

Although *W. succinogenes* is able to survive in microoxic conditions (less than 2% oxygen), it grows only by anaerobic respiration and does not ferment carbohydrates. Fumarate, nitrate, nitrite, nitrous oxide (N_2O), polysulfide or dimethyl sulfoxide (DMSO) can serve as terminal electron acceptors with formate as the electron donor ((Einsle, 2000, Simon & Einsle, 2000). Molecular hydrogen and, at least in fumarate respiration, sulfide are alternative electron donor substrates (Simon, 2006 and Simon & Einsle, 2004). The

annotation of the *W. succinogenes* genome resulted in the identification of 30 *nif* genes and related genes, which code for the structural subunits of nitrogenase, as well as for accessory proteins and regulators involved in activation and repression of the *nif* regulon (Baar, 2003).

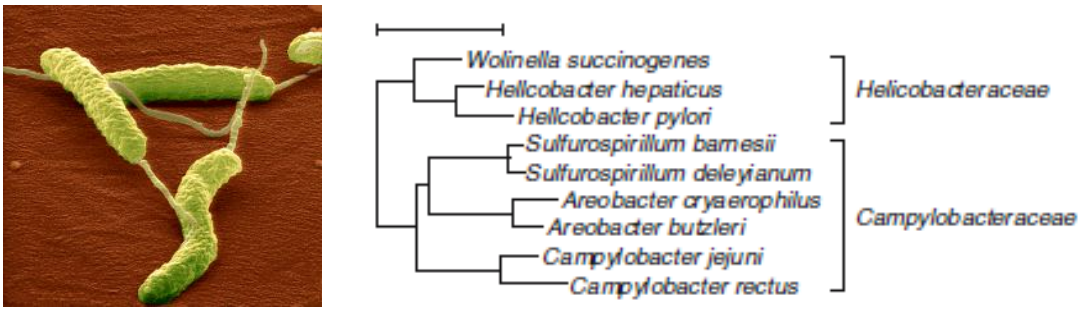

Figure (1-8): Left: *Wolinella succinogenes* cells. Right: The phylogenetic relationships of *W. succinogenes* compared to other members of the families Helicobacteraceae and Campylobacteraceae. The Marker bar represents a 5% difference in nucleotide sequences

The most commonly studied genera of the Epsilon-proteobacteria, *Helicobacter* and *Campylobacter*, are often associated with the gastrointestinal tract of mammals as pathogens. Other major phylogenetic groups within the Epsilon-proteobacteria include *Arcobacter*, *Wolinella*, *Sulfurospirillum*, and *Thiovulum*, commonly found in natural settings as living cells or in symbiotic association with animals (Schumacher, 1992 and On, 2001).

Although all members of the ε-proteobacteria generally have small genomes, a feature that has been observed as an adaptive trait for various other host adapted bacteria, the commensal *W. succinogenes* has a genome consisting of one 2.11 Mbp, circular chromosome that is larger than the genomes of its close, pathogenic relatives. This increased genome size corresponds to a greater number of predicted ORFs (2046) compared with the revised annotations of its relatives (around 1600). The genomes of *W. succinogenes* and some of its relatives have a high gene density with a coding area of 94% versus 91% for *H. pylori*. The overall GC content of the *W. succinogenes* genome (48.5%) is greater than that of its relatives *C. jejuni* (30.6%), *H. hepaticus* (35.9%) and *H. pylori* (39.0%). This indicates that all four genomes are derived from a larger ancestral genome (Eppinger, 2004).

1.4. The nitrogenase system:

Nitrogenase system is composed of two pairs of oxygen-labile metalloproteins, figure (1-9): Dinitrogenase (also called MoFe protein or component I) and dinitrogenase reductase (also called Fe protein, or component II) (Vandecasteele, 1970 and Bulen, 1966). These two components may occur independently of each other in different concentrations within the cell. The iron protein hydrolyzes MgATP and uses the required energy to provide electrons by its iron suphur cluster to the molybdenum iron protein, which contains the active site of nitrogen reduction, the iron molybdenum cofactor (FeMo-co) and additional unique iron sulphur cluster through which the electrons flow to the active site (Burgess, 1996). It has been possible to stabilize the complex of MoFe protein and Fe protein, crystallize it, and determine its structure by replacing MgATP by MgADP.AlF4 and thus

suppressing the hydrolysis of ATP, which is followed by dissociation of the two components (Schmid, 2002a).

Nitrogenase gene expression is highly regulated at levels ranging from transcription to post-transcriptional protein modification (Chen, 1998, Kim, 1999, and Dodsworth, 2007).

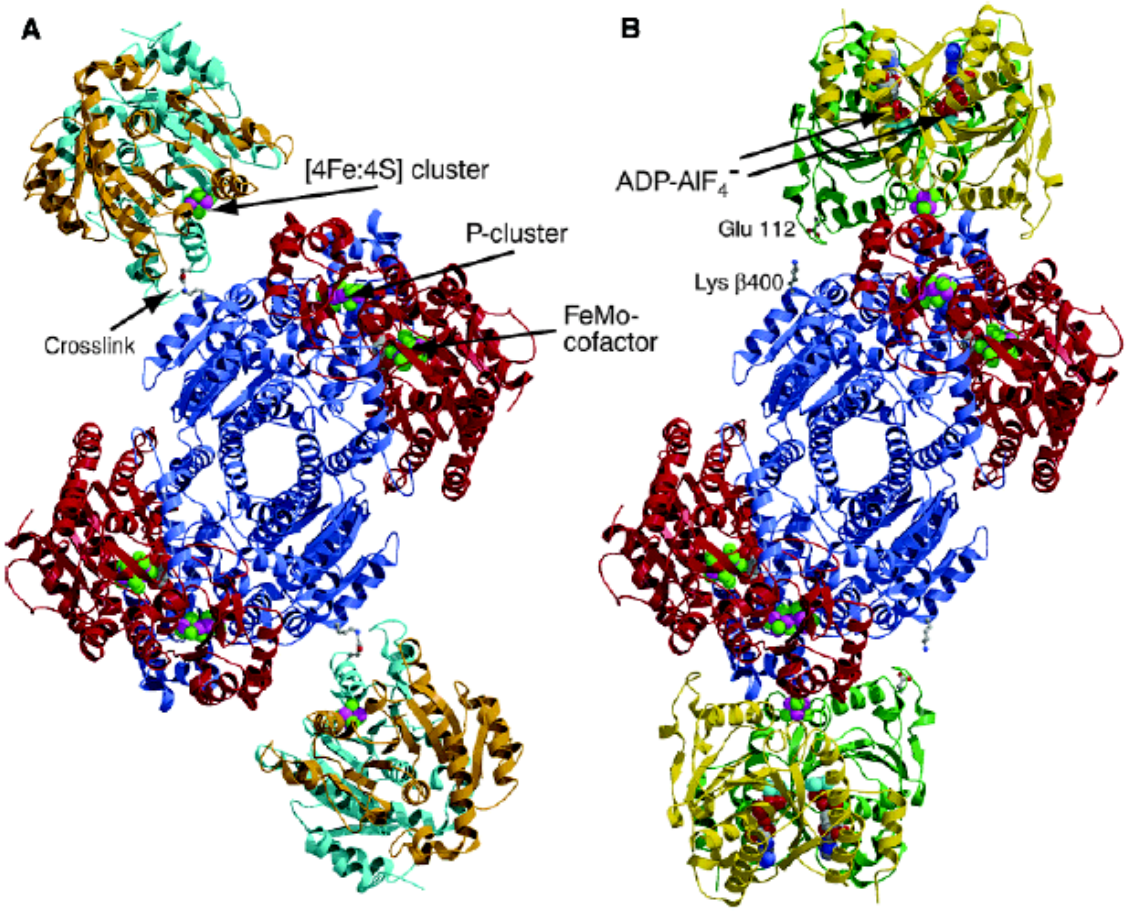

Figure (1-9): Ribbon diagrams of the α_2, β_2, γ_4 complexes between the nitrogenase component proteins Av1 and Av2, stabilized by (A) the 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC)-mediated cross-link between Glu 112 and Lys β400 and (B) ADP-AlF4$^-$. The MoFe protein α-subunits are in red; the β-subunits are in blue, and the individual γ-subunits of each Fe protein are in orange and cyan for the cross-linked complex and in yellow and green in the ADP-AlF4$^-$-stabilized complex. The residues participating in the cross-link in panel A are depicted in ball-and-stick mode. The cofactors and nucleotides are represented with space-filling models, color-coded by atoms with Fe in purple, S in green ([4Fe-4S] cluster, P-cluster, and FeMo-cofactor; the Mo atom is not visible), O in red, and C in gray (homocitrate, ADP) as well as N in blue and F in cyan (ADP-AlF4$^-$; P, Al, and Mg atoms not visible) (Schmid & Einsle, 2002a)

1.4.1. The MoFe protein:

Dinitrogenase is an $\alpha_2\beta_2$ heterotetramer of the *nifD* and *nifK* gene products, α subunit (55 kDa) is encoded by *nifD* and β subunit (59 kDa) by *nifK*, with a total mass of approximately 240-250 kDa, each αβ unit of the MoFe protein comprises a single catalytic unit that contains one P-cluster and one FeMo-cofactor, which are separated by approximately 17 Å (Kim, 1992b). Each of the homologous α- and β-subunits comprises three domains, designated αI, αII, and αIII and βI, βII, and βIII, respectively, all of which exhibit a parallel β-sheet/ α-helical type of polypeptide fold. The P-cluster is located

between domains αI and βI, whereas FeMo-cofactor occupies a cavity formed among domains αI- αIII (Schmid & Einsle, 2002b).

MoFe protein contains two identical pairs of unique metal clusters, known as the iron-molybdenum cofactor (FeMo-co) and the P-cluster. The P-cluster is associated with the protein and it assembled on the protein during the folding process, whereas the FeMo-cofactor is synthesized separately and then inserted into the folded apo-protein (Shah, 1977 and Bolin, 1993). The tetramer interface is stabilized by two Ca^{2+} cationsthat bind to sites created by ligands from both β subunits (Burgess, 1996). The structure was first solved in 1992 for the *A. vinelandii* protein (Kim, 1992a). Later, more structures were solved from *Clostridium pasteurianum* and *Klebsiella pneumoniae* (Kim, 1993 and Mayer, 1999).

1.4.1.1. Assembly of the MoFe protein:

Assembly of nitrogenase MoFe protein is arguably one of the most complex processes in the field of bioinorganic chemistry, requiring, at least, the participation of *nifS*, *nifU*, *nifB*, *nifE*, *nifN*, *nifV*, *nifQ*, *nifZ*, *nifH*, *nifD*, and *nifK* gene products (Dos Santos, 2004). Multiple events occur during this process, including the biosynthesis of clusters, the incorporation of clusters into the polypeptide matrices, and the coordination of these events that eventually leads to the formation of a mature protein.

Recently, characterization of a number of assembly-related intermediates was established providing significant insight into this biosynthetic "black box". Hu, *et al.* proposed a refined mechanism of MoFe protein assembly on the basis of these intermediates (Figure 1-10), which consists of (1) "*ex situ*" assembly of FeMo-cofactor on NifEN, (2) incorporation of FeMo-cofactor into MoFe protein, (3) "*in situ*" assembly of the P-cluster on MoFe protein, and (4) stepwise assembly of MoFe protein (Hu, 2008). More recent investigations of the same group showed that NifEN undergoes a significant conformational rearrangement upon the heterometal insertion, which allows the subsequent NifEN–MoFe protein interactions and the transfer of the cofactor between the two proteins (Yoshizawa, 2009).

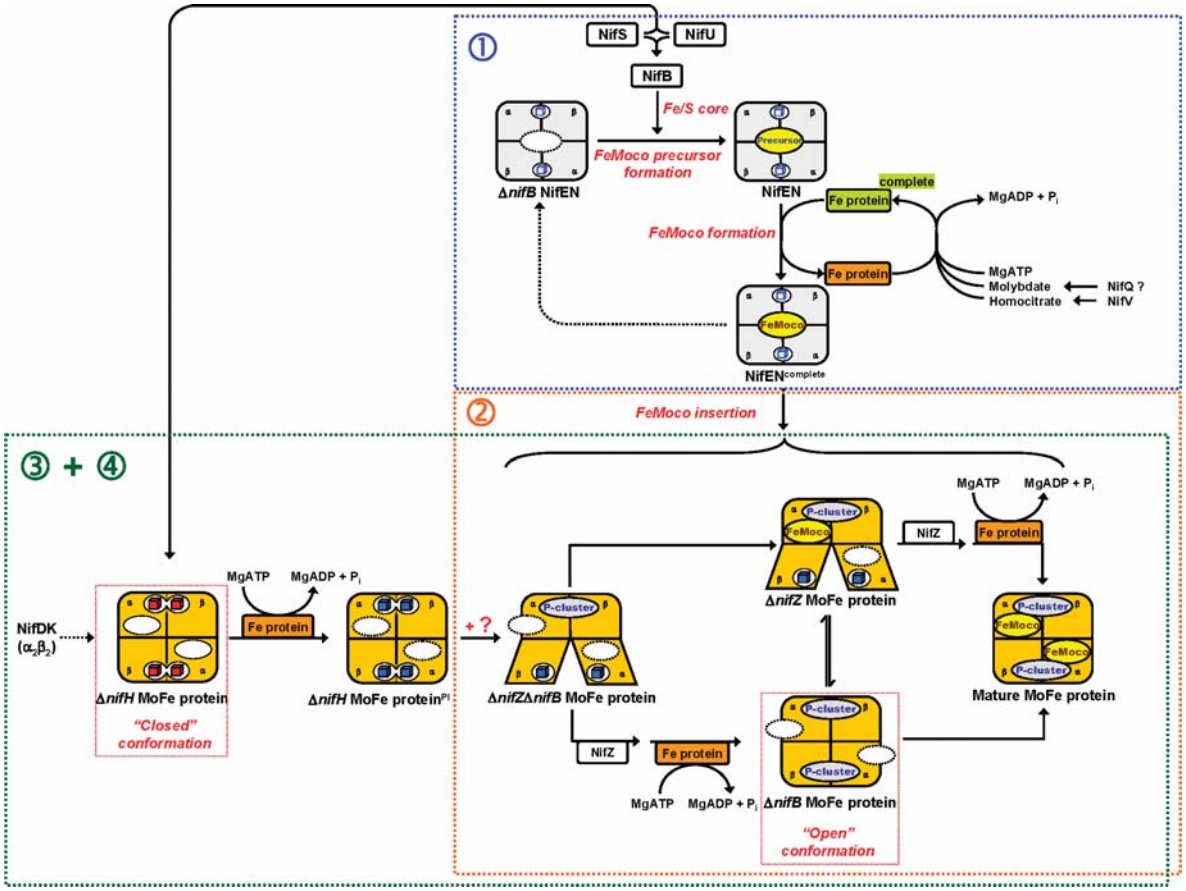

Figure (1-10): Refined model of the assembly of nitrogenase MoFe protein. (1) *Ex situ* assembly of FeMoco on NifEN, which involves a series of events leading to the conversion of the precursor (as in NifEN) to "FeMoco" (as in NifEN^{complete}) upon addition of Mo and homocitrate by Fe protein/MgATP (as in Fe protein^{complete}). (2) Insertion of FeMoco into MoFe protein, which involves the concomitant conversion of the FeMoco binding site to an accessible conformation upon formation of the P-cluster, the direct transfer of FeMoco from NifEN to MoFe protein by protein–protein interactions, and the insertion of FeMoco into its binding site through a positively charged funnel. (3) and (4) *In situ* assembly of the P-cluster on MoFe protein and concurrent stepwise assembly of MoFe protein, which involve the sequential conversion of paired [Fe4S4]-like clusters to [Fe8S7] P-clusters, one at a time, and the concomitant assembly of MoFe protein upon P-cluster formation and FeMoco insertion, one αβ-subunit half prior to the other (Hu, 2008)

1.4.1.2. Metalloclusters of the MoFe protein:

The P-cluster, which mediates electron transfer from the Fe protein to the FeMo-cofactor (Kim, 1992 and Lanzilotta, 1996), is an [8Fe:7S] cluster composed of two [4Fe:3S] subclusters that are bridged by a hexacoordinate sulphur atom, with the overall assembly coordinated to the protein through six Cysteine ligands, three from the α-subunit and three from the β-subunit (Chan, 1993). It locates about 10 Å below the protein surface at the interface between α and β subunits and bridges them. The protein environment around the P-cluster is mainly provided by hydrophobic residues (Kim, 1992b). Spectroscopic studies indicate that the P-cluster is in the all-ferrous state (designated P^N) as isolated in dithionite (McLean, 1987). The P^N state of the P-cluster can be two-electron oxidized to the P^{2+} state (designated P^{OX}) with oxidants like indigo disulfonate (IDS) (Watt, 1980). However, P^N and P^{OX} are structurally different in that one half of the P-cluster in the P^{OX} state is present in a more open conformation. The interconversion between P^N and P^{OX}

involves two Fe atoms of the P-cluster, and changes in the core structure of the cluster are accompanied by changes in the ligation between the cluster and the protein (Peters, 1997).

Biosynthesis of nitrogenase P-cluster has attracted considerable attention because it is biologically important and chemically unprecedented. Recent studies suggested that P-cluster is formed from a precursor consisting of paired [4Fe–4S]-like clusters (figure 1-11 left) and that P-cluster is assembled stepwise on MoFe protein, i.e., one cluster is assembled before the other. In addition, it was shown that the maturation of the second P-cluster requires the concerted action of NifZ, Fe protein, and MgATP and that the action of NifZ is required before that of Fe protein/MgATP, suggesting that NifZ may act as a chaperone that facilitates the subsequent action of Fe protein/MgATP (Hu, 2007 and Lee, 2009).

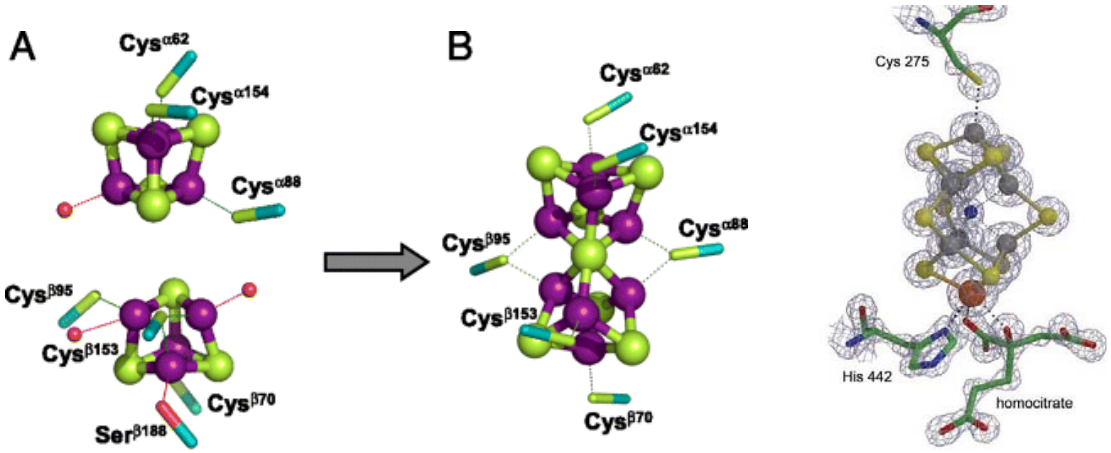

Figure (1-11): Left: Structural models for the cluster species in the ΔnifH MoFe protein before (A) and after (B) P-cluster maturation. Fe in purple and S in green (Lee, 2009). Right: Stereo representation of the FeMo-cofactor with the central ligand modeled as a nitrogen atom. Mo in red, Fe in gray, S in yellow, and the central N in blue (Einsle, 2002)

FeMo-cofactor is composed of molybdenum, iron, sulphur, and homocitrate in a ratio of 1:7:9:1 (Hoover, 1989). Homocitrate is coordinated to the molybdenum atom through its 2-hydroxy and 2-carboxyl groups. Recently, Einsle *et al.* reported a new model, at an improved resolution of 1.16 Å, which includes an internal hexa- coordinate light atom within the FeMo-cofactor cluster that is bounded to each of the six iron atoms and they suggested that the light atom is most likely to be nitrogen (figure 1-11 right) or may be oxygen or carbon (Einsle, 2002). FeMo-cofactor is located within the α subunit, at least 10 Å below the protein surface, and anchored to the protein by α-Cys-275 to an iron atom at one end of the cofactor and by α-His-442 to the molybdenum atom. These two residues as well as α-Ser-278, which is hydrogen bounded to the Sγ of α-Cys-275, are strictly conserved in all known MoFe protein sequences. The protein environment around the FeMo-cofactor is mainly provided by hydrophilic residues, although there are some hydrophobic residues (Kim, 1992b). FeMo-cofactor can be studied when it is buried within the MoFe protein or extracted into N-methyl formamide (NMF) (Shah, 1977 and Burgess, 1990).

The core charge of FeMo-cofactor is proposed to be +1 or +3 in the resting state (Lee, 1997 and Yoo, 2000), yet isolated FeMoco is known to be anionic. The overall negative charge of FeMo-cofactor is supplied by homocitrate, an organic terminal ligand of Mo,

which is -4 if the OH group is deprotonated. FeMo-cofactor can undergo reversible one-electron oxidation and reduction (Burgess, 1990 and 1996)

1.4.2. The Fe protein:

The iron protein is indispensable for nitrogenase catalysis and biosynthesis. Deletion of nifH gene results in the formation of a MoFe protein with disrupted P-clusters or precursor fragments comprising [4Fe4S]-like clusters, indicating that the Fe protein my facilitate the fusion of these fragments into a fully assembled [8Fe:7S] P-cluster (Hu, 2006). The Fe protein is an integral component of the nitrogenase reaction in a way that other low-redox-potential electron donors do not support dinitrogen reduction, undoubtedly due to the requirement for coupled ATP hydrolysis. In contrast, a variety of electron donors (ferredoxins and flavodoxins) provide a connection between cellular metabolism and the reduction of the Fe protein (Howard, 1994).

The Fe protein (figure 1-12) is an α_2 homodimer of the *nifH* gene product with a molecular mass of approximately 60-64 kDa and contains a single [4Fe4S] cluster bridged between its identical subunits and two nucleotide (MgATP or MgADP)-binding sites (Hausinger, 1983 and Rangaraj, 1997). The MgATP-binding sites and the [4Fe4S] cluster are separately located within the Fe protein, and they are unlikely to come within intimate contact with each other at any stage of catalysis (Rees & Einsle, 2005).

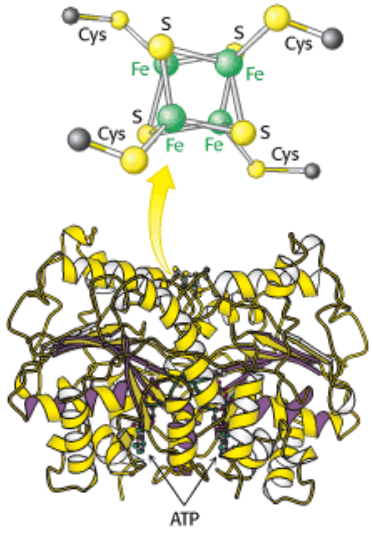

Figure (1-12): Fe protein with its iron sulphur cluster and the ATP binding sites (Berg, 2002)

The [4Fe4S] cluster of the Fe protein hydrolyzes MgATP to MgADP and uses the required energy to provide a single electron to the MoFe protein in a process that involves association and dissociation of the protein components and no MgATP hydrolysis or intermolecular electron transfer occurs without formation of this complex (Burgess, 1996). This cycle is repeated until enough electrons have accumulated in the MoFe protein for substrate reduction (Hageman, 1978).

In addition to its role as electron donor to the MoFe protein, the Fe protein has at least two and possibly three other functions. First, it is required for the initial biosynthesis of P-

cluster and FeMo-cofactor, and also for mobilizing Mo/homocitrate for the maturation of FeMo-cofactor precursor on NifEN (Hu, 2006). Second, it is required for the insertion of preformed FeMo-cofactor into the MoFe apoprotein, a process that may involve modification of the latter. Third, it has been implicated as being possibly important in the regulation of the alternative systems (Burgess, 1996).

1.4.3. Biosynthesis of FeMo-cofactor, the active site of nitrogenase:

Early studies showed that the products of at least six nitrogen fixation (*nif*) genes, including *nifQ*, *nifV*, *nifB*, *nifH*, *nifN* and *nifE*, are required for the biosynthesis of FeMo-co (Shah, 1986). Recently, it was established the requirement of altogether twenty proteins (Table 1-1) for assembly and insertion of FeMo-cofactor (Dos Santos, 2004). Interestingly, the genes that encode dinitrogenase (*nifD* and *nifK*) are not required for FeMo-cofactor biosynthesis (Ugalde, 1984 and Imperial, 1987), suggesting that FeMo-cofactor is assembled elsewhere in the cell and is then inserted into FeMo-co-deficient dinitrogenase (Shah, 1994 and Allen, 1995).

Table (1-1): *nif* gene products and other components involved in the overall FeMo-cofactor biosynthesis and insertion (Dos Santos, 2004)

gene	product/function(s)
nifH	Fe protein subunit
nifD	MoFe protein R-subunit
nifK	MoFe protein â-subunit
nifB	involved in the production of an Fe/S-containing FeMo cofactor precursor, designated NifB-cofactor
nifQ	involved in FeMo-cofactor biosynthesis, probably at an early step
nifV	homocitrate synthase
nifX	probably an intermediate carrier in FeMo-cofactor biosynthesis
nifY	probably an intermediate carrier in FeMo-cofactor biosynthesis
nifN	subunit of NifN2E2; appears to provide a transient site upon which one or more events related to FeMocofactor assembly occur
nifE	subunit of NifN2E2
nifU	complements NifS in the mobilization of Fe and S for metallocluster assembly; required for the synthesis of active Fe protein and MoFe protein
nifS	pyridoxal-*dependent* cysteine desulfurase; required for the synthesis of active Fe protein and MoFe protein
nifW	required for the synthesis of a fully active MoFe protein
nifZ	required for the synthesis of a fully active MoFe protein
nifT	function unknown
nifF	flavodoxin
nifJ	pyruvate:flavodoxin oxidoreductase
nifA	positive regulatory element
nifL	negative regulatory element
nafY	probably an intermediate carrier in FeMo-cofactor biosynthesis

Early investigations on the nitrogenase biosynthesis pathway suggested that [Fe-S] cluster substrates are provided essentially by NifU and NifS (Jacobson, 1989). The first intermediate defined in the biosynthesis pathway of FeMo-cofactor was the B-cofactor (renamed later as NifB-cofactor) that is considered as FeMo-cofactor precursor. The

formation of NifB-cofactor, which is composed of an iron-sulphur core that does not include molybdenum or homocitrate, is catalyzed by NifB using [Fe-S] cluster substrate produced by NifS and NifU (Shah, 1994). Recent studies showed that the role of NifU and NifS could be compensated by other non-*nif* machinery for the assembly of [Fe-S] clusters, indicating that NifUS are not essential to synthesize active NifB and subsequently active nitrogenase but they are required for full activity of nitrogenase (Zhao, 2007 and Dos Santos, 2007). More recent studies using EXAFS spectroscopy revealed that NifB-cofactor comprises a 6Fe core with an interstitial light atom (George, 2008).

NifE and NifN form an $\alpha_2\beta_2$ tetramer, homologous to MoFe protein subunits, that is able to bind the B-cofactor providing a scaffold for one or more steps in FeMo-cofactor biosynthesis (Allen, 1995). NifQ appears to have a role in the activation and mobilization of molybdenum for FeMo-cofactor assembly (Imperial, 1984 and Hernandez, 2008), and NifV is a homocitrate synthase that provides the organic constituent of the FeMo-cofactor (Hoover, 1989). The Fe protein and the product of another gene [NifY in the case of *K. pneumoniae* (White, 1992) and a protein called γ in the case of *A. vinelandii* (Homer, 1995)] also appear to have some role in the incorporation of the FeMo-cofactor into the apo-MoFe-protein (Goodwin, 1998).

Lately, a new intermediate was isolated of the FeMo-cofactor biosynthesis pathway, VK-cluster. Despite NifEN and NifX were found to exchange the VK-cluster, several experimental evidences suggested that this interaction is transient, and NifEN does not form a stable complex with NifX. In contrast to NifB-cofactor, the VK-cluster is electronic paramagnetic resonance (EPR)-active in the reduced and oxidized states (Hernandez, 2007).

Using purified proteins, a minimal *in vitro* system, containing NifB, NifEN, and NifH proteins, together with Fe^{2+}, S^{2-}, $MoO4^{2-}$, R-homocitrate, S-adenosyl methionine, and Mg-ATP, was found to be sufficient for the synthesis of FeMo-cofactor and the activation of apo-dinitrogenase under anaerobic-reducing conditions (13% activation) while activation of 48% of apo-NifDK was established in a composition-defined reaction mixture containing pure NifB, NifX, NifEN, NafY, NifH, and apo-NifDK as protein factors, along with Na_2MoO_4, R-homocitrate, $(NH_4)_2Fe(SO_4)_2$, Na_2S, S-adenosyl methionine (SAM), Mg-ATP, and sodium dithionite (DTH). Subsequently, an integrative model of the current understanding of the pathway for FeMo-cofactor biosynthesis was suggested (figure 1-13) (Curatti, 2007).

Quite recently, Yoshizawa, *et al*. reported the heterologous incorporation of vanadium and iron into the NifEN-associated FeMoco precursor implying that, instead of being assembled by the previously postulated mechanism that involves the coupling of [Fe4:S3] and [Mo:Fe3:S3] subclusters, the FeMo-cofactor is assembled by having the complete Fe/S core structure in place before the insertion of molybdenum. Besides, they showed that vanadium and iron can be inserted at much reduced efficiencies compared with molybdenum, and incorporation of both vanadium and iron is enhanced in the presence of homocitrate. An optimization of FeMo-cofactor maturation on NifEN was achieved by increasing the concentration of the reducing agent, dithionite, up to 20 mM. At 2 mM dithionite, the conversion of NifEN-associated precursor to full FeMo-cofactor was

incomplete and molybdenum was only loosely associated with NifEN-bound "FeMo-cofactor" (Yoshizawa, 2009a and 2009b).

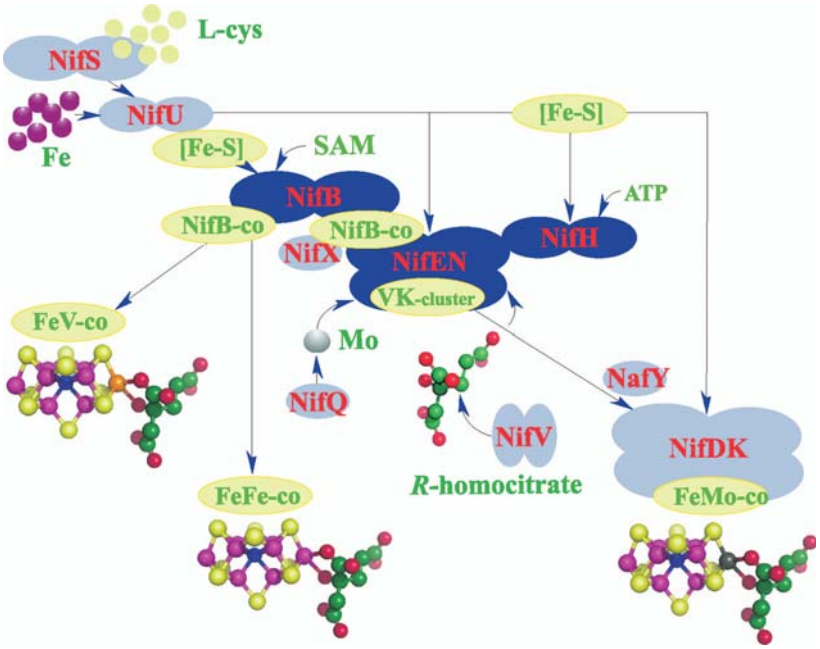

Figure (1-13): Current model for FeMo-cofactor biosynthesis and insertion into apo-NifDK. The model shows in dark blue the core components of the FeMo-cofactor biosynthesis pathway: NifB, NifEN, and NifH. The other Nif/Naf proteins known to participate in FeMo-co biosynthesis and insertion into apo-NifDK are shown in light blue (Curatti, 2007)

1.4.4. Mechanism of substrate reduction on the active site of nitrogenase:

Nitrogenase has proved to be an unusually versatile enzyme that could bind a lot of substrates and inhibitors (Dilworth, 1966, Li, 1982, Vaughn, 1989, and Seefeldt, 1995). Although the subtle differences among these substrates and inhibitors, reduction of each them carries an absolute requirement for ATP and a strong reductant as well as the presence of both components of nitrogenase. In general, the mechanism consists of two parts (Mortenson, 1979):

- A cycle involving the ATP-dependent electron transfer between the two protein components of nitrogenase.
- Substrate reduction on the MoFe protein when sufficient cycles of intermolecular electron transfer occurred.

The Fe protein cluster has the unique property of undergoing reversible redox reactions between three oxidation states, unlike classical [4Fe-4S] clusters that use only two states.

$$[2Fe^{2+}:2Fe^{3+}:4S^{2-}]^{+2} \leftrightarrow$$

$$[3Fe^{2+}:1Fe^{3+}:4S^{2-}]^{+1} \leftrightarrow [4Fe^{2+}:4S^{2-}]^{0}$$

Dithionite-reduced Fe protein is well established to exhibit the +1 oxidation state of the cluster and to serve as a single electron donor to the MoFe protein in figure (1-14).

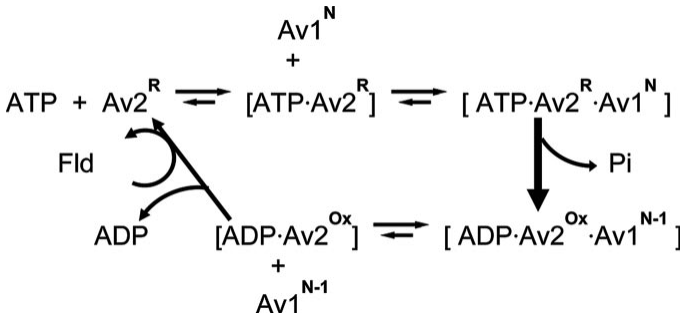

Figure (1-14): Kinetic scheme depicting ATPdependent electron transfer between the component proteins of nitrogenase. Av1 and Av2 denote the MoFe protein and Fe protein, respectively, from *Azotobacter vinelandii*. The superscripts R and Ox denote reduced and oxidized states of Av2, and the superscripts N and N-1 indicate the oxidation levels of Av1 before and after electron transfer from Av2. Fld, flavodoxin (Howard, 2006)

If the Fe protein is reduced to the all ferrous 0 state during turnover, each cycle could be a two-electron process *in vivo*, at least when flavodoxin is the Fe protein electron donor (Howard, 2006 and Lowery, 2006). Although the direct electron donor to nitrogenase in diazotrophic bacteria has been proposed to be either flavodoxins or ferredoxins, other pathways were found to be involved in the electron transfer to nitrogenase (Edgren, 2006).

Kinetic studies of substrate reduction by nitrogenase show that N_2 reduction is distinct from binding and reduction of all other substrates, including alkynes (Liang, 1988). Many of the observations from such kinetic studies are embodied in the classic Thorneley and Lowe model (figure 1-15) (Thorneley and Lowe, 1984).

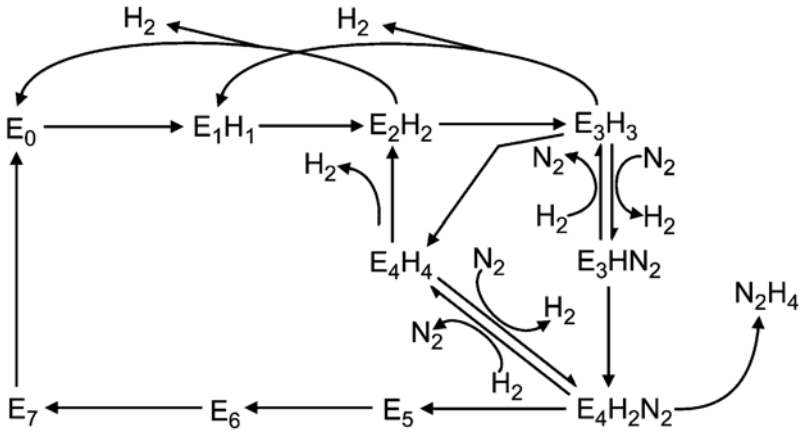

Figure (1-15): Thorneley–Lowe cycle for dinitrogen binding and reduction by nitrogenase (Thorneley and Lowe, 1984)

In this model, the addition of electrons to the MoFe protein results in successive E_n states, where n represents the number of [H^+, e^-] delivered to the MoFe protein, starting from E_0 as the resting state of the MoFe protein. Possible involvement of the P-cluster is ignored in this model. As indicated, N_2 binds to the more highly reduced states of the MoFe protein than other substrates (e.g., acetylene or protons). N_2 binds to the E_3 and E_4 states whereas acetylene and protons bind to the less reduced E_1 and E_2 states (Liang, 1988). The binding of N_2 furthermore appears to be unique in requiring an obligatory reduction of two H^+ to H_2 (Simpson, 1984). The existence of fundamental differences between the interactions of N_2 and other substrates with FeMo-cofactor is also supported by the non-

reciprocal inhibition patterns of alternative substrates vs. N_2 (Dos Santos, 2005). For example, while acetylene is a competitive inhibitor of N_2 reduction, N_2 is a non-competitive inhibitor of acetylene reduction. Another difference is that N_2 reduction is inhibited by H_2, whereas no other nitrogenase substrate is inhibited by H_2 (Hwang, 1973 and Jensen, 1986). Moreover, N_2 cannot fully suppress H_2 evolution, whereas acetylene can. Altogether, these observations point to fundamental differences in the mechanism of N_2 activation catalyzed by nitrogenase when compared to all other substrates. Using a combination of mutant proteins, magnetic spectroscopy, and isotopically labeled compounds, potential intermediates were identified and evidence was provided that for the substrate methyldiazene, only the terminal nitrogen atom binds to an iron of the FeMo-cofactor and that protons are added in an alternating fashion between the two nitrogen atoms with the first proton added to the non-iron-bound nitrogen (Barney, 2006).

In contrast to the current state of knowledge for N_2 binding and reduction on organometallic complexes outside of proteins, little is known about the specific nature of the reduction mechanism taking place at the active site of nitrogenase (Pickett, 1996 and MacKay, 2004). The current paradigm for N_2 activation by metal complexes is the Chatt cycle at a mononuclear Molybdenum metal complex (Chatt, 1978). In this cycle N_2 is activated at a mononuclear Molybdenum atom contained in an organometallic complex. A number of intermediates in the cycle have been structurally characterized (Yandulov, 2003 and Schrock, 2003). Other metal complexes containing both Mo and Fe have been shown to reduce hydrazine, *cis*-Dimethyldiazene, and acetylene (Malinak, 1997 and Demadis, 1996) and some Fe-centered metal complexes have recently been shown to bind various nitrogenous molecules (Vela, 2004).

1.4.5. Transcriptional and posttranscriptional regulation of nitrogenase system:

Nitrogen fixation is regulated at the transcriptional level in response to environmental oxygen and ammonium levels. Because the nitrogenase components are oxygen labile, it is advantageous for bacteria to repress transcription when oxygen levels are high. It is also advantageous to repress the expression of the metabolically expensive nitrogenase system when the cellular level of fixed nitrogen is sufficient. The regulation level is characteristic of each particular diazotroph. While expression of nitrogenase in symbiotic diazotrophs is fairly insensitive to ammonium because export of ammonium to their symbiont keeps ammonium levels low, the expression of *nif* genes in free-living diazotrophs is more sensitive to cellular ammonium levels (Halbleib, 2000).

In the *K. pneumoniae* model system, the control of *nif* gene expression focuses on NifA, a member of the enhancer-binding protein (EBP) family, together with the RNA polymerase sigma factor, σ^{54} or (*rpoN* gene product), that are responsible for control of all major *nif* gene cluster transcription events. Transcription of *nifA* is under the control of the *ntrBC* gene products, which comprise a global two-component transcriptional activator system, responsible for cellular nitrogen regulation. NtrB is a histidine kinase that phosphorylates NtrC under nitrogen limiting conditions and can also act as a phosphatase to dephosphorylate NtrC under nitrogen-excess conditions. NtrB activity is regulated by PII protein in response to the carbon/nitrogen balance in the cell. The phosphorylated form of

NtrC acts as a transcriptional activator of *glnA*, *nifLA*, *glnK*, *amtB1* and other operons involved in nitrogen fixation and assimilation (Zou, 2008).

nifA gene is co-transcribed with *nifL*, which encodes a redox- and nitrogen-responsive regulatory flavoprotein (NifL). NifL acts as a negative regulator of NifA, adding effectively another level of regulation in response to oxygen and fixed nitrogen. Oxidized NifL is also sensitive to the presence of nucleotides *in vitro*, with increased inhibition especially in response to ADP. Deviations from the *K. pneumoniae* paradigm exist in nearly all nitrogen fixation, e.g. in *A. vinelandii* and *Rhodospirillum rubrum* expression of *nifA* is not under the control of the *ntrBC* gene products (Hill, 1996).

Because of the metabolically demanding nature of nitrogen fixation, an additional layer of nitrogenase regulation is present in a few free-living diazotrophs. To prevent unproductive nitrogen fixation during energy-limiting or nitrogen-sufficient conditions, the nitrogenase complex is rapidly, reversibly inactivated by ADP-ribosylation of Fe protein. The ADP-ribosylation system has been first identified in *R. rubrum* (Lowery, 1988). Another regulation mechanism was lately suggested where NifI inhibits nitrogenase by competing with the Fe protein for binding to the MoFe protein (Dodsworth, 2007).

1.5. Bacterial growth phases in a batch system:

In the laboratory, under favourable conditions, a growing bacterial population doubles at regular intervals by geometric progression: 2^0, 2^1, 2^2, 2^3.....2^n (where n = the number of generations) resulting in so-called exponential growth. In reality, exponential growth is only part of the bacterial life cycle, and not representative of the normal pattern of growth of bacteria in nature.

When a fresh medium is inoculated with a given number of cells and the multiplication of population is monitored over a period of time, plotting the data will yield a typical bacterial growth curve, Figure (1-16).

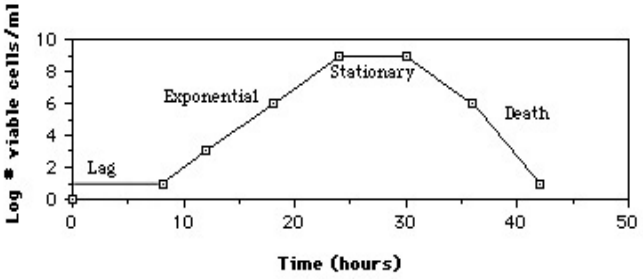

Figure (1-16): Typical bacterial growth curve for batch culture.

Four characteristic phases of the growth period are recognized:

1. **Lag phase:** Immediately after inoculation of the cells into a fresh medium, the population remains temporarily unchanged. Although there is no apparent cell division occurring, the cells may be growing in volume or mass, synthesizing enzymes, proteins, RNA, etc., and increasing in metabolic activity. The length of the lag phase is apparently dependent on a wide variety of factors including the size of inoculums, time necessary to recover from physical damage or shock in the transfer, time required for synthesis of essential coenzymes or division factors, and

time required for synthesis of new (inducible) enzymes that are necessary to metabolize the substrates present in the medium.

2. **Exponential (log) phase:** The exponential phase of growth is a pattern of balanced growth wherein all cells are dividing regularly by binary fission, and are growing by geometric progression. Cells division occurs at a constant rate depending on the composition of the growth medium and the conditions of incubation.

3. **Stationary phase:** Exponential growth cannot be continued forever in a batch culture (e.g. a closed system such as a test tube or flask). Population growth is limited by one of three factors: Exhaustion of available nutrients, accumulation of inhibitory metabolites or end products, and exhaustion of space; in this case called a lack of "biological space". During the stationary phase, if viable cells are being counted, it cannot be determined whether some cells are dying and an equal number of cells are dividing, or the population of cells has simply stopped growing and dividing. The stationary phase, like the lag phase, is not necessarily a period of quiescence.

4. **Death phase:** If incubation continues after the population reaches stationary phase, a death phase follows, in which viable cell population declines. During the death phase, the number of viable cells decreases geometrically (exponentially), essentially the reverse of growth during the log phase.

1.6. Diauxy, bacterial growth on multiple sources of one substrate:

The word diauxy (di- + Greek: auxein, to increase in size) is used in cell biology to describe the growth phases of a bacteria as it metabolizes a mixture of sugars; one sugar is mitabolized before a second begins to be metabolized. When *E. coli* is grown on glucose and lactose for example, it starts first to utilize the glucose, which needs less energy for metabolism, and upon exhausting the glucose of the medium, the second source of carbon, meaning the lactose, will be executed.

Diauxic growth was discovered by Jacques Monod in 1941 as working with *E. coli* and *Bacillus subtilis*. Firstly it was believed that a single enzyme could adapt to metabolize different sugars. Later the idea of enzyme adaptation was replaced with the concept of induction. A molecule induces expression of a gene or operon by binding to a repressor protein and preventing the repressor from attaching to the operator. Organisms have genes to metabolize common nutrients, but if they use energy to make enzymes to utilize uncommon nutrients they may be at a disadvantage. Evolution has resulted in the ability of organisms to change their genetic activity so that only those genes needed at the time are expressed.

Diauxic growth is not restricted to carbon source, but also may be dependent on nitrogen source. It is well known that utilizing nitrogen from dinitrogen, in nitrogen fixing organisms, is much more energetically expensive than other fixed sources such as ammonium or nitrate. Therefore, when a nitrogen-fixing organism grows in nitrogen-limiting medium, it will first use the fixed nitrogen available in the medium and then obligately convert to use dinitrogen from atmosphere; figure (1-14).

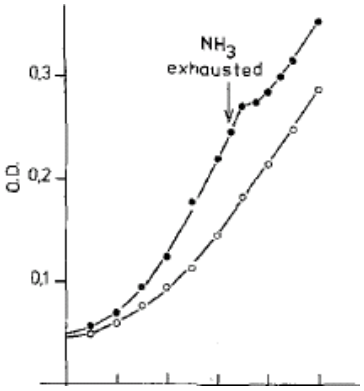

Figure (1-14): Influence of ammonia on growth of *A. vinelandii*, • - - • growth with $(NH_4)_2SO_4$ (5 μg N/ml), o - - o with N_2.

1.7. Goals of the work:

A complete set of *nif* genes was found in the genome of *Wolinella succinogenes*. So far there is no indication about the origin of these genes and the capability of *W. succinogenes* to live as a diazotroph. As an enteric bacterium, *W. succinogenes* is not expected to be subject to nitrogen-limiting conditions, a fact that raises questions about the purpose of such genes for the organism. Moreover, *W. succinogenes* belongs to the epsilon-proteobacteria, a subgroup whose members are not known formally to fix dinitrogen.

The main goal of this study is to uncover whether the *nif* genes can be functionally expressed in *Wolinella succinogenes*. First, an analysis of nitrogenase-associated genes has to be carried out to make sure that all essential products for nitrogenase biosynthesis and function are encoded within the *nif* gene cluster. An alignment comparison of the structural subunits of nitrogenase is the second task to be done aiming at the ascertainment of the capability of *W. succinogenes* to express intact nitrogenase components and to have all conserved residues that affect the enzyme functionality. Investigation of nitrogenase expression in *W. succinogenes* and optimization of the enzymatic activity should be established. In line with *Azotobacter vinelandii*, the model diazotroph, a protocol for purification of nitrogenase components from *W. succinogenes* will be developed and then characterization of the purified components is to be attempted.

2. Materials and methods:

2.1. Materials:

2.1.1. Chemicals:

All standard chemicals were of analytical purity grade (p.a.) and were obtained from the following companies: Applichem (Darmstadt, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany) and Sigma-Aldrich (Deisenhofen, Germany).

2.1.2. Culture media and additives:

For growth of *Wolinella succinogenes* on Formate / Fumarate medium:

NaFormate	100	mM	6.80	g
Fumaric acid	90	mM	10.45	g
Buffer*	20-50	mM		
K_2HPO_4	20	mM	3.50	g
NaAcetate · 3 H_2O	20	mM	2.75	g
$(NH_4)_2SO_4$**	5	mM	0.66	g
NH_4Cl**	5	mM	0.27	g
Glutamic acid (autoclave separately)	1	mM	0.15	g
$MgCl_2$ · 6 H_2O	0.25	mM	0.05	g
$CaCl_2$ · 2 H_2O	0.05	mM	0.01	g
Trace Element Solution PT	0.2	ml		
Deionized water		dH_2O	1000.00	ml

Medium is adjusted to pH 7.9 – 8.0 using NaOH and H_2SO_4 and then autoclaved (121°C for 20 min) in Widdel Flask (or in fermentor). While still hot, medium is flushed for 1 min with inertal20 (60–100 mbar) and then left to cool down to room temperature under positive inert gas pressure. Then, 3ml/l 2M cysteine is added for reduction and dispensation, and 500µl/l (25mg/ml) kanamycin (only for nosZ/6 strain) to inhibit contamination.

* Three kinds of buffer were tried for keeping pH stable at 7.9 – 8.0 during cells growth: Tris/HCl (50mM), $NaHCO_3$ (20mM), and KH_2PO4/K_2HPO_4 (10mM of each).

** $(NH_4)_2SO_4$ and NH_4Cl were used for growth with no ammonium-limitation conditions, and they were replaced with Na_2SO_4 and NaCl for growth under ammonium-limiting conditions.

Trace elements solution PT:

$Na_2EDTA \cdot 2\ H_2O$		5.200	g
$FeCl_2 \cdot 4\ H_2O$		1.500	g
$MnCl_2 \cdot 4\ H_2O$		0.100	g
$CoCl_2 \cdot 6\ H_2O$		0.190	g
$ZnCl_2$		0.070	g
$CuCl_2 \cdot 2\ H_2O$		0.017	g
H_3BO_3		0.062	g
$Na_2MoO_4 \cdot 2\ H_2O$		0.036	g
$NiCl_2 \cdot 6\ H_2O$		0.025	g
$Na_2SeO_3 \cdot 5\ H_2O$		0.500	mg
$Na_2WO_4 \cdot 2\ H_2O$		0.400	mg
distilled water	dH_2O	1000	ml

(*from J. Simon, Inst. f. Mikrobiologie, Universität Frankfurt; after Pfennig & Trüper, 1981, modified*)

Solution is modified from original by addition of selenate and tungstate salts.

For growth of *Geobacter sulfurreducens* on NBAF medium:

NH_4Cl*	3.7 mM	0.20	g
KH_2PO_4		0.42	g
K_2HPO_4		0.22	g
KCl		0.38	g
Na-acetate	(anhydrous)	1.25	g
Trace Element Solution m141+		10.00	ml
Vitamin Solution m141		10.00	ml
Yeast extract		1	g
$NaHCO_3$		1.80	g
Na_2CO_3		0.50	g
Fumarate		6,4	g
Distilled water	dH_2O	980.00	ml

Medium is autoclaved in Widdel flask (or in fermentor) (120 C° for 20 min). While it is still hot, it is flushed for 1 min with Inertal20 (60 – 100 mbar) and then it is left to cool down to room temperature under positive inert gas pressure. 2ml/l (2M cysteine) is added for reduction and as a sulphur source. 1 ml/l (1M filter-sterilized HCl) is added to reach a pH of 6.8 and then medium can be dispensed in autoclaved bottles.

- NH_4Cl is used for growth with no ammonium-limitation conditions and it is replaced with NaCl upon growth under ammonium-limitation conditions.

Vitamin Solution m141 (DSMZ):

Biotin	Biotin	2.0	mg
Folic acid	Folsäure	2.0	mg
Pyridoxin-HCl	Pyridoxin-HCl	10.0	mg
Thiamine-HCl · $2H_2O$	Thiamin-HCl · $2H_2O$	5.0	mg
Riboflavin	Riboflavin	5.0	mg
Nicotinic acid	Nikotinsäure	5.0	mg
D-Ca-panthothenate	*D*-Ca-Panthothenat	5.0	mg
Vitamin B_{12}	Vitamin B_{12}	0.1	mg
p-Aminobenzoic acid	*p*-Aminobenzoesäure	5.0	mg
Lipoic acid	Liponsäure	5.0	mg
distilled water	dH_2O (degassed!)	1000	ml

Trace elements solution m141+:

First, nitrilotriacetic acid is dissolved in water and pH is adjusted to 6.5 with KOH. Then, minerals are added and final pH is adjusted again to 7.0 with KOH.

Nitrilotriacetic acid	Nitrilotriessigsäure	1.500	g
$MgSO_4 \cdot 7\ H_2O$		3.000	g
$MnSO_4 \cdot 2\ H_2O$		0.500	g
NaCl		1.000	g
$FeSO_4 \cdot 7\ H_2O$		0.100	g
$CoSO_4 \cdot 7\ H_2O$		0.180	g
$CaCl_2 \cdot 2\ H_2O$		0.100	g
$ZnSO_4 \cdot 7\ H_2O$		0.180	g
$CuSO_4 \cdot 5\ H_2O$		0.010	g
$KAl(SO_4)_2 \cdot 12\ H_2O$		0.020	g
H_3BO_3		0.010	g
$Na_2MoO_4 \cdot 2\ H_2O$		0.010	g
$NiCl_2 \cdot 6\ H_2O$		0.025	g
$Na_2SeO_3 \cdot 5\ H_2O$		0.500	mg
$Na_2WO_4 \cdot 2\ H_2O$		0.400	mg
distilled water	dH_2O	1000	ml

For growth of *Azotobacter vinelandii* on Burke's medium:

Scrose		20	g
0,9M $CaCL_2$		1	ml
1,67M $MgSO_4$		1	ml
2M Phosphate7,5	Autoclaved separately	5	ml
3M NH_4Cl	Autoclaved separately	3.5	ml
Distilled water	dH_2O	1000	ml

Autoclave (120 C° for 20 min) in flasks (or in fermentor); pH must be 7.3 - 7.5.

- NH_4Cl was used for growth with no ammonium-limitation.
- Phosphate7.5 is a mix of 2M KH_2PO_4 and 2M K_2HPO_4 with pH of 7.5.

For agar plates: 0.5 % agar with Burke's medium was autoclaved at120°C for 20 min.

Let cool to 60°C.

Add 1ml 2M PO4

Add 0.7ml 3M NH4Cl (only for N+).

Shake and pour in plates.

2.1.3. Gases:

- Inertal20 gas (80% N_2 & 20% CO_2) Air Liquide, Krefeld, Germany
- Acetylene gas (purity grade 2.6) Air Liquide, Krefeld, Germany
- Acetylene gas (purified in the inorganic chemistry institute)
- Nitrogen gas (purity 4.6) Air Liquide, Krefeld, Germany
- Formier gas (95% N_2 & 5% H_2) Air Liquide, Krefeld, Germany
- Argon gas (95%Ar & 5% H_2) Air Liquide, Krefeld, Germany
- Oxygen gas (99,5% O_2) Air Liquide, Krefeld, Germany
- Ethylene gas (≥99.95%) Sigma-Aldrich GmbH, Germany

2.1.4. Bacteria strains:

- *Wolinella succinogenes nosZ/6* from J. Simon, Frankfurt
- *Wolinella succinogenes wt* from DSMZ (Nr.DSM1740)
- *Geobacter sulfurreducens* from DSMZ (Nr. DSM12127)
- *Azotobacter vinelandii* from DSMZ (Nr. DSM2289)

2.1.5. Equipments:

- 12L bioreactor (fermentor); Applikon Biotek, Knuellwald, Germany
- Centrifuges (Avanti J-20XPI; J-30I); Beckmann Coulter, Krefeld, Germany
- PH-Meter (HI 8314 Membrane); Hanna Instruments, Kehl am Rhein, Germany
- Gas chromatography (Agilent 6890, FID detector); USA

- Biophotometer; Eppendorf AG, Hamburg, Germany
- Aekta prime chromatography system; GE Healthcare, Freiburg, Germany
- Chromatography columns; GE Healthcare, München, Germany
- Ultrafiltration membranes; Vivaspin, Sartorius, Germany
- Thermocycler (*Tpersonal*); Whatman Biometra, Göttingen, Germany
- Microfluidizer (S100); Microfluidics, Newton, USA
- centrifugal filter device (0,22 μm); Millipore, Schwalbach, Germany
- Hoefer miniVE SDS gel system; GE Healthcare, München, Germany
- Hoefer (big one) SDS gel system
- Gel imaging system (GelDoc 2000); Biorad, München, Germany
- spectrophotometer (Ultrospec^{TM} 2100 pro); GE Healthcare, München, Germany
- Mass spectrometer (Q-TOF Ultima Global) Micromass, Manchester, UK

2.1.6. Anaerobic equipments:

- Vacuum Pumps (Model RV8); BOC Edwards, Crawley Sussex, England
- Pressure meters (Modell VAP 5); Vacubrand, Wertheim, Germany
- Reductant columns; Ochs, Bovenden, Germany
- Peristaltic pump,
- Anaerobic tent; Coy, Grass lake, USA

Because nitrogenase is an oxygen-labile enzyme, it is essential to work under anaerobic conditions. All flasks, bottles, and solutions must be free of oxygen prior to use in the purification procedures. Anaerobic equipment is glass or metal closed system that provides vacuum and inert gas. Vacuum pumps are protected by a cryo trap that is cooled by liquid nitrogen (-196°C). Therefore, any substances that may come from the apparatus are condensed in the cryo trap.

Anaerobizing process may be attained either automatically or manually. Automatic process (the left part of figure2-1) is achieved by switching between vacuum and gas for eight cycles (each cycle includes 4min vacuum and 1min N_2). Manual process (the right part of figure2-1) is performed with the same protocol (4min vacuum & 1min N_2 repeated six times) but switching between vacuum and gassing must be carried out manually. Nitrogen gas used for gassing is passed through a reductant column that contains a copper catalyst to remove traces of oxygen. Traces oxygen, that may be present in the nitrogen gas, oxidizes the copper catalyst according to the following equation (producing a green-coloured material):

$$Cu + ½\ O_2 \rightarrow CuO$$

Regeneration of the column is carried out through handling with H_2 according to the following equation:

$$CuO + H_2 \rightarrow Cu + H_2O$$

The catalyst is reduced again and a black-coloured material is produced.

Bottles and flasks are connected to the system through gas-tight tubes and then turning the vacuum and gas lines leads to getting free of the oxygen. In the case of solutions, a reductant (dithionite) is used to completely removal of oxygen traces. All anaerobized materials are held under positive pressure (100 mbar) of nitrogen gas.

The anaerobic chamber is a transparent plastic tent filled with forming gas (95% N_2 and 5% H_2); it is very important to complement the work under anaerobic conditions, i.e. steps where protein solutions must be exchanged between flasks for dilution, concentration or other purposes such as screens. The tent is accessible through an automatic airlock, which provides exchange of materials without direct contact with air.

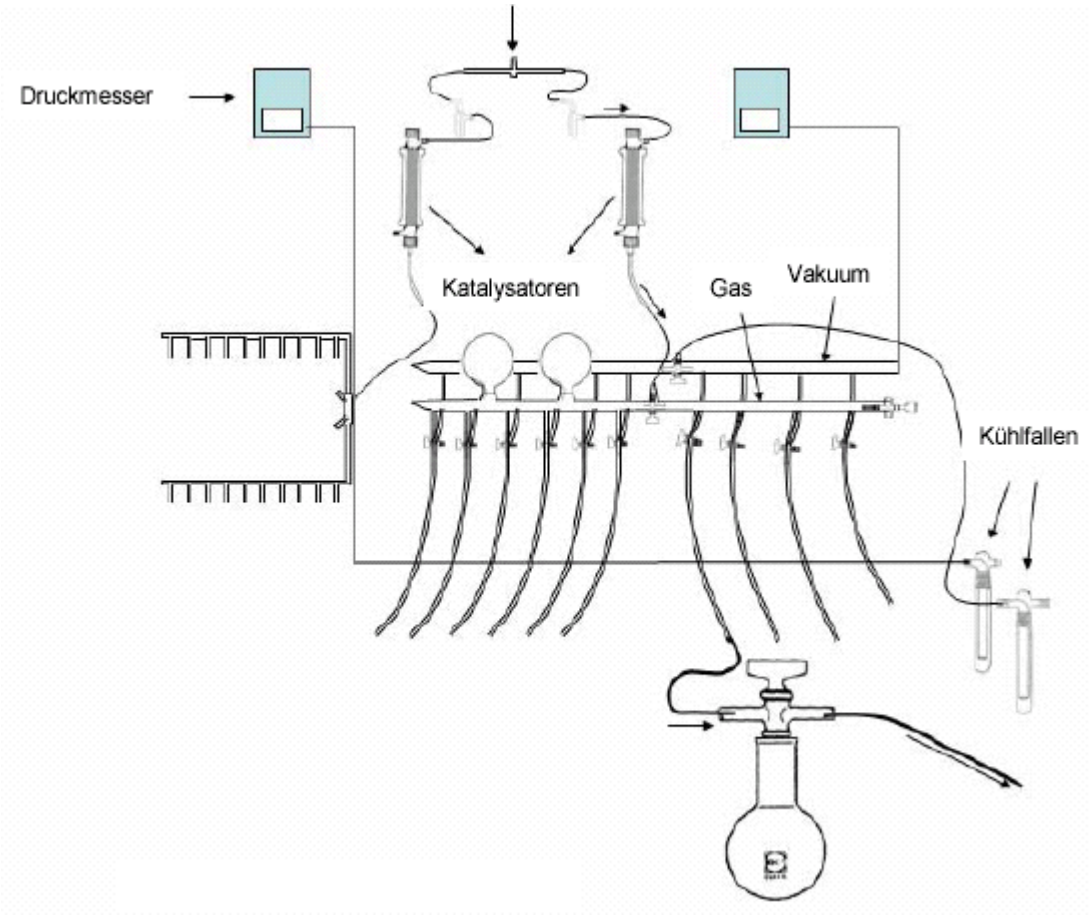

Figure (2-1): Anaerobic equipment (Swen Engelmann)

2.1.7. Enzymes and markers:

For decomposition of DNA during RNA-prep, DNAseI wsa applied. The Reverse Transcriptase M-MuLV (MBI Fermentas, St. Leon-Rot, Germany) was used for reverse

transcription of mRNA. As DNA polymerase, Taq (MBI Fermentas, St. Leon-Rot, Germany) was imployed for synthesis of DNA.

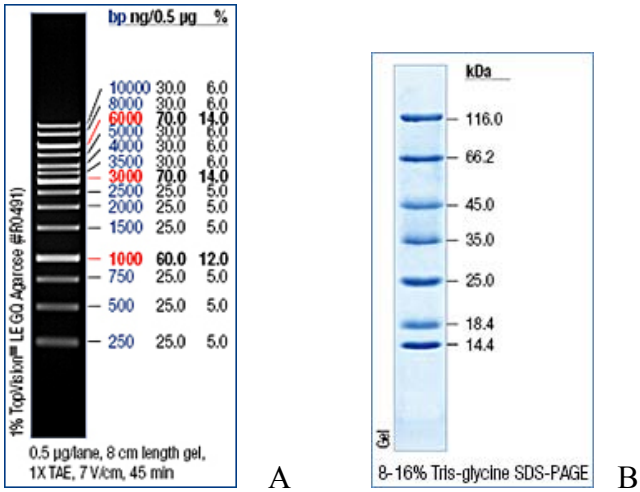

Figure (2-2): A, the geneRuler 1kb DNA; B, unstained molecular weight marker for protein sizing

The GeneRuler™ 1kb DNA Ladder (MBI Fermentas, St. Leon-Rot, Germany) served as DNA marker. For sizing of proteins by SDS-PAGE, the unstained protein molecular weight marker from MBI Fermentas was utilized (figure 2-2).

2.1.8. Primers:

Primers were designated using the MWG-Biotech software and then tested for self-complementarity via ”Oligonucleotide Properties Calculator” software from the ``Northwestern University Medical School`` website. Proper primers were accordingly obtained from MWG-Biotech AG, Ebersberg, Germany (figure2-3).

For_*nifD* 5’-CCG ATG ATG AGA GCC AAA AG-3’ **Tm** 57,3°C

Rev_*nifD* 5’-TGC CCA TAC AGG AGA GTT G-3’ **Tm** 56,7°C

For_*nifH* 5’-ATC GAC TAC TTC TCA AAA CAC C-3’ **Tm** 56,5°C

Rev_*nifH* 5’-CGA ACT GCT CAG CGA TAC-3’ **Tm** 56,0°C

Figure (2-3): Primers of *nifH* and *nifD* genes from *W. succinogenes*

2.2. Methods:

2.2.1. Cultivation of bacteria:

2.2.1.1. Stock cultures:

It is necessary to maintain cells for a long time to be available for laboratory work because fresh cultures can not be used simultaneously for two reasons. The first one is decreasing the purity because of repeated refresh of the culture where other organisms may infect the culture or some mutations in the same culture may occur. The second is that fresh cultures are not stable in the case of long-time storage.

Azotobacter vinelandii and *Wolinella succinogenes* were maintained as glycerol stocks, which were prepared in the following way:

- Let 50 ml culture grow to mid-log phase.
- Centrifuge 2 ml of the culture for 30 min @ 16000 ×g and 4°C.
- Resuspend with 250 µl fresh sterilized medium and 250 µl sterilized glycerol.
- Aliqout to 50 µl, freeze immediately in liquid nitrogen, and store at –80°C.

Geobacter sulfurreducens was maintained in DMSO as the following:

- Let 400 ml culture grow to mid-log phase (OD_{600} 0.2 – 0.35, $\cong 10^8$ cells/ml).
- Centrifuge 8 min @ 4300 ×g and 4° C.
- Wash cells twice with 400 ml Electroporation Buffer (1 mM HEPES/NaOH pH 7.0, 1 mM $MgCl_2$, 175 mM sucrose), centrifuge as above.
- Resuspend cells in 2 ml Electroporation Buffer (~ 10^{11} cells/ml).
- Add 1/5 volume (400 µl) of (60 % DMSO / 40% Electroporation Buffer) to achieve a final DMSO concentration of 10%.
- Aliquot to 25 µl, freeze immediately in liquid nitrogen, and store at –80° C.

Because *G. sulfurreducens* is an anaerobe, all manipulations are to be carried out on ice in the anaerobic chamber. Anoxic ice-cold buffers must be used. Cells are sensitive to shearing, therefore, the pipetting process should be carefully achieved and glass pipettes are recommended (Coppi, 2001).

2.2.1.2. *Azotobacter vinelandii* cultivation:

A. vinelandii, which is an obligate aerobe, was cultivated in flasks at 37°C with agitation of 210 rpm or in 12L-fermentor at 37°C, rotor 100 rpm, 20% O_2 and pH 7.5. Stock cultures were prepared in 250ml-flasks (50ml Burke's medium + 250µl 2M PO_4 + 175µl 3M NH_3Cl), inoculated with glycerol stock, and incubated for 2-3 days. Precultures were prepared in 300ml-flasks (100ml Burke's medium + 500µl 2M PO_4 + 350µl 3M

NH_3Cl) and inoculated with 2ml stock culture. All precultures were incubated at 37°C with agitation of 210 rpm for 18 h and then used directly as inoculums or stored at +4°C for further use. Main cultures were cultivated either in 2L-flasks (1L Burke's medium + 5ml 2M PO_4) + 3.5ml 3M NH_3Cl (for cultures that were cultivated without ammonium-limitation conditions) and inoculated with 20ml preculture, or in 12L-fermentor (10L Burke's medium + 50ml 2M PO_4) and inoculated with 100 ml preculture.

Agar plates (-NH_3) are important to make sure that bacteria are still able to fix nitrogen and to eliminate infections. The grown bacteria from plate were cultivated first in test tubes (3ml Burke's medium + 15µl 2M PO_4 + 10,3µl 3M NH_3Cl) for 18 h and then used for glycerol stock preparation or as preculture.

2.2.1.3. *Wolinella succinogenes* cultivation:

W. succinogenes, which is an anaerobe with ability to grow in the presence of 2% oxygen, was cultivated in fermentor that allows working under anaerobic conditions in addition to controlling temperature, pH, and rotation speed by a control unit. Precultures were prepared in gastight screw-cap vials with different sizes (50, 100, 500, and 1000ml).

At the beginning, a 50ml-bottle containing formate/fumarate medium was inoculated with a glycerol stock and incubated at 30°C for 2-4 days till cells grow notably. The last stock culture might be used directly or stored at +4°C but it must be consumed at the latest within one week; otherwise it must be refreshed. In this way, culture may still active for 2-3 months; after that a new preculture must be prepared from glycerol stock.

Formate/fumarate medium was prepared and filled in the fermentor. After autoclaving, the fermentor was connected to the control unit and left to reach the desired conditions: temperature 30°C, pH 7.7-7.9, and rotation 60 rpm. Then, 3ml/l (2M) Cysteine and 500µl/l (25mg/ml) Kanamycin (for nosZ/6 strain) were added aseptically to the medium; after that the preculture was added.

2.2.1.4. *Geobacter sulfurreducens* cultivation:

G. sulfurreducens, which is an microaerophile, was cultivated in fermentor under Inertal 20 or Aragon milieu at 30°C with rotor 100 rpm and pH 7.0 ± 0.1. Precultures were prepared in glass bottles with different sizes.

11L NBAF medium (without NH_4Cl for nitrogen fixation experiments) was prepared in the fermentor and autoclaved at 120°C for 20 min; after connecting to the control unit and arriving to the suitable conditions, the medium was inoculated with 1L preculture after adding cysteine aseptically (by microfilter). The preculture was first cultivated in 100ml-bottle, which was inoculated with 10 ml stock culture, over night at 30°C on a roller and then in 1L inoculated with 100ml preculture at the same conditions over night too. In contrast to *Wolinella, Geobacter* is incubated on roller because it is non-motile and the stock culture must be refreshed weekly.

2.2.2. Growth curves:

Growth curves were obtained by measuring the optical density (OD) with a photometer at a wavelength of 600 nm. For this purpose, samples of 1ml were taken from cultures at intervals of about one hour for *Wolinella* and 2-3 hours for *Geobacter* and *Azotobacter* and then OD600 was directly measured. As a blank, 1ml of the medium before inoculation was used.

2.2.3. Nitrogenase activity assay:

The ability of nitrogenase to reduce other substrates in addition to dinitrogen, allows in determination of its activity. Until now, the easiest method used for this purpose is the acetylene-ethylene assay, which bases on the nitrogenase-catalyzed reduction of acetylene to ethylene and then quantitative measurement of the two gases by gas chromatography (Dilworth, 1966).

14ml wheaton vials with rubber septum (gas proof) were used in the test that was performed as the following:

- Transfer 10ml culture into a vial and cover it directly under flushing of the proper gas (Argon or dinitrogen).
- Inject 0.1ml highly pure acetylene (> 99.6 % vol.) through the rubber septum into the vial.
- Incubate at 37°C and 210 rpm agitation for 30 min.
- Inject 50µl acetic acid (100%) into the vial.
- Take 0.1ml gas from the vial for GC.

Samples were taken for the activity assay at appropriate intervals up the point of cultivation initiation until the death phase. Thus, it would be possible to assign the stage at which the enzyme has the highest value of activity, or in other words, the highest enzyme concentration. Activity was estimated as a percentage of the produced ethylene.

2.2.4. Reverse transcription polymerase chain reaction (RT-PCR):

RT-PCR is an effective technique to check whether genes are expressed under certain conditions or not. The RNA transcript of the target gene is first reverse-transcribed into its DNA complement (*complementary DNA, or cDNA*) using the enzyme reverse transcriptase, and the resulting cDNA is subsequently amplified using traditional PCR. The two-step RT-PCR processes for converting RNA to DNA subsequent PCR amplification of the reversely-transcribed DNA are:

- First strand reaction: complementary DNA (cDNA) is made from an mRNA template using dNTPs, primers and reverse transcriptase. The components are combined in a reverse transcriptase buffer for an hour at 37°C.

- Second strand reaction: after the reverse transcriptase reaction is complete, cDNA has been generated from the original single strand mRNA, standard PCR (called the "second strand reaction") is initiated.

2.2.4.1. RNA-prep with phenol from *W. succinogenes*:

Wolinella succinogenes cells were grown on FuFo medium (under ammonium-limited condition when needed) till the mid of log phase and then aliquoted to 10-15ml culture and harvested at 4800rpm and +4°C; pellet was used directly for RNA-prep or stored at -20°C for further use. For RNA-prep, the following protocol was applied:

- Resuspend cell pellet (from 10-15ml medium OD=0.2-0.3) in 125 µl Suc/NaAc (0.3 M Sucrose & 10 mM NaAc pH 4.5 – 5.2) (cooled)
- Add 125 µl SDS/NaAc (2% SDS & 10mM NaAc pH 4.5 – 5.2)
- Sample for 1.5 min at 65°C
- After that 3x times:
    - Add 400 µl Phenol (65°C)
    - 3 min at 65°C
    - Sample for 30 sec in liquid nitrogen
    - 13.000 rpm for 10 min at room temperature
    - Transfer the supernatant to a new e-cup
- Add 40 µl 3M NaAc, pH 5,2 and 1 ml EtOH (cooled) and incubate over night at -20°C or for 3 h at -70°C (not more than -70°C)
- Centrifuge (13.000 rpm) at 4°C for 20 min
- Wash pellet with 70% EtOH
- Centrifuge for 2min (13.000 rpm)
- Dry Pellet for 2 min under sterile conditions (speed-vac)
- DNAse-treatment
    - Dissolve pellet in 180 µl (RNAse free or DEPC [0.1% DEPC in ddH2O, mix overnight on roller and autoclave 120°C for 20min])
    - Add 20 µl 10x DNAse buffer
    - Add 2 µl DNAseI (RNAse free)
    - Incubate for 30 min at room temperature
- After that 3x times:

- Add 200µl chloroform/isoamylalcohol (24:1) or just chloroform, vortex shortly
- Centrifuge (13.000 rpm) for 2 min at 4°C
- Transfer the supernatant (the aqueous phase; being careful not to transfer any of the protein at the phase interface) to a new e-cup

- Add 25 µl 3M NaAc, pH 5,2 and 900 µl EtOH and incubate at -20°C over night or at -80°C for 3 h
- Centrifuge (13.000 rpm) at 4°C for 20 min
- Wash pellet with 70% EtOH
- Centrifuge for 2min (13.000 rpm)
- Dry Pellet for 2 min under sterile conditions (speed-vac)
- Store @ -20°C or dissolve pellet in 80 µl H2O (RNAse free or DEPC) for direct use.

2.2.4.2. RNA quantification:

Dissolved RNA was quantified spectrometrically using absorption at 260 nm (A260). An A260 reading of 1 is equivalent to 40µg/ml of single strand RNA. A_{260}/A_{280} and A_{260}/A_{230} were measured to control the purity grade of extracted RNA where the first value indicates contaminations of protein and the second one refers to salts concentration.

2.2.4.3. First strand cDNA synthesis:

A protocol from Fermentas was applied to perform reverse transcription of extracted RNA:

- Add into sterile nuclease-free tube on ice in the indicated order:
  - Template RNA (10ng – 5µg)
  - Primer: Oligo$(dt)_{18}$ (0.5µg or 100pM)
  - DEPC-treated water: to 12.5µl
- Optional: If RNA template is GC rich or is known to contain secondary structures, mix gently, centrifuge briefly and incubate at 65°C for 5min, chill on ice, briefly centrifuge and place on ice
- Add the following components in the indicated order:
  - 5X reaction buffer: 4µl
  - RiboLock RNase inhibitor: 0.5µl (20u)
  - dNTP mix, 10mM each: 2µl (1mM final concentration)
  - M-MuLV Reverse Transcriptase: 2µl (40u)

- Mix gently and centrifuge briefly
- Incubate 60min at 37°C
- Terminate the reaction by heating at 70°C for 10min.
- The reverse transcription reaction product can be directly used in PCR or stored at -20°C.
- Use 2µl of the reaction mix to perform PCR in 50µl of reaction volume

2.2.4.4. Polymerase chain reaction (PCR):

The Polymerase chain reaction (Mullis and Faloona, 1987; Saiki et al., 1988) is an *in vitro* enzymatic method for the exponential amplification of any DNA fragment. This technique enables the selective amplification of a short, exactly defined part of a DNA strain out of a complex DNA mixture.

All PCR applications employ a heat-stable DNA polymerases, such as Pfu and Taq polymerases. These DNA polymerases enzymatically assemble a new DNA strand from DNA building blocks, the nucleotides, by using single-stranded DNA as a template and DNA primers, which are required for initiation of DNA synthesis. A basic PCR set up requires several components and reagents. These components include:

- DNA template that contains the DNA region (target) to be amplified.
- Two primers, which are complementary to the DNA regions at the 5' and 3' ends of the DNA region.
- DNA polymerase with a temperature optimum at around 70°C.
- Deoxynucleoside triphosphates (dNTPs), the building blocks from which the DNA polymerases synthesize a new DNA strand.
- Buffer solution, providing a suitable chemical environment for optimum activity and stability of the DNA polymerase.
- Divalent cations, magnesium or manganese ions; generally Mg^{2+} is used.

PCR usually consists of a series of 20 to 40 repeated temperature changes called cycles; each cycle typically consists of 2-3 discrete temperature steps. Most commonly PCR is carried out with cycles that have three temperature steps (figure2-4). Temperatures and length of time that are applied in each cycle depend on a variety of parameters. These include the enzyme used for DNA synthesis, the length of amplified gene, the concentration of divalent ions and dNTPs in the reaction, and the melting temperature (Tm) of the primers. Steps required for the whole process are:

- **Initialization step:** This step consists of heating the reaction to a temperature of 94-96°C, which is held for 1-9 minutes. It is only required for DNA polymerases that require heat activation by hot-start PCR.

- **Denaturation step:** This step is the first regular cycling event and consists of heating the reaction to 94-98°C for 20-30 seconds. It causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.

- **Annealing step:** The reaction temperature is lowered to 50-65°C for 20-40 seconds allowing annealing of the primers to the single-stranded DNA template. Typically the annealing temperature is about 3-5°C below (Tm) of the primers used.

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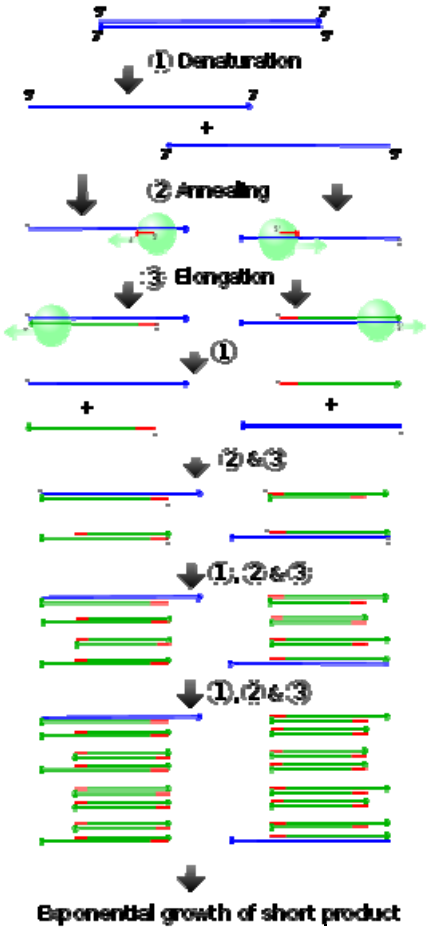


Figure (2-4): Schematic drawing of the PCR cycle. (1) Denaturing at 94-96°C. (2) Annealing at ~65°C (3) Elongation at 72°C. Four cycles are shown here. The blue lines represent the DNA template to which primers (red arrows) anneal that are extended by the DNA polymerase (light green circles), to give shorter DNA products (green lines), which themselves are used as templates as PCR progresses.

2.2.5. Protein purification:

All procedures were carried out under anaerobic conditions and all buffers were anaerobized and supplied with dithionite to remove traces of oxygen when needed.

Swelling buffer	Cracking buffer	Buffer A	Buffer B	Buffer G
500 ml ddH2O + 8,8 g NaCl + 6 g Tris Adjust with HCl to pH 8,2 Add 500 g Glycerol Adjust to 1L with ddH2O	800 ml ddH2O + 9 g NaCl + 6 g Tris Adjust with HCl to pH 8,2 Adjust to 1L with ddH2O (+ 2 mM Dithionite)	50 mM Tris pH 7.75 (+ 2 mM Dithionite)	50 mM Tris pH 7.75 + 500 mM NaCl (+ 2 mM Dithionite)	50 mM Tris pH 7.75 + 100 mM NaCl (+ 0,5 mM Dithionite)

2.2.5.1. Cell disruption:

A combination of two disruption techniques was employed to achieve the minimum levels of exposure to oxygen. First one is osmotic shock and the second is fluidization. Cells were suspended in 3-4 fold swelling buffer inside an anoxic glove box and left with stirring for 20min and then centrifuged at 10000 rpm and 4°C for 10-20 min; gas-tight tubes were used during centrifugation outside the anoxic glove box. Supernatant was discard and cells were resuspended in 3-4 folds cracking buffer supplemented with 2 mM sodium dithionite and left for 20 min with stirring inside the anaerobic glove box. The Lysate was placed on ice and transported anaerobically to a microfluidizer (S100, Microfluidics, Newton, USA), which was previouslyy washed with buffer A (+ 2mM dithionite), where it was subjected to one passage through the microfluidizer and collected directly in an anaerobized gas-tight flask that was cooled on ice. After centrifugation for 1 h at 30000 rpm and 4°C, the dark brown supernatant was ready for purification by ion-exchange chromatography.

2.2.5.2. Ion-exchange chromatography:

Ion exchange is a process in which one type of ion in a compound is exchanged for a different type; cation for cation and anion for anion.

The supernatant was positioned on ice under positive inert gas pressure and loaded on DEAE sepharose column (GE Healthcare, Germany) equilibrated anaerobically with buffer A (+2 mM sodium dithionite) at room temperature. Eluted proteins were monitored by measuring the absorbance at 280 nm. After washing the column with the equilibrating buffer, an additional washing step with 20% buffer B (100 mM NaCl) was applied until the bottom of the column turned white. A dark band of bound proteins was formed on the top of the column during loading of supernatant and was still there after the washing steps; that was a good sign for binding of nitrogenase components. Elution was achieved by a linear gradient of buffer B till 100% of buffer B (100 till 500 mM NaCl). Fractions were collected in anaerobized flasks that were cooled on ice and maintained under positive inert gas pressure. The molybdenum iron protein fraction has a dark green-brownish color and eluted at about 240 mM NaCl while the iron protein has a yellow color and is eluted at about 320 mM NaCl. Molybdenum iron protein fraction was diluted 2-3 fold with buffer A and loaded

on 5 ml HiTrap Q column (GE Healthcare, Germany) for concentration. After washing with buffer A, the protein was eluted with buffer B by reversing the flow direction of the buffer. Concentrated protein was divided to aliquot of 1 ml, frozen in liquid nitrogen, and stored at – 80°C.

2.2.5.3. Size exclusion chromatography:

Size exclusion chromatography is a chromatographic technique in which the separation is based on differences in the size of the sample molecules. The column material consists of a porous gel. The size of the pores determines the molecular weight range of the compounds that can be separated. A general rule is that compounds that differ by 10% in size can be separated on the same column. A series of columns, each fitted with a different exclusion limit gel, can be used to separate a multiple-component mixture.

When molecules of various sizes are loaded into the column, the large molecules cannot fit at all and will be directly washed out of the column with the solvent. The smaller molecules can permeate the gel; the smaller the molecule, the farther the penetration and the greater the retardation. This results in a separation because it requires more solvent to wash the smaller molecules out of the gel.

A further purification step was carried out by size exclusion chromatography whereby the protein was simultaneously rebuffered and separated from impurities and misfolded protein. A Superdex 200 16/ 60 column (GE Healthcare, München, Germany) was anaerobically equilibrated with buffer G (+ 0.5 mM sodium dithionite) and then 0.5 – 1 ml Molybdenum iron protein was loaded into the column. The purified protein solution was collected in an anaerobized flask positioned on ice under positive inert gas pressure. Then the protein solution was transported into the anoxic glove box and concentrated anaerobically by ultrafiltration membrane with cut-off size of 100 kDa (Vivaspin, Sartorius, Germany). Samples for defining the protein concentration and analyzing by SDS-PAGE were taken before freezing the protein in liquid nitrogen and storing it at – 80°C for further use or using it directly for crystallization trials.

2.2.5.4. SDS-PAGE:

Proteins were separated according to their electrophoretic properties by SDS-PAGE (Raymond, 1959 and Davis, 1959). The principle of this method lies in denaturing of proteins to its primary peptide chains and charging it negatively by boiling with the loading buffer. The negatively-charged peptide chains are left to run through the matrix of gel, which is positioned between cathode and anode. The shortest peptide chains run more quickly than the long ones meaning that peptide chains will be separated according to its molecular weight.

Protein samples were mixed with loading buffer and boiled for 5 min at 95°C. After centrifugation for 1 min at 4°C, samples were loaded into the stacking gel pockets and the gel was connected to the power unit.

SDS gels were prepared with the Hoefer miniVE SDS gel system (GE Healthcare, München, Germany). The separation was carried out at 300 V and 25 mA for one gel and at

300 V and 50 mA for two gels. By application of an electric field, molecules are separated according to their charge and size, so that the negatively charged peptide chains migrate across the electric field to the anode.

Subsequently, the gels were incubated in 20 ml Coomassie staining solution for about 30 minutes. After destaining with 10% ethanol over night, gels were analyzed and documented with a GelDoc 2000 (Biorad, München, Germany).

For sizing of proteins by SDS-PAGE, the unstained protein molecular weight marker from MBI Fermentas was utilized (figure 2-2).

Resolving gel 12.5%	**Volume**
30% bis-acrylamid	3.8 ml
1.88 mM Tris pH 8.8	1.8 ml
0.5% SDS	1.8 ml
TEMED	16 µl
10% APS	45 µl
ddH_2O	1.6 ml

Stacking gel 5%	**Volume**
30% bis-acrylamid	0.5 ml
0.625 mM Tris pH 8.8	0.6 ml
0.5% SDS	0.6 ml
TEMED	6 µl
10% APS	15 µl
ddH_2O	1.3 ml

Coomassie Staining solution	**Destaining solution**
10% ethanol 5% acetic acid 0.002% Coomassie (G250:R250 = 4:1)	10% ethanol

5 X loading buffer	**1 X running buffer**
0.5 M Tris/HCl pH 6.8	25 mM Tris base
40% glycerol	192 mM glycine
8% (w/v) SDS	0.1% (w/v) SDS
0.004% bromphenol blue	

2.2.5.5. Determintation of protein concentration:

Protein concentration was determined by the bicinchoninic acid method (BCA), which is based on the biuret reaction (Smith et al., 1985). The biuret test is based on the reduction of Cu(II) to Cu(I) upon contact with the peptide bonds of proteins. The total protein concentration is measured as the color change of the sample solution from green to purple in proportion to protein concentration.

2.2.5.6. Measurement of absorption spectra:

Iron sulphur clusters have a characteristic absorbance at 400 nm. Therefore, absorption spectra of purified protein were recorded to assure the presence of nitrogenase components, which possess such clusters, in reduced form. With a wavelength range of 200-600 nm, spectra were recorded using an *Ultrospec™ 2100 pro* spectrophotometer (GE Healthcare, München, Germany). All measurements were carried out under strictly anaerobic conditions. Cuvettes of 1ml volume with air-tight cover were utilized for both of the sample and the blank. As a blank, 1ml of the same buffer, which is used for the purification process, was used.

2.2.6. Mass spectrometry:

Mass spectrometry is an important, emerging method for the characterization of proteins. The two primary methods for ionization of whole proteins are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI). Two approaches are used for characterizing proteins. In the first, intact proteins are ionized by either of the two techniques described above, and then introduced to a mass analyzer. This approach is referred to as "top-down" strategy of protein analysis. In the second, proteins are enzymatically digested into smaller peptides using proteases such as trypsin or pepsin, either in solution or in gel after electrophoretic separation. Other proteolytic agents are also used. The collection of peptide products are then introduced to the mass analyzer. When the characteristic pattern of peptides is used for the identification of the protein, the method is called peptide mass fingerprinting (PMF), if the identification is performed using the sequence data determined in tandem MS analysis it is called de novo sequencing. These procedures of protein analysis are also referred to as the "bottom-up" approach.

2.2.6.1. Solutions:

All solutions are made in microcentrifuge tubes. Stock solutions are pipetted directly from bottles into microcentrifuge tubes for further use.

- Stock solutions:
  1) Make fresh weekly in plastic bottles:
     a) 1 M $CaCl_2$ (14,7 g/100ml ddH_2O)
     b) 1 M Ammonium Bicarbonate (7,906 g/100ml ddH_2O)
  2) Make fresh monthly in glass bottles:
     a) 2% Trifluoroacetic acid (TFA) in water (0,5ml TFA in 25ml ddH_2O)
     b) 0,1% TFA in water (0,5ml TFA in 500ml ddH_2O)
- Solutions for daily use:
  - 50% Acetonitrile (ACN) in water (250 µl of ACN + 250 µl ddH_2O)

- 100 mM Ammonium Bicarbonate (100 µl of 1 M Ammonium Bicarbonate in 900 µl ddH_2O)
- Digestion buffer without Trypsin (according to the protocol from Sigma): 40 mM Ammonium Bicarbonate in 9% ACN
- Trypsin digestion buffer (according to the protocol from Sigma): solution is prepared by adding 100 µl of 1mM HCl to one vial of Trypsin (containing 20 µg Trypsin). The vial is mixed briefly to ensure dissolving of the Trypsin. 900 µl of 40 mM ammonium bicarbonate in 9% acetonitrile solution is added to the vial and mixed. The final concentration of Trypsin is 20 µg/ml.
- 30% ACN + 70% trifluoroacitic acid (TFA): 300 µl ACN + 700 µl TFA
- 60% ACN + 40% trifluoroacitic acid (TFA): 600 µl ACN + 400 µl TFA

2.2.6.2. Coomassie-stained gel preparation:

SDS gel of 10% for resolving gel and 5% for stacking gel was prepared with the Hoefer SDS gel system (GE Healthcare, München, Germany). The separation was carried out at 100 mA and 300 V for one gel.

2.2.6.3. In-gel digestion:

- Washing and De-staining:

1) Before excising bands, gel is washed in ddH_2O for 15 min.
2) Bands are excised in form of cubes 1 × 1 mm (cut should be as close to the band as possible to minimize excess gel material) and placed in clean 1ml-tubes.
3) 100 µl ddH_2O are added to each cube in the 1ml-tube and incubated for 15 min (washing should be repeated 2×).
4) dd ddH_2O is pulled off and 40 µl of 50/50 (ACN/ ddH_2O) are added and incubated for 15 min.
5) Solution is pulled off and 40 µl of ACN are added and incubated until the gel pieces become white and sticky.
6) ACN is pulled off and 40 µl of 100 mM Ammonium Bicarbonate are added and incubated for 5min.
7) 40 µl of ACN are added to make 1:1 solution and then incubated for 15 min.
8) Solution is pulled off and samples are dried in a speed vacuum system for approximately 15 min (samples must be very dry).

- Digestion:

a) 20 µl of Trypsin digestion solution (containing 0,4 µg Trypsin) are added to the dried samples and incubated 45 min on ice (according to the protocol from Sigma). More solution is added if the samples absorbed the whole solution.

b) Solution is pulled off and discarded. 20 - 60 µl (enough to keep gel pieces covered overnight) of digestion buffer without Trypsin are added and incubated at 37°C over night.

- Extraction of peptides:
  - Solution is removed from the incubated samples into new clean microcentriguge tube and dried in a speed vacuum system.
  - The gel pieces are covered with 25 – 70 µl (for each single gel slice) of 0,1% TFA and placed in a floating rack in sonicating water bath. Extraction with sonication is applied for 30 min. Solution is combined with the last one (in step 1) and dried in a speed vacuum system too.
  - The last step is repeated with 30% of ACN + 70% of 0,1% TFA.
  - The last step is repeated with 40% of ACN + 60% of 0,1% TFA.
  - The last step is repeated with 100% of ACN.
  - The speed vacuum system is applied on the pooled solutions to remove TFA and ACN traces and then the dried peptide samples are stored at –20°C until analysis by MS.

2.2.6.4. Peptide sequence analysis:

Peptides were reconstituted in an aqueous solution of 0.1% v/v formic acid for separation by nano LC followed by analysis with ESI-QTOF-MS/MS.

One microliter of sample was introduced onto a µ-precolumn cartridge (C18 pepMap, 300 µm × 5 mm; 5 µm partical size) using a CapLC autosampler (Waters) and further separated through a C18 pepMap100 nano Series (75 µm × 15 cm; 3 µm partical size) analytical column (LC Packings). The mobile phase consisted of solution A [0.1% FA in 5% ACN] and solution B (0.1% FA in 95% ACN). The single sample run time was set to 60 min and the pump flow rate to 5 µL/min. An exponential gradient was initiated 5 min after sample loading beginning with 10% B/90% A and ending with 95% B/5% A at 50 min. A flow splitter was used to achieve a tip flow rate of 250 nL/min. The chromatographically separated peptides were analysed using a Q-TOF Ultima Global (Micromass, Manchester, UK) mass spectrometer equipped with a nanoflow ESI Z-spray source in the positive ion mode. The nanospray needle was held at 2 kV and the source temperature at 40 °C. Multiply charged peptide parent ions were automatically marked and selected in the quadrupole, fragmented in the hexapole collision cell and their fragment patterns were analysed by time-of-flight. Data acquisition was performed using MassLynx (v 4.0) software on a Windows NT PC. Following data acquisition, data files were initially processed using the Protein-Lynx-Global-Server (v 2.1), (Micromass, Manchester, UK) module to generate Mascot-searchable *.pkl files. Processed data were searched against MSDB and Swissprot databases through a Mascot search engine using a peptide mass tolerance of 100 ppm.

3. Results:

3.1. Comparison of *nif* genes products:

An alignment of the three structural subunits of nitrogenase NifH, NifD, and NifK from *Wolinella* in comparison to other known nitrogen-fixing bacteria reveals a lot of similarity especially in regions that contain motifs and ligands that are important for functionality of the enzyme (figure3-1).

Regarding NifH, residues 9-16 exhibit the Walker motif A (GXXXXGKS) that represents the nucleotide-binding site. Residues 125-128 exhibit the second Walker motif (DXXG) that in combination with motif A completes the Mg-phosphate protein interactions. Switch I (residues 39-46) and switch II (residues 139-150) regions that are crucial for functionality because they are involved in the conformational changes upon hydrolysis of the ATP, are also conserved. Cys97 and Cys132 are ligands of the 4Fe4S cluster. Arg100 is involved in the inhibition of nitrogenase activity in the presence of ammonium by posttranscriptional modification of the iron protein by DraT, which adds an ADP-ribose moiety to the Arg100 (Lowery, 1988). This positively charged residue, an invariant surface residue, has been identified in the structure as being close to the [4Fe:4S] cluster on the same face as Gln112 and is most likely to have a critical role in the formation of a bridge with other negatively charged residues of the molybdenum iron protein. Iron protein variants of R100 are either completely inactive or have greatly reduced activity. All the last residues were found to be conserved in NifH of *W. succinogenes*. However, *Wolinella*'s NifH has more amino acids than the others and these additional amino acids are depicted in the alignment as insertion region (residues 85-96), which seems to be common among the epsilon-proteobacteria members, *Sulfurospirillum multivorans* and *Arcobacter nitrofigilis*..

The important residues in NifD (α subunit) and NifK (β subunit) are αCys275 and αHis442 that ligate the cofactor in addition to αCys62, αCys88, αCys154, βCys70, βCys95, and βCys153 which ligate the P-cluster. These residues are conserved in NifD and NifK of *W. succinogenes*. More important conserved residues, which affect the environment around the FeMo-cofactor and subsequently substrate reduction, are αHis274, αHis451, αTrp444, αGly69, αGly356, αGly357, αArg359, αArg361, αLys426, αHis362, and αHis195. When the last residue is substituted by Glu the resulted enzyme does hardly reduce N_2 but still reduces acetylene and protons at near wild-type rates.

Conservation of the important residues in the three structural subunits of *Wolinella's* nitrogenase, in addition to the high similarity with the other nitrogenase sequences of known nitrogen-fixing bacteria, implies that *Wolinella* is able to build an active nitrogenase system.

Furthermore, all nif genes that are responsible for expression of products involved in the FeMo-cofactor biosynthesis and insertion are present in Wolinella (Table 1-3). The only missed gene is nifQ, which is believed to mobilize molybdenum during assembly of FeMo-cofactor on NifEN (Imperial, 1984 and Hernandez, 2008). However, the role of NifQ could be compensated by increasing the molybdenum supplement (Rodriguez-Quinones, 1993). In the case of W. succinogenes, increasing the molybdenum concentration did not affect the

rate of acetylene reduction (data are not shown) suggesting that the role of NifQ could be compensated by alternative mechanism.

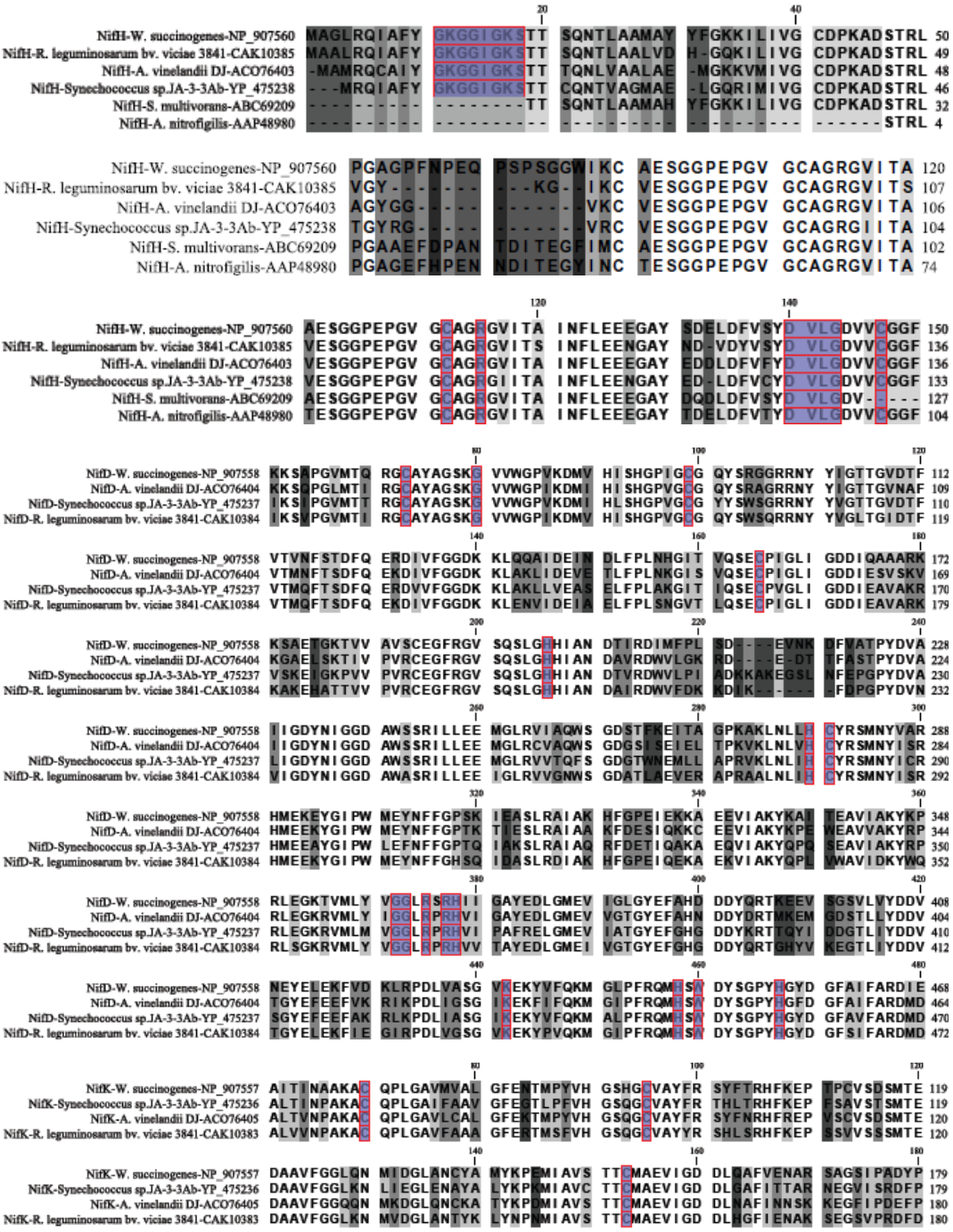


Figure (3-1): Alignment comparison of NifH, NifD, and NifK from *Wolinella succinogenes, Azotobacter vinelandii DJ, Synechococcus sp. JA-3-3Ab, Rhizobium leguminosarum bv. Viciae 3841, Sulfurospirillum multivorans, and Arcobacter nitrofigilis*. Only sections that contain the important ligands and motif regions are shown. Only NifH alignment of the last two bacteria is used, because sequences of NifD and NifH are not available on NCBI database yet.

Gene	Description
nifB	FeMoco biogenesis
nifV	homocitrate synthase
nifE	FeMoco maturation, α subunit
nifN	FeMoco maturation, β subunit
nifS	[Fe:S] biogenesis; cysteine desulfurase
nifU	[Fe:S] biogenesis; cluster assembly
nifH	Fe protein
nifD	MoFe protein, α subunit
nifK	MoFe protein, β subunit
nifX	regulation of the *nif* regulon
nifW	possibly O_2-protection ?
nifZ	P-cluster maturation
rpoN	σ^N transcription factor
glnK	P_{II} family regulator protein
glnB	P_{II} family regulator protein

Table (3-1): Genes involved in biosynthesis and regulation of nitrogenase in *W. succinogenes*

3.2. Expression of *nif* genes in *W. succinogenes*:

Wolinella is not known to be able to grow diazotrophically; therefore it was important to make sure that its *nif* genes could be expressed under nitrogen-limiting conditions. RT-PCR was used to detect mRNA of *nifH* and *nifD* genes in a culture of *Wolinella* grown without afixed-nitrogen source. Harvested cells from *Wolinella* cultures grown under nitrogen-limiting conditions were subjected to RNA isolation and then cDNA was obtained using reverse transcription. As template for next PCR step, the last cDNA was used with primers for the *nifH* and *nifD* genes. The presence of bands on an Agarose gel of the PCR products indicates subsequently the presence of mRNA of the corresponding genes (lane1 and 3 in figure3-2).

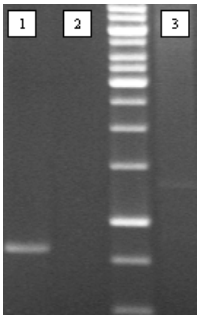


Figure (3-2): 1% agarose; marker: 1kbp ladder from Fermentas; lane 1: nifH; lane 2: nifH and nifD control (cells grown with ammonium); lane 3: nifD

In parallel, a negative control was prepared using cells obtained from a culture of *Wolinella* grown under excess of ammonium chloride; no bands were detected on the gel (lane 2 in figure3-2) suggesting that no expression of *nifH* and *nifD* had occurred in this case.

3.3. Growth models and nitrogenase activity:

Different media and growth conditions were utilized to optimize concurrently the yield of cells and the enzyme activity. In the case of *A. vinelandii* and *G. sulfurreducens*, which are known to be capable of diazotrophic growth, cells were routinely left to grow in media with nitrogen deprivation using ammonium chloride as sole nitrogen source. But for *W. succinogenes* that is not considered a diazotroph, growth under nitrogen-limiting conditions was a challenge in the term of cells yield and the enzyme activity, respectively.

Expression of the nitrogenase system was monitored by assaying cells at different points of growth period. The routine assay of nitrogenase, reduction of acetylene to ethylene, was carried out by gas chromatography with flame ionization detector (Agilent, USA). Figure (3-3) shows one chromatogram of a sample with acetylene and ethylene peaks.

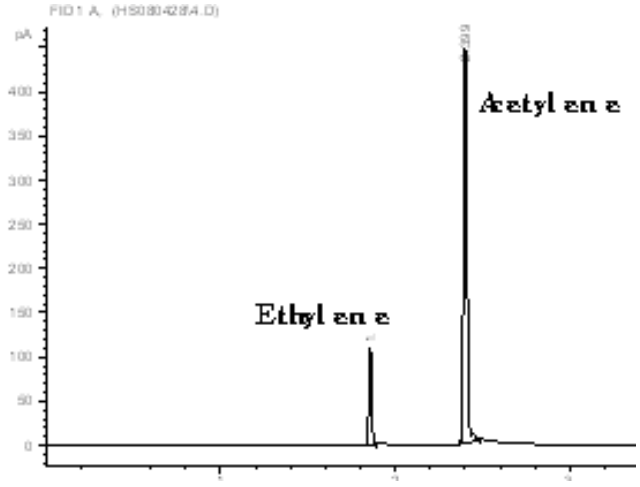


Figure (3-3): Chromatogram of gas chromatography

3.3.1. Growth of *A. vinelandii* in Burke's medium:

Figure (3-4) shows the growth curve of *Azotobacter vinelandii* grown in a batch system. Cells were grown in 1L Burke's medium after inoculation with 10ml preculture and incubation at 37°C and 210rpm agitation for 2.5 days.

The lag phase is noticeable in this case, nearly 10 hours, and the exponential growth has taken 30 hours wherein cells are dividing regularly by binary fission, and are growing by geometric progression. Then, curve seems to reach the stationary phase where cells start to die because of nutrients exhaustion and accumulation of inhibitory metabolites or end products.

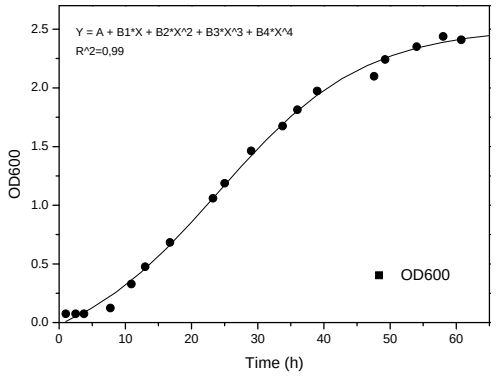


Figure (3-4): growth curves of *Azotobacter* with fixed nitrogen source; 1L medium in 2L-flask

3.3.2. Growth of *A. vinelandii* diazotrophically:

Cells were grown in Burke's medium with low ammonium chloride concentrations and diauxie was obtained in different modes according to the fixed nitrogen concentration. In the first stage of growth, all available fixed nitrogen is consumed and upon exhaustion of this source of ammonium, another stage of growth is initiated where bacteria utilize gaseous nitrogen as a source of ammonium. After a second lag phase, an exponential growth was observed and simultaneously nitrogenase activity was detected. The second lag phase is necessary for bacteria to adapt with the new style of life. In this period, bacteria start to synthesize enzymes that are required for the new metabolic pathways, which compensate the lack in nutrient sources.

3.3.2.1. Growth of *A. vinelandii* with 10.5mM ammonium chloride:

Bacteria were grown in a batch system of 1L medium that contains 10.5mM ammonium chloride as a sole fixed nitrogen source and inoculated with 5ml of preculture. The culture was incubated at 37°C and 210 rpm agitation for 7 days (figure 3-5).

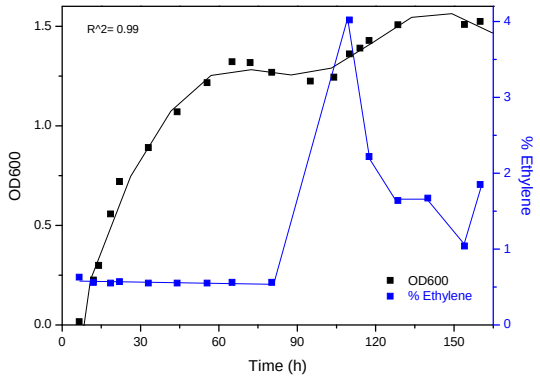


Figure (3-5): growth curve and nitrogenase activity of *Azotobacter;* 1L medium in 2L-flask with 10.75mM NH4CL

Diauxie and enzyme activity were observed after long time of growth and relatively at high OD value. As expected, the highest nitrogenase activity was obtained at the beginning of the second exponential growth phase. The activity in this case is relatively low because the long of the first phase of growth (nearly 4 days) where most of nutrients are consumed and a lot of end products are accumulated meaning that the batch is all over.

3.3.2.2. Growth of *A. vinelandii* with 0.1 and 0.2mM ammonium chloride:

One litre of Burke's medium without ammonium chloride in a 2L-flask was inoculated with 10ml preculture (containing 10.5mM NH_4Cl) and incubated at 37°C and 210rpm agitation for 25 h. The first stage of growth was short (~ 20 h) in comparison to the last one (~ 110 h). Nitrogenase was synthesized at the beginning of the first exponential growth but its activity reached the highest value not until the beginning of the second exponential growth phase (figure 3-6).

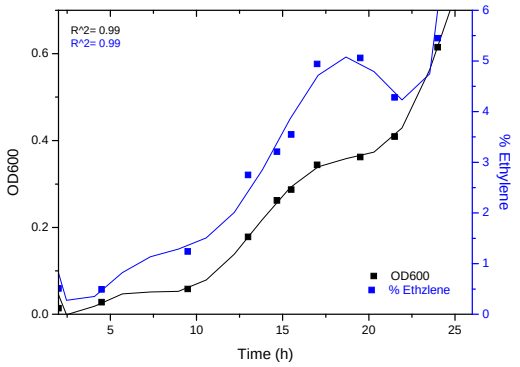


Figure (3-6): growth curves and nitrogenase activity of Azotobacter; 1L medium in 2L-flask with 0.1mM NH_4CL

Cultivation of *Azotobacter* in a fermentor is advantageous because it allows of controlling all parameters permanently and effectively during the growth period. Besides, a considerable amount of cells could be obtained at the same time. For that purpose, 10L Burke's medium was used and parameters were adjusted as the following: Temperature 37°C, pH 7.5, rotor 100rpm, and oxygen 20%. The medium was inoculated with 200ml preculture (containing 10.5mM NH_4Cl) and the OD was measured during 25 hours of growth (figure 3-7).

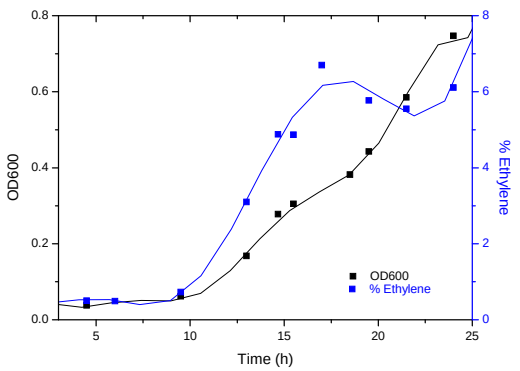


Figure (3-7): growth curve and nitrogenase activity assay of *Azotobacter;* 10L medium in fermentor with 0.2mM NH_4Cl

3.3.3. Growth of *G. sulfurreducens* in NBAF medium:

Figure (3-4) shows a growth curve of *Geobacter sulfurreducens* in the presence of 3.7mM ammonium chloride and 1 g/l yeast extract as source of fixed nitrogen.

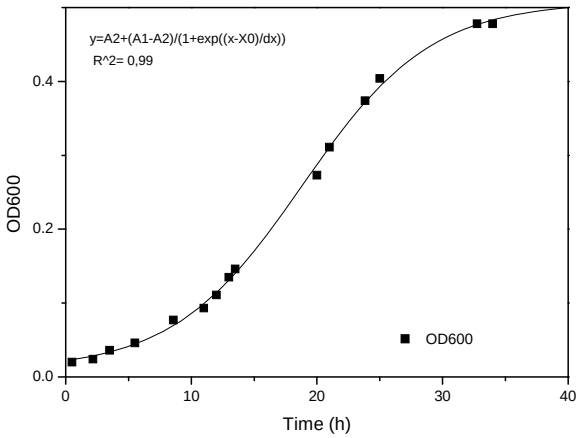


Figure (3-8): growth curve of *Geobacter* with fixed nitrogen source (NH_4Cl & YE); under inertal20 milieu

3.3.4. Growth of *G. sulfurreducens* diazotrophically:

Cells were grown in NBAF medium with limited amounts of ammonium chloride. Inertal20 and Argon/H_2 were served as over-medium gas to compare the behaviour of bacteria in the presence of N_2 and without it.

3.3.4.1. Growth with 170nM NH_4Cl and 45mg/l YE under Inertal20 milieu:

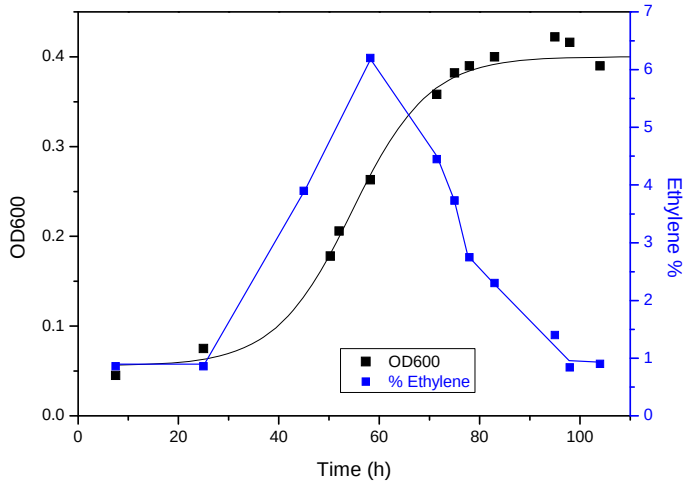


Figure (3-9): growth curve of *Geobacter* with 0.17mM NH_4CL and 45mg/l YE under inertal20 milieu

3.3.4.2. Growth with 68nM NH_4Cl and 18mg/l YE under Argon/H_2 and Inertal20 variously:

11L NBAF medium without ammonium chloride was inoculated with 200ml preculture (containing 3.7mM ammonium chloride and 1g/l yeast extract) and incubated under Argon/H_2 and N_2 variously for 7 days. During the first three days, the culture was incubated under Argon/H_2 and upon the beginning of stationary phase; N_2 was used as over-medium gas instead of Argon/H_2. During the next two days, another cycle of growth was initiated.

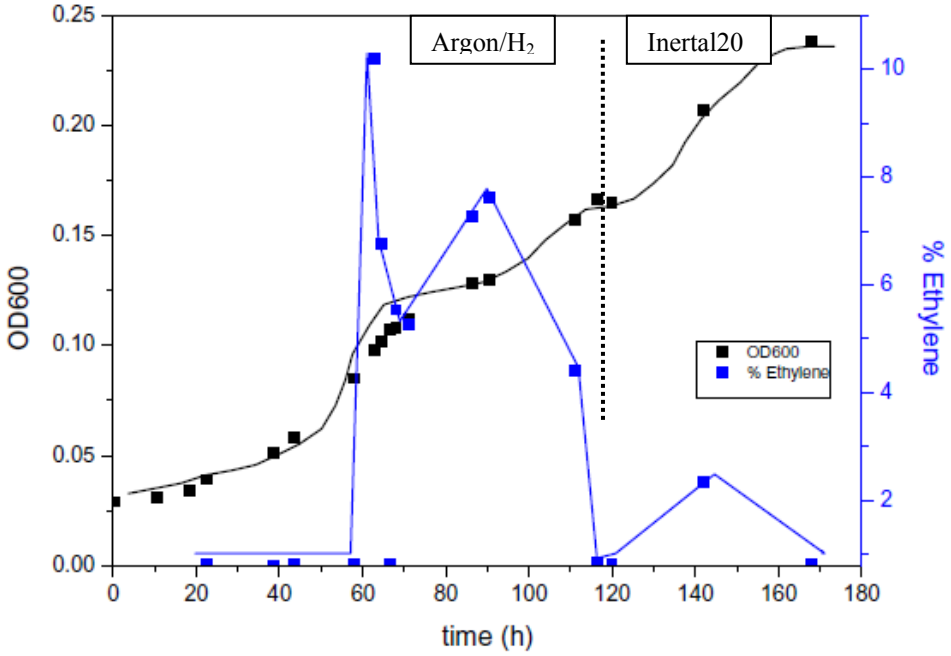


Figure (3-10): growth of *Geobacter* with 68nM ammonium chloride and 18mg/l YE under Argon/H_2 and Inertal20 variously

3.3.4.3. Growth with 34nM NH_4Cl and 9mg/l YE under Inertal20:

11L NBAF medium without fixed-nitrogen source was inoculated with 100ml preculture and incubated for 4 days at 30°C, pH 6.9, and 100rpm rotation.

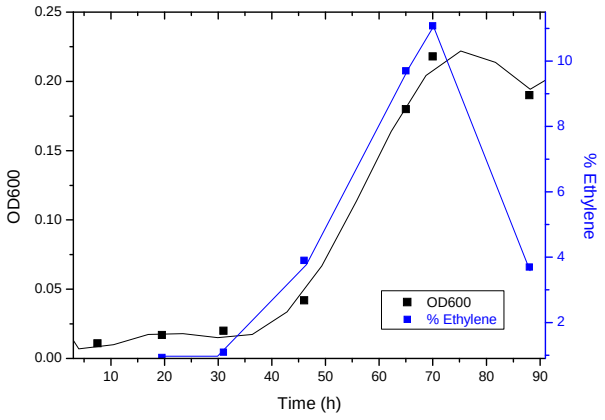


Figure (3-11): growth curve of *Geobacter* with 34nM ammonium chloride and 9mg/l YE; under Inertal20

3.3.5. Inhibition of nitrogenase activity by ammonium chloride in *Geobacter*:

11L NBAF medium without fixed-nitrogen source was inoculated with 100ml preculture and incubated for three days at 30°C, pH 6.9, and 100rpm rotation.

Upon reaching the highest enzyme activity, parallel samples were taken and supplemented with variant concentrations of ammonium chloride before injecting the acetylene into them. Then, produced ethylene was measured to check the mode of nitrogenase inhibition by NH_4Cl. Ammonium chloride concentrations were varied in the range of 0.2-0.02mM. A typical inhibition was noticed (figure 3-12) where the enzyme activity dropped gradually with increasing NH_4Cl concentration.

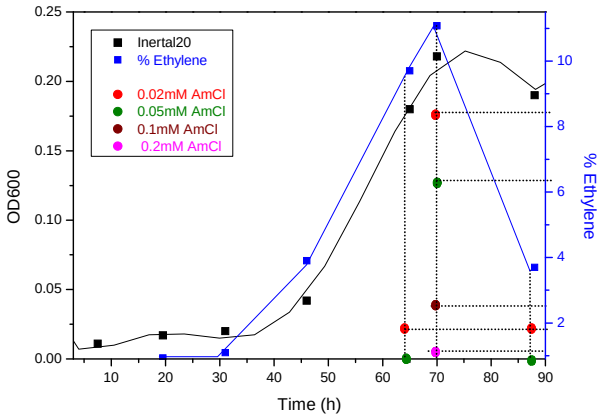


Figure (3-12): growth curve of *Geobacter* with 34nM ammonium chloride and 9mg/l YE; under Inertal20

3.3.6. Growth of *W. succinogenes* in Fumarate/Formate medium:

Wolinella was cultivated in fermentor using 11L FuFo-medium. As inoculums, 1L preculture grown in the same medium and supplemented with 5 g/l yeast extract was used. Growth conditions in fermentor were adjusted to 30°C, pH 7.8, and 60rpm rotation.

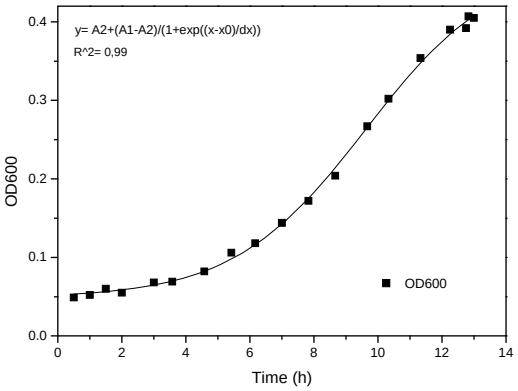


Figure (3-13): growth curve of *Wolinella* with 5mM ammonium chloride and 5mM ammonium sulfate; under inertal20 milieu

3.3.7. Growth of *W. succinogenes* at limited fixed-nitrogen conditions:

A wide range of concentrations of ammonium sulphate were tried in order to check the response of *Wolinella* to the decrease of a fixed nitrogen source. Results in figure (3-14) show growth curves of *Wolinella* in FuFo media without yeast extract and with different concentrations of ammonium sulphate (as a sole source of fixed nitrogen) that are used under inertal20 milieu and inoculated with precultures of 1L FuFo-medium without yeast extract as well. Cells were grown at 30°C, pH 7.8, and 60rpm rotation. It seems clear that *Wolinella* grows normally with 5mM ammonium sulphate and starts to be affected at lower concentrations where the half value of OD was obtained upon cultivation in a medium without ammonium sulphate. In the last case, cells utilize fixed nitrogen left over from the preculture medium (1L preculture used for inoculation contains 5mM AmCl and 5mM $AmSO_4$), *i.e.* 1.2mM ammonium will be available in the whole culture.

Similar results were obtained when the same concentrations of ammonium sulphate were applied for cultures grown under Argon/H_2 milieu.

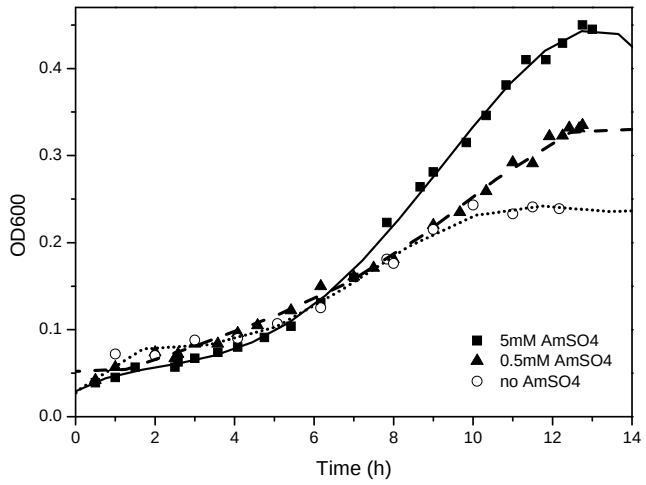


Figure (3-14): growth curves of *Wolinella* with different ammonium sulfate concentrations; under inertal20 milieu; precultures contain 5mM AmCl and 5mM $AmSO_4$

At very low fixed-nitrogen concentrations, cells grow badly, indicating inability of diazotrophic growth. Special media containing lower fixed-nitrogen concentrations were used to prepare precultures that were utilized as inoculum. Figure (3-15) shows two of growth curves that were obtained from *Wolinella* cultures grown in 11L FuFo-medium without any fixed-nitrogen source in a fermentor under Inertal20 milieu, 30°C, pH 7.8, and 60rpm rotation. Concentrations of 3mM and 10mM ammonium chloride in the 1L inoculums were used, *i.e.* 0.25mM and 0.83mM AmCl will be available for the whole culture respectively.

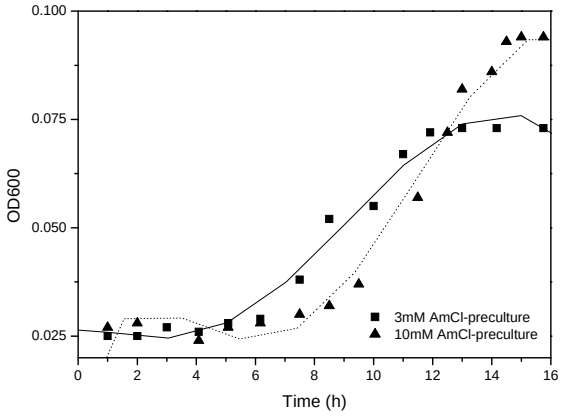


Figure (3-15): growth curves of *Wolinella* with different ammonium chloride concentrations in inocula; under inertal20 milieu; media were free of fixed-nitrogen

3.3.7.1. Growth of *Wolinella* with yeast extract as sole source of nitrogen:

Wolinella grows very badly without yeast extract. Therefore, yeast extract was utilized as a source of nitrogen instead of ammonium chloride and varying amounts were used to optimize the growth of cells and expression of the enzyme. Growth curves and nitrogenase activity were tried in *Wolinella* cultures (FuFo medium, $NaHCO_3$ buffer, 30°C, pH 7.8) grown under Inertal20 at three different yeast extract concentrations (175[+170nM AmCl and $AmSO_4$], 434, and 833 mg/L). Samples for enzyme activity were flushed with Inertal20. Results in figure (3-16) show that growth with 170nM of ammonium chloride and ammonium sulphate, in addition to 175mg/l yeast extract, gives the same maximum OD as the growth with only 833 mg/l yeast extract but the enzyme activity in the second case seems to be higher. Besides, the culture with only yeast extract seems to grow more rapidly, where it reaches the stationary phase earlier than the culture with ammonium and the enzyme activity peak is shifted in the same way, respectively.

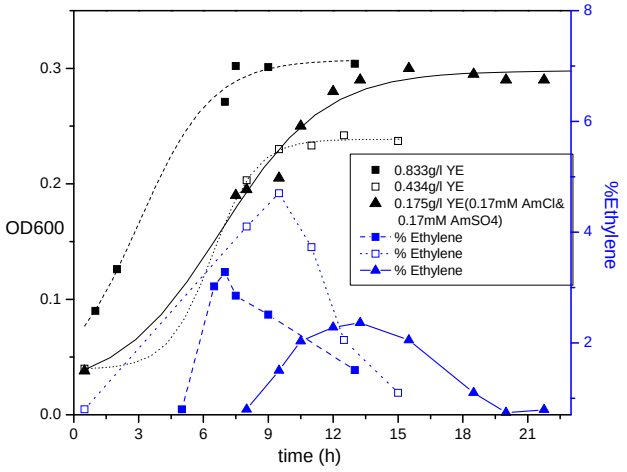


Figure (3-16): growth of *Wolinella* with yeast extract as sole of nitrogen source; under inertal20 milieu

3.3.7.2. Growth of *Wolinella* with different buffer systems:

Using $NaHCO_3$ was problematic because of the continuous change of the medium during cultivation under Inertal20, N_2 or Argon. In the case of Inertal20, the CO_2 concentration will increase in the medium gradually and the pH value goes down. To keep the pH constant, addition of a base is required; a matter that leads to increasing salt concentration along the growth period. Upon using N_2 or Argon/H_2, the same problem with reverse effect on the pH will be produced because of CO_2 migration from the medium to the gaseous phase. Instead, using a PO_4 buffer system yielded a higher enzymatic activity.

Growth curves and nitrogenase activity shown in figure (3-17) were obtained from *Wolinella* cultures (FuFo medium without NH_4, with 1.25g/l yeast extract, 25°C, and pH 7.8) grown under N_2 with $NaHCO_3$ and with PO_4 buffer. Samples for enzyme activity were flushed with N_2.

Moreover, Tris buffer [tris(hydroxymethyl)aminomethane] was tried and found to be suitable for use in *Wolinella* cultures. Although Tris contains an ammonium moiety, it was established that cells are not able to metabolize it as a source of fixed nitrogen.

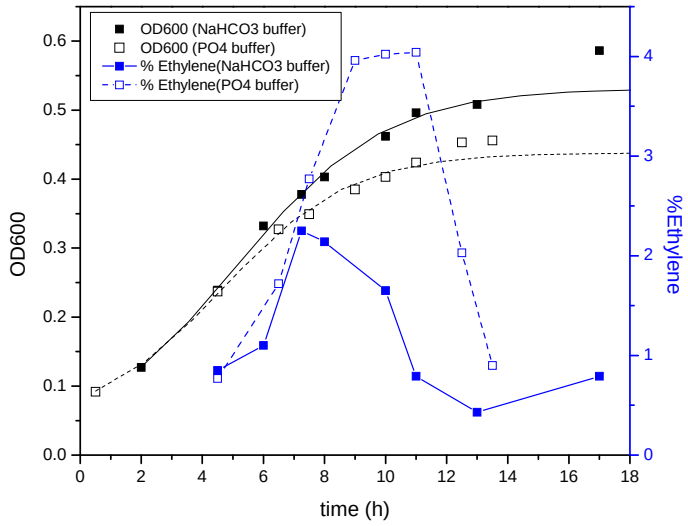


Figure (3-17): growth of *Wolinella* with different buffer system at nitrogen-limiting conditions; under N_2, 25°C, 1.25g/l YE, pH 7.8

3.3.7.3. Growth of *Wolinella* at different temperatures:

Because *Wolinella* is an enteric bacterium, it was essential to test its ability to express the acetylene-reducing enzyme at body temperature. Enzyme activity seems to be widely affected with temperature at which cultivation takes place. No activity was detected at 37°C and the activity at 23°C was higher than the one at 30°C (figure 3-18). Loss of activity at high temperature is not supposed to be posttranscriptional inhibition because all samples (taken for assay of enzyme activity) were incubated at 37°C.

Wolinella was cultivated in FuFo medium with 833mg/l yeast extract as a sole source of fixed nitrogen, $NaHCO_3$ buffer, and pH 7.8 under Inertal20 at three different temperatures 23, 30, and 37°C. Samples for enzyme activity were flushed with Inertal20.

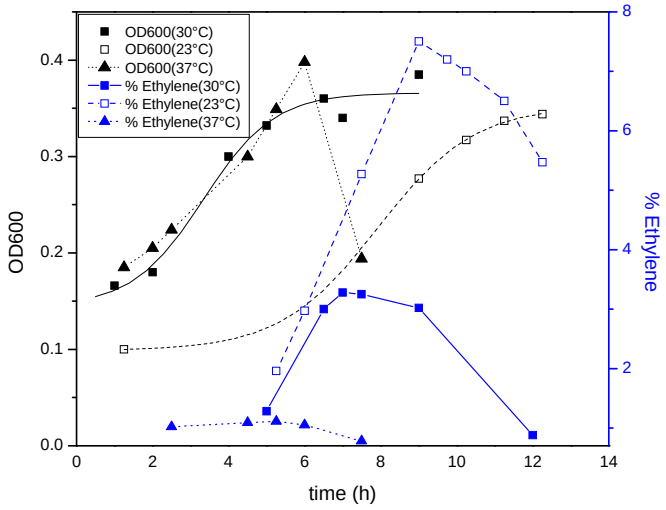


Figure (3-18): growth of *Wolinella* at different temperatures under nitrogen-limiting conditions; under Inertal20, 833mg/l YE, pH 7.8

3.3.7.4. Growth of *Wolinella* under different gasses:

Enzyme activity was unaffected upon cultivation under N_2 or Inertal20 while cultivation under Argon/H_2 raised the activity dramatically. The same results were obtained upon using Tris or $NaHCO_3$ buffer systems with different yeast extract concentrations. Besides, samples for enzymatic activity, taken from one culture grown under N_2 but flushed separately with N_2 and with Argon/H_2, showed a wide difference in enzyme activity. The highest enzymatic activity was obtained from samples flushed with Argon/H_2.

The growth curves and enzymatic activity presented in figure (3-19) were obtained from *Wolinella*'s culture grown in FuFo medium with 833mg/l yeast extract as a sole fixed nitrogen source and Tris buffer under N_2 and under Argon/H_2. Samples from cultures grown under N_2 were flushed with N_2 and samples from cultures grown under Argon/H_2 were flushed with Argon/H_2 as well.

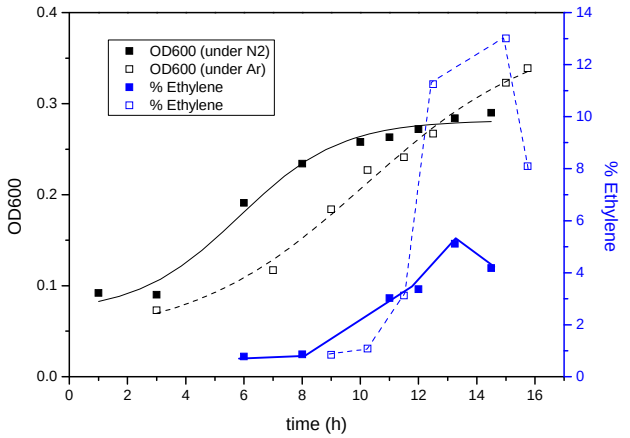


Figure (3-19): growth of *Wolinella* with different over-medium gases at nitrogen-limiting conditions; Tris buffer, 833mg/l YE, 25°C, pH 7.8

In figure (3-20), growth curves and nitrogenase activity were obtained from *Wolinella* cultures grown in FuFo medium with 1.25g/l yeast extract as sole fixed nitrogen source and $NaHCO_3$ buffer at 25°C and pH 7.8 under N_2 and under Argon/H_2. Samples from culture grown under N_2 were flushed with N_2 and samples from culture grown under Argon/H_2 were flushed with Argon/H_2.

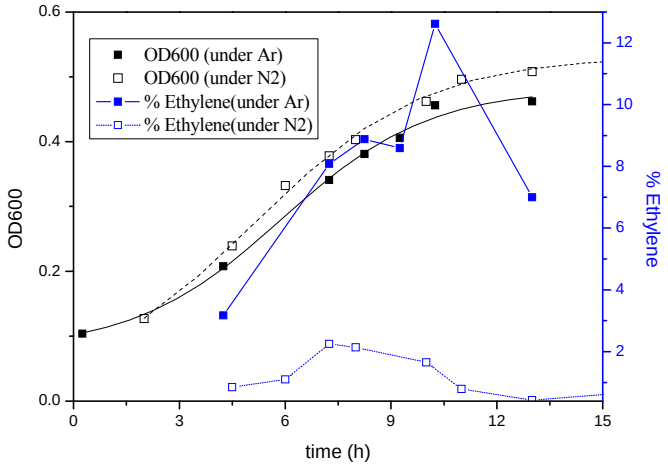


Figure (3-20): growth of *Wolinella* with different over-medium gases at nitrogen-limiting conditions; $NaHCO_3$ buffer, 1.25g/l YE, 25°C, pH 7.8

Wolinella cultures were grown under N_2 in FuFo medium with 833mg/l yeast extract, PO_4 buffer, 25°C, and pH 7.8. In parallel, two samples for an assay of enzymatic activity were taken at different phases of growth. One of them was flushed with N_2 and the second with Argon/H_2 and then both were handled in the same way. The difference in activity level is clear (figure 3-21).

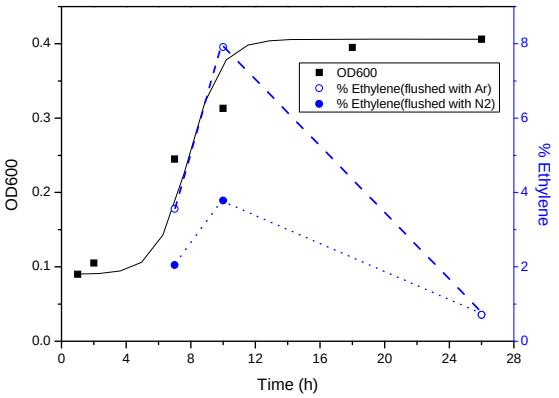


Figure (3-21): growth of *Wolinella* at nitrogen-limiting conditions (samples for activity assay were separately flushed with Argon/H_2 and with N_2)

3.3.7.5. Inhibition of acetylene reductase by ammonium chloride:

Since the enzyme was expressed only under conditions of nitrogen-limitation, it was necessary to check its posttranscriptional regulation by ammonium. For that, different amounts of ammonium chloride were directly added to samples, taken from culture around the point of maximal enzymatic activity, before incubation with acetylene. The enzymatic

activity decreased proportionally with increase of the ammonium concentrations in the samples. In comparison to other known nitrogen-fixing bacteria, the inhibition mode of enzymatic activity in *Wolinella* seems to be similar to that in *G. sulfurreducens* and *A. vinelandii*.

For determining the inhibition of enzymatic activity, a culture of *Wolinella* was grown in FuFo medium under Argon/H_2 with 1.25g/l yeast extract as sole nitrogen source, $NaHCO_3$ buffer, pH 7.8, and 25°C. Samples for an assay of enzymatic activity were flushed with Argon/H_2 and ammonium chloride was added to samples directly before acetylene injection. Results are shown in figure (3-22). Upon addition of 0.1mM ammonium chloride, enzymatic activity was decreased to ~50% and to ~10% with 0.2mM ammonium chloride.

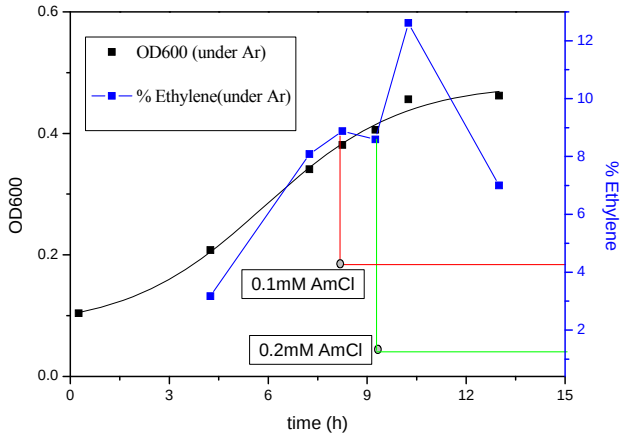


Figure (3-22): Inhibition of acetylene reduction in a culture of *Wolinella* grown at nitrogen-limited conditions under Argon/H_2

For a comparison of the inhibition type of the enzyme in *Wolinella* and in both *Azotobacter* and *Geobacter*, samples were taken from the cultures and supplemented with different amounts of ammonium chloride before incubation with acetylene and then the activity was measured at each ammonium concentration (figure 3-23). The three cultures were grown under nitrogen-limited conditions and the samples were taken around the point of maximal enzymatic activity. Two cultures of Wolinella were tested; one of them was grown under N_2 and the other under Argon/H_2.

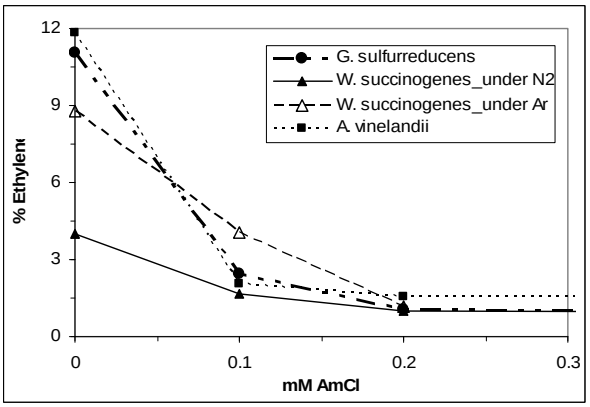


Figure (3-23): Inhibition of enzymatic activity by AmCl in cultures of *W. succinogenes*, *A. vinelandii*, and *G. sulfurreducens* grown at limited fixed-nitrogen conditions

3.3.7.6. Ethylene production during long time of incubation:

To check the enzyme vitality, samples of *Wolinella* cultures taken around the point of maximal enzymatic activity were incubated with acetylene and the activity was recorded at frequent intervals. Two sets of samples were taken from culture grown under N_2; the first set of samples was flushed and incubated under Argon/H_2 and the second one under N_2 (figure3-24). Higher ethylene reduction rates were obtained from the first set than from the second one supporting the assumption that N_2 binds competitively to the acetylene-reducing enzyme. Furthermore, in a culture grown under Argon/H_2 where the samples for the assay were flushed and incubated under Argon as well, the production of ethylene was increased relative to the previous case (figure3-25). However, acetylene reduction rates in *Wolinella* cultures are still low compared to those in *A. vinelandii* and *G. sulfurreducens* cultures (figure3-26).

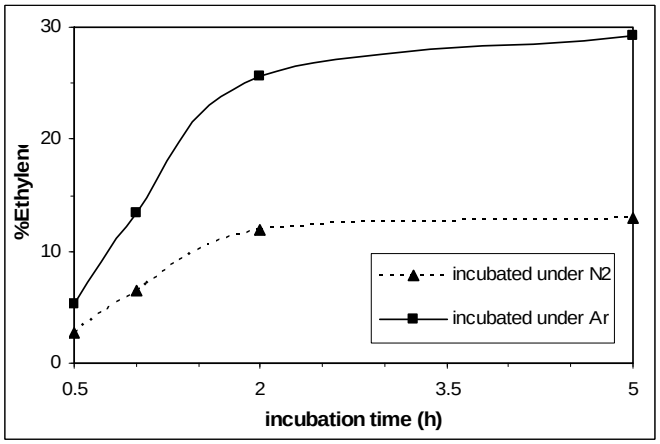


Figure (3-24): Production of ethylene during long time incubation of samples from a *Wolinella* culture with limited fixed-nitrogen source (samples were flushed with N_2 and with Argon/H_2); 1.25g/l YE, PO_4 buffer, 25°C, under N_2, pH 7.8

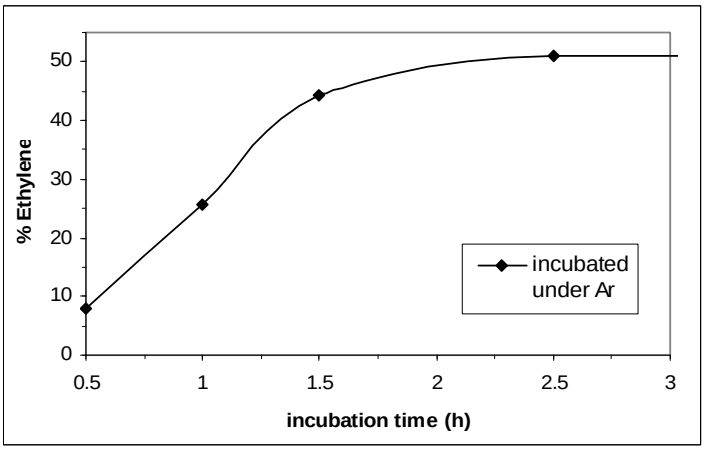


Figure (3-25): Production of ethylene during long time incubation of one sample from a *Wolinella* culture with nitrogen-limiting source (the sample was flushed with Argon/H_2 too); 1.25g/l YE, PO_4 buffer, 25°C, under Argon/H_2, pH 7.8

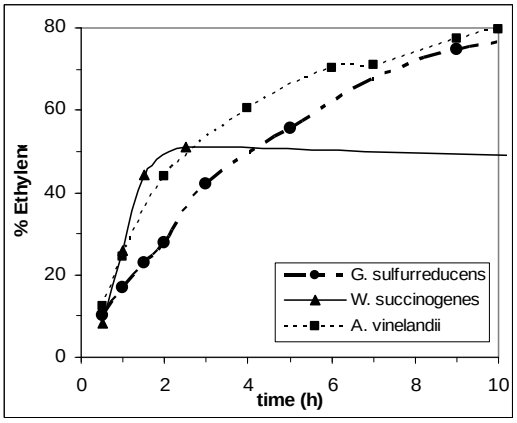


Figure (3-26): Production of ethylene during long time of incubation in sample from a *Wolinella* culture with nitrogen-limiting source comparison to samples from *G. sulfurreducens* and *A. vinelandii*

3.3.7.7. Optimization of cell productivity and enzyme expression:

As a result of the previous trials, the best conditions for cell productivity and enzymatic activity could be determined. Increasing the yeast extract concentration to 1,25 g/l and cultivation at 25°C in the medium gave the highest OD value of the culture together with the highest enzymatic activity. Upon decreasing the yeast extract concentration to 833mg/l, the same activity was obtained but the cell yield was clearly lower.

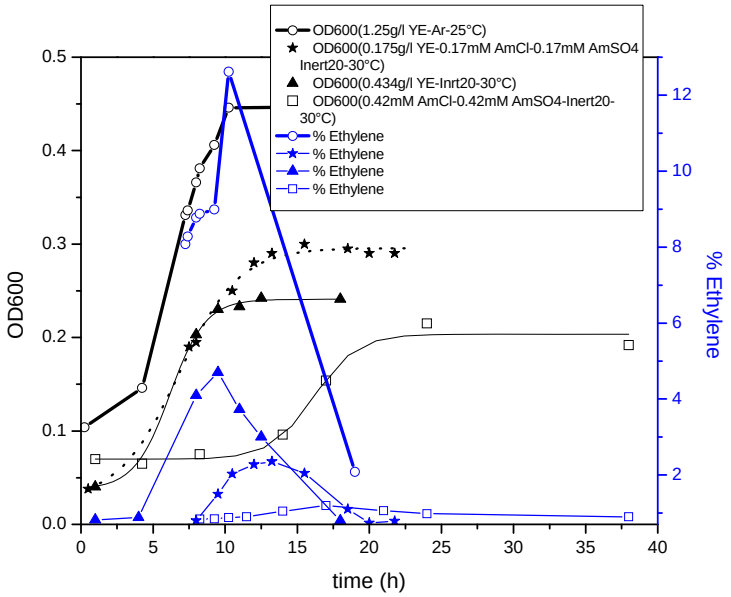


Figure (3-27): growth of *Wolinella* in nitrogen-limiting medium with different fixed-nitrogen sources and different growth conditions

3.4. Anaerobic purification of nitrogenase components:

The classic protocol for the purification of nitrogenase components from *Azotobacter vinelandii* was utilized and then applied to the other two bacteria, *Wolinella succinogenes* and *Geobacter sulfurreducens*.

3.4.1. Anaerobic purification of nitrogenase components from *Azotobacter*:

Cells were cultivated and disrupted as described in material and methods (2.2.1.2 and 2.2.5.1); 11.79g cells were used in the described case. After centrifugation in gas-tight tubes, the supernatant was loaded onto a 54ml Sepharose DEAE column (see 2.2.5.2). Two fractions were anaerobically eluted with a linear gradient (100- 500mM) of NaCl (figure 3-28). The first fraction was dark brown, a typical color of Molybdenum iron protein and the second one was yellow, the color of iron protein. The two fractions were concentrated using a 1 or 5ml HiTrap Q column and stored at -80°C. Furthermore, molybdenum iron protein was loaded again onto 5ml HiTrap Q column and eluted in the same way (figure 3-29). The second ion exchange purification step was necessary for improvement of the MoFe protein purity.

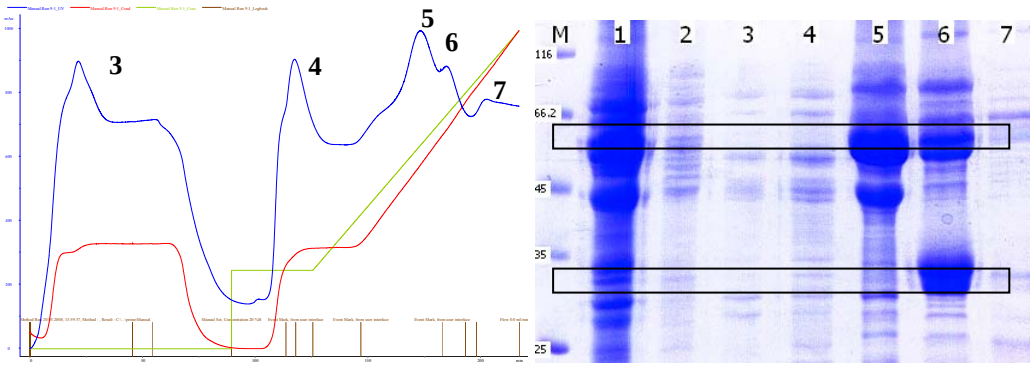


Figure (3-28): Left: Chromatogram of the 54ml Sepharose DEAE column; Right: Coomassie-stained gel; M: marker with corresponding molecular weight in kDa, 1: supernatant loaded on DEAE, 2: pellet, 3: first wash step, 4: second wash with 100mM NaCl, 5: first colored fraction (MoFe protein), 6: second colored fraction (Fe protein), 7: late gradient.

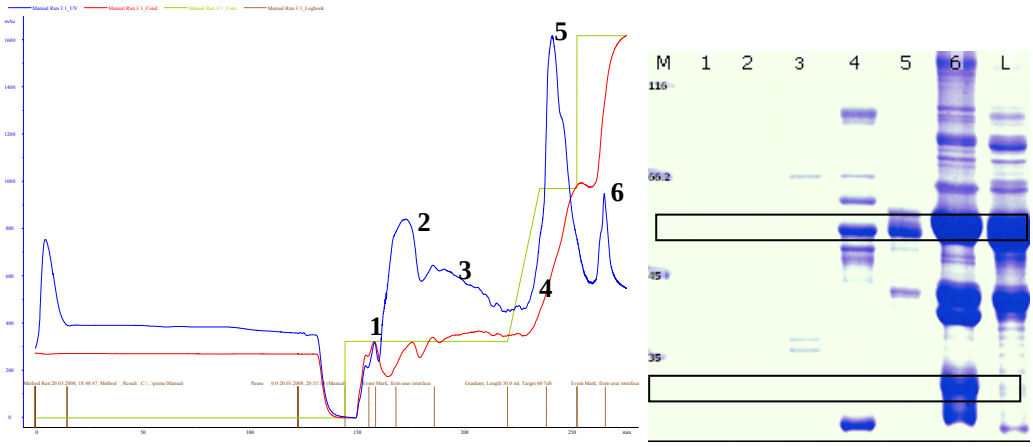


Figure (3-29): Left: Chromatogram of the 5ml HiTrap column; Right: Coomassie-stained gel; M: Marker with corresponding molecular weight in kDa, L: Load (fraction 5 from the last purification with DEAE column), 1: the tiny peak at the beginning of the wash step with 100mM NaCl, 2: the second peak in the wash step, 3: the third peak in the wash step, 4: sample of the very beginning of the gradient step, 5: the first colored fraction contains MoFe protein, 6: the second colored fraction contains Fe protein with significant amount of MoFe protein

The next purification step was done by size exclusion chromatography, where 0.5-1ml of concentrated molybdenum-iron protein was loaded onto a 120ml Superdex200 column

(see 2.2.5.3). As shown on the chromatogram, three peaks were obtained (figure 3-30). The dark brown fraction was anaerobically collected and concentrated by ultrafiltration with a molecular weight cut-off of 100 kDa. The yield of the protein was 1mg/g of wet cells. Protein of the right size could be separated from misfolded protein and other unwanted proteins which elutes in the exclusion volume making the size exclusion chromatography an essential step in the purification process. Simultaneously, the protein could be rebuffered in this step and maintained in the desired buffer.

The molybdenum-iron protein concentration was adjusted to 30mg/ml and the protein was subjected to crystallization trials in an anoxic glove box. Crystals appeared after one day in the following conditions: 14% PEG8000, 0.96-1.16M NaCl, and 50-120 mM Tris/HCl pH 8. The crystals were small and clustered.

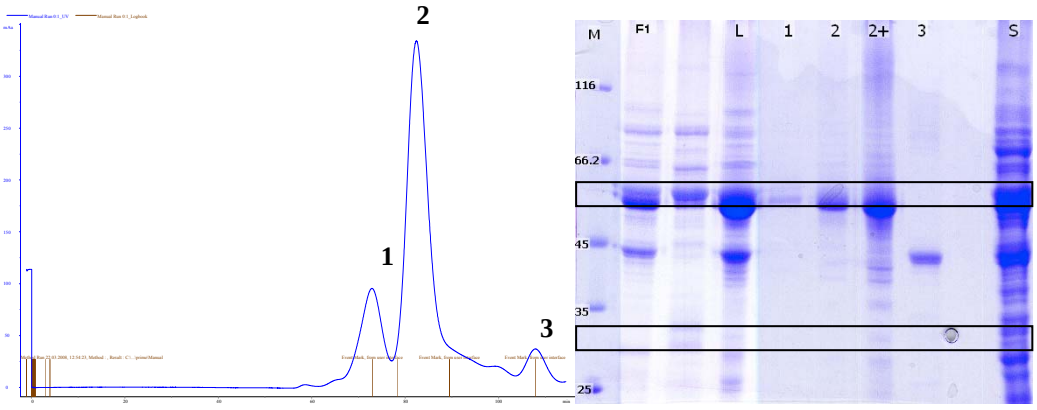


Figure (3-30): Left: Chromatogram of the 300ml Superdex 200 column; Right: Coomassie-stained gel; M: Marker with corresponding molecular weight in kDa, S: Supernatant loaded on DEAE column, F1: Fraction 5 from DEAE column (loaded on the HiTrap column), L: Load (fraction 5 from the 5ml HiTrap column, 1: first peak on the chromatogram, 2: MoFe protein fraction (2+: the same fraction after concentrating with ultrafiltration), 3: The last peak on the chromatogram

After the size exclusion chromatography step, the spectrum of the pure protein was recorded to establish the presence of iron sulfur clusters. The scan showed an absorbance shoulder around 400nm (figure 3-31), which is characteristic of iron sulfur clusters. SDS-PAGE was used to check the purity of the fractions at each purification step. The negative peak at 300 nm originates from a higher $Na_2S_2O_4$ concentration in the reference cell.

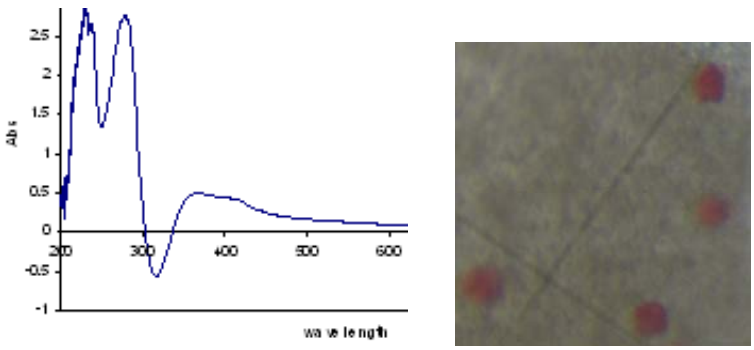


Figure (3-31): Left: UV/Vis spectrum of the MoFe protein fraction from the gel filtration step (2.3mg/ml protein, 50mM Tris/HCl pH 7.75, 100mM NaCl, and 0.5mM dithionite); Right: crystal clusters of MoFe protein

3.4.2. Anaerobic purification of nitrogenase components from *Wolinella*:

The purification process was carried out in analogy to the one in *Azotobacter*. Both Molybdenum-iron protein and iron protein characteristically show a strongly negative electrostatic surface potential, and consequently anion exchange chromatography at slightly basic pH was carried out for an initial purification of the proteins from crude extract of *W. succinogenes*. 9.8 g cells were used in the described case. 5ml HiTrap Q column was used in the first anion exchange purification step because of the low amount of broken cells and the low yield of the protein (figure 3-32). Besides, it was not possible to apply the second ion exchange step because the low yield of the protein too. Size exclusion chromatography was made by 120ml Superdex200 column (figure 3-33). The protein yield was 0,43mg/g of wet cells.

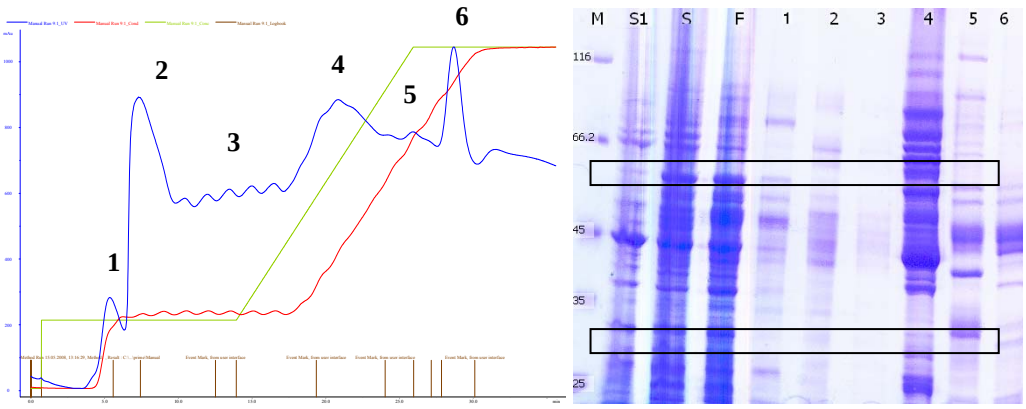


Figure (3-32): Left: Chromatogram of the 5ml HiTrap column; Right: Coomassie-stained gel; M: Marker with corresponding molecular weight in kDa, S1: Supernatant 10k rpm (after incubation of cells with swelling buffer), S: supernatant 30k rpm, 1: the tiny peak at the beginning of the wash step with 100mM NaCl, 2: the second peak in the wash step, 3: the small peaks in the late wash step, 4: first colored fraction of the gradient step (it would be corresponding to MoFe protein), 5: the second colored fraction (it would be corresponding to Fe protein), 6: the last colored fraction

After the gel-filtration step, typically two bands of NifD and NifK were detectable on the SDS-PAGE (figure 3-33). Molecular weights of the two peptides calculated from the sequence are 53.8 kDa for NifD and 57.0 kDa for NifK.

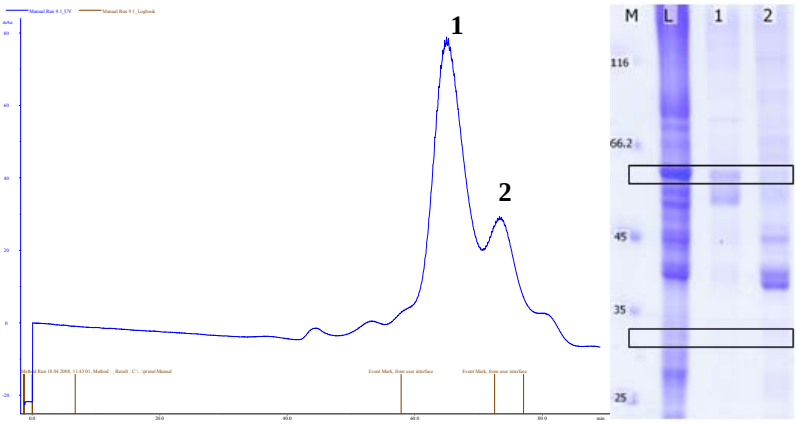


Figure (3-33): Left: Chromatogram of the 120ml Superdex 200 column; Right: Coomassie-stained gel; M: Marker with corresponding molecular weight in kDa, L: Load (fraction 4 from the 5ml HiTrap column, 1: The first colored fraction (MoFe protein fraction), 2: The second peak on the chromatogram

UV/visible absorption spectra of the protein obtained from gel-filtration showed an absorbance shoulder around 400nm demonstrating the presence of iron sulfur clusters (figure 3-34). In comparison to the spectrum of molybdenum iron protein in *Azotobacter*, the absorbance of *Wolinella* protein is considerably low. The difference could be raised of the high impurities in *Wolinella* protein, which are clear on the polyacrylamide gel too.

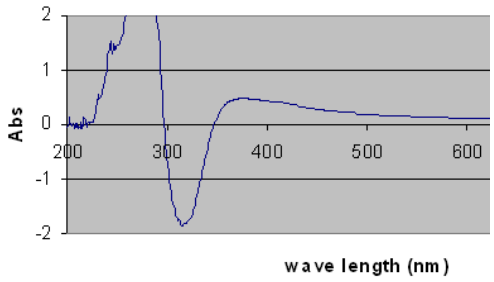


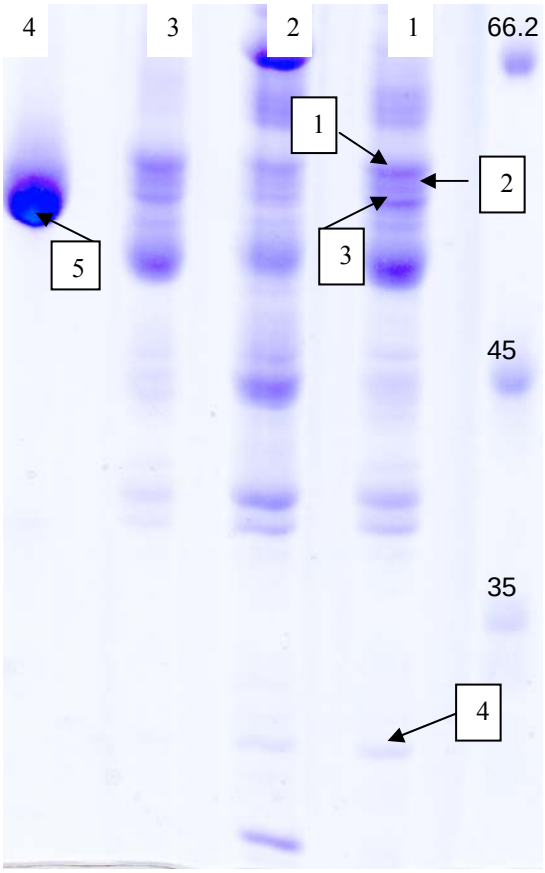
Figure (3-34): UV/Vis spectrum of the MoFe protein fraction from gel filtration step (6.4mg/ml protein, 50mM Tris/HCl pH 7.75, 100mM NaCl, 0.5mM dithionite)

3.5. Confirmation of nitrogenase expression in *Wolinella* by Q-TOF/MS:

An alignment comparison of the structural subunits of the nitrogenase system in *Wolinella* showed high similarity to the one of *Azotobacter*. Besides, RT-PCR confirmed the presence of mRNA of *nifH* and *nifD* in *Wolinella* cells grown under nitrogen-limiting conditions. Moreover, it was shown previously that the nitrogenase system is expressed and functional in terms of acetylene reduction, the assay commonly used for nitrogenase.

In order to confirm the presence of nitrogenase protein, mass spectrometry was employed. Cells were harvested around the point of maximal acetylene reductase activity. All following steps were carried out under strictly anaerobic conditions using modified Schlenk techniques and glove boxes for handling of buffers and protein solutions. After anion exchange and size exclusion chromatography purification, the brownish fraction was loaded onto SDS-PAGE, which was prepared using a self-made electrophoresis apparatus; the length of the gel was ca. 20cm in order to get better separation of the double band of the molybdenum iron protein (figure 3-35). 1×1mm cubes from the bands of the corresponding size that exclusively appeared in cells showing acetylene reductase activity were excised from the polyacrylamide gel and subjected to in-gel digestion (see material and methods 2.2.6.3).

Peptides were subsequently analyzed by mass spectrometry. Spots 1 and 3 in lane 1 were taken from bands that were expected to be corresponding to α and β subunits of molybdenum iron protein and spot 3 in lane 1 was taken of the middle of the last double band, where a very weak band appeared, to make sure that no other impurities hidden in between. Spot 4 in lane 1 was taken of a band that was expected to be corresponding to the iron protein. Spot 5 in lane 4 was taken from band corresponding to molybdenum iron protein of *Azotobacter* and it was considered as a control to assure that the whole procedure was working well.



Fugure (3-35): SDS-PAGE of nitrogenase components from *Wolinella* and *Azotobacter*; lanes 1,2, and3: (20-26µg) MoFe protein from three distinct purification trails from *Wolinella*; lane 4: MoFe protein (20µg) from *Azotobacter*; arrows refer to the points where cubes excised for mass spectrometry analysis

Starting with spot 5, results of the mass spectrometry analysis showed that this band was really corresponding to α and β subunits of molybdenum iron protein of *Azotobacter*, meaning that everything was working well.

Spots 1, 2, and 3 were all corresponding to α and β subunits of molybdenum iron protein of *Wolinella* (table 3-2), where spot 2 was a mix of the tow subunits.

Spot 4 was not related to the iron protein.

spot	Acc	Name	Mass	Ip	Score	Peptide Matched	Coverage	
1	NP_907558	hypothetical protein WS1392 [Wolinella succinogenes DSM 1740]	53807	5.92	938	32	48	**1** MRAEELKALQ KEAIEEVLAA YPEKTAKNRS KHLGVGAPDD ESQKTCGGVR **51** SNKKSAPGVM TQRGCAYAGS KGVVWGPVKD MVHISHGPIG CGQYSRGGRR **101** NYYIGTTGVD TFVTVNFSTD FQERDIVFGG DKKLQQAIDE INDLFPLNHG **151** ITVQSECPIG LIGDDIQAAA RKKSAETGKT VVAVSCEGFR GVSQSLGHHI **201** ANDTIRDIMF PLSDEVNKDF VATPYDVAII GDYNIGGDAW SSRILLEEMG **251** LRVIAQWSGD STFKEITAGP KAKLNLLHCY RSMNYVARHM EKEYGIPWME **301** YNFFGPSKIE ASLRAIAKHF GPEIEKKAEE VIAKYKAITE AVIAKYKPRL **351** EGKTVMLYVG GLRSRHIIGA YEDLGMEVIG LGYEFAHDDD YQRTKEEVSG **401** SVLVYDDVNE YELEKFVDKL RPDLVASGVK EKYVFQKMGL PFRQMHSWDY **451** SGPYHGYDGF AIFARDIEMA VNSPVWAHNT APWD **Start - End Observed Mr(expt) Mr(calc) Delta Miss Sequence** **12 - 24 731.3790 1460.7434 1460.7398 0.0036 0 K.EAIEEVLAAYPEK.T** **12 - 24 731.3841 1460.7536 1460.7398 0.0138 0 K.EAIEEVLAAYPEK.T** **30 - 44 523.2612 1566.7618 1566.7638 -0.0020 1 R.SKHLGVGAPDDESQK.T** **32 - 44 451.5493 1351.6261 1351.6368 -0.0107 0 K.HLGVGAPDDESQK.T** **32 - 44 676.8270 1351.6394 1351.6368 0.0026 0 K.HLGVGAPDDESQK.T** **55 - 63 473.7383 945.4620 945.4702 -0.0082 0 K.SAPGVMTQR.G** **55 - 63 481.7406 961.4666 961.4651 0.0015 0 K.SAPGVMTQR.G** Oxidation (M) **55 - 63 481.7414 961.4682 961.4651 0.0031 0 K.SAPGVMTQR.G** Oxidation (M) **125 - 132 425.7215 849.4284 849.4232 0.0052 0 R.DIVFGGDK.K** **125 - 133 489.7638 977.5130 977.5182 -0.0052 1 R.DIVFGGDKK.L** **125 - 133 489.7670 977.5194 977.5182 0.0012 1 R.DIVFGGDKK.L** **191 - 206 568.9616 1703.8630 1703.8703 -0.0074 0 R.GVSQSLGHHIANDTIR.D** **191 - 206 568.9672 1703.8798 1703.8703 0.0094 0 R.GVSQSLGHHIANDTIR.D** **207 - 218 704.3521 1406.6896 1406.6752 0.0145 0**

								R.DIMFPLSDEVNK.D **244 - 252 537.3042 1072.5938 1072.5950 -0.0012 0** **R.ILLEEMGLR.V** **244 - 252 545.2997 1088.5848 1088.5900 -0.0051 0** **R.ILLEEMGLR.V** Oxidation (M) **253 - 264 669.8268 1337.6390 1337.6616 -0.0225 0** **R.VIAQWSGDSTFK.E** **253 - 271 679.0233 2034.0481 2034.0422 0.0059 1** **R.VIAQWSGDSTFKEITAGPK.A** **282 - 288 420.7083 839.4020 839.3959 0.0061 0** **R.SMNYVAR.H** **293 - 308 982.9618 1963.9090 1963.8815 0.0276 0** **K.EYGIPWMEYNFFGPSK.I** **293 - 308 982.9697 1963.9248 1963.8815 0.0434 0** **K.EYGIPWMEYNFFGPSK.I** **293 - 308 990.9568 1979.8990 1979.8764 0.0227 0** **K.EYGIPWMEYNFFGPSK.I** Oxidation (M) **319 - 326 478.7416 955.4686 955.4763 -0.0077 0** **K.HFGPEIEK.K** **337 - 345 458.2710 914.5274 914.5437 -0.0162 0** **K.AITEAVIAK.Y** **354 - 363 554.8129 1107.6112 1107.6111 0.0002 0** **K.TVMLYVGGLR.S** **354 - 363 562.8123 1123.6100 1123.6060 0.0041 0** **K.TVMLYVGGLR.S** Oxidation (M) **354 - 363 562.8203 1123.6260 1123.6060 0.0201 0** **K.TVMLYVGGLR.S** Oxidation (M) **366 - 393 807.3838 3225.5061 3225.4713 0.0348 0** **R.HIIGAYEDLGMEVIGLGYEFAHDDDYQR.T** **366 - 393 811.3811 3241.4953 3241.4662 0.0291 0** **R.HIIGAYEDLGMEVIGLGYEFAHDDDYQR.T** Oxidation (M) **394 - 415 849.0814 2544.2224 2544.2119 0.0105 1** **R.TKEEVSGSVLVYDDVNEYELEK.F** **416 - 430 548.6519 1642.9339 1642.9406 -0.0068 1** **K.FVDKLRPDLVASGVK.E** **420 - 430 577.8470 1153.6794 1153.6819 -0.0025 0** **K.LRPDLVASGVK.E** **466 - 484 1077.0015 2151.9884 2151.9684 0.0201 0** **R.DIEMAVNSPVWAHNTAPWD.-**
3	NP_907557	nitrogenase BETA subunit [Wolinella succinogenes DSM 1740]	56970	5.54	346	10	19	**1 MQTIENIVTG** **K**DLFLRPEYQ EIFTQKRAEF EGMPSCDEVA KVADWTKSWE **51** YREKNLAREA ITINAAKACQ PLGAVMVALG FENTMPYVHG SHGCVAYFRS **101** YFTRHFKEPT PCVSDSMTED AAVFGGLQNM IDGLANCYAM YKPEMIAVST

								151 TCMAEVIGDD LQAFVENARS AGSIPADYPV PYANTPSFKG SHITGYDNMM **201** YSIITQLSNV SQSADKKDR**I NIIPGFEPYI GSLR**EVKEIV EAFGADYIML **251** GDHSEQWDMP AGK**YQMFSGG TK**LDDARDAG ASKVTITLQK YTTPRTMKSF **301** AKKFKQETLE LNPIGLAGTD AFVSAISKLT GKSVPQKLKD ERGRLVDAMQ **351** DSYPYMHGKS FAIWGDPDFL IGAVSFLIEM GAEPKHVLCH NAPDGWEEEM **401** K**ALLATSPAK** DLHVWAGKDL WHMR**SLLFTE PVDFMIGNTY GK**ELERDTGI **451** PLIR**VGFPIF DR**HHLHRYSI SGYK**GTLNLL TWIVNKVLDK MDEDTK**QIAK **501 TDFFFDAVR** **Start - End Observed Mr(expt) Mr(calc) Delta Miss Sequence** **1 - 11 617.3292 1232.6438 1232.6435 0.0004 0 -.MQTIENIVTGK.D** **220 - 234 844.9755 1687.9364 1687.9297 0.0067 0 R.INIIPGFEPYIGSLR.E** **264 - 272 509.7351 1017.4556 1017.4590 -0.0033 0 K.YQMFSGGTK.L** **402 - 410 436.2629 870.5112 870.5174 -0.0062 0 K.ALLATSPAK.D** **425 - 442 1016.5078 2031.0010 2031.0023 -0.0013 0 R.SLLFTEPVDFMIGNTYGK.E** **455 - 462 475.7546 949.4946 949.5022 -0.0075 0 R.VGFPIFDR.H** **475 - 486 686.4017 1370.7888 1370.7922 -0.0033 0 K.GTLNLLTWIVNK.V** **475 - 486 686.4037 1370.7928 1370.7922 0.0007 0 K.GTLNLLTWIVNK.V** **487 - 496 597.2861 1192.5576 1192.5645 -0.0069 1 K.VLDKMDEDTK.Q** **501 - 509 559.2672 1116.5198 1116.5240 -0.0042 0 K.TDFFFDAVR.-**

Table (3-2): Identification report of spot 1 and 3; peptides that were detected are shown in bold red

4. Discussion:

4.1. *Wolinella succinogenes*, a perplexing bacterium:

Since *Wolinella succinogenes* was first isolated in the year 1961, it has proved to be a surprising bacterium. It was classified first with the genus *Vibrio* based on morphology and the presence of cytochromes (Wolin, 1961). Twenty years later, *W. succinogenes* was considered among others as a new genus including anaerobic, asaccharolytic, rod-shaped bacteria with single polar flagella and G + C content of 42-49 % (Tanner, 1981). Further phylogenetic studies then took the other grouped bacteria out, leaving *W. succinogenes* as a single species within the genus *Wolinella* (Vandamme, 1991 and Dewhirst, 2005). Many of *Wolinella*'s unique features were uncovered or ascertained upon sequencing its genome, which was found to be 25% larger than its two epsilon-proteobacteria relatives, *Helicobacter pylori* and *Campylobacter jejuni* (Baar, 2003):

- Simon, *et. al.* hypothesized that the unprecedented *nos* gene cluster of *W. succinogenes* encodes a complete electron transport chain, which catalyses N_2O reduction by menaquinol, a pathway that was found to be relevant to other bacteria (Simon, 2004 and Kern, 2009).

- Although *W. succinogenes* is more related to *H. pylori*, the pathogenicity-related genes rather resemble the pattern found in *C. jejuni* instead of the one of *H. pylori*. However, the most striking example is the secreted *C. jejuni* invasion antigen B (*ciaB*), which is a key pathogenicity factor of *C. jejuni* and is essential for invading the host cell in the infection process. This protein is exclusively found in both *C. jejuni* and *W. succinogenes* but not in any other organism (Konkel, 1999). Nevertheless, *W. succinogenes* is considered nonpathogenic to its bovine host and no adverse effects on the health of humans or animals were reported so far.

- In contrast to other host-adapted bacteria, *W. succinogenes* does harbor the highest density of bacterial sensor kinases found in any bacterial genome (Baar, 2003)

- Although *W. succinogenes* is a microaerophilic bacterium, it was established that its ATP synthase is similar to those of aerobic and phototrophic bacteria, mitochondria, and chloroplasts (Bokranz, 1985).

- Acrylate and methacrylate reductase activity was detected in *W. succinogenes* with unknown physiological role of the enzyme that is proposed to play a role in the the reduction process that is coupled to the oxidation of benzyl viologen radical (Gross, 2001).

- A complete set of *nif* genes was discovered in the genome of *W. succinogenes* implying that *it* could be capable of a diazotrophic lifestyle (Baar, 2003).

As an enteric, host-associated bacterium, *W. succinogenes* does not require diazotrophy for survival inside intestines, a matter that leads to wonder about the presence of such genes

and the capability to express an active nitrogenase system. Indeed, presence of nitrogen-fixing bacteria was reported in human and animal intestine (Bergersen, 1970).

4.2. *Wolinella*'s *nif* gene products: A comparison to other known diazotrophs:

Starting from the three *nif* gene products that represent the structural subunits of nitrogenase, we can clearly observe the high similarity of NifH, NifD, and NifK of *Wolinella* in comparison to those of other known diazotrophs (see results 3.1) including *Azotobacter vinelandii*, a model-diazotroph whose nitrogenase components are structurally and biochemically well characterized, *Synechococcus*, a member of the cyanobacteria whose *nif* genes are syntenic to the *nif* gene cluster of *W. succinogenes*, and *Rhizobium leguminosarum*, a symbiont whose *nif* genes are taxonomically related to the *nif* gene cluster of *W. succinogenes*. In addition, two known diazotrophs of the subclass epsilon-proteobacteria were used in the comparison, *Sulfurospirillum multivorans* and *Arcobacter nitrofigilis* (formerly known as *Campylobacter nitrofigilis*) (McClung, 1983, Vandamme, 1991 and Ju, 2007). More species of the genus *Arcobacter*, such as *Candidatus Arcobacter sulfidicus*, were found to be capable of nitrogen fixation but no sequences of their *nif* genes are available so far (Wirsen, 2002).

The iron protein of *W. succinogenes* seems to share all critical features of functionality with other sequences. The two cysteine residues that ligate the iron sulphur cluster and the P-loop region with the switch I and II regions, which are essential for binding and hydrolysis of ATP, are fully conserved. Moreover, the important residue R100, which is involved in the complex formation and posttranscriptional regulation of nitrogenase, is conservedas well. Nevertheless, the characteristic insertion (residues 85-96) of *Wolinella* NifH is the only discerned difference in comparison to other sequences. Interestingly, this insertion seems to be common among the epsilon-proteobacteria members implying that they may comprise a distinct sub-group of diazotrophs and that the *nif* genes in *W. succinogenes* may not be transferred from other diazotrophs *via* horizontal gene transfer as supposed before.

The MoFe protein of *W. succinogenes* seems to be functional according to the alignment comparison. All cysteine residues of the α and β subunits that are involved in ligating the P-cluster are conserved. Both Cys275 and His442, which ligate the FeMo-cofactor, are conserved as well. All other known conserved residues that affect the function of the enzyme are conserved in the *Wolinella* MoFe protein.

As reported lately by Hernandez, *et. al*., the minimal set of *nif* genes required for the synthesis of a functional FeMo-cofactor is *nifH*, *nifEN*, and *nifB* (Hernandez, 2008). All *nif* genes required for the biosynthesis and insertion of FeMo-cofactor are present in the *Wolinella nif* gene cluster (table 3-1), implying the possibility of substrate reduction to take place at the active site of dinitrogenase.

4.3. Expression and activity of nitrogenase in *W. succinogenes*:

RT-PCR results showed that the *nifH* and *nifD* genes were expressed in *Wollinella* cells under nitrogen-limiting conditions while no expression could be detected under excess of

ammonium (see results 3.2), indicating the presence of a mechanism to sense the level of fixed nitrogen and respond to its change. Although NifA, the transcriptional activator of the expression of all other *nif* genes, was not found in *W. succinogenes*, expression of *nif* genes could be detected. However, activity of NifA was not reported in all known diazotrophs (Rudnick, 1997). Furthermore, the *rpoN* gene product (σ^N transcription factor), which acts together with NifA to initiate the transcription of the other *nif* genes, was found in *Wolinella succinogenes* (table 3-1).

In addition to the regulation of *nif* gene expression by ammonium, another temperature-depending regulation mechanism was observed. Cells grown at 37°C showed no enzymatic activity (see results 3.3.7.3) and activity at 23°C was higher than activity at 30°C concurrently. Such regulatory effect may explain the requirement of *nif* genes to survive in various environmental habitats when *W. succinogenes* exists outside intestines as a free-living microorganism.

Posttranscriptional regulation of nitrogenase in *W. succinogenes* cells was also established, whereby the reduction of acetylene in cultures grown under nitrogen-limiting conditions was inhibited in a way similar to that of *A. vinelandii* and of the anaerobe *G. sulfurreducens* (see results 3.3.5 and 3.3.7.5). Acetylene reduction was completely inhibited upon addition of 0.2 mM ammonium chloride to cultures of the last three bacteria that were able to reduce acetylene actively, whereas lower ammonium concentrations resulted in partially inhibition on the enzymatic activity (see figure 3-23).

4.3.1. Fixed nitrogen as the growth-limiting factor for *W. succinogenes*:

Concentrations of up to 5mM ammonium sulphate were enough for normal growth of *Wolinella succinogenes* and lower concentrations could be considered as growth-limiting factor where the half value of OD was obtained upon cultivation of cells in a medium containing 1.2 mM ammonium sulphate (see results 3.3.6 and 3.3.7). The same growth-limiting levels of fixed nitrogen could be detected in the case of *Azotobacter vinelandii* (see results 3.3.2.1), although the produced biomass was much higher than in the case of *W. succinogenes*.

Because *Wolinella succinogenes* was found to grow badly without yeast extract, a modified medium using yeast extract as a fixed-nitrogen source instead of ammonium chloride or ammonium sulphate was used, aiming at an increase of cell productivity and enzyme yield. The genome of *Wolinella succinogenes* was predicted to contain amino acid and oligopeptide transporters (Baar, 2003), a fact that could be supported by our results where growing cells on yeast extract as sole source of fixed-nitrogen increased the biomass of cells and shortened the growth phases (see results 3.3.7.1). The lag phase was clearly shortened upon growing cells on yeast extract as sole source of fixed nitrogen and the growth attained the stationary phase earlier, a matter that is of advantageous because the highest enzymatic activity was obtained only in the early stages of the stationary phase.

4.3.2. Diauxy, growth on gaseous nitrogen:

Diazotrophs are known to be able to utilize nitrogen in the form of fixed nitrogen (nitrate or ammonium) and gaseous nitrogen. When both sources are available diazotrophs tend to utilize the less energy-demanding source (fixed nitrogen) and only upon exhausting it they begin to adapt to the new conditions by expressing their unique, but highly energy-demanding system, nitrogenase.

As a model diazotroph, *Azotobacter vinelandii* was cultured in Burke's medium containing 10mM ammonium chloride. During the first 90 h, a normal growth curve was obtained and at some point of the stationary phase, another stage of growth was initiated where second logarithmic and stationary phases could be observed. In parallel with this second stage of growth, nitrogenase activity could be detected (see results 3.3.2.1). At lower ammonium chloride concentrations, the same phenomena could be observed but the first stage of growth would be shortened in this case and much higher values of enzymatic activity could be observed because higher amounts of nitrogen would be required for growth (see results 3.3.2.2).

Anaerobic diazotrophs may show triphasic growth when they grow under nitrogen-limiting conditions and another gas than dinitrogen is used over the culture. This may be due to the ubiquity of dinitrogen where traces of dinitrogen left over in the medium or in the gas phase can be utilized to achieve a second stage of growth. When dinitrogen is supplied instead of the other gas, a third stage of growth can be accomplished. An example of the last case could be shown upon growing *Geobacter sulfurreducens* at nitrogen-limiting conditions with Argon/H_2 and N_2 variously as head-space gas (see results 3.3.4.2). During the first five days of growth, diauxy was obtained with a short second stage of growth and high nitrogenase activity. At the beginning of the second stationary phase, nitrogenase activity dropped dramatically off to become completely absent. At this point, Inertal20 was supplied instead of the Argon/H_2 for further two days. During this period, nitrogenase activity was partially restored and a third stage of growth was attained.

If fixed-nitrogen concentration was very low, the first stage of growth would be short and diauxy could be hardly detectable (see results 3.3.4.3).

In the case of *Wolinella succinogenes*, no diauxy was observed upon growth in limited-nitrogen medium although nitrogenase activity, in terms of acetylene reduction, was detectable. Growth curves obtained from culturing *Wolinella* under Inertal20 and under Argon/H_2 at nitrogen-limiting conditions were similar (see results 3.3.7.4), indicating that cells were not able to survive in the presence of dinitrogen as source of nitrogen.

The inability of *W. succinogenes* to fix dinitrogen leads to the assumption that a disturbance in the cascade of nitrogenase expression or substrate reduction mechanism may be present. The nitrogenase system in *W. succinogenes* seems to be fundamentally complete and functional. Structurally, the three subunits of nitrogenase in *W. succinogenes* showed high similarity to those of known diazotrophs and all essential residues were found to be conserved. In addition, the biosynthesis pathway of the active site was found to be

complete. Thus, a likely scenario for the inability of *Wolinella* to grow diazotrohically may be explained by a disorder in mechanism of substrate reduction.

According to the Thorneley-Lowe model, dinitrogen binds to more highly reduced states of the MoFe protein than do the other substrates (e.g., acetylene or protons). MoFe protein is reduced by the Fe protein through a cycle involving ATP-dependent electron transfer between the two proteins. In turn, Fe protein must be reduced by a potent reductant such as ferredoxins or flavodoxins, which gain their electrons from another source in the cell. If this electron transfer chain was corrupted or ineffective, MoFe protein would not be able to reach the highly reduced state that is required for binding and reduction of dinitrogen.

4.3.3. Variables affect the enzymatic activity and vitality in *W. succinogenes*:

The enzymatic activity in culture of *W. succinogenes* grown under nitrogen-limiting conditions was affected by the kind of the buffer system (see results 3.3.7.2), temperature (see results 3.3.7.3), and head-space gas (see results 3.3.7.4).

Enzymatic activity was enhanced in cultures supplemented with phosphate buffer more than in those with bicarbonate buffer (see figure 3-17). Cells grew similarly in the two cases but the enzymatic activity was higher in the case of phosphate buffer. The reason could be the increased salt concentration during cultivation in the case of bicarbonate buffer (see results 3.3.7.2).

The growth temperature impacted the enzymatic activity to a high extent. Cultures grown at 37°C showed no enzymatic activity; and activity in cultures grown at 23°C was higher than one in cultures grown at 30°C (see figure 3-18). These results are in agreement with the suggestion that *W. succinogenes* may require its *nif* gene cluster for sustaining in various environments outside intestines, where temperatures are generally lower.

The enzymatic activity in cultures grown under Argon/H_2 was higher than the activity in cultures grown under N_2 (see figures 3-19, 3-20, 3-21, 3-24, and 3-25). Although competitive inhibition of acetylene reduction by N_2 was excluded (Dos Santos, 2005), it seems here that acetylene reduction is inhibited in the presence of N_2. It is possibly that N_2 binds on the active site and suppresses the reduction of acetylene and then released without reduction or it may be reduced at a very low rate.

During the first hour of incubation, the rate of acetylene reduction by the enzyme of *W. succinogenes* was equal to that in the case of *A. vinelandii* (see figure 3-26). Then, the ability of the *Wolinella* enzyme to reduce acetylene decreased very quickly to be eliminated within one hour; about 50% of the acetylene was reduced during the whole incubation time. In contrast, in the case of *A. vinelandii*, acetylene reduction went on until almost the whole amount of acetylene was reduced. This behaviour of the *Wolinella* enzyme supports the assumption that a disorder in the electron transfer pathway is reason of inability to reduce dinitrogen.

4.4. Dinitrogenase from *W. succinogenes*:

The reduction of acetylene only under nitrogen-limiting conditions and inhibition of this process by ammonium, together with the evidence obtained from RT-PCR of *nifH* and *nifD* expression under nitrogen-limiting conditions and the absence of such expression under non-limiting concentrations of fixed-nitrogen, gave high indication to the presence of functional MoFe protein under these conditions. Thus, different biochemical techniques were employed to assure the presence of this protein.

4.4.1. Preliminary evidences for presence of dinitrogenase in *Wolinella*'s crude extract:

Cells of *Wolinella succinogenes* grown under nitrogen-limiting conditions and harvested around the point of maximal acetylene reduction value were subjected to a strictly anaerobic purification protocol.

The purification protocol was first tested on *Azotobacter vinelandii,* where MoFe protein was obtained at a purity grade that was enough to show its biochemical and spectroscopical characteristics and even to get crystals at the previously defined conditions (see results 3.4.1)

In analogy to the procedures that were applied for *Azotobacter vinelandii*, the purification trials were performed on *Wolinella succinogenes*. Chromatograms obtained from the ion exchange chromatography step seemed to be similar in the two cases (see figures 3-28 and 3-32) where peaks corresponding to the MoFe protein fraction appear at the beginning of the gradient stage. The peak in the case of *W. succinogenes* is very small in comparison to that one in the case of *A. vinelandii* because of the big difference in protein yield between the two bacteria. Yield of MoFe protein in *A. vinelandii* was 1mg/g of wet cells and 0.43mg/g in *W. succinogenes*. Moreover, protein purity in the case of *A. vinelandii* was much higher than the protein of *W. succinogenes*.

Bands corresponding to the MoFe protein subunits can be seen on the SDS-PAGE (see figures 3-28 and 3-32) although many more impurities seem to be present in the case of *W. succinogenes*.

The size exclusion chromatography step is of advantage not only for increasing the purity grade of proteins but also for ascertainment of the presence of defined protein within a mixture, because each protein according to its molecular mass will elute at a definite volume. In the case of MoFe protein from *W. succinogenes*, the elution volume was 65ml (see figure 3-33), and according to the calibration curve of the column (S200/120ml) this volume is equivalent to molecular mass of about 200 kDa. Theoretically, MoFe protein of *W. succinogenes* is around 220-230 kDa.

Iron-sulphur clusters have characteristic absorption at wavelengths around 400nm (Okura, 1979). The MoFe protein contains iron-sulphur clusters, a matter that helps in ascertainment of its presence in solution by recording the absorption. The fraction that was eluted from the size exclusion chromatography step at the particular size of MoFe protein

was subjected to a wave scan on a UV-vis spectrophotometer and the resulting spectra revealed a shoulder around 400nm (figure 3-31 and 3-34) raising the possibility that MoFe protein of *W. succinogenes* is present in this fraction.

The second peak in the gradient stage would be corresponding to the Fe protein. In the case of *W. succinogenes*, a distinct peak can be observed while this peak appears as a shoulder in the case of *A. vinelandii* (see figures 3-28 and 3-32). The explanation of this state is the same as before, where the high yield of MoFe protein in *A. vinelandii* leads to a wide peak during elution from the columnthat consequently overlaps with the Fe protein peak. The very low yield of the Fe protein in the case of *W. succinogenes* in addition to its high sensitivity and instability make it difficult to get significant amounts of this protein.

4.4.2. Unambiguous evidence for expression of dinitrogenase in *W. succinogenes*:

The purification protocol that was applied to crude extract of *Wolinella*'s cells grown under nitrogen-limiting conditions led to separation of partially pure protein, which was eluted from gel-filtration column at volume that is covalent to the molecular mass of MoFe protein. Besides, double band with size corresponding to both subunits of MoFe protein, NifD and NifK, could be detect upon loading the protein on polyacrylamide gel. Moreover, the protein showed absorbance around 400nm indicating the presence of iron-sulphur-containing protein.

Up to here, decisive evidence is still lacking that the purified protein is really MoFe protein. Therefore, cubes from bands that corresponding to both α and β subunits on the polyacralamide gel were excised and subjected to mass spectrometry analysis. Both bands were found to be substantially correlated to α and β subunits of MoFe protein (see table 3-2).

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Acknowledgement

The studies of the presented work have been carried out from April 2006 till March 2010 at the department of Molecular Structural Biology, George-August-University Göttingen, Germany.

A dissertation is not just a result of scientific work. Behind, there are many events and long time of common life in labs with a lot of people. In Arabic culture there is a nice proverb: “Who lives with a group for 40 days, he becomes one of them”. Accordingly, I would like to thank some of my new family members who supported me during my work on this dissertation:

Prof. Dr. Oliver Einsle, my super supervisor for encouragement and being a continuous source of inspiration. I really have learned a lot from you. I was never such positive and optimistic one before meeting you. I like also to express my heartfelt thanks to you for your special support and enthusiasm during writing this dissertation.

Prof. Dr. Ralf Ficner for your support and acceptance of the Korreferat of my thesis.

My other exam committee members for agreeing to evaluate my dissertation and participating in examination: Prof. Dr. Franc Meyer, PD. Dr. Rolf Daniel, Prof. Dr. Stefanie Pöggeler, and PD. Dr. Michael Hoppert.

Lab colleagues Anja Pomowski and Dr. Maren Hoffmann for the continuous help and the friendly conduct. Thanks to all other group members Nikolai Biermann, Dr. Daniel Heitman, Wie Lü, Sarah Helfmann, Andreas Kirscht, Peer Lukat. My special gratitude goes to Dr. Susana Andrade for her support and encouragement and a lot of ideas and discussion during our seminars.

All the people at MSB, thanks for your help in a lot of different matters, for nice parties, and for making this place a nice place to work. Special thanks to Dr. Ernst Achim Dickmans, Dr. Kristina Lakomek, Dr. Thomas Monecke, Angela Bindseil, Annette Berndt, Chrissoula Greulich, Sohail Khoshnevis, and Jens Brinkmann.

Prof. Dr. Ivo Feußner and Dr. Cornelia Göbel for help with the GC equipment. I will never forget your friendly aid, Dr. Cornelia.

Prof. Dr. Franc Meyer, Dr. Michael Fuchs, and Dr. Joachim Ballmann from the Inorganic Chemistry department for help with purification of the acetylene for the enzyme activity.

International Research Training Group (IRTG) for the scholarship and the IRTG members for the nice and helpful seminars and for the enjoyable time during our workshops, special thanks to Tomislav Argirevic, Prinson Samuel, Simone Woeckel, Alexandra-Zoi Andreou, and Dr. Florian Brodhun.

Romia, Masa, and Aos, my great and lovely family. It is really meaningful to struggle together for better future.

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