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TÍTULO: Study of metalloproteins involved in nitrogen fixation in sugarcane

Trabajo de integración curricular presentado como requisito para la obtención del título de QUÍMICO

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Dedicatoria

A mi mamá Susi quien me apoyado en las decisiones que he tomado,
quien me ha dado fuerza en los momentos más duros,
quien me aconsejado en los momentos adecuados y
a quien he amado incondicionalmente.

Michelle Beatriz Chicaiza Lema

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Michelle Beatriz Chicaiza Lema

Resumen

La fijación biológica de nitrógeno es un proceso donde una bacteria diazótrofa reduce el nitrógeno molecular a amonio por la acción de la enzima nitrogenasa. El estudio de la bacteria diazótrofa es de gran importancia ya que pude proporcionar información sobre la enzima nitrogenasa como una alternativa biológica para la fertilización de cultivos. La nitrogenasa está compuesta por dos metaloproteínas, la proteína MoFe y la proteína Fe. Este trabajo se centró en la purificación y caracterización de la enzima nitrogenasa de una bacteria diazótrofa de la raíz de la caña de azúcar. La bacteria se aisló del ingenio azucarero de Urcuqui y se identificó como *Azospirillum amazonense*. Además, la actividad nitrogenasa de *Azospirillum amazonense* se midió mediante el ensayo de reducción de acetileno y se detectó que el oxígeno inhibió la actividad de nitrogenasa.

La purificación de la nitrogenasa de *Azospirillum amazonense* se realizó con columnas cromatográficas de Q-Sepharose y Sephacryl S-200. Las fracciones obtenidas de la purificación se analizaron mediante el ensayo de ácido bicinconínico para determinar la concentración de las proteínas. Se utilizó electroforesis en gel de dodecil sulfato de sodio-poliacrilamida para estimar las bandas de las proteínas MoFe y Fe con respecto al peso molecular. En el gel de electroforesis, la proteína MoFe se identificó a aproximadamente 35 kDa y la proteína Fe a 57 kDa. Para confirmar la presencia de la nitrogenasa, se realizó una resonancia paramagnética electrónica para caracterizar las metaloproteínas de la nitrogenasa. El espectro de resonancia paramagnética electrónica correspondió al cofactor FeMo de la proteína MoFe con estado de espín S = 3/2 y valores g de g_x = 4.503, g_y = 4.268 y g_z = 1.993. Estos valores comparados con la literatura confirmaron la presencia de la nitrogenasa de la bacteria *Azospirillum amazonense* de la raíz de la caña de azúcar.

Palabras claves: bacteria diazótrofa, nitrogenasa, fijación biológica de nitrógeno, resonancia paramagnética electrónica.

Abstract

Biological nitrogen fixation is a process where diazotroph bacteria reduce molecular nitrogen to ammonium by the action of the nitrogenase enzyme. The study of diazotroph bacterium is of great importance as it can provide information about the nitrogenase enzyme as a biological alternative for crop fertilization. The nitrogenase is composed of two metalloproteins, MoFe protein and Fe protein. This work was focused on the purification and characterization of the nitrogenase enzyme from the diazotroph bacterium from the sugar cane root. The bacterium was isolated from sugar mill from Urcuqui and was identified as *Azospirillum amazonense*. Also, the nitrogenase activity of *Azospirillum amazonense* was measured by acetylene reduction assay and was detected that oxygen inhibited the nitrogenase activity.

The purification of the nitrogenase from *Azospirillum amazonense* was performed with chromatographic columns of Q-Sepharose and Sephacryl S-200. The fractions obtained from purification were analyzed by the bicinchoninic acid assay to determine protein concentration. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was used to estimate the bands of MoFe and Fe proteins with respect to molecular weight. In the electrophoresis gel, the MoFe protein was identified at approximately 35 kDa and Fe protein at 57 kDa. To confirm the presence of the nitrogenase, electron paramagnetic resonance was done to characterize metalloproteins of the nitrogenase. Electron paramagnetic resonance spectrum corresponded to the FeMo cofactor of MoFe protein with spin state S=3/2 and g-values of g_x = 4.503, g_y =4.268 and g_z=1.993. These values compared with literature confirmed the presence of the nitrogenase from the *Azospirillum amazonense* bacterium from sugar cane root.

Keywords: diazotroph bacteria, nitrogenase, biological nitrogen fixation, electronic paramagnetic resonance.

CONTENT

List of abbreviations

GDP: Gross Domestic Product

N_2: molecular nitrogen

BNF: Biological Nitrogen Fixation

MoFe protein: molybdenum-iron protein

Fe protein: iron protein

FeMoco: iron-molybdenum cofactor

ARA: Acetylene Reduction Assay

SDS-PAGE: Sodium Dodecyl Sulfate-Polyacrylamide Gels

EPR: Electronic Paramagnetic Resonance spectroscopy

NFb: Nitrogen Fixing bacteria media

ATP: adenosine triphosphate

Mo-nitrogenase: molybdenum-based nitrogenase

V-nitrogenase: vanadium-based nitrogenase

Fe-nitrogenase: iron alone nitrogenase

EPR: Electronic Paramagnetic Resonance

LB: Luria Bertani media

BMS: Bristol-Myers Squibb media

PDA: Potato Dextrose Agar

MT: Motility Test agar

FID: Flame Ionization Detector

GC: Gas Chromatography

DT: sodium dithionite

PMSF: phenylmethylsulfonyl fluoride

BCA: Bicinchoninic acid assay

BSA: Bovine Serum Albumin

1. INTRODUCTION

Sugarcane production is important in the economic development of Ecuador since it represents 3,30% of agricultural Gross Domestic Product (GDP)[1,2]. According to the National Institute of Statistics and Censuses, in Ecuador, the production of sugarcane reached a total of 7.5 million tons in 2018. Guayas, Cañar and Imbabura provinces generated 95.6% of total sugarcane production. In the Urcuqui canton of Imbabura province, sugarcane is the most abundant crop and has high profitability given the presence of the sugar industry in this area[3,4].

According to the Ministry of Agriculture and Livestock of Ecuador, production costs that are invested in one hectare of sugarcane are distributed as follows: 28% are destined for labor, 20% for machinery, 11% for land administration and 41% for inputs such as fertilizers and insecticides[5]. Fertilizers supply nutrients in assimilable compounds to the plant to increase the growth and productivity of crops. One of the most important nutrients is nitrogen and fertilizers provide nitrogen as ammonia compound[6].

Ammonia is obtained from the reduction of molecular nitrogen (N_2) by nitrogen fixation processes such as the Haber-Bosch process and biological nitrogen fixation (BNF). The Haber-Bosch is an industrial process that produces ammonia but needs an iron catalyst and extreme conditions as the pressure of 200 - 1000 atm and temperature 400 - 6000 ºC. Unfortunately, the Haber-Bosch process presents some disadvantages such as high equipment costs for a factory implementation, consumes a large amount of fossil fuels and contributes to environmental pollution[7–9].

The BNF is a process that occurs in nature by a catalytic enzyme called nitrogenase. Diazotroph bacteria have the nitrogenase enzyme that catalyses the reduction of N_2 to ammonia[10,11]. The ammonia produced by the enzyme is assimilable for the plant. The nitrogenase enzyme is composed of two metalloproteins, the molybdenum-iron protein (MoFe protein) and the iron protein (Fe protein). MoFe protein is constituted by two metalloclusters: the iron-molybdenum cofactor (FeMoco) and P-cluster. Fe protein is composed of [4Fe:4S] cluster[8].

So far, it has only been possible to obtain information about the nitrogenase of *Azotobacter vinelandii* and *Gluconacetobacter diazotrophicus* bacteria[7,12]. The study of

diazotroph bacterium is of great importance as it can provide information about the nitrogenase enzyme in the BNF process. And the study of BNF can solve the disadvantages of the Haber-Bosch process and reducing the use of harmful fertilizers for the environment.

This work was focused on the purification and characterization of the nitrogenase enzyme of a diazotroph bacterium from sugar cane in the Urcuqui canton. The bacterium was isolated from the root of the sugarcane. The identification of the bacterial genus was made by morphological and biochemical characterization. Acetylene reduction assay (ARA) was performed to evaluate nitrogenase activity from a bacterium. The nitrogenase enzyme from bacterium was purified and analyzed by sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE). The MoFe protein and Fe protein obtained in the SDS-PAGE electrophoresis gel were characterized by electronic paramagnetic resonance spectroscopy (EPR). This technique was used to analyze the electronic properties of paramagnetic metals of MoFe and Fe protein.

1.1. Nitrogen

Nitrogen is a non-metallic element of group 15 of the periodic table, has atomic number 7. The nitrogen atom has an electronic configuration of [He] $2s^2 2p^3$. Under normal conditions, N_2 is a colorless, odorless, tasteless diatomic gas. From the molecular orbital diagram for N_2, the bond order is 3, which means that the molecule has a triple bond and is pretty stable (Figure 1). N_2 is a small molecule because it has a bond length of 1.10 Å (110 pm). Also, N_2 is an unreactive molecule by the strong triple bond that has a bond energy of 946 kJ mol^{-1}[13].

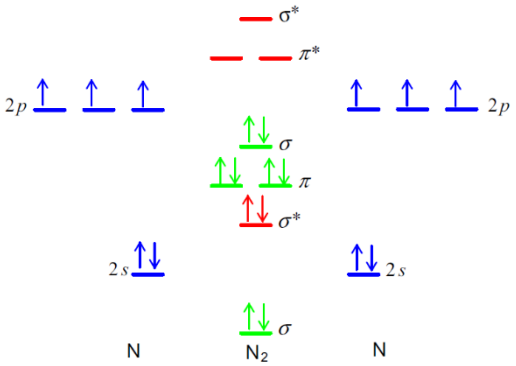


Figure 1. Molecular orbital diagram for N_2[13].

1.2. The nitrogen fixation

Nitrogen is an essential element for all living organisms since it is a component of amino acids, nucleic acids and proteins. N_2 is the most abundant molecule in the atmosphere and occupies 78% by volume. But, N_2 is an inert gas and cannot be used directly by animals or plants[8,14]. Nitrogen fixation consists of the reduction of N_2 to produce ammonia, which is an assimilable chemical component for the development of life on earth. The most important nitrogen fixation processes are: atmospheric, industrial, and BNF.

Atmospheric nitrogen fixation: is a natural mechanism that fixes nitrogen through thunderstorms. Lightning of thunderstorm generates enough energy to break the triple bond of N_2 that reacts with oxygen to form nitrogen oxides (Figure 2). However, nitrogen oxides produced from this process are less assimilable for the plant than ammonia produced by nitrogen-fixing organisms [15,16].

$$N_2 + O_2 \xrightarrow[thunder]{lighting} 2NO \qquad (1)$$

$$2NO + O_2 \xrightarrow{oxidation} 2NO_2 \qquad (2)$$

$$2NO_2 + H_2O \rightarrow HNO_3 + HNO_2 \qquad (3)$$

$$4NO_2 + H_2O + O_2 \rightarrow 4HNO_3 \qquad (4)$$

$$HNO_3 \rightarrow H^+ + NO_3^- \qquad (5)$$

$$HNO_2 \rightarrow H^+ + NO_2^- \qquad (6)$$

Figure 2. Atmospheric nitrogen fixation reactions. (1) N_2 dissociated by thermal energy from lighting reacts with oxygen to form nitric oxides. (2) Nitric oxide is oxidized in the presence of oxygen to form nitrogen dioxide. (3) Nitrogen dioxide may react with water and produce nitric and nitrous acids. (4) Nitrogen dioxide can react with rain and atmospheric oxygen to form nitric acid. (5) In the soil, nitric acid reacts with rainwater and alkaline substances to produce a proton and forming nitrate ion. (6) Nitrous acid reacts with rainwater and forms nitrite ion.

Industrial nitrogen fixation: one of the most used processes is called Haber-Bosch. This process prepares ammonia using N_2 and hydrogen gas at high temperatures near 400-6000 ºC and high pressures of 200-1000 atm with the help of an iron catalyst in an exothermic process (Figure 3). These conditions are necessary for the activation of the inert triple bond of N_2[7].

$$N_2 + 3H_2 \xrightarrow[Fe\ catalyst]{\substack{6000^{\circ}C \\ 1000\ atm}} 2NH_3 \qquad \Delta H^{\circ} = -91{,}8\ kJ/mol$$

Figure 3. Haber-Bosch process conditions. The chemical reaction of industrial production of ammonia and energy required for the reaction, enthalpy of an exothermic reaction.

The Haber-Bosch process is highly expensive because the equipment price for a factory has an estimated $255 million. Also, for the synthesis of ammonia, the raw material is expensive and needs high fossil fuels imput[9].

The global production of ammonia by the Haber-Bosch process is approximately 150 million metric tons per year and 80% of ammonia is used for nitrogen fertilizers that increase the crop yield. Nitrogen fertilizers are not very efficient, only 30% end in plant tissues. The rest of the fertilizer that the plant does not absorb accumulates in the soil and converts to nitrates, which cause negative changes in the environment since it contributes to the contamination of soil, water, and natural areas[6,17].

The excessive use of ammonia as fertilizers becomes a problem since it affects the balance of the ecosystem, human health and produces the formation of greenhouse gases such as nitrous oxide and nitric oxide. In the soil, the ammonia can form nitrates, which can contaminate groundwater and surface water. An example is the intensive growth of algae in lakes or rivers, which limits the amount of oxygen available for the development of aquatic organisms. On the other hand, drinking groundwater contaminated with nitrates can cause diseases such as thyroid cancer, hypertension and stomach cancer[6].

Therefore, the study of a process such as BNF, which is more efficient, would provide better biological inspiration for crop fertilization. BNF can solve the problems of the Haber-Bosch process such as extreme conditions of pressure and temperature, high equipment costs and contribution to environmental pollution.

Biological nitrogen fixation: is a process by which plants obtain nitrogen for growth. This process can provide until 60% of the nitrogen that is necessary for crop growth, indicating that it may have economic potential[11,18].

A group of bacteria and archaea called diazotroph have a nitrogenase enzyme that is responsible for nitrogen fixation. The nitrogenase enzyme is the only natural system capable of catalyzing the reduction of N_2 to ammonia[11,17]. In contrast to specific

conditions required in the Haber-Bosch process, the BNF takes place under ambient conditions[7].

The equation of the nitrogen fixation of the enzyme nitrogenase shows that eight electrons and eight protons are delivered for the reduction of one molecule N_2 to produce two ammonia molecules and one hydrogen molecule (Figure 4). Moreover, the reaction consumes 16 adenosine triphosphate (ATP) molecules[14].

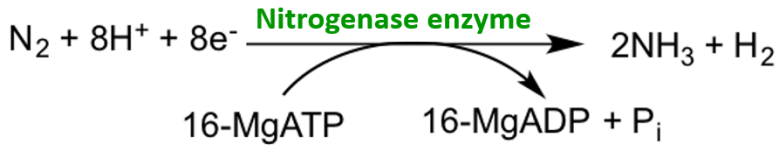


Figure 4. The nitrogen-fixing reaction by nitrogenase enzyme. In this reaction, 16 ATP molecules are used to fix one molecule of N_2 into two molecules of ammonia.

1.3. Nitrogen-fixing bacteria

Nitrogen fixation occurs in the soil by diazotroph organisms. These nitrogen-fixing organisms exist as free-living diazotroph and symbiotic diazotroph. Free-living diazotrophs are aerobic as *Azotobacter,* anaerobic as *Clostridium* and photosynthetic bacteria as C*yanobacteria*. Symbiotic diazotrophs as rhizobia are found in the nodules of plants. The symbiosis occurs when the host plant provides energy source as carbon to the bacterium. And the bacterium provides nitrogen for the growth of the plant.

To study nitrogen fixation, *Azospirillum, Gluconacetobacter diazotrophicu*s, and *Herbaspirillum* are the main diazotroph bacteria identified in sugarcane [11].

***Azospirillum*:** was discovered in 1922 by Beijerinck, who isolated it using nitrogen, malate, or lactate free media. This bacterium is symbiotic and can colonize the surface or the inside of the root, stems or leaves [18].

The bacterium is micro-aerobic, non-fermentative and Gram-negative to Gram variable from the family of Rhodospirillaceae. Some studies have reported that the beneficial effect it brings to the plant is by the fixation of nitrogen and the production of growth hormones. Three species of this genus have been isolated from the sugarcane. Three species of this genus have been isolated from the sugarcane: *Azospirillum amazonense, Azospirillum brasilense* and *Azospirillum lipoferum* [19,20].

***Gluconacetobacter diazotrophicus*:** was isolated by Cavalcante and Dobereiner in 1989, from the sugar cane from Brazil. This bacterium is of great interest in agriculture since it can fix up to 150 kg nitrogen/hectare in sugarcane crops [21,22]

Gluconacetobacter diazotrophicus is Gram-negative, micro-aerobic and rod-shaped bacterium[22]. This bacterium is characterized by growing at acid pH and produced acetic acid from ethanol. The optimal growth of this bacterium is at pH 5,5 and nitrogen-fixing activity at pH 2,5. This bacterium does not possess nitrate reductase, and the activity of the nitrogenase enzyme is not inhibited by nitrates, but partially inhibited by ammonium ion[19]. This bacterium inoculated on potato agar supplemented with 10% sucrose has dark brown color colonies[21,23].

***Herbaspirillum*:** is one of the most important diazotrophic bacteria found in the sugarcane. This bacterium is Gram-negative, curved rods with polar flagella. The optimal growth and nitrogen-fixing activity are performed at a pH range of 5.3 – 8[19]. Two species of this genus have been isolated from the sugarcane: *Herbaspirillum seropedicae* and *Herbaspirillum rubrisubulbicans*

Herbaspirillum seropedicae was isolated from roots and stems of sugarcane. This bacterium inoculated in the nitrogen fixing bacteria (NFb) agar has small white colonies[18,24]. *Herbaspirillum rubrisubalbicans* is pathogenic and produces mottled stripe disease in sugarcane[25].

1.4. Nitrogen-fixing systems

Biological nitrogen fixation of diazotroph bacteria is mostly done in root nodules (Figure 5 a). These nodules represent the symbiosis between the plant and the bacterium. When both come into contact, there is a change of differentiation in the bacterium to form a bacteroid. The bacteroid is responsible for expressing nitrogenase activity by its nitrogenase enzyme complex. The invasive process of the bacterium becomes a beneficial process for both the plant and the bacterium[26].

For the formation of root nodules, a plant that requires nitrogen will go through a series of stages. The first stage consists of a process of cell recognition by the bacterium. The plant emits chemoattractant signals such as carbohydrates, organic acids, vitamins, amino acids or flavonoids. The bacterium picks up these signals and looks for points to invade the root of the plant. Subsequently, adhesion between the plant and bacterium occurs and deformations in the root hairs are produced[26].

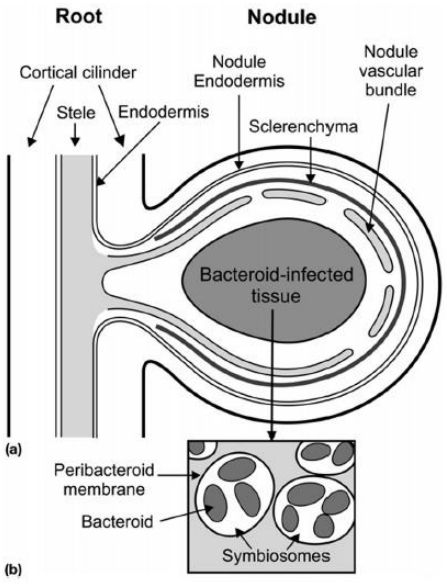


Figure 5. Formation of root nodule. a) Schematic drawing of root nodule parts when bacterium infects a root b) Symbiosomes [14].

The second stage takes place when the bacterium invades the root and forms an infection tube, through which it advances until it reaches the root cortex. The infection tube is a cell wall deposit in reaction to the infection caused by the invading bacterium. This infection tube advances through the root hair to the root bark. In the cortex, the bacterium colonizes the cytoplasm of the plant cell. The colonizing bacterium undergoes a series of morphological changes and differentiates, giving rise to a bacteroid. On the cytoplasm of the plant cell, the bacteroid is wrapped in a peribacteroid membrane. This membrane allows to avoid direct contact with the cytoplasm of the cell, forming what is called symbiosome (Figure 5 b)[14].

In the third stage, within the symbiosome, abnormal growth of the bacterium produces the break the root surface and allows the formation of **root nodules**. Once the cell division is finished, nitrogen fixation begins. Both processes cannot be performed at the same time. Also, mature nodules have developed structures around infected tissue: endodermis, vascular system and a layer of oxygen hampering cells[14,26].

1.5. Nitrogenase enzyme

Nitrogenase enzyme catalyzes the reduction of N_2 to ammonia in BNF process under ambient conditions (Figure 4). This enzyme is anaerobic and is inhibited by contact with molecular oxygen. There are two types of nitrogenases. The Group-I nitrogenases

are closely but genetically distinct enzymes: molybdenum-based nitrogenase (Mo-nitrogenase), the vanadium-based enzyme (V-nitrogenase) and iron alone (Fe-nitrogenase). Each enzyme has a different heterometal either Mo, V, Fe. On the contrary, Group-II nitrogenase was isolated from a thermophilic organism *Streptomyces thermoautotrophicus*. This nitrogenase is insensitive in the presence of oxygen, carbon monoxide, and hydrogen gas[27].

Most nitrogen-fixing organisms have Mo-nitrogenase. V-nitrogenase or Fe-nitrogenase appears randomly. The expression of one or another nitrogenase depends on the availability of Mo, V, Fe metals in the growth. Mo-nitrogenase is the most efficient enzyme for nitrogen fixation.

Mo-nitrogenase is an enzyme that consists of two proteins: **MoFe protein** and **Fe protein**. MoFe protein is an $(\alpha\beta)_2$ heterotetramer and contains two metalloclusters, the FeMoco and P-cluster. Fe protein is a γ_2 homodimer composed of [4Fe:4S] cluster and two ATP-binding sites (Figure 6)[7].

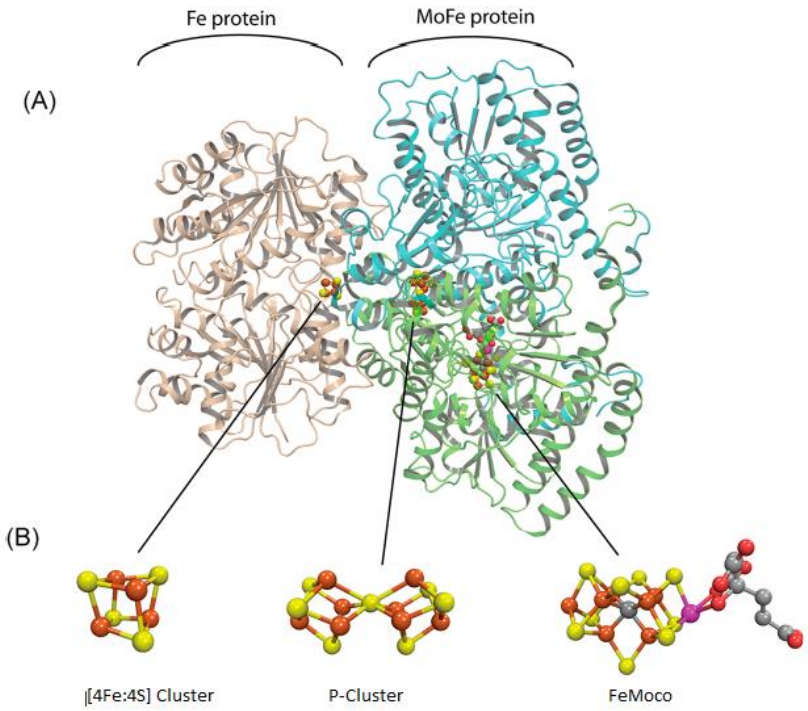


Figure 6. Molybdenum nitrogenase. (A) One catalytic half of the Fe protein:MoFe protein complex with the Fe protein homodimer shown in pink, the MoFe protein α subunit in green, and the β subunit in cyan. (B) Space-filling and stick models for the 4Fe−4S cluster, P-cluster, and FeMoco[28].

Fe protein is a homodimer of 64 kDa molecular mass and has [4Fe - 4S] cluster joins by two identical subunits, which have their own MgATP / MgADP-binding site. Fe protein is a specific reducer for MoFe protein[7]. MoFe protein is an $\alpha_2\beta_2$ heterotetramer with 230 kDa molecular mass. Each αβ subunit has a copy of FeMoco and P-cluster. FeMoco is [7Fe:9S:Mo:C] complex, which is the site of union and reduction of the substrate[29]. P-cluster is [8Fe:7S] metal center and cannot be isolated since it is disrupted when removed from MoFe-protein[27,30].

For the study in vitro of this enzyme, either flavodoxin or ferredoxin (a small iron-sulfur protein) is used and acts as a Fe protein reductant. Later, electrons are transferred in just one step to MoFe protein with 2MgATP being hydrolyzed and the consumption rate of MgATP is reduced to half. In artificial conditions, sodium dithionite is the most used. The Fe protein accepts only one sodium dithionite electron, which will be transferred to the MoFe-protein, along with two MgATP being hydrolyzed [27]. MgATP has the primary function of binding and hydrolysis to regulate the transfer of an electron from [4Fe:4S] cluster to MoFe protein. The electron acceptor of the MoFe protein is FeMoco, where the substrate binds and is reduced. After the transfer of electrons, the Fe protein dissociates from MoFe protein. Later, oxidized Fe protein is reduced by replacing MgADP with MgATP. Taking this as a reference to the process of hydrolysis of MgATP to MgADP seems to regulate the affinity of the association between Fe protein and MoFe protein. Studies of electron transfer, electron flow, the ratio of electrons transferred to MgATP hydrolyzed is not confirmed to occur in vivo [27].

Mo-nitrogenase catalyses the reduction of other small molecules (Figure 7). These must meet at least five requirements: an anaerobic environment, a consistent source of MgATP, a reductant, a buffer system compatible with the product-quantification protocol to be used, and the desired substrate. The most used is acetylene, the nitrogenase reduces acetylene to ethylene. When there is no other substrate in the assay, the flow of electrons is directed for the reduction of protons to H_2. The production of H_2 inhibits the reduction of N_2 but does not affect the reduction of another small molecule [27,31,32].

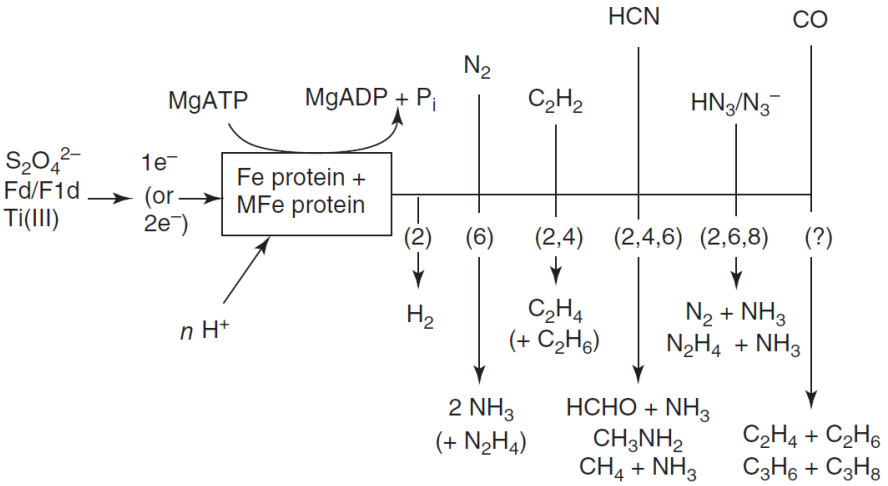


Figure 7. Reduction of small molecules by nitrogenase enzyme. Numbers in parentheses are the number of electrons used to form the products shown. CO is not only a substrate but also a potent reversible inhibitor of all nitrogenase-catalyzed substrate reductions except for that of protons to H_2. Fe-p represents the Fe protein, MFe-p is the MoFe protein; reductants are dithionite ($S_2O_2^{-4}$), titanium (III) citrate (Ti(III)), Fld (flavodoxin hydroquinone), or Fd (ferredoxin)[27].

1.5.1. Study of nitrogenase metalloclusters by electron paramagnetic resonance

EPR spectroscopy is one of the most used tools in the fields of inorganic and bioinorganic chemistry to study paramagnetic metals. Only paramagnetic species can be detected by EPR since it has a small number of unpaired electrons, while diamagnetic (no-unpaired electrons) and ferromagnetic (a lot of unpaired electrons) are not detected. The interaction of unpaired electrons of paramagnetic species align with an external magnetic field (B_0) is called Zeeman interaction (Figure 8). The applied field can be varied with fixed microwave frequency, and the species will resonate, resulting in absorption energy. Energy absorption data (relative units) are plotted on the *Y-axis*, while on the *X-axis* the magnetic field (gauss). EPR spectrum is reported as the first derivative of the absorption spectrum. An inflection of the absorption data is assigned as *g-values* (g_x, g_y, g_z). To get g, the (Equation 1 is used, where v is microwave frequency (GHz), B magnetic field (Gauss).

(Equation 1) $$g = \frac{714.48x\ v}{B}$$

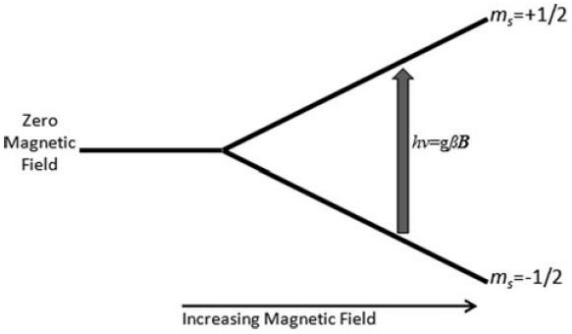


Figure 8. The Zeeman interaction. Resonance would be obtained when the energy of the magnetic field (increasing to the right) matches the energy difference (between the $m_s = -1/2$ and $m_s = +1/2$ states) at a fixed microwave frequency arrow), resulting in the absorption of energy equal to gβB, where β is Bohr magneton and B is the magnetic field that satisfies the resonance conditions [32].

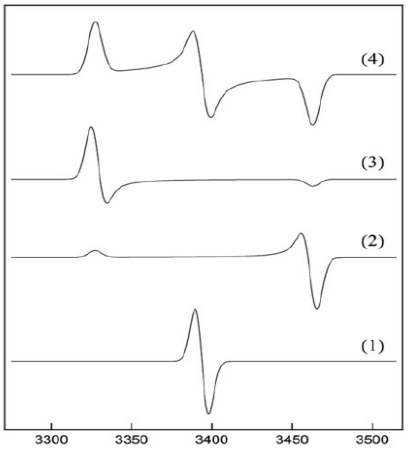


Figure 9. Simulated derivative EPR spectra. (1) Isotropic EPR signal when $g_x = g_y = g_z$ (2), axial signal when $g_y = g_z \neq g_x$ (3), axial signal when $g_x = g_y \neq g_z$, and (4) rhombic EPR signal when $g_x \neq g_y \neq g_z$ [32].

Also, between the unpaired electrons of the atom, there is an interaction that produces to the splitting of the signal in three separate transitions (g_x, g_y, g_z). When all values of g have identical energy, the EPR spectrum is called **isotropic** (Figure 9, trace 1). If two of the three values of g are identical, the signal is **axial** (Figure 9, trace 2, 3). And when the values of g are different, the signal is **rhombic** (Figure 9, trace 4) [33].

The nitrogenase enzyme contains three metalloclusters: [4Fe-4S] cluster from Fe protein, P cluster, and FeMoco from MoFe protein. The cluster [4Fe-4S] of the Fe protein is a paramagnetic species, in the presence of dithionite and has a state of oxidation 1+, with $3Fe^{2+}$, $1Fe^{3+}$ species that has a mixed spin state of S = 1/2, and S = 3/2 in EPR spectrum. The spin state of S = 1/2 shows a rhombic signal in g = 2 region, with a spin state of S = 3/2 in g = 3-4 region has inflections (Figure 10, trace 1). When the cluster [4Fe-4S] of the Fe protein undergoes oxidation by oxygen, Fe protein acquires a 2+ oxidation state, which is diamagnetic and cannot be detected by EPR[33–35].

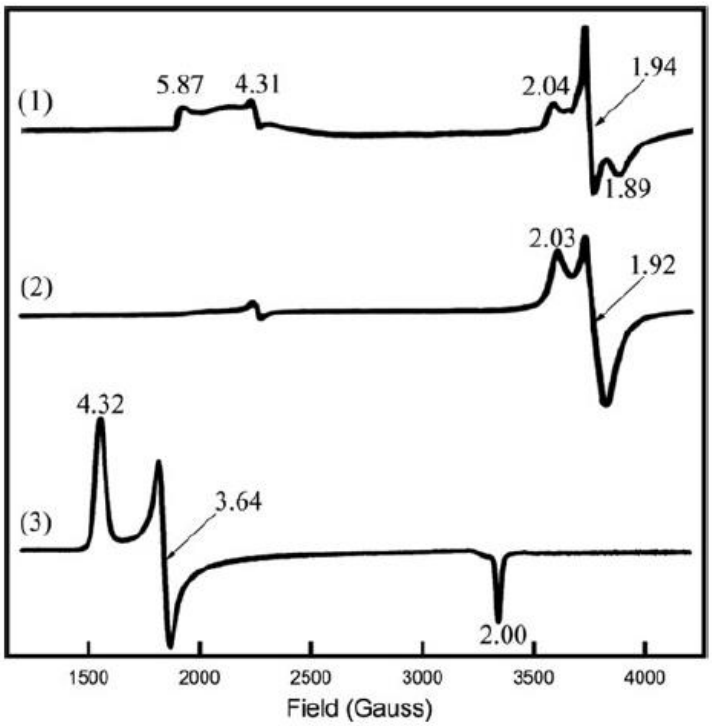


Figure 10. Derivative EPR spectra of Fe protein and MoFe protein. Trace 1 shows the EPR spectrum of Fe protein in the as-isolated state. Spectral conditions were 9.44 GHz microwave frequency, 1.26 modulation amplitude, and 15 mW microwave power at 12 K. Trace 2 shows the Fe protein in the presence of MgATP under the same spectra conditions as trace 1. Trace 3 shows the EPR spectrum of the MoFe protein in its as-isolated state. Spectral conditions were 9.65 GHz microwave frequency, 1.26 modulation amplitude, and 1.0 mW microwave power at 4.8 K [33].

On the other hand, MoFe protein can be detected by EPR because they have paramagnetic metals. The FeMo cofactor has an S = 3/2 signal, rhombic signal g = 4.32, 3.64, 2 (Figure 10, trace 3). Some MoFe proteins that varied amino acids in the FeMoco values g = 4.32, 3.64 have been shifted, causing changes in the electronic properties of the cofactor and EPR spectrum. Also, P-Cluster is diamagnetic and is not detected by EPR[33].

2. PROBLEM STATEMENT

Nitrogen is an important nutrient to increase the growth and production of crops. Fertilizers provide nitrogen in the assimilable compound of ammonia for the plant. The ammonia is produced by nitrogen fixation processes such as BNF and Haber-Bosch process. BNF is a process of reduction of N_2 to ammonia by the nitrogenase enzyme from diazotroph bacteria. The study of the BNF process can solve the disadvantages of the Haber-Bosch process such as extreme conditions of temperature and pressure, high equipment costs for factory implementation and contribution to environmental pollution. In contrast to Haber-Bosch process, the BNF takes place in nature under ambient conditions and may have economic potential in the future. The study of diazotroph bacterium is of great importance as it can provide information about the nitrogenase enzyme as a biological alternative for crop fertilization. I present this work in order to purify and characterize the nitrogenase enzyme of the sugarcane diazotrophic bacterium.

3. OBJECTIVES

General objective

The main objective of this work is the purification and characterization of the nitrogenase enzyme of diazotroph bacterium from the sugar cane extracted in the Urcuqui canton.

Specific objectives

- Isolating a diazotrophic bacterium from the sugarcane root.
- Identifying a bacterium's genus thought the morphological and biochemical characterization
- Evaluating the nitrogen activity of the bacterium by ARA
- Purification of the nitrogenase enzyme by column chromatography.
- Analyzing the MoFe protein and Fe protein of nitrogenase enzyme by SDS-PAGE.
- Characterization of metalloproteins of nitrogenase by EPR

4. METHODOLOGY

The following methodology was divided into three sections:

- Materials
- The isolation and identification of diazotroph bacteria
- The purification and characterization of the nitrogenase enzyme.

4.1. Materials

Reagents

The following **chemical reagents** were used: dipotassium phosphate, ≥98%, Sigma-Aldrich; potassium dihydrogen phosphate, 99.99%, trace metals basis Sigma-Aldrich; magnesium sulphate heptahydrate, ≥98%, Sigma-Aldrich; calcium chloride, ≥99.9%, Sigma Aldrich; sodium molybdate dihydrate, 98+%, Sigma; iron III chloride hexahydrate, 98%, Sigma-Aldrich; potassium chloride, ≥85.0%, Fisher; sodium chloride, 99,5 %, Sigma-Aldrich; iron II sulphate heptahydrate, ≥99.0%, Fisher; manganese sulphate, ≥99.99%, Sigma; ammonium chloride, 99.998%, Sigma; polyacrylamide, Sigma-Aldrich; sodium dodecyl sulphate, 98,5%, Sigma-Aldrich; sodium dithionite, ≥82%, Merck; L-malic acid, 95%, Sigma; hydrogen peroxide, 30%, Fisher; bromothymol blue, 95 %, Merck; crystal violet, 1%, Sigma; iodine, Sigma-Aldrich; safranine Sigma-Aldrich; ethanol 70%, Fisher chemical; acetone, 95%, Sigma-Aldrich; Q-Sepharose, GE Healthcare Life Sciences; Sephacryl S-200, GE Healthcare Life Sciences; Coomassie blue, Sigma-Aldrich; methanol, ≥99.8%, Fisher chemical; acetic acid, ≥99.7% w/w, Fisher chemical.

The following **biological reagents** were used: Luria Bertani media, TM media; agar, Sigma; yeast extract, Sigma-Aldrich; agar papa dextrose, TM media; sucrose, Crystalline/Certified ACS Fisher Chemical; glucose, Fisher Chemical; glycerol, 99.5%, Sigma; D-fructose extra pure, Fisher Chemical; peptone, Sigma-Aldrich; Tris-base Molecular Biology grade, Merck; Dnase I-Ampliification grade, 100U 1U/ul Invitrogen; PMSF Biochemica, Applichem; protease cocktail, Sigma Aldrich; BCA kit Pierce™ BCA Protein Assay Kit, Thermo Scientific; mercaptoethanol, Thermo Scientific TM; glycine ≥99%, Sigma Aldrich.

Microorganism

A commercial strain *Gluconacetobacter diazotrophicus* 'Dobereiner PA1 5' was used in the morphology characterization.

Equipment

Major equipment and instrument needed: NanoDrop 2000/2000c spectrophotometer, Thermo Fisher Scientific; DU-650 UV-Vis spectrophotometer, Beckam; ultrasonic homogenisers, Sonoplus; Sorvall™ ST 16 Centrifuge Series, Thermo Scientific; Microscope DM300, Leica; Autoclave, JP SELECTA; MaxQ™ 4450 Benchtop Orbital Shakers, Thermo Scientific; 7890A Gas chromatograph equipped with a flame ionization detector (FID), Agilent Technologies; electrophoresis Cell System-Mini-protean II, BIORAD; ELEXYS E500 EPR device with ER-4102ST resonator, Bruker.

Culture media preparation

The composition of culture media is shown in the appendix section. The culture media that were prepared in this study are the following: nitrogen-fixing bacteria media (NFb) (Appendix 1), LGI media (Appendix 2), motility test agar (MT) (Appendix 3); Bristol-Myers Squibb media (BMS) (Appendix 4), SYP media (Appendix 5). The commercial mediums used in this work were the following: potato dextrose agar (PDA), Luria Bertani media (LB).

The culture media prepared was sterilized by the autoclave at 121 °C for 15 minutes at 15 psi. The glass material, micropipette tips were sterilized by the autoclave at 121 °C for 15 minutes at 15 psi.

4.2. Isolation and identification of diazotroph bacterium

4.2.1. Study site and sample collection

Sugarcane plants were extracted from crops of the sugar mill in the Urcuqui canton, Imbabura province, Ecuador. Geographically the sample collection site is 0 ° 25'19.2 "N 78 ° 11'52.8" W. Sugarcane plants were randomly chosen from a homogeneous soil plantation that was cultivated in a grid pattern and had a length of less than one hectare. Later, roots were cut from the sugarcane plant. Sugarcane roots were stored in sealed plastic bags and stored at 4 °C in the refrigerator until analysis in the laboratory.

4.2.2. Surface sterilization of root sample

The sugarcane roots were left at room temperature and were washed under tap water to eliminate the excess of soil. Nodules were cut from the rest of the sugarcane root.

Nodules were sterilized by immersion in ethanol (95 % v/v) for one minute and then washed ten times with distilled water for ten minutes[36].

4.2.3. Isolation of diazotroph bacterium from sugarcane root

Nodules were individually cut into small fragments with a sterilized knife. The fragments were inoculated in LB agar plate. This Petri dish was incubated at 27 ºC and monitoring every 24 hours to isolate the colonies formed. Once the isolated colony was identified, it was inoculated on a second LB agar plate and incubated at 27 ºC. A colony was picked from the second LB agar plate and was subcultured into the NFb agar plate. NFb media is a selective medium to isolate only nitrogen-fixing bacterium[36].

In this work, the bacterium that grew in the NFb agar plate was called as an **isolated bacterium**. The isolated bacterium was subcultured every 15 days in NFb agar plate to prolong the bacterium life.

4.2.4. Determination of bacterial growth

The optical density method is useful to measure the growth rate of the bacterial population over time with the use of a spectrophotometer. Increased turbidity of inoculated liquid media indicates the index of bacteria growth. The light transmitted decreases as the bacteria population increases[37]. The isolated bacterium was inoculated on 100 mL liquid LGI media and 100 mL NFb media and respectively incubated at 37 ºC. The optical density of culture media was measured at 600 nm on the UV-VIS spectrophotometer.

4.2.5. Characterization of the isolated bacterium

The isolated bacterium was characterized to identify the bacterium´s genus based on the morphological and biochemical properties.

4.2.5.1. Morphological characterization

Morphological characterization was performed with two bacteria:

- Isolated bacterium from sugarcane root.
- *Gluconacetobacter diazotrophicus* commercial strain. This bacterium had previously been isolated from the sugarcane root[10,22].

The *Gluconacetobacter diazotrophicus* bacterium was used with the objective of analyzing their morphological characteristics and comparing them with the

morphological characteristics of the isolated bacterium from the sugar cane. This comparison allowed identifying if the isolated bacterium belonged to the genus G*luconacetobacter diazotrophicus*.

The following morphological characterizations are based on previous studies for the identification of a bacterium[3,20,38,39].

(i) Gram staining

Gram staining method was performed to observe the cell morphology to the isolated bacterium and *Gluconacetobacter Diazotrophicus*. The Gram-positive or Gram-negative staining was observed under a microscope with 100x objective lens and oil immersion.

(ii) Colony morphology

The colony morphology method was used to describe the colony characteristics from the isolated bacterium and *Gluconacetobacter diazotrophicus*. LGI, NFb, PDA sterilized media were poured into Petri dishes separately. Isolated bacterium and *Gluconacetobacter diazotrophicus* were inoculated on their respective Petri dish and were incubated at 37ºC for five days. After the incubation, the shape, size, color and texture from colonies in Petri dishes were observed.

4.2.5.2. Biochemical characterization

Biochemical characterization was performed to demonstrate that the isolated bacterium from the sugarcane root belonged to the genus of *Azospirillum*. The following tests were based on Bergey´s Manual of Systematic Bacteriology [20].

(a) Bristol-Myers Squibb media

Four Petri dishes with sterilized BMS medium were prepared. The first Petri dish contained as a carbon source the L-malic acid. The second Petri dish, the carbon source was malic acid and sucrose. The third Petri dish was prepared with glucose as a carbon source. And the fourth Petri dish, sucrose as a source of carbon. The isolated bacterium was inoculated on the Petri dishes that were prepared with BMS media. All Petri dishes were incubated at 37ºC for five days.

(b) Motility

To determine if the isolated bacterium was mobile or immobile, two test tubes with 10 mL of MT media were prepared and sterilized. The isolated bacterium was inoculated

on the first test tube. The inoculation was performed with an inoculation needle to a depth of 1.2 cm in MT media. The second test tube was not inoculated and was used as an assay control. Tubes were incubated at 37 °C. The motility of the bacterium was evaluated every 24 hours for five days to verify bacterial growth. If the bacterium grew from the inoculum line and spread in the medium, causing turbidity, it shows a positive test. Otherwise, a negative test.

(c) Utilization of carbon source

The growth of the bacterium in a specific culture medium with different carbon sources was used to identify a bacterium. Semi-solid LGI medium was prepared, but sucrose as carbon source was replaced by glucose (1% v/v) and glycerol (1% v/v). For each carbon source, two test tubes were made. One tube was inoculated with the isolated bacterium and the other tube was not inoculated and was used as a control assay. All test tubes were incubated at 37 °C for three days. After incubation, if the bacterium formed a veil or disk near the surface of the medium, the test was positive. Otherwise, the test was negative.

(d) Acid production test

LGI media has bromothymol blue as an indicator of pH change (Figure 11). The liquid LGI media was used, but sucrose was replaced by glucose (1% v/v) and fructose (1% v/v). For each sugar, two test tubes were made, one inoculated with the isolated bacterium, the other as a control. The acid production was observed after five days of incubation at 37°C. If the color of the medium changed from green to yellow, the test was positive. Otherwise, the test was negative.

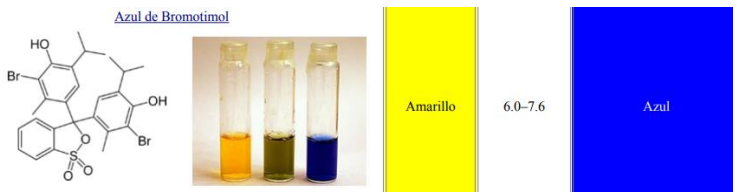


Figure 11. Bromothymol blue pH range[40].

(e) Catalase test

To determine the presence of catalase enzyme, a colony of the isolated bacterium was placed on a glass slide and added drops of hydrogen peroxide (3 % v/v). The presence of bubbles indicated a positive catalase test[41].

(f) Growth with NaCl

Growth in the presence of 3% NaCl had been observed in some species. Therefore, the LGI medium was prepared in a Petri dish with an increased concentration of 3 % NaCl. The isolated bacterium was inoculated and incubated for five days at 37°C. Colony growth of the isolated bacterium in this medium corresponds to a positive test, otherwise a negative test.

4.3. Purification and characterization of the nitrogenase enzyme

4.3.1. Nitrogenase activity by acetylene reduction assay

ARA is one of the most used techniques to measure the BNF of nitrogen-fixing bacteria. This technique focuses on the ability of the nitrogenase enzyme to break the triple bond (reduction reaction) of acetylene (H─C≡C─H) to ethylene gas (C_2H_4). The ethylene gas produced by nitrogenase can be quantified by gas chromatography (GC)[42]. This assay was performed on a gas chromatograph Agilent 7890A equipped with FID. The following conditions for ARA: nitrogen carrier gas (flow 1 mL/min), FID hydrogen gas (flow 35 mL/min), GS-GasPro column 60 m x 0.32 mm, oven temperature: Ramp 180 - 250 ºC.

(a) Calibration curve

The ethylene calibration curve was performed because the nitrogenase activity of the *Azospirillum amazonense* was calculated as moles of ethylene per culture. Different dilutions of ethylene with nitrogen were prepared for each reference point of the calibration curve, using a static mixer system and previously removing 30 mL of the air contained in the measuring tubes.

(b) Nitrogenase activity

Azospirillum amazonense bacterium was grown in LGI medium and incubated at 30 °C in a shaker overnight. 350 µL of the culture medium was taken with a sterile Pasteur pipette and transferred into four tubes. These tubes were incubated at 30ºC without agitation and cotton plugs are used to facilitate the exchange of atmospheric gases. After 20 hours of incubation, tubes were sealed with sterile rubber stoppers to prevent the exchange of gases from the atmosphere.

The effect of atmospheric oxygen concentrations on the acetylene reduction activity was evaluated. Of the 4 tubes prepared, the gas phase of the 3 tubes was modified as the following:

- Tube 1: control, unmodified gas phase.
- Tube 2: 15% gas phase was extracted and 15% acetylene was added.
- Tube 3: total gas phase was removed under vacuum.
- Tube 4: total gas phase was removed under vacuum and added 15% acetylene.

All tubes were incubated overnight at 30 °C. Ethylene production was quantified by taking 250 µL of the gas phase of each of the tubes with a gas-tight syringe and injecting them into the GC. The area of the ethylene peak obtained from each tube was converted to ethylene nmol per culture using the standard curve equation.

4.3.2. Purification of nitrogenase enzyme

The following methodologies were performed to purify the nitrogenase enzyme of the *Azospirillum amanzonense* bacterium that was isolated from the sugarcane root.

Three buffers solutions were prepared. The buffer R was used for the procedures of bacterial growth and cell lysis. The buffer E and buffer GF were used in the nitrogenase purification procedure. Buffers were prepared as the composition shown in Table 1. The buffers were sterilized by autoclave at 121 °C for 15 minutes at 15 psi and stored at 4 °C. It is important to mention that the sodium dithionite (DT) was added before the use of a buffer and cannot be stored for more than 48 hours[7,22,43].

Table 1. Buffer composition for nitrogenase enzyme purification.

Buffer solution	Composition
Buffer R	50 mM Tris (pH 8), 100 mM NaCl, 5 mM DT*
Buffer E	50 mM Tris (pH 8), 500 mM NaCl, 5 mM DT*
Buffer GF	50 mM Tris (pH 8), 200 mM NaCl, 5 mM DT*

*DT must be added before use of the buffer, and cannot be stored for more than 48 hours.

Growth media

For the *Azospirillum amazonense* growth, two methodologies were performed in order to choose the procedure that shows the greatest bacterial growth. The direct growth, the bacterium was inoculated directly in the LGI medium. The indirect growth, the bacterium was inoculated in a SYP media as preculture media.

The direct growth: 500 mL of LGI medium in 1L flask was prepared. The *Azospirillum amazonense* was inoculated in the medium and incubated at 37°C at 200 rpm for five hours.

The indirect growth: 25 mL of liquid SYP medium was prepared in a 50 mL flask and 500 mL of liquid LGI medium in a 1L flask. *Azospirillum amazonense* was inoculated into the SYP medium and incubated at 37 °C at 200 rpm for 24 hours. After incubation, the SYP medium was transferred to the LGI medium under sterile conditions to avoid contamination. This medium was incubated at 37°C at 200 rpm for 48 hours. After incubation time, the LGI medium was transferred to Falcon tubes and centrifuged at 4°C at 4000 rpm for 30 min. The supernatant was discarded, and the pellet was resuspended with cooled Buffer R (1g cell /1.4 mL) and stored at -20ºC until the time of use.

Cell lysis

The following procedure was based on the research of Fisher et al.[22] with some modifications. For the lysis procedure, the bacteria pellet was thawed and 500 µL of 0.01M phenylmethylsulfonyl fluoride solution (PMSF) was added. The bacterial pellet was placed on an ice bath. The cell rupture was performed with a sonicator with an amplitude of 40% for five cycles of five seconds, and a break of 30 seconds to control the temperature.

Later, 30 µL of DNase was added to the lysis fraction for one hour at room temperature. The lysis fraction solution was heated for five minutes at 25 °C in a water bath followed by immersion on an ice bath. This fraction was centrifuged 4000 rpm for 60 minutes at 4°C. The pellet was discarded and the supernatant had total protein content and was denominated as a **crude extract.** The crude extract was used for nitrogenase enzyme purification.

Nitrogenase enzyme purification

The following procedure was based on the research of Owens et al.[7] with some modifications taking into account the availability of reagents and equipment. The first chromatographic column was prepared with the Q-Sepharose resin, which was pre-equilibrated and washed with the Buffer R. The crude extract was loaded on the chromatographic column. Buffer E was used for the elution of the nitrogenase enzyme

with a linear gradient of 100 - 500mM NaCl. The collected fraction from this column was denominated as **Q-Sepharose fraction**. This fraction was stored at 4ºC.

The second chromatographic column was prepared with the Sephacryl S-200 resin, which was equilibrated and washed with Buffer GF. The collected fractions from the first column were loaded onto the second column and eluted with Buffer GF. Two fractions were collected and were denominated as **S-200 Frac 1, S-200 Frac 2**. These fractions were stored at 4ºC.

4.3.3. Protein concentration

The following procedure was based on the procedure of Thermo Scientific[7]. Bicinchoninic acid assay (BCA) was used to determine the protein concentration of four samples: the crude extract, the Q-Sepharose fraction, S-200 Frac 1 and S-200 Frac 2. The Thermo Scientific ™ Pierce ™ BCA Protein Assay had bovine serum albumin (BSA) as standard protein. BSA was diluted to prepare a set of diluted standards with a final concentration in the range 0 - 30µg/mL. Then, BCA working reagent was prepared in a 50: 1 ratio, reagent A: B. These reagents were included in the kit. Next, 20 mL of each standard and the unknown solution (sample) were added in the test tubes. BCA working reagent (2mL) was added to each tube and stirred well. Tubes were subsequently incubated for 30 minutes at 60 ° C and allowed to cool to room temperature. All samples were measured within 10 minutes in a Beckam spectrophotometer (DU650) at 562nm.

4.3.4. Sodium dodecyl sulfate-polyacrylamide gels

SDS-PAGE was performed to analyze the presence of FeMo and Fe protein in 4 samples: the crude extract, the Q-Sepharose fraction, S-200 Frac 1 and S-200 Frac 2. Each sample was denatured boiling and using sodium dodecyl sulfate. Then, denatured sample were resuspended in 10 µL of loading buffer x2 (20% glycerol, 0.125 M Tris / HCl pH 6.8, 4% SDS, 0.02% bromophenol blue, 10% mercaptoethanol). A Mini-protean II (BIORAD) was used for electrophoresis procedure with a running buffer (14.4% glycine, 0.3% Trizma base, 0.1% SDS) for 50 minutes at 150 V. The separated proteins were observed by staining the gel with Coomassie Blue (240 mg Coomassie blue, 120 mL methanol, 40 mL acetic acid, cbp water 500 mL).

4.3.5. Electron paramagnetic resonance spectroscopy of metalloproteins of the nitrogenase

Once the purified FeMo and Fe proteins had been identified. The bands of MoFe and Fe proteins were cut from the SDS-PAGE gel and diluted with a phosphate buffer. Then the MoFe and Fe protein bands were placed in a quartz EPR tube of 5mm (external diameter). Each EPR tube was subjected to flash freeze in liquid N_2. The EPR spectrum was obtained on a Bruker Elexys E500 device with ER-4102ST resonator, in the USAII of the Faculty of Chemistry of the UNAM.

The EPR experiments were performed at 10 K, an X-band with the following conditions: microwave frequency 9.4GHz, modulation frequency 100 kHz, modulation amplitude 3.00 G, microwave power 2.033 mW, attenuation 20.0 dB, a time constant 20.48 ms, conversion time 81,92 ms.

5. RESULTS AND DISCUSSION

5.1. Isolation of diazotroph bacterium

Figure 12. Sugarcane extracted from sugar mill of Urcuqui. (Left) Sugarcane plants. (Right) the root of the sugarcane plant collected.

The sugar cane plant was 18 months of cultivation when it was collected (Figure 12). The nodule growth zone was clearly observed when the root fragments were washed and sterilized. The nodules were inoculated on the LB agar plate (Figure 13 a). After 48 hours of incubation at 27ºC, colonies appeared near nodules fragments. One colony had filamentous morphology, and the second colony was smooth, raised, and circular (red circle in Figure 13b). The second colony was inoculated on a new LB agar plate because it had characteristics of nitrogen-fixing bacteria (Figure 13 c). Later, the colony from the second LB agar plate was inoculated on NFb agar plate. The NFb media is a selective medium to isolate only nitrogen-fixing bacteria. This isolated bacterium grew up after six days of incubation at 27 ºC (Figure 14a). The colonies were smaller and white. After 35 days, the colonies had undulate margins, whitish color, raised elevation, smooth and wet.

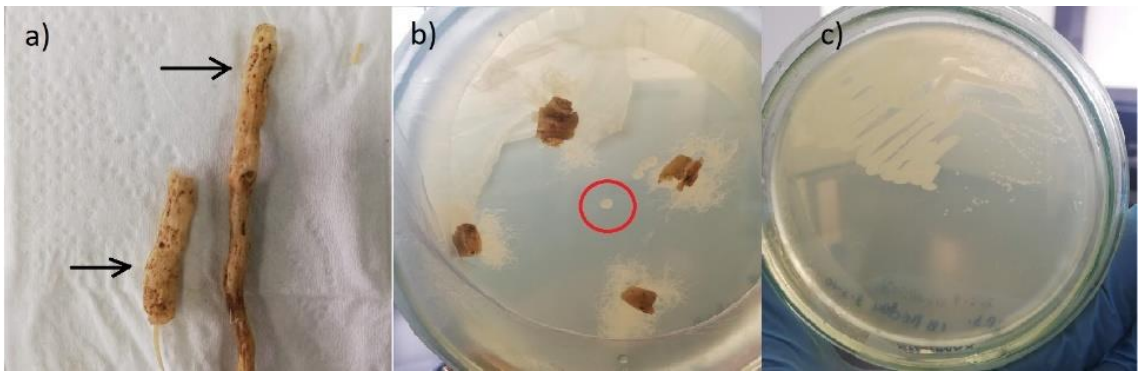


Figure 13. Isolation of diazotroph bacterium from the sugarcane root. a) Black arrows indicate the nodule growth zone after the sterilization procedure. b) Fragment of nodules inoculated on LB agar plate; red circle indicates the isolated colony. c) Isolated colony inoculated on LB agar plate.

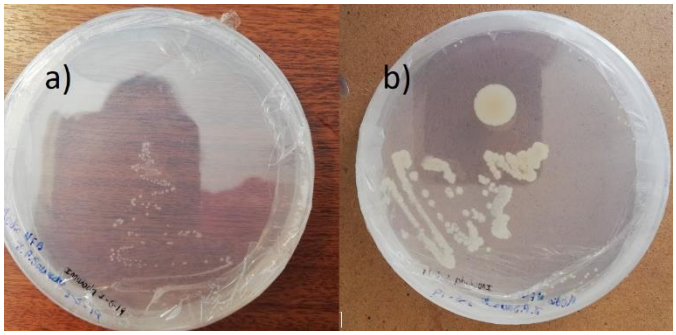


Figure 14. Isolated bacterium inoculated on NFb agar plate. a) Isolated bacterium of sugar cane root on NFb media at six days of incubation. b) Isolated bacterium at 35 days of incubation.

5.2. Bacterial growth

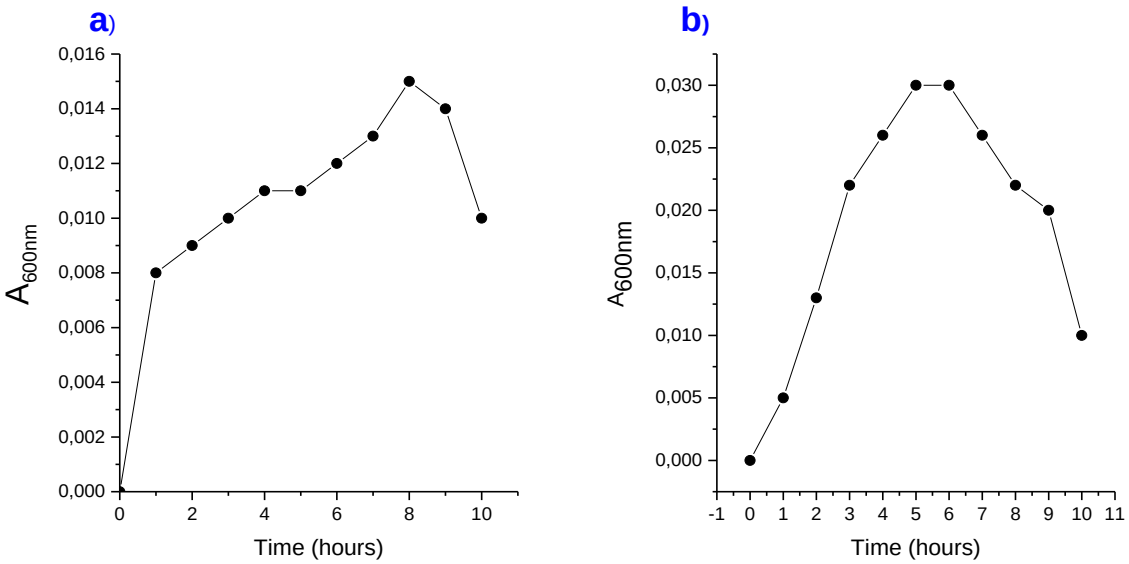


Figure 15. Isolated bacterium growth curve. a) Isolated bacterium inoculated on NFb media. b) Isolated bacterium inoculated on LGI media.

The bacterial growth curve of the isolated bacterium on the NFb liquid medium reached the highest absorbance of 0.015 at the eighth hour of incubation (Figure 15a). From the eight hours, the absorbance measurements decreased because the bacterium began to die. The bacterial growth curve of the isolated bacterium on the LGI medium and reached an absorbance of 0.03 in the stationary phase between the fifth and sixth hours of bacterial growth (Figure 15 b). This fact is important as it gives an estimate of the culture time of the bacterium for the following assays. From the sixth hour, the absorbance measurements decreased because the bacterium began to die. The isolated bacterium growth was evidenced in a more significant proportion in the LGI medium than in NFb. Therefore, for the tests of biochemical characterization, the LGI medium was chosen to evaluate the characteristics of the bacterium.

5.3. Morphological characterization

The Gram staining and observations of colony morphology were performed to identify if the isolated bacterium from sugarcane had similar features to *Gluconacetobacter diazotrophicus*.

The Gram staining of both bacteria showed gram-negative staining because of the coloration of the bacteria had in pink tones. The microscopic observation at 100X distinguished that isolated bacterium did not possess the same shape of *Gluconacetobacter diazotrophicus*. The isolated bacterium had a vibrioid shape, while the commercial strain of *Gluconacetobacter diazotrophicus* presented small rod-shaped (Figure 16).

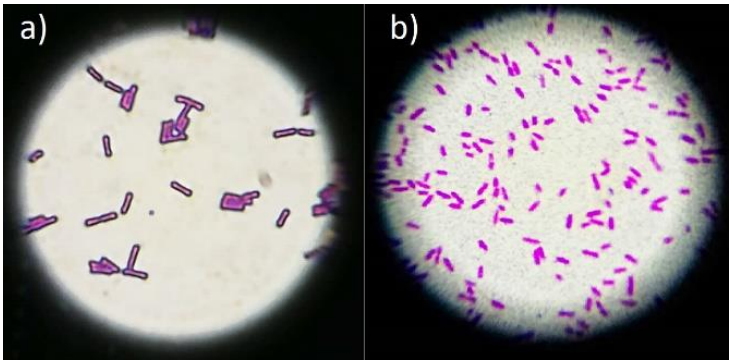


Figure 16. Gram staining of bacteria observed under a microscope with a 100X objective lens. a) The isolated bacterium. b) Gluconacetobacter diazotrophicus.

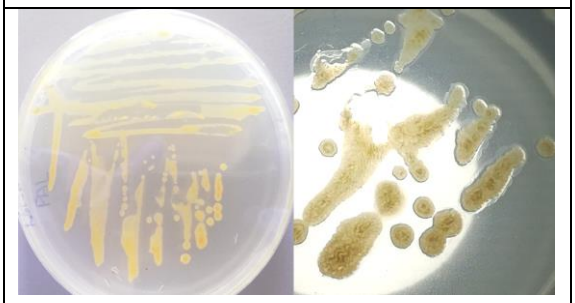
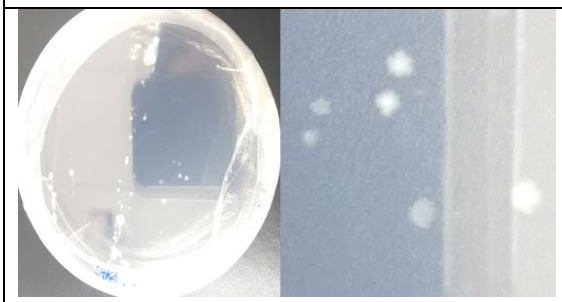
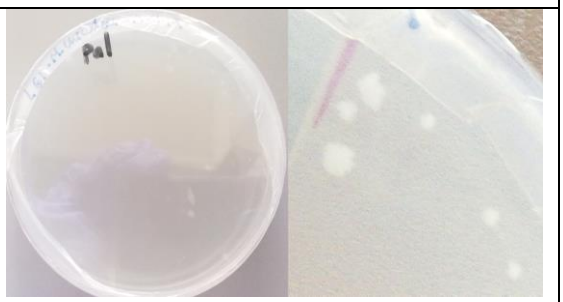
The colony morphology method was used to identify the genus of the isolated bacterium. The colonies of *Gluconacetobacter diazotrophicus* on LGI agar plate were orange and the culture medium changed from green to yellow. The culture medium LGI had a bromothymol blue indicator that was sensitive to pH change. In this case, the color was initially green at pH 6.8 and the bacterium produced acetic acid and the medium changed to yellow pH= 5. The isolated bacterium inoculated on the LGI agar plate showed white colonies and there was no color change in the culture medium (Table 2). This fact ruled out the possibility that the isolated bacterium belonged to the genus of *Gluconacetobacter diazotrophicus*.

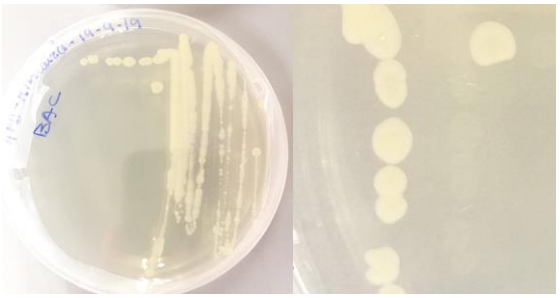
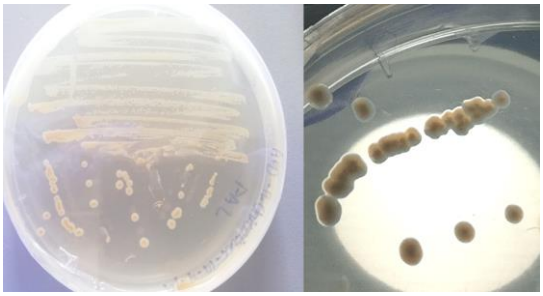
The NFb medium was used for the selective growth of the bacterium that was capable of fixing nitrogen. The growth of the isolated bacterium in the NFb medium confirmed that the bacterium was a nitrogen fixer. According to the literature, species of the genus of *Azospirillum* or *Herbaspirillum* grew up in the NFb medium[44]. And this information

allowed us to focus on gender *Azospirillum* or *Herbaspirillum* for biochemical characterization.

The isolated bacterium was inoculated in the PDA medium. The colonies were smooth and creamy. In this case, the literature mentioned that the colony of the genus *Herbaspirillum* was brownish, small, smooth, and raised colonies on PDA 46. Therefore, the possibility that the bacterium isolated belonged to the genus *Herbaspirillum* was ruled out since the morphological characteristics of the colonies do not match. Therefore, morphologically the isolated bacterium did not resemble *Gluconacetobacter diazotrophicus* and *Herbaspirillum* species and it was necessary to focus the biochemical characterization in the genus *Azosprillum.*

Table 2. Colony characteristics of the isolated bacterium and Gluconacetobacter diazotrophicus on different culture media.

Isolated bacterium Colony Characteristic	***Gluconacetobacter Diazotrophicus* Colony Characteristic**
 LGI media White colonies Filamentous form Flat elevation Small colonies No color change of media	 LGI media Orange colonies Circular form Raised elevation Well-delineated border The color change of media (yellow)
 NFb media Small colonies Irregular form Flat elevation Poor growth	 NFb media Small colonies Flat elevation Poor growth White colonies

 PDA media Cream color Circular form Flat elevation Smooth	 PDA media Dark brown colonies Small Circular Raised elevation

5.4. Biochemical characterization of *Azospirillum*

The isolated bacterium was inoculated on four BMS agar plates with different carbon sources to identify if isolated bacterium corresponded to *Azospirillum*. The most notable features were small, smooth and white colonies on BMS agar plate with L-malic acid (Figure 17a). In the BMS agar plate with malic acid and sucrose, the colonies were flat, had elevated borders and were white color (Figure 17 b). The colonies of the isolated bacterium on BMS agar plate with glucose as carbon source were big, flat, had well-delineated borders and were white color (Figure 17 c). The colonies on BMS media with sucrose were flat and white (Figure 17 d). These features were similar to the *Azospirillum amazonense* specie described by Brenner et al.[20].

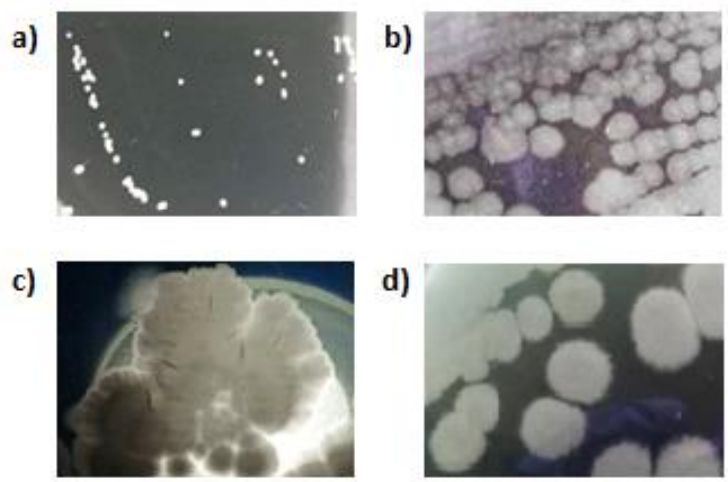


Figure 17. Isolated bacterium inoculated on BMS agar with different carbon source. a) BMS media with L-malic acid. b) BMS media with L-malic acid and sucrose. c) BMS media with glucose. d) BMS media with sucrose.

The results of the biochemical characterization are presented in Table 3, where the isolated bacterium was compared with the characteristics of *Azospirillum amazonense* described by Brenner et al.[20]. The biochemical characterization results resembled with the distinctive characteristics of the *Azospirillum amazonense* genus. Therefore, through morphological analysis, inoculation on different culture media, and biochemical characterization, the isolated bacterium was identified with the genus *Azospirillum amazonense*. However, it is necessary to combine the methods used in this investigation with molecular techniques to affirm the identification of the bacterium.

Table 3. Comparison of characteristics of isolated bacterium and Azospirillum amazonense

Characteristic	*Azospirillum amazonense*	Isolated bacterium
Cell shape		
Vibrioid	+	+
Helical	-	-
Catalase	+	+
Motility	+	+
Growth with 3%NaCl	-	-
pH range for growth	6.0 - 7.8	6.0 - 7.8
Carbon sources		
Glucose	+	+
Glycerol	-	-
Colony type on		
BMS agar medium	White, flat, raised margin	White, flat, raised margins
Acid from:		
Glucose	-	-
Fructose	-	-

Symbols: – negative test, + positive test

5.5. Nitrogenase activity of *Azospirillum amazonense*

The bacterium *Azospirillum amazonense* had a feature of being a nitrogen-fixing bacterium. The study of nitrogenase activity by ARA was necessary. A calibration curve was made with pure ethylene, in a range of 0 - 1000 nmol and described with (Equation 2 (Figure 18). A coefficient of determination was 0,9994 that showed a good fitting of the data.

(Equation 2) $$y = 0.2638x - 0.1114$$

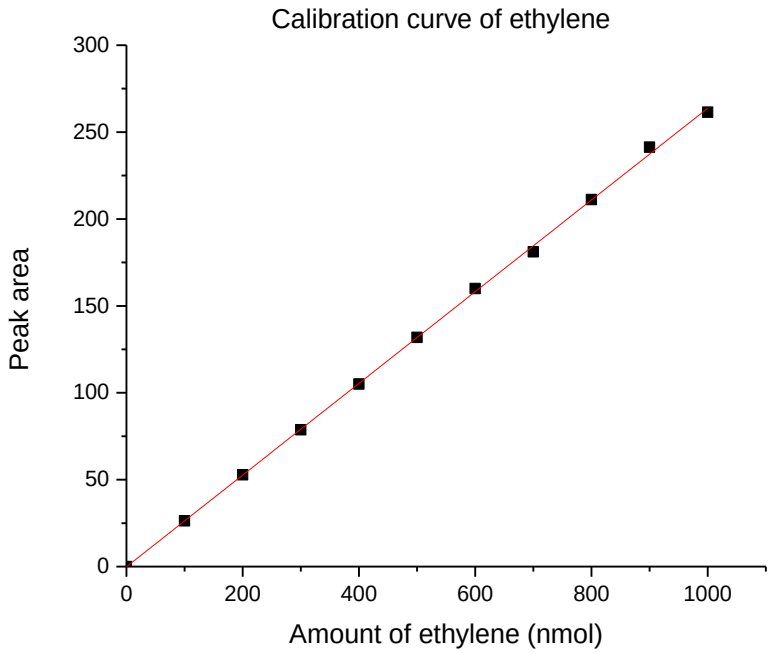


Figure 18. Calibration curve of ethylene for ARA to evaluate the nitrogenase activity of Azospirillum amazonense.

Results from the nitrogenase activity of the *Azospirillum amazonense* is presented in Table 4. Tube 1 had an unmodified gas phase and ethylene was not detected. Tube 3 neither had activity because the gas phase was removed. In tubes 2 and 4, the ethylene was detected. The presence of nitrogenase was affirmed since it was responsible for the reduction of acetylene to ethylene. In tube 4 the nitrogenase activity was more than tube 2. The tube 4 did not have oxygen because it was subjected to vacuum while the tube 2 had oxygen from the air. The nitrogenase enzyme is sensible to molecular oxygen and inhibits the nitrogenase action. Thus, it is convenient to try a test with different oxygen conditions to understand the oxygen tolerance of nitrogenase of *Azospirillum amazonense*.

Table 4. Nitrogenase activity of Azospirillum amazonense nmol of ethylene of test tubes measured by GC.

Test Tube	**nmol of Ethylene**
Tube 1: unmodified gas phase	No activity
Tube 2: 15 % gas phase was extracted and 15% acetylene was added	510 nmol
Tube 3: total gas phase was removed under vacuum	No activity
Tube 4: total gas phase was removed under vacuum and 15% acetylene	750 nmol

5.6. Nitrogenase purification of *Azospirillum amazonense*

For *Azospirillum amazonense* growth two methodologies were performed. The direct growth and indirect growth. According to the bacterial growth curve of *Azospirillum amazonense*, the bacterium reaches an optimal growth at five hours of incubation in the LGI medium (Figure 15 b). In the direct growth, no bacterial growth or turbidity was observed in 500 mL of LGI medium at five hours of incubation. So, the medium was left for 24 hours at 37 °C at 200 rpm. Unfortunately, in the medium no turbidity was observed.

The indirect growth, the *Azospirillum amazonense* was inoculated on the SYP medium and grew in higher proportion due to the enrichment of the medium with nutrients (Figure 19 a). Then, the SYP medium was transferred to the LGI medium and turbidity was observed after 48 hours at 37ºC at 200 rpm (Figure 19 b). After being centrifuged at 4ºC for 30 minutes at 4000 rpm, bacteria pellet had whitish color and viscosity (Figure 19 c). It was stored at -20ºC with 1 mL of buffer R. Thus, the indirect growth of *Azospirillum amazonense* was chosen because its method showed the greatest bacterial growth. After the sonication and centrifugation process, **the crude extract** had a total protein content of bacterium and was stored at 4ºC.

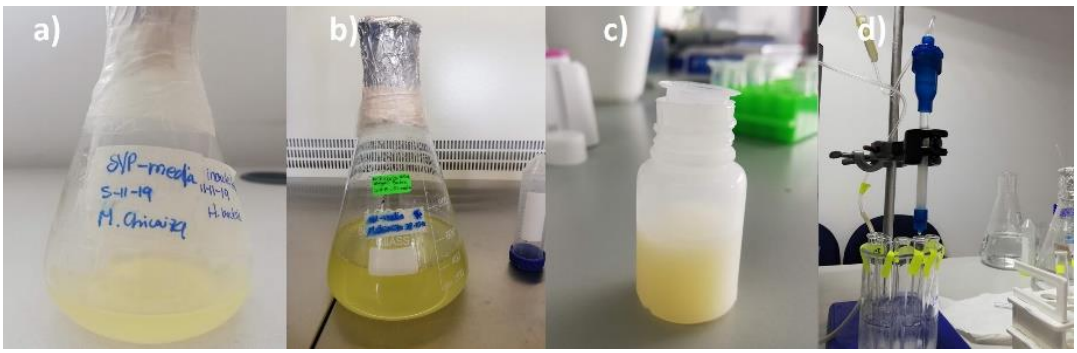


Figure 19. Purification of nitrogenase from Azospirillum amazonense. a) Azospirillum amazonense inoculated on SYP media. b) LGI media growth. c) bacterial pellet of Azospirillum amazonense. d) Chromatographic column for nitrogenase purification.

For the purification process of the nitrogenase enzyme, two chromatographic columns were used. The first column of Q-Sepharose acted as strong anion exchange chromatography (Figure 19 d). The gradient of NaCl allowed protein separation, MoFe and Fe proteins eluted at approximately 0.4 - 0.6 mM NaCl gradient. The fraction obtained from the first purification was denominated as **Q-Sepharose fraction**.

The Q-Sepharose fraction was loaded on the second chromatographic column of Sephacryl S-200 that acted as size exclusion chromatography. The fractions obtained from purification were measured at 280 nm and an elution purification profile was obtained (Figure 20). Two high absorbance peaks were observed. **S-200 Frac 1** eluted approximately from 10 to 20 mL and **S-200 Frac 2** eluted 20 to 45 mL.

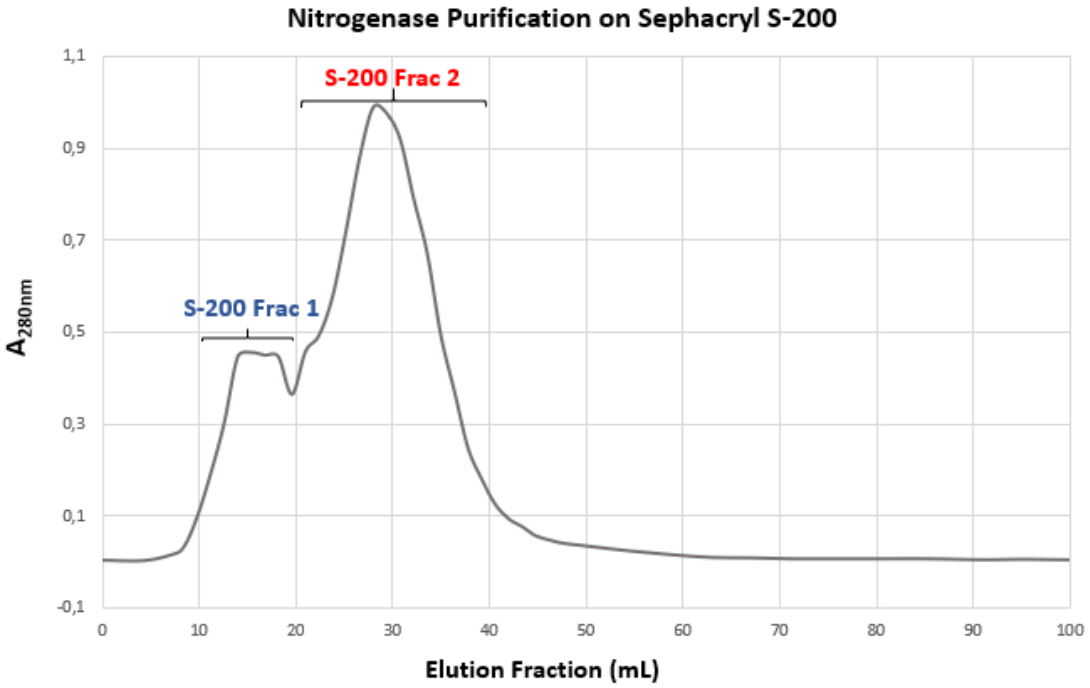


Figure 20. Elution purification profile of nitrogenase enzyme on Sephacryl S-200 measured at 280nm.

The BCA method allowed the determination of protein concentration with a set of standard solutions was prepared twice. BSA standard curve was plotted (Figure 21). The (Equation 3 was used for the quantification of protein concentration of the fractions obtained from the chromatographic column purification.

(Equation 3) $$y = 0{,}0425x + 0{,}046$$

Four samples were analyzed by the BCA method: the crude extract, the Q-Sepharose fraction, S-200 Frac 1 and S-200 Frac 2. The results of protein concentration are presented in Table 5. In the case of the crude extract had a high protein concentration due to this fraction contained a total protein content from the lysis procedure. The Q-Sepharose, S-200 Frac 1, S-200 Frac 2 fractions had a lower protein concentration due to the purification process.

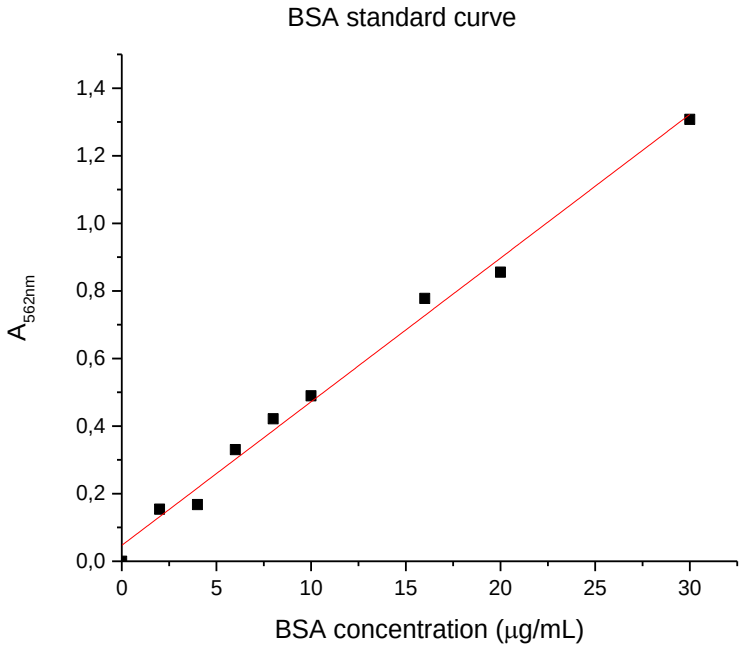


Figure 21. BSA calibration curve. concentration to estimate protein concentration.

Table 5. Results from BCA to determine the protein concentration of fractions for nitrogenase purification

Fraction	Absorbance	µg Protein	µg Protein Concentration
crude extract	1,257	28,085	14,043
Q-Sepharose	0,273	5,224	2,612
S-200 Frac 1	0,257	4,852	1,129
S-200 Frac 2	0,145	2,257	2,426

The SDS-PAGE was performed with 4 samples: the crude extract, the Q-Sepharose fraction, S-200 Frac 1 and S-200 Frac 2. In the electrophoresis gel, the crude extract presented a wide range of bands because this fraction contained a total protein content from lysis procedure (Figure 22, lane 3). The Q-Sepharose fraction showed two bands that stand out from the others in approximately 35 kDa and 57 kDa (Figure 22, lane 2). According to the research of Fisher et al.[22] the molecular weight of the MoFe protein had an estimated 53 and 57.5 kDa, and 33 kDa for the Fe protein. Therefore, the Q-Sepharose fraction may contain the nitrogenase proteins, but it was necessary to continue with the purification with Sephacryl S-200 to obtain a clearer band in the electrophoresis gel.

From the second purification with Sephacryl S-200, the S-200 Frac 1 had a band at 57 kDa that probably corresponded to the MoFe protein (Figure 22, lane 4). The fraction S-200 Frac 2 had a band at approximately 35 kDa, which could correspond to the Fe protein (Figure 22, lane 1). To ensure that the bands selected correspond to the FeMo and Fe protein, EPR was used.

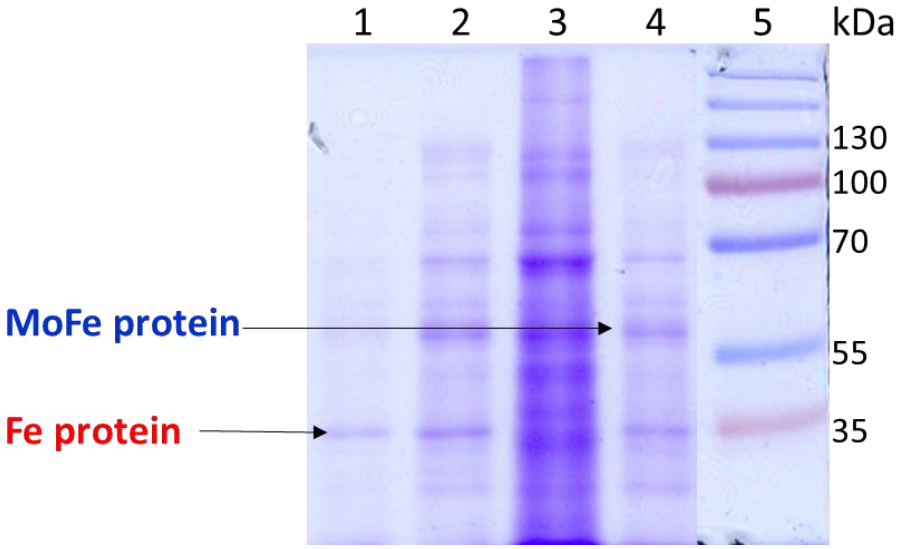


Figure 22. SDS-PAGE of nitrogenase metalloproteins from Azospirillum Amazonense. Lane 1: correspond to S-200 Frac 2 obtained from purification and the presence of Fe protein. Lane 2: Q-Sepharose fraction from nitrogenase purification. Lane 3: the crude extract. Lane 4: S-200 Frac 1 with the presence of MoFe protein. Lane 5: molecular weight marker standard.

5.7. Electron paramagnetic resonance spectra

In order to study the metallocluster of nitrogenase enzyme from *Azospirillum amazonense*, the MoFe and Fe proteins bands obtained from SDS-PAGE were analyzed by EPR.

The MoFe protein had two metalloclusters: FeMoco and P-cluster. According to Karamatullah et al.[33] as-isolated state the FeMoco is paramagnetic with spin state of S = 3/2 with g-values of of g_x = 4.32, g_y = 3.32 and g_z = 2. The P-cluster is diamagnetic at resting state so EPR does not detect it. The EPR spectrum of purified MoFe protein showed three different g-values of g_x = 4.503, g_y = 4.268 and g_z = 1.993 (Figure 23). These *g*-values coincided with g-values of FeMoco from the researches of Fisher et al.[22], Owens et al.[7] and Karamatullah et al.[33].

The different g-values are explained by spatial information contained in g-tensor (matrix) that includes values of g_x, g_y and g_z. When a sample interacts with an external magnetic field the *g*-values are different (Appendix 6). The *g-tensor* is denominated as rhombic when $g_y \neq g_z \neq g_x$ (Appendix 7). According to Smith at al.[45] the spin state of S=3/2 from FeMoco is due to a system of iron atoms with oxidation state Fe^{3+} that is detected by EPR.

The EPR spectrum of MoFe protein showed a rhombic pattern signal, spins state of S = 3/2 and *g*-values that affirmed that spectrum corresponded to FeMoco from MoFe protein (Figure 23). The characterization of FeMoco confirmed the presence of the nitrogenase enzyme from *Azospirillum amazonense* that was isolated from the sugarcane root.

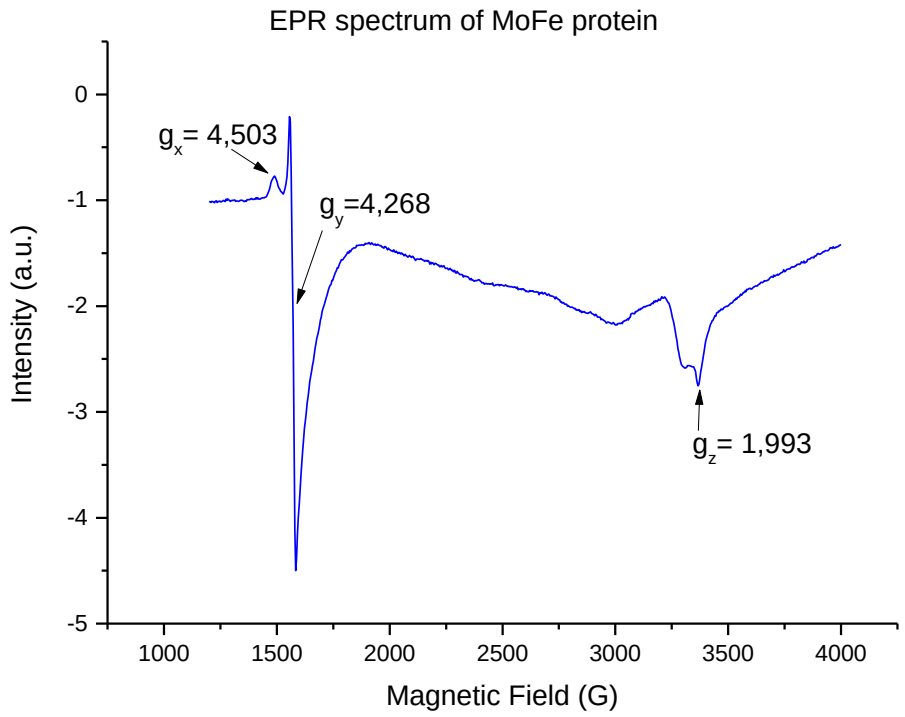


Figure 23. EPR spectra obtained from the MoFe protein of Azospirillum amazonense. Spectral conditions were 9.4GHz microwave frequency, 2.033 mW microwave power at 10K.

The EPR spectrum of Fe protein was not obtained, probably because there was not enough protein concentration or [4Fe-4S] cluster was oxidized by dioxygen. The oxidized $[4Fe\text{-}4S]^{2+}$ cluster was diamagnetic and was not detected by EPR. The nitrogenase enzyme is sensitive to oxygen concentration. The characterization of the nitrogenase enzyme should be performed with argon gas to avoid oxidation[33].

6. CONCLUSIONS AND RECOMMENDATION

The main objective of the work has been accomplished because the isolation of at least one nitrogen-fixing bacteria was achieved. The morphology and biochemical characterization of the isolated bacteria lead us to conclude that we had a bacterium of Azospirillum *Amazonense* genus. One of the future perspectives is to carry out a genetic sequencing to determine the genus and species of the isolated microorganism without any unambiguously.

The acetylene reduction assay measured the nitrogenase activity of *Azospirillum amazonense*. The results showed that *Azospirillum amazonense* could fix molecular nitrogen. But oxygen inhibited the nitrogenase activity. It is convenient to try a test with different oxygen conditions to understand the oxygen tolerance of the nitrogenase of *Azospirillum amazonense*.

Following a purification objective, the use of Q-sepharose and Sephacryl S-200 resins allowed to isolate the two main components of the nitrogenase enzyme, which are the MoFe protein and the Fe protein.
The characterization of the MoFe protein and the Fe protein was carried out by SDS-PAGE, in electrophoresis gel the MoFe protein was identified approximately at 57 kDa and Fe protein at 35 kDa.

Finally, it was possible to make the spectroscopic characterization of the MoFe protein by electron paramagnetic resonance by determining the values of rhombic signals at g_x = 4.503, g_y =4.268 and g_z=1.993 and spin state of S = 3/2 that are characteristic of the FeMo cofactor in its basal state.

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Appendix

Appendix 1. Composition of NFb media.

NFb media (pH 7)	**1L**
Malic acid	10 g
K_2HPO_4 10%	5 mL
$MgSO_{4*7}H_2O$ 10%	2mL
NaCl 1%	1mL
$CaCl_2$ 1%	2mL
$FeSO_4$ 4mg/mL	1mL
$Na_2MoO_4*_2H_2O$ 0,1%	2mL
$MnSO_4$ 0,001%	1mL
KOH	4,8g
NH_4Cl	0,2g

Appendix 2. Composition of LGI media.

LGI media (pH 6.8)	**1L**
K_2HPO_4	0,2g
KH_2PO_4	0,6g
$MgSO_{4*}7H_2O$	0,2g
CaCl2	0,02g
$Na_2MoO_4*2H_2O$	0,002g
$FeCl_3*6H_2O$	0,01g
Bromothymol blue	2mL
KOH 2N	5mL
Agar	1,8g
Sucrose	5g

Appendix 3. MT media composition.

MT media	**1L**
Peptone	10g
Beef extract	3 g
NaCl	5 g
Agar	4 g
Water	1000 mL

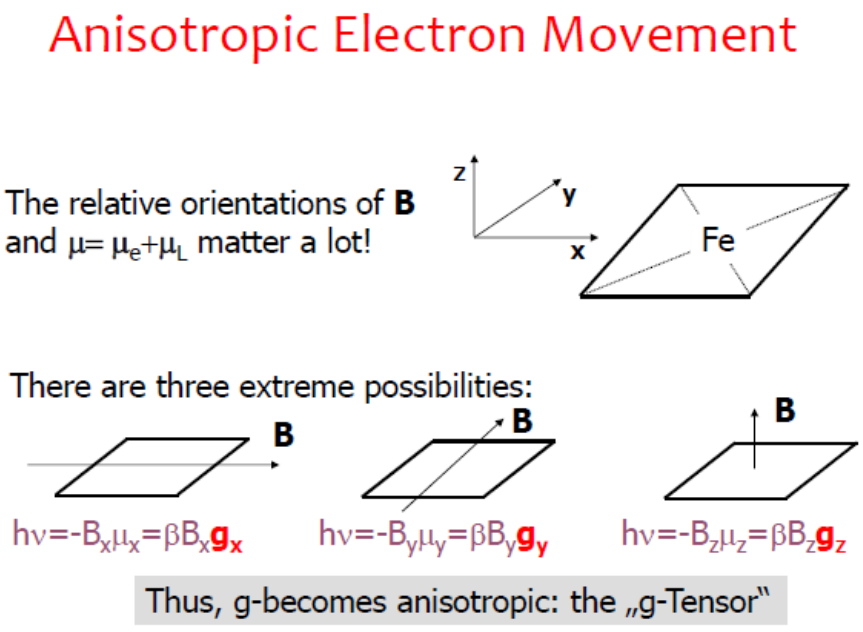
Appendix 4. Composition of BMS media.

BMS media	**1L**
Washed, peeled, sliced potatoes	200 g
L-malic acid,	2,5g
KOH	2 g
Raw cane sugar	2,6 g
Bromothymol blue (0.5% alcoholic solution)	2 drops
Agar	15 g

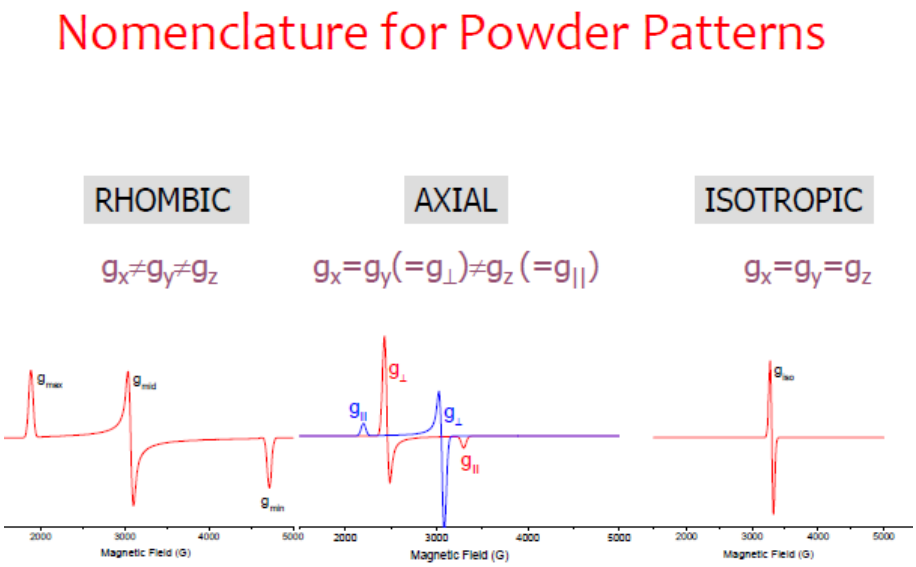
Appendix 5. SYP media composition.

SYP media	**1L**
Yeast Extract	3g
Sucrose	10g
K_2HPO_4	1g
KH_2PO_4	3g
Water	1000mL

Appendix 6. Anisotropic electron movement in Electron Paramagnetic Resonance[46].



Appendix 7. Derivative EPR spectra of rhombic, axial, isotropic signals[46].



Appendix 8. Scientific contribution in the Bionatura Journal. Jiménez-Guailla, S.; Chicaiza-Lemus, M.; Saucedo-Vázquez, J. P. Synthesis and Characterization of a Nitrogenase-Cofactor Biomimetic Based on Molybdenum Complexes with a Polydentate-N5 Ligand[16].

RESEARCHS / INVESTIGACIÓN

Synthesis and characterization of a Nitrogenase-cofactor biomimetic based on molybdenum complexes with a polydentate-N_5 ligand.

Steven Jiménez-Guailla[1], Michelle Chicaiza-Lemus[1] and Juan Pablo Saucedo-Vázquez[1*].
DOI. 10.21931/RB/2019.04.03.6

Abstract: Nitrogen fixation is an outstanding process in which atmospheric molecular nitrogen is reduced to ammonia, which is easier to assimilate by the plants. Due to the challenge to understand the nitrogen fixation process *in vivo* conditions, biomimetic compounds have been synthesized to perform the reduction of nitrogen in softest environments than in the Haber process. Thus, the purpose of this work is the synthesis and characterization of new molybdenum complexes with polydentate nitrogenated ligands and the evaluation of those complexes as possible dinitrogen reductants.

Key words: Nitrogenase, nitrogen fixation, biomimetic, molybdenum complex.

Introduction

Nitrogen fixation is a fundamental process to obtain ammonia and sustain life. There are at least three forms of nitrogen fixation in the earth: atmospheric, bacterial, and industrial production. The low concentration of ammonia is located in the troposphere, and most of it occurs in the agricultural process. By Keywords, nitrogen is fixed in the troposphere by photochemical reactions[1] of dinitrogen in the presence of lightning; however, nitrogen oxides NO_x produced in such reactions are less assimilable than ammonia or ammonium and do not contribute significantly to the nitrogen assimilation by the plants.

Haber Fritz proposed a production method of ammonia more than 100 years ago, then taken by Carl Bosch and converted into an industrial process[2]. In such a process, hydrogen required for the reduction of N_2 is synthesized by a redox reaction between CO and H_2O, nitrogen is taken from air through distillation towers, then, both gases are directed to a chemical reactor and treated with an iron catalyst at high pressure (~150 bar) and high temperatures (500-600 ºC) in order to obtain NH_3 (equation 1). Haber-Bosch process is expensive and pollutant, however, about 40% of the earth population depends on the production of fertilizers coming from this process[3].

$$3H_{2(g)} + N_{2(g)} \rightarrow 2NH_{3(g)} \qquad \text{eq. 1}$$

In biological nitrogen fixation, Nitrogenase corresponds to a complex enzyme responsible of the fixation of atmospheric dinitrogen to a reduced form of nitrogen (i.e., amino, amido, imido, azido, nitrite or ammonia). In biology, only a specific group of microorganisms contains such an enzyme to make this a successful process. The first structural description of a nitrogenase enzyme corresponds to that of *Azobacter vinelandii*[4].

The structure founded for its active site corresponds to a molybdenum (III) complex with an octahedral geometry on Mo with three sulfurs of the Fe-S cluster, two oxygen from the bidentate homocitrate and the imidazole group of a histidine (Figure 1)[5].

To contribute to a full understanding of the nitrogen fixation mechanism *in vivo*, synthesis of new functional biomimetic compounds suitable to reduce dinitrogen in softer conditions than the Haber-Bosch method have been reported. The synthesis of such nitrogenase cofactor biomimetics began with the synthesis of molybdenum-dinitrogen complexes, which were further functionalized to produce amides, hydrazide, and imido compounds because these compounds could be transformed to ammonia easily. In the first synthetic biomimetic complex, the oxidation state of molybdenum was zero, but, further investigations have improved the synthesis of new ligands, which tune the oxidation state of molybdenum (Mo^{III}, Mo^{V}, and Mo^{VI}) to increase their reactivity[6]. Besides, the nitrogenase mechanism according to the model of Thorneley and Lowe suggests that monoatomic nitrogen in the active center of the enzyme is reduced to form a nitride (N^{3-}) before the release of ammonia[7]. However, later ESEEM studies complemented the crystallographic results showed the presence of an interstitial carbon instead of a nitrogen atom[8]. Later is a clear example of the complexity of the mechanism of reduction of N_2.

As an effort to contribute to the unraveling reaction mechanism of nitrogen fixation, we will propose the synthesis and characterization of molybdenum (III) complexes with ligands oxygen and nitrogen electron donors.

Materials and methods

All the reactants were of an analytical grade of purity and used without further purification. However, 2-pirydinecarboxylaldehyde and diethylenetriamine which were distilled before the reactions. UV-Vis spectra were obtained in a Perkin Elmer Lambda 1050 spectrophotometer; cyclic voltammograms were performed in a Metrhom Autolab PGSTAT302N potentiostat and NMR was obtained in a Varian 400 MHz NMR Unity-Inova spectrometer.

Synthesis of tris (acetylacetonato) molybdenum (III)

The synthesis of tris (acetylacetonato) molybdenum (III) (Figure 3a) was performed according to the procedure reported previously[9] but with slight changes and implementing two types of synthesis by changing the presence of inert (He) atmosphere by air atmosphere. In a general procedure, 100 mg of hexacarbonyl molybdenum (0) were dissolved in 5ml of acetylacetone. This mixture was stirred and refluxed for 2 hours, after that, the reaction was kept in a warm bath at 100 °C for 1 hour more, and then let it cooled at RT. Later, the solvent was removed and finally the solid was sublimated at around

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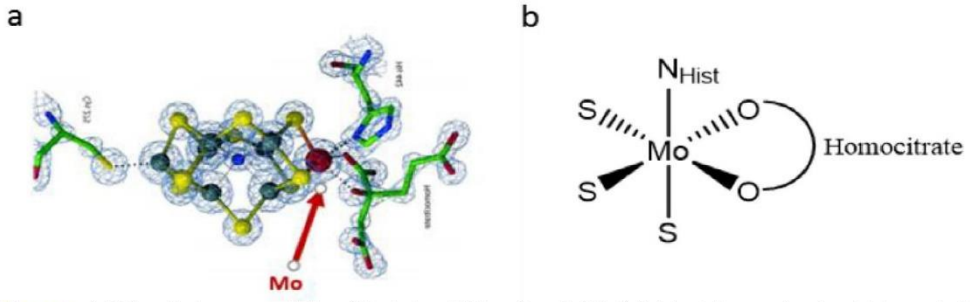

Figure 1. a) FeMo cofactor representation of A. vinelandii. Taken from Ref. 7. b) Octahedral geometry of molybdenum in the cofactor.

150°C until the remnants of hexacarbonyl molybdenum do not sublimate anymore. The later procedure was performed under the exclusion of air by helium atmosphere and air without inert atmosphere.

Synthesis of the ligand

The synthesis of penta-amine ligand 1,9-bis(2'-pyridyl)-2,5,8-triazonane was carried out according to the procedure previously reported[10, 11]. In a round flask 25 mL of anhydrous ethanol, 2 mL (0.021 mol) of 2-pyridinecarboxaldehyde and 1.13 mL (0.0105 mol) of diethylenetriamine were placed together and the reaction mixture was heated to 55 °C with constant stirring. The reaction was followed by thin layer chromatography tests and using methanol: chloroform: hexane 1: 5: 3 mixture as an eluent to verify the disappearance of the aldehyde. Then, a catalytic (Pd/C) hydrogenation of the imine obtained in such synthesis was performed (Figure 2). The reduced polyamine pentadentate ligand was used without the formation of the hydrochloride as is described in (10, 11).

Synthesis of the complex

In a round flask, 25 mL of anhydrous ethanol, 2,5 mL of a 0,42 M of the ligand and 414 mg (1,05 mmol) were placed and stirred at 60 ºC during 10 hours. After this time, a yellow-brown solid was obtained.

Results and Discussion

Two procedures were performed for the synthesis of tris (acetylacetonato)molybdenum(III), yields for both procedures (65% He, 25% air) and purity of the final product showed that helium atmosphere provides better results probably because inert sphere prevents the formation of oxo-molybdenum species.

Obtained product from the reaction under inert atmosphere was a dark purple solid (Figure 3b) with the physical characteristics of the previously reported for such compound, a melting point of 225-226 ºC compared with those obtained in the literature[9] indicates the success in the synthesis of the desired molybdenum complex.

From the reaction of 2-pirydinecarboxylaldehyde and diethylenetriamine we obtained the condensation product (imine) and the corresponding reduced amine via catalytic hydrogenation with 81% of yield. The obtained compound was characterized using NMR-H[1] and assigned unambiguously to the pentadentate ligand (Figure 4).

The yellow-brown product obtained from the reaction between tris (acetylacetonate)molybdenum(III) and the pentadentate ligand 1,9-bis(2'-pyridyl)-2,5,8-triazonane presents a UV-Vis electronic spectra with three maximum in the UV region, two of them at high energy (206, 260 nm) with large molar extinction coefficients (7,760 and 5,560 $M^{-1}cm^{-1}$) that corresponds to charge transfer electronic transitions. The other transition is located at 309 nm and has a lower extinction coefficient (890 $M^{-1}cm^{-1}$) that could be assigned to a d-d transition. In the case of our product, we expect a hexacoordinated octahedral complex, in which, five of the coordination positions are occupied by the pentadentate ligand and a labile solvent molecule in the sixth position. Thus, for an octahedral d^3 High Spin species, we expect three electronic transitions following the Tanabe-Sugano diagram[12], however, as in the case of the d^3 first row transition metals, the third transition $^4T_{1g}(P) \leftarrow {}^4A_{2g}$ for Mo (III) is expected to be at high energy and then obscured by the charge-transfer transitions. By the other hand, the literature is reported that the second and the third electronic transitions for Mo (III) HS are located in the UV region. For example, in the case of the $[Mo(H_2O)_6]^{3+}$ such transitions are located at 310 nm for the second one $^4T_{1g}(F) \leftarrow {}^4A_{2g}$ and 380 nm for the first one $^4T_{2g} \leftarrow {}^4A_{2g}$[13]. In our case, we can assign the signal at 309 nm as the first electronic transition for the d^3 species; however, the second transition is most probably obscured by the charge transfer located at 260 nm (Figure 5a).

From cyclic voltammetry (Figure 5b), we observed a reversible process and determined a midpoint $E_{m} = 0{,}07$ V (Fc/Fc^+) for our complex, such redox potential is in the same order that other for octahedral Mo (III) complexes, for example $MoCl_3(pyridine)_3$ shows an $E^o = 0{,}49$ VSCE [Mo(III)/Mo(IV)][14].

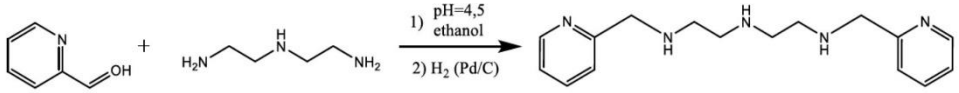

Figure 2. The reaction of Schiff base formation.

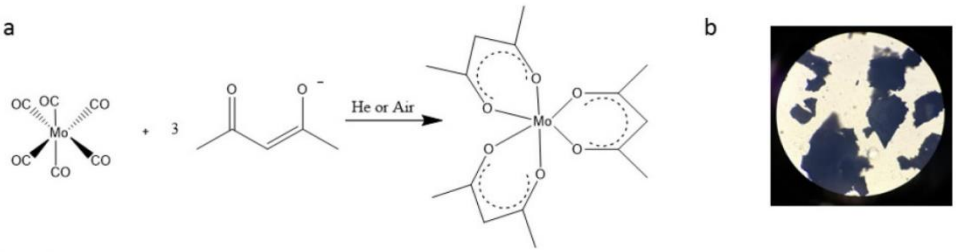

Figure 3. a) Synthesis reaction of tris (acetylacetonato) molybdenum (III) and b) Dark purple crystals obtained.

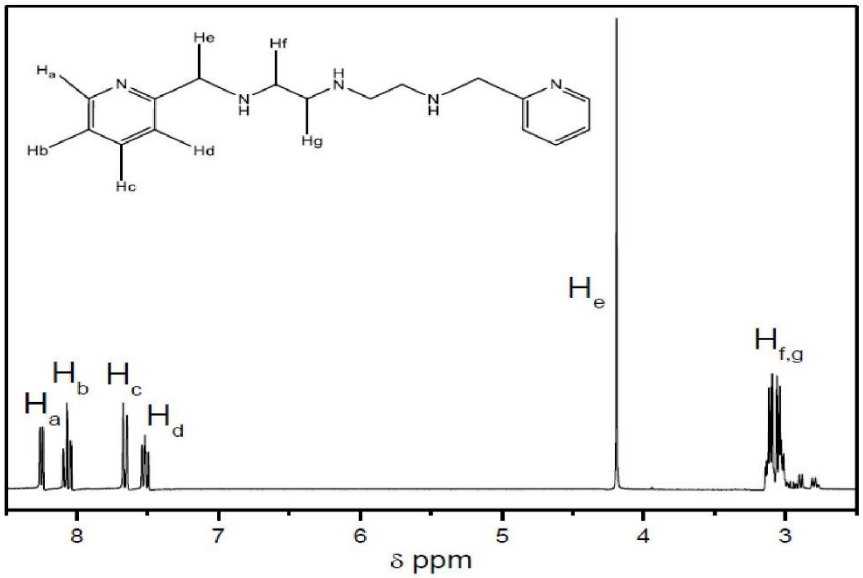

Figure 4. 400 MHz ^{1}H NMR spectra of the pentadentate ligand in D_2O.

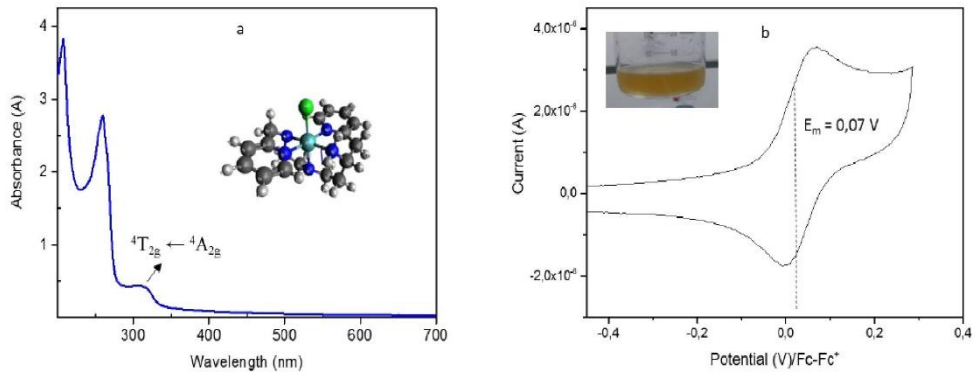

Figure 5. a) UV-vis spectrum and b) voltammogram of the molybdenum-polydentate complex in EtOH, 50 mV/s.

Conclusions

We performed the synthesis and partial characterization of a molybdenum complex coordinated with a pentadentate ligand. UV-Vis and electrochemical studies helped us to determine the oxidation state of Mo as 3+. The strategy of the synthesis was to have a complex with molybdenum in an octahedral environment with a pentadentate ligand and one labile position occupied by a solvent molecule. Such a labile position is expected to be a good site for the coordination of dinitrogen to induce the redox chemistry (nitrogen fix) between molybdenum and N_2. Further characterization of the molybdenum complex is required to perform the next step of the biomimetic system. The reactivity of the synthesized compound with N_2 is in the process of evaluation.

Acknowledgements

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