Instituto Tecnológico de Costa Rica
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"pH effects on the reaction catalyzed by *Aspergillus fumigatus* Siderophore A (*Af*SidA)"

Proyecto de graduación para optar por el grado de Bachiller en Biotecnología

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Cartago, Agosto 2011

"pH effects on the reaction catalyzed by *Aspergillus fumigatus* Siderophore A (*Af*SidA)"

Informe presentado a la Escuela de Biología del
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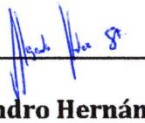
para optar al título de Bachiller en Ingeniería en Biotecnología.

Miembros Del Tribunal

Pablo Sobrado, PhD.
Virginia Tech Tutor



Alejandro Hernández, MSc.
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Lector

Dedicatoria

A Dios
A mis padres
A mis hermanos
A mis abuelos y tíos

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RESUMEN

Aspergillus es el nombre dado a una familia de hongos saprófitos y ubicuos, que son importantes en el área de la industria microbiológica ya que son responsables de la producción a gran escala de varios metabolitos y enzimas. De este género, *Aspergillus fumigatus* es uno de los más importantes, ya que es conocido como el agente patógeno líder en los pacientes inmunodeprimidos, causando la aspergilosis, que es una infección que afecta al sistema respiratorio. Varios estudios revelaron que un paso crucial de la infección es la adquisición de nutrientes, como hierro, cuya disponibilidad en el suero humano es muy limitada. Por esa razón, *A. fumigatus* biosintetiza sideróforos, para superar el problema de obtención hierro. Para la síntesis de estas moléculas, *A. fumigatus* hace uso de una enzima llamada N^5-ornitina hidroxilasa (*AfSidA*), que cataliza el primer paso comprometido en la síntesis. La información de la enzima es muy limitada, sin embargo el sistema de sideróforo ha llamado mucho la atención, por lo tanto la enzima, lo que representa un posible objetivo para las terapias de hongos, es por eso que los análisis bioquímicos son importantes. El objetivo principal fue la evaluación in vitro de la actividad enzimática de la ornitina hidroxilasa, variando la concentración de compuestos seleccionados y el pH. Para estas evaluaciones, primeramente se expresó y purificó *AfSidA*, a continuación, de acuerdo con la actividad y la estabilidad de la enzima en una serie de buffers, un sistema de amortiguación fue seleccionad para realizar ensayos de consumo de oxígeno, con concentraciones constantes de coenzima NADH, 1 mM, y la enzima, 2µM, y variando las concentraciones de los sustratos seleccionados, L-ornitina y L-lisina. También un análisis de un posible efecto causado por el NADH se llevó a cabo. Los resultados obtenidos en los dos perfiles, usando los sustratos, fueron muy similares. En el perfil de k_{cat}, se obtuvo un pK, relacionados con la formación de especie activa, mientras que en los perfiles de k_{cat}/K_M, dos pKs se presentaron, las más bajas representando la formación de especies activas y los valores más altos, la transición a una especie menos activa. En el caso del perfil de NADH, no se observó efecto de *uncoupling*, y dos grupos ionizables fueron identificados. Los pKs resultantes de los diferentes análisis pueden ser relacionados con una deprotonación de los sustratos, o a la activación de enzima; también se pueden relacionar los pKs de protonación a la formación del intermediario, C4a-peroxiflavina.

Palabras Claves: *Aspergillus fumigatus*, *AfSidA*, perfil de pH, cinética enzimática, pKa

ABSTRACT

Aspergilli is the name given to a family of saprophytic and ubiquitous fungus, that are important as industrial microorganisms, responsible of the large-scale production of several metabolites and enzymes. From this genus, *Aspergillus fumigatus* is one of the most important, because it is known as the leading mold pathogen in immunocompromised patients, causing aspergillosis, which is an infection that affects the respiratory system. Several studies revealed that one crucial step of the infection is the acquisition of nutrients, like iron, which availability in the human serum is really limited. For that reason, *A. fumigatus* biosynthesizes siderophores, to overcome the problem of limited iron accessible. For the synthesis of these molecules, *A. fumigatus* use an enzyme called N^5-ornithine hydroxylase (*AfSidA*), which catalyzes the first committed step in the synthesis. The information of the enzyme is really limited, however the siderophore system have attracted much attention, so the enzyme, which represents a possible target for fungal therapies, that is why biochemical analysis are important. The principal objective was the evaluation *in vitro* of the enzymatic activity of the ornithine hydroxylase, by varying the concentration of selected compounds and pH. For these evaluations, *AfSidA* was first, expressed and purified; then according to the activity and stability of the enzyme in a series of buffers, a buffer system was selected to performed oxygen consumption assays, using constant concentrations of coenzyme, NADH 1mM, and enzyme, 2uM; and varying the concentrations of the selected substrates, L-ornithine and L-lysine. Also an analysis of a possible effect caused by NADH was done. The results obtained in both profiles using the substrates were very similar, obtaining for the k_{cat} profile one pK related to the formation of an active species, while in both k_{cat}/K_M profiles, two pKs were presented, the lower one representing the formation of an active species and the higher one, the transition to a less active species. In the case of the NADH profile, no uncoupling effect was observed, and tow ionizable groups were identified. The pKs resultants form the analysis can be highly related to a deprotonation on the substrates, leading to the formation of active specie, or the activation of the enzyme, when it reacts with NADH; and the protonation pKs can be linked to the formation of the protonated intermediate, C4a-peroxyflan.

Key Words: *Aspergillus fumigatus*, *AfSidA*, pH profile, enzyme kinetics, dissociation constant.

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1. INTRODUCTION

Aspergilli are ubiquitous and saprophytic fungus, first described by Micheli in 1729; known because of its important role in the recycling cycle of carbon and nitrogen. This genus consists in more than 200 individuals, comprising a particularly important group of filamentous ascomycetes species. They are important industrial microorganisms for the large-scale production of both homologous and heterologous enzymes. Among them, there are some species recognized by the Food and Drug Agency of the United States of America (FDA) as safe or *GRAS.* On the other side, this genus also includes the major filamentous fungal pathogen of humans, *Aspergillus fumigatus*.

Aspergillus fumigatus is considered the leading mold pathogen among immunocompromised patients, especially bone marrow, leukemia and solid organ transplant recipients, and is responsible for a wide range of human diseases, its severity depends on the immune status of the host. The term aspergillosis is referred to large spectrums of fungal diseases, primarily affecting the lungs and respiratory system, caused by members of the genus *Aspergillus*. The common clinical manifestations are allergic bronchopulmonary aspergillosis, pulmonary aspergilloma and invasive aspergillosis. The disease is a leading cause of death in acute leukemia and hematopoietic stem cell transplantation.

The infectious life cycle of *Aspergillus*, begins with the production of conidia, which easily spreads into the air. In humans, the primary route of infection is via inhalation, followed by conidial deposition in the bronchioles or alveolar spaces. In healthy individuals, conidia are removed by mucociliary clearance encounter epithelial cells or alveolar macrophages. Therefore, in an immunocompromised person the fungus settles in the pulmonary system, and the infection begins.

Several studies in *A. fumigatus* metabolic pathways mutants have allowed the identification of the nutritional needs of the fungus during infection; one of the most important is iron, which is a necessary component of many biosynthetic pathways, acting as a cofactor in enzymatic reactions and as a catalyst in electron transport systems. However, the instability of free iron and sequestration *in vivo* by host defense mechanisms severely limit iron availability. For many human pathogens, including *A. fumigatus*, the ability to acquire iron from the host is a necessary virulence determinant.

Iron levels in the human body are very low, that is why fungi have developed various high-affinity mechanisms for acquisition of this metal. Under iron starvation, the fungi synthesize and secrete siderophores, which are low molecular-weight, ferric iron-specific chelators. Two genes involved in siderophore biosynthesis have been characterized in *Aspergillus fumigatus*, namely *sidA* (*L*-ornithine-N^5-oxygenase), which catalyzes the first committed step in siderophore biosynthesis, and *sidC*, which encodes a non-ribosomal peptide synthetase (NRPSs) involved in ferrichrome (FC) biosynthesis.

Ferrichrome; is a siderophore categorized as hydroxamate-containing and is related to the virulence of *A. fumigatus*. The biosynthesis of this siderophore requires the incorporation of the derivative of L-Ornithine, N^5-hydroxyornithine, into the backbone of the siderophore where it directly coordinates the iron. L-Ornithine hydroxylation is catalyzed by the enzyme mentioned, *Af*SidA, which is a flavindependent monooxygenase that requires NAD(P)H for activity.

The information available of the enzyme *SidA* of *Aspergillus fumigatus* is very limited, however, several *in vivo* studies using knockout mutants revealed that this enzyme is important for growth of the fungi and more importantly the infection, because this mutants are unable of producing siderophores and therefore are non-virulent.

The siderophore systems have attracted much attention, especially the *AfSidA* enzyme, because this enzyme catalyzes the committed step of the biosynthesis of this siderophores, and that is why it represents a possible target for fungical therapies, because without siderophore production, the virulence is affected.

The performance of biochemical studies in this enzyme may permit us learn and elucidate more information about it and may allow, in the future, the development of new drugs or treatment to fight the different infections that this fungi produces in immunocompromised patients.

2. LITERATURE REVIEW

2.1. Aspergillus sp.

Aspergillus is the name used for one of the oldest genus of molds described since 1729, which reproduce only by asexual means. The different species of *Aspergillus* can be seen in all natural environments, and their ease of cultivation on different type of laboratory media and the huge economic importance of several of its species ensured that many scientists and industrial microbiologists were attracted to their study. The Aspergilli grow abundantly as saprophytes, primordially on decaying vegetation, like organic compost piles, leaf litter and soil. Most species are adapted for the degradation of complex plant polymers, but they can also dine on substrates as diverse as dung, human tissues and antique parchment.

The morphology of the conidiophore, the structure that abides asexual spores or conidia, is the most important taxonomic character used in the taxonomy of this fungus. *Aspergillus* spores can easily drift on air currents and disperse themselves, and this ability to disperse globally in air currents and to grow almost anywhere when appropriate food and water are available means that 'ubiquitous' is among the most common adjectives used to described this molds (Bennett, 2010).

Aspergilli are known for using a wide variety of substrate for growth and have the ability to switch between biochemical pathways, depending of the substrate, to improve the assimilation. The decomposition process carried out by these molds is important in driving natural cycling of chemical elements, particularly in the carbon and nitrogen cycle where they contribute to replenishment of the supply of carbon dioxide and other inorganic compounds, because they are adept at recycling starches, hemicelluloses, celluloses, pectins and other sugar polymers, and in the case of nitrogen these species are capable of utilizing a wide range of nitrogen-containing compounds as a sole nitrogen source, including ammonia, nitrate, nitrite, purines, amides, and most amino acids, but preferentially glutamine and glutamate (Brakhage *et al*, 1999).

2.1.1. Biotechnological Importance

Several studies in *Aspergillus*, has demonstrated a huge biotechnological potential, not only for producing numerous extracellular enzymes and organic acids, these molds also produce secondary metabolites of importance in biotechnology.

2.1.1.1. Primary and Secondary Metabolite Production

The biosynthesis by *A. nidulans* represents the most advanced model system for studying the regulation of the biosynthesis of a secondary metabolite (Ward *et al, sf*). Penicillin is synthesized from three amino acids as precursors, L-α-aminoadipic acid, L-cysteine, and L-valine, which are critical in the regulation of biosynthesis (Trip *et al*, 2004).

Also, there have been classified different strains of *Aspergillus terreus*, which have the ability to produce cyclosporine A, which is an immunosuppressant drug used in organ transplantation to reduce the risk of rejection; and lovastatin, which is a drug used for lower cholesterol. The occurrence of indole alkaloids among secondary fungal metabolites was studied by Zelenkova *et al* (2003), in different Aspergillus species; for example, *Aspergillus* fumigatus reported the formation of fumigaclavine B, α-clyclopiazonic acid and diketopiperazine alkaloids.

The production of citric acid is another example (Karaffa *et al*, 2003), because the use of *Aspergillus niger* for its production has been reported since 1917. The importance of citric acid goes from the food industry to the pharmaceutical and cosmetics industries. Other technical applications of citric acid are as a hardener in adhesive and for retarding the setting of concrete. *A. niger* also has found use in the industrial production of gluconic acid, which is used as an additive in certain metal cleaning applications, as well as for the therapy for calcium and iron deficiencies (Liu *et al*, 2003).

Aspergillus fumigatus produces many secondary metabolites including fumagillin, fumitremorgin, fumigaclavine, gliotoxin, helvolic acid, verruculogen, and sphingofungins, and it has been suggested that the virulence of the strain may be enhanced by these metabolites (Latge, 1999).

2.1.1.2. Food Fermentations and Extracellular Enzymes

The technology of food fermentations is an ancient process that permits the use of microorganisms and their enzymes to produce several types of nourishments and therefore improve the human diet, this also allows the enhancement of different characteristics of the aliments, like flavors, textures and aromas, and may also possess certain health benefits including superior digestibility. Exemplifying this, there are some types of Aspergilli, called koji molds (*A. oryzae* and *A. sojae*), and are employed in many ways, such as the release of amylases that break down rice starch which in turn can be fermented to make rice wine. Also, this fungus has being recognized as high capacity secretors, and this means that their extracellular enzymes can easily be exploited for the production of several industries process, like baking, beverage and brewing industries. *Aspergillus* species, especially the GRAS-designated strains, produce and secrete a variety of industrial enzymes including α-amylases, glucoamylases, cellulases, pectinases, xylanases and other hemicellulases and proteases (Ward *et al*, 2006).

2.1.1.3. Biotransformations and Environmental Applications

The biotransformation consists in the chemical conversion of a substance that is mediated by living organisms or enzyme preparations derived from them, and it exploits the versatility and high reaction rates achievable with the enzymes under mild conditions to catalyze reactions that are highly region- and stereo-specific. This high selectivity is particularly useful in implementing synthesis or modification of complex chemical structures into bioactive molecules or their precursors.

Aspergillus species have been employed extensively for implementation of specific steroil hydroxylations, oxidations, hydrolysis, esterification, isomerisations, and racemic resolutions. In addition, some species have been employed in biotransformations of alicyciclic insecticides, aromatic and phenoxy herbicides, organophosphorus and other pesticides, cyclic hydrocarbons, terpenes, and alkaloids (Ward *et al*, 2006).

Environmental applications of Aspergillus are rather limited, however it has been reported that *A. terreus* metabolizes polycyclic aromatic hydrocarbons (PAHs), by hydroxylation performed by cytochrome P-450 monooxygenase followed by conjugation with sulfate ion. *A. terreus* also degraded pyrene and benzo[a]pyrene (Capotorti *et al*, 2004).

In addition, *A. niger*, has shown an environmental application with the bioabsorption of heavy metal. The effect of copper (II), lead (II), and chromium (VI) ions on the growth and bioaccumulation properties of *A. niger* was studied by Dursun *et al* (2003), as a function of initial pH and metal ion concentration.

2.2. Nutritional Needs of Aspergillus fumigatus

Every organism must find in its environment all of the substances required for energy generation and cellular biosynthesis, and these substances are called nutrients. At an elementary level, the nutritional requirements of a microorganism are revealed by the cells elemental composition, which consists of C, H, O, N, S. P, K, Mg, Fe, Ca, Mn, and small quantities of Zn, Co, Cu, and Mo. These elements are found in the form of water, inorganic ions, small molecules, and macromolecules which serve either a structural or functional role in the cells.

2.2.1. Salts

It has been reported by Lavigne *et al* (2005) that magnesium is one of the nutrients that many species require in high amount to grow *in vitro*. They described that the magnesium acquisition of magnesium *in vivo* in the phagolysosome is an essential prerequisite for bacterial pathogens such as *Mycobacterium tuberculosis* or *Salmonella typhimurium*. Magnesium acquisition is under the control of *mgtC*, which encodes a membrane protein of unknown function that is known to be essential for pathogenicity of bacteria, but it has been shown that this gene is also present in *A. fumigatus*.

Other important salt in the fungal growth is phosphate, which amount present in the human serum is really low, affecting *A. fumigatus* growth. The human serum contains around 1 mM of phosphate, but *Aspergillus fumigatus* require ten times more P_i than this (Bernard *et al*, 2002). Zinc is another macroelement that *A. fumigatus* must acquire from the environment, and this has been shown to be important for microbial infection (Vicentefranqueira *et al*, 2005).

2.2.2. Iron Needs

Iron is an essential nutrient for all eukaryotes and nearly all prokaryotes, and is one of the most abundant elements on Earth, and can adopt either of two ionic forms; an oxidized ferric (Fe^{3+}) and as reduced ferrous (Fe^{2+}). Iron is indispensable for a variety of cellular processes including respiration, oxidative stress detoxification, as well as synthesis of amino acids, desoxyribonucleotides, lipids, and sterols.

If iron is present as a reduced form (Fe^{2+}), it has the potential to catalyze the formation of cell-damaging hydroxyl radicals; however, iron is principally present in the oxidized form and is required for an enormous variety of metabolic processes in virtually all organisms, but it presents low solubility under aerobic conditions at near neutral pH.

The concentration of Fe^{3+} in water is very low, around 10^{-18} M, and the presence of mammalian iron-binding proteins reduce even more the concentration present in the human serum, near 10^{-24} M (Fischbach *et al*, 2006). This proposes that microorganism that depend on iron have developed some strategies to fix this problem, evolving tactics to steal iron from their host, and one of the most studied and important mechanism developed for iron uptake are the siderophores.

2.3. Fungal Iron Acquisition Systems

According to Haas *et al* (2008), there are four different mechanisms for iron uptake in fungi, these are: (*i*) siderophore-mediated Fe^{3+} uptake, (*ii*) reductive iron assimilation (RIA), (*iii*) heme uptake, and (*iv*) direct Fe^{2+} uptake. The first two are classified as high-affinity iron uptake systems and are important in conditions of limited iron availability; the third one represents the utilization of a special iron source typically found within hosts and the last one is a low affinity iron acquisition system, used only when iron is plenty in the host.

2.3.1. High-Affinity Fe^{2+} Uptake

When the Fe^{2+} available is very limited, it is taken up via a high-affinity transport complex consisting of a ferroxidase and an iron permease, termed *Fet3p* and *Ftr1p* respectively. The iron is first oxidized by the ferroxidase and subsequently moved to the cytosol as Fe^{3+} by *Ftr1p* (Kwok *et al*, 2006). If the iron permease is missing, the Fet3p is mislocalized, and vice versa, signifying that the association Fet3-Ftr1 is mandatory.

2.3.2. Heme Uptake and Direct Fe^{2+} Uptake

The heme uptake mechanism consists in the direct use of host iron proteins such as transferrin, lactoferrin, ferritin, and heme-proteins by binding to receptors on the cell surface, followed by extraction of the iron and import into the cytoplasm. In fungi binding and uptake of only heme has been found. Notably, a number of fungi lack heme oxygenase, like *Aspergillus* spp., and are therefore probably unable to use or recycle heme-iron (Haas *et al*, 2008).

In the case of the direct uptake of reduced iron, this mechanism has been studied, at the molecular level, in *S. cerevisiae*, where the Fe^{2+} is taken up by the permease. This low affinity system is not specific for Fe2+, also transports other metals, like zinc and copper (Hassett *et al*, 2000).

2.3.3. Siderophore-Mediated Fe^{3+} Uptake

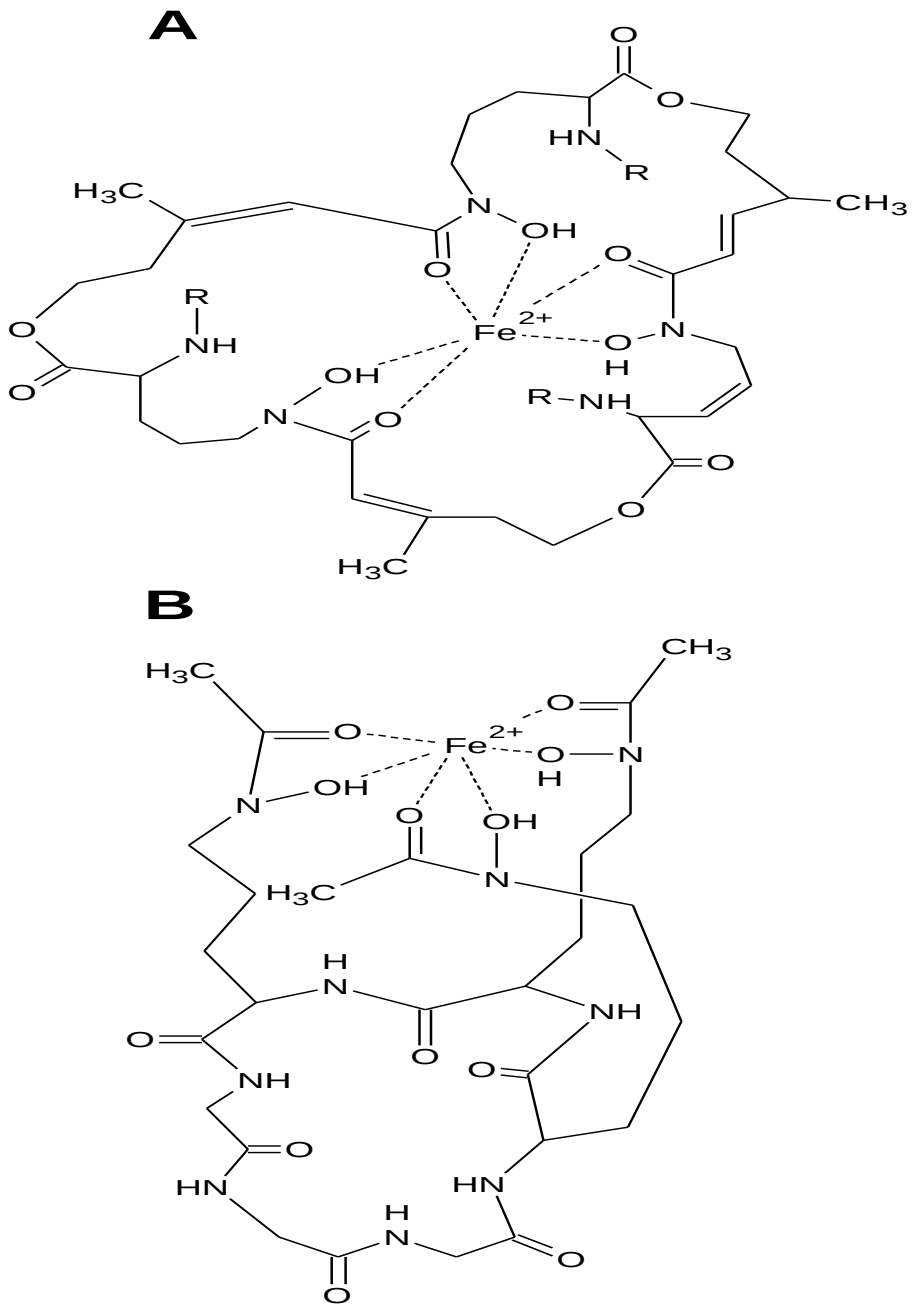
A siderophore is a relatively low molecular weight, ferric ion specific chelating agent, that are secreted by different types of fungi and bacteria, that are developing in situations with very limited iron conditions, forming tight complexes with Fe^{3+} to overcome the problem of low bioavailability by solubilization. These chelating agents can be classified, according the chemical nature of the moieties donating the oxygen ligands, into three main groups: aryl caps, carboxylates, and hydroxamates (Haas *et al*, 2008), but in case of fungi, the siderophores known so far are hydroxamates.

2.3.3.1. Hydroxamate Siderophores

Fungal hydroxamates are derived from the nonproteinogenic amino acid ornithine and different acyl groups and can be grouped into four structural families: (*i*) rhodotorulic acid, (*ii*) coprogens, (*iii*) fusarinines, and (*iv*) ferrichromes (Haas *et al*, 2008). The last two are the ones present in *Aspergillus fumigatus*. The fusarinine prototype, *fusarinine C*, consists of three N^5-*cis*-anhydromevalonyl-N^5-hydroxyornithine residues, linked by ester bonds in a head-to-tail fashion, and by the N^5-acetylation of this compound, the Triacetylfusarinine C (*TAFC*) is formed (Fig. 1A). The ferrichrome**,** such as ferricrocin (*FC*), are cyclic hexapeptides consisting of three N^5-acyl-N^5-hydroxyornithines and three aminoacids (glycine, serine and alanine) (Wallner *et al*, 2009) (Fig. 1B). *A. fumigatus* excrete mainly TAFC for iron acquisition and contain a ferrichrome for intracellular handling of iron.

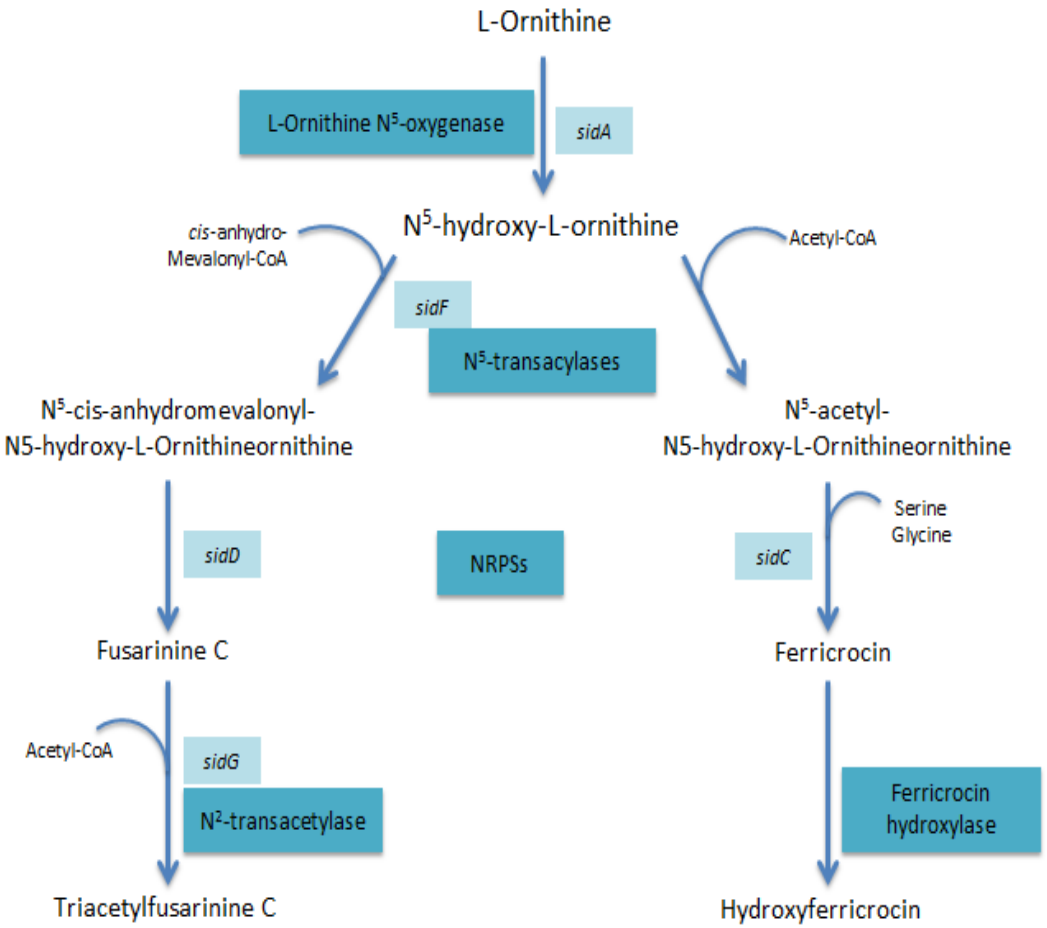
2.3.3.2. Siderophore Biosynthesis

The general steps of fungal siderophore biosynthesis, specifically *Aspergillus fumigatus*, are described in Figure 2. The first step in the biosynthesis of fungal siderophores is N^5-hydroxylation of ornithine catalyzed by ornithine-N^5-monooxygenase, which was first described in *Ustilago maydis* (*sid1*), followed by the characterization of orthologs, like *sidA* from *Aspergillus fumigatus*.



Source: Eisendle *et al*, 2003.

Figure 1. Representative fungal siderophores. *A.* Fusarinine Tryacetylfusarinine C (*TAFC*), *B.* Ferrichrome (*FC*). Both present in *Aspergillus fumigatus* siderophore iron uptake system.



Source: Haas *et al*, 2008.

Figure 2. Postulated siderophore biosynthetic pathway in *Aspergillus fumigatus*. Genes encoding enzymes necessary in the biosynthetic steps are shown in light blue.

Fungal ornithine-N^5-monooxygenases show significant similarity at the protein level to siderophore-biosynthetic bacterial ornithine-N^5-monoxygenases (PvdA from *Pseudomonas aeruginosa*), also both enzymes require oxygen as substrate and NAD(P)H and FAD as cofactors (Meneely *et al*, 2007). If a deletion occurs in the gene that encodes these enzyme, the biosynthetic steps will be blocked, for all siderophores studied in *Aspergillus*, *Ustilago* and *Fusarium*, and therefore, axenic growth of cells of *A. fumigatus*.

The second step in the siderophore biosynthesis results in the formation of the hydroxamate group which, depending on the choice of the acyl group, would take a different pathway, leading to the third biosynthetic step. Here the hydroxamates are covalently linked via ester or peptide bonds, accomplished by nonribosomal peptide synthetases (NRPSs), which are large multifunctional enzymes that synthesize peptides from proteinogenic and nonproteinogenic precursors independently of the ribosome.

2.4. Aspergillus fumigatus and Health

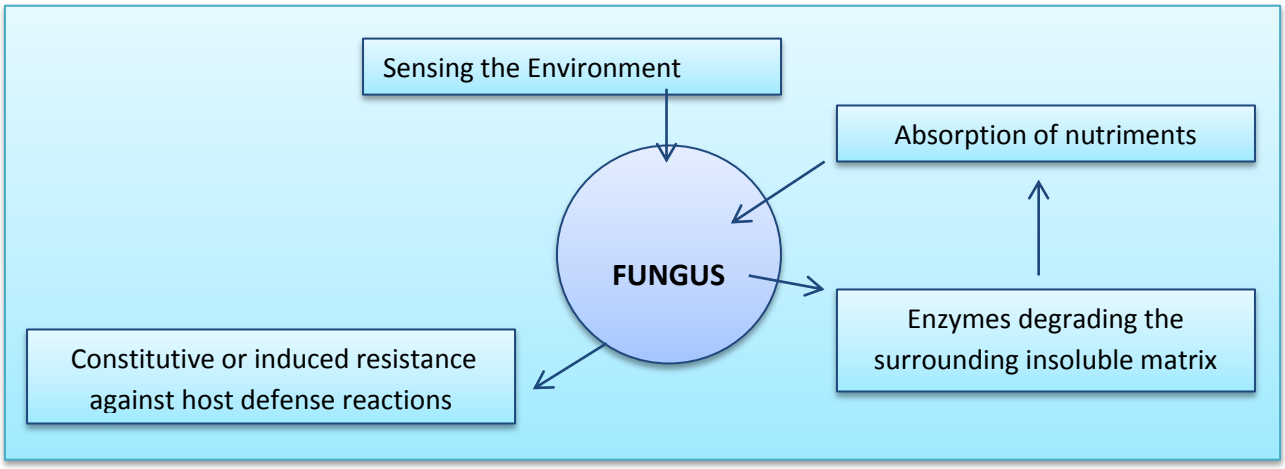
As mentioned before, *Aspergillus fumigatus* is responsible for Invasive Aspergillosis (IA), a life-threatening disease that usually only occurs in immunocompromised patients. The incidence of this disease has increased enormously during the past 20 years, because there are some medical practices that are becoming more common, for example the immunosuppression in the transplant patient and aggressive anti-cancer chemotherapy, both predisposing patients to IA (Tekaia & Latge, 2005), despite a better understanding of the epidemiology of *Aspergillus* infections, important diagnostic limitations persist.

The incidence of IA varies among the patient population and can infect 15% of allogeneic transplant and the mortality rates associated with the ailment caused by *Aspergillus* species range from 60-90%, again depending upon the type of patients infected (Steinbach *et al*, 2005). As most of the *Aspergillus* infections are caused by *Aspergillus fumigatus*, the majority of studies have focused on this species. *Aspergillus flavus* is the second leading cause of invasive and non-invasive aspergillosis (Pasqualotto, 2008).

2.4.1. Responses to a hostile environment

Following inhalation of conidia by the immunocompromised host, the pathogen senses the environment before it starts to germinate (Fig. 3). The innate cellular immune system (comprised of alveolar macrophages and neutrophils) is responsible for the killing of the conidia, and this anti-fumigatus activity of phagocytes primarily requires oxidative mechanisms to function (Philippe *et al*, 2003).

In *A. fumigatus*, melanins have been shown to play a protective role in pathogenicity, for example, Langfelder *et al* (2003) presented that dihydroxynaphthalene-melanin has been also recognized as a virulence factor, because the melanine protects the conidia against phagocituc defense reactions by quenching reactive oxygen. There is also a theory that suggests that these pathogenic microorganisms developed a network of oxidoreductases and metabolites that neutralize the reactive oxygen intermediates of the phagocyte (Tekaia & Latge, 2005).



Source: Tekaia & Latge, 2005.

Figure 3. Key biological traits and steps during infection of *Aspergillus fumigatus*

2.4.2. Siderophores and Interaction with Animal Hosts

The microbial quest for iron in mammalian hosts is crucial for successful pathogenesis as, in this environment, iron is tightly bound by carrier proteins such as transferrin, ferritin and lactoferrin, leaving free iron concentrations insufficient for sustained microbial growth. A pathogens requirement for iron is the basis for an elaborate mammalian defense system against microbial infection, which relies upon iron-withholding mechanisms to deny access to iron for invading microorganisms.

The crucial role of siderophores in virulence is reflected by the fact that mammals possess at least two proteins, the lipocalins Lcn2 and Lcn1, which are able to sequester siderophores (Fluckinger *et al*, 2004). Lcn2 binds its substrates with high affinity but has rather narrow substrate specificity by recognizing only particular catecholate but not hydroxamate siderophores. Lcn1 has broader substrate specificity by recognizing a variety of bacterial and fungal catecholates and hydroxamate siderophores and therefore has bacteriostatic and fungistatic activity, but binds its substrates with lower affinity.

Recent studies have determined that the ability of *A. fumigatus* to acquire iron and survive in serum involves siderophore-mediated removal of iron from host transferrin, implying a role for the siderophore biosynthesis *in vivo* (Hissen *et al*, 2004). Also, studies from Schrettl *et al* (2004) revealed that, mutants of *Aspergillus fumigatus* with deficiencies on the siderophore system were not able to grow on blood agar plates. Inactivation of the gene *sidA* prevents initiation of mammalian infection using a mouse model for pulmonary aspergillosis.

3. OBJECTIVES

3.1. General

Evaluate the *in vitro* enzymatic activity of the ornithine hidroxylase (*SidA*), from *Aspergillus fumigatus*, isolated and purified from a transformed culture of *E. coli*, varying the concentration of selected compounds and pH.

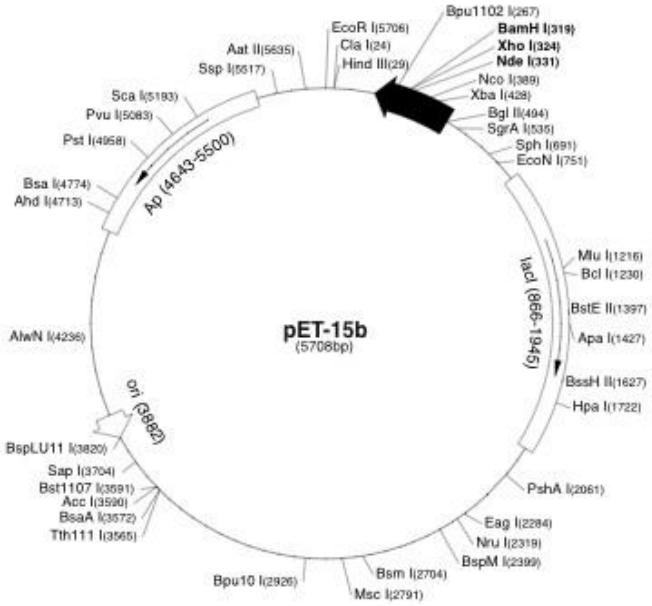
3.2. Specific

- Expression of the protein *SidA*, from *A. fumigatus*, using *E. coli* BL21TI^{R} (DE3).
- Purify *Af*SidA, by using IMAC (Immobilized Metal Ion Affinity Chromatography) in a three in-tandem 5 mL HisTrap columns.
- Evaluate enzyme activity and stability in a series of selected buffers, and design a stable buffer system.
- Quantify the pH activity of the enzyme, by varying the concentration of substrates L-Ornithine and L-Lysine.
- Determine and study the enzymatic kinetics values, k_{cat} and $k_{cat}/K_{M,}$ of each saturation curve obtained after the pH activity quantification.
- Design pH profiles, for each substrate; using k_{cat} and k_{cat}/K_{M} data, and determine possible dissociation constants present, according to formulas derivated from the Michaelis-Menten equation.
- Study the possible presence of an enzyme coupling effect with NADH.

4. METHODOLOGY AND MATERIALS

4.1. Protein Expression and Purification

Protein expression of N^5-ornithine hydroxylase protein from *Aspergillus fumigatus (Af*SidA), was performed using strains of *Escherichia coli* BL21TI^R (DE3), the gen been previously transformed with pET15b plasmid (Fig. 4). This plasmid poses resistance to the antibiotic ampicillin and more importantly, a His-Tag coding sequence. In addition, the strains of *E.* coli selected, have another plasmid that gives them chloramphenicol resistance.



Source: Novagen, 2011

Figure 4. The pET-15b vector.

The cells were cultivated in a Luria – Bertani (LB) plate, with antibiotics, and were incubated overnight, at 37°C, in a Barnstead Lab-Line incubator. Later, the cells were pre-inoculated in 50 ml LB broth, supplemented with ampicillin and chloramphenicol, and cultivated overnight at 37°C, at 250 rpm, using the Innova 4230 Incubator (New Brunswick Scientific).

This culture was used to inoculate six 2.8 L flasks of Terrific Broth (TB) media. The TB media was prepared using tryptone and yeast extract, and autoclaved at 121°C for 30 min. When these were at room temperature, the autoinduction solution was added, consisting of $MgSO_4$ 1M, succinic acid 15%, phosphate buffer, 30X80155 solution (Glycerol, glucose and lactose), and antibiotics ampicillin (1 ml) and chloramphenicol (1.5 ml). Once added, the culture was incubated in an Excella E25 Incubator, at 37°C during day and 25°C overnight, always at 250 rpm, and controlling the OD_{600}, to a value near 0.6. Finally, the cells were harvested by centrifugation and were frozen in a -80°C fridge, until their use.

To purify the protein, the pellets obtained by centrifugation were resuspended in *buffer A*, which contains HEPES, NaCl and imidazole, at pH 7.5. The suspension was done in ice, with the presence of DNase I, RNase and lysozyme. Always in ice, the cells were lysed by sonication, using the program of 10 seconds on and 20 seconds to a final time of 5 min on. Then, the lysated was clarified by centrifugation at 4°C, 15000 rpm during 15 minutes.

For the purifications steps, the clarified solution was loaded onto three in-tandem 5mL His•Trap columns, previously equilibrated with *buffer A*. The enzyme was loaded in the loop to be injected into the columns, in which the tag will permit the protein to stick in it, to finally been eluted with *buffer B*, which is the same *buffer A*, only with and increased concentration of imidazole; every load was at a flow rate of 5mL/min. Selected fractions of enzyme were loaded in a SDS-PAGE 12%, to judge purity. Then, the most pure fractions were mixed and concentrated using AMICON stirred cell concentrator and dialyzed with buffer phosphate, pH 7.5, using the His-Tag desalting column or the Millipore filters.

The concentration of enzyme was measure using the Agilent 8453 spectrophotometer, at wavelength of 450 nm. Also, the Bradford was performed at wavelength 595 nm, in triplicate. Later, the enzyme was frozen with liquid nitrogen and stored at -80°C.

4.2. pH Testing and Buffer Selection

The functionality of the enzyme in different buffers was evaluated. The buffer tested were *MES* [2-(N-morpholino) ethanesulfonic acid], *PIPES* [piperazine-N,N′-bis(2-ethanesulfonic acid)], Potassium phosphate buffer, *TRIS* [*tris*(hydroxymethyl)aminomethane], *HEPES* [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid], *CHES* (N-Cyclohexyl-2-aminoethanesulfonic acid), Borate buffer, Tricine buffer and *CAPS* (N-cyclohexyl-3-aminopropanesulfonic acid).

Each buffer was chosen so that the accessible pHs of each buffer overlapped with another on each side of the pH range, looking to cover a pH range from 5.5 to 10.0. In these overlap regions, the rate measurements were taken at identical pHs in each of the buffers to check for buffer-specific effects on the V_{max} values.

The test consisted in monitoring the amount of molecular oxygen consumed by *AfSidA*, using the Hansatech Oxygraph. The standard assay consisted of a 1000 µL reaction, saturated with 1 mM NADH, 15 mM L-Ornithine and using 100 mM of each buffer at different pHs and the reaction was initiated adding 2µM of enzyme. The results obtained were tabulated and according to the values the buffers were selected.

4.3. Determination of pH Effects with L-Ornithine

The assay consisted in measuring the oxygen consumption of the reaction involving the enzyme. This evaluation was done at constant concentration of NADH (1mM) and the respective buffer (100mM); varying the concentration of substrate L –Ornithine. The reaction was initiated with 2µM of enzyme. The reaction proceeded for 3 min at room temperature, with constant stirring.

4.4. Determination of pH Effects with L-Lysine

The assay was done similar to the one using L-Ornithine, utilizing the Hansatech Oxygraph, at constant concentration of NADH (1 mM), *AfSidA* (2µM), but in this case, varying the concentration of substrate L-Lysine, in 100 mM of buffer, at different pHs. The reaction proceeded for 3 min at room temperature, with constant stirring.

4.5. NADH Control Assay

The development of this test was based on the pH effects assays. Using the Oxygraph, the oxygen consumption rates were measured, with the only difference that no substrate was added. Constant concentration of NADH (1 mM) and enzyme (2 µM) were used. The reaction was done in the same buffer system settled for the previous assays, and proceeds for 3 min at room temperature, with constant stirring. Every test was done by triplicate.

4.6. Enzyme Stability Assay

This assay was done to evaluate the stability of the enzyme in the selected buffers. For the performance of this assay, enzyme previously frozen was used. The enzyme was defrosted, and then concentrated up to 400 µM, then using the desalting column and the AKTA, a potassium phosphate buffer concentration exchange was done, lowering the concentration from 100 mM to 5mM.

The assay consisted in the measurement of the oxygen consumption, using the Oxygraph. The reaction comprised of 1 mL, with 1mM NADH and initiating the reaction with 2µM of enzyme, all in buffer potassium phosphate 5mM, pH 7.5. The measurements were made at time zero, one hour, two hours and finally after five hours, each one by duplicate. All results were plotted using the Kaleidagraph.

4.7. Data Analysis

The obtained data using the oxygen consumption assay was analyzed using the program KaleidaGraph. Initial rate data will fit to the different derivations, already published (Sobrado & Fitzpatrick, 2003), of the Michaelis-Menten equation to obtain k_{cat} and K_M values.

$$\log y = log\left(\frac{C}{1 + \frac{H}{K_1}}\right) \qquad Equation\ 1$$

$$\log y = log\left(\frac{Y_L + Y_H\left\{\frac{K_a}{H}\right\}}{1 + \frac{K_a}{H}}\right) \qquad Equation\ 2$$

$$\log y = log\left(\frac{C}{1 + \frac{H}{K_1} + \frac{K_2}{H}}\right) \qquad Equation\ 3$$

5. RESULTS

5.1. AfSidA Expression and Purification.

After autoinduction treatment, cells of *Escherichia coli* BL21TI^{R} (DE3) were harvested, obtaining 65 g, in average, of cells. Later, the cells were lysed and recombinant *Af*SidA was expressed containing an 8xHis tag at the N-terminus was purified in two chromatographic steps.

The UV-visible absorbance spectrum indicates the presence of flavin cofactor bound to the purified enzyme with peaks at 380 and 450 nm. The stoichiometry of flavin incorporation was determined to be 40-50%. This was determined by dividing the protein concentration calculated using the flavin extinction coefficient by the protein concentration using the Bradford assay.

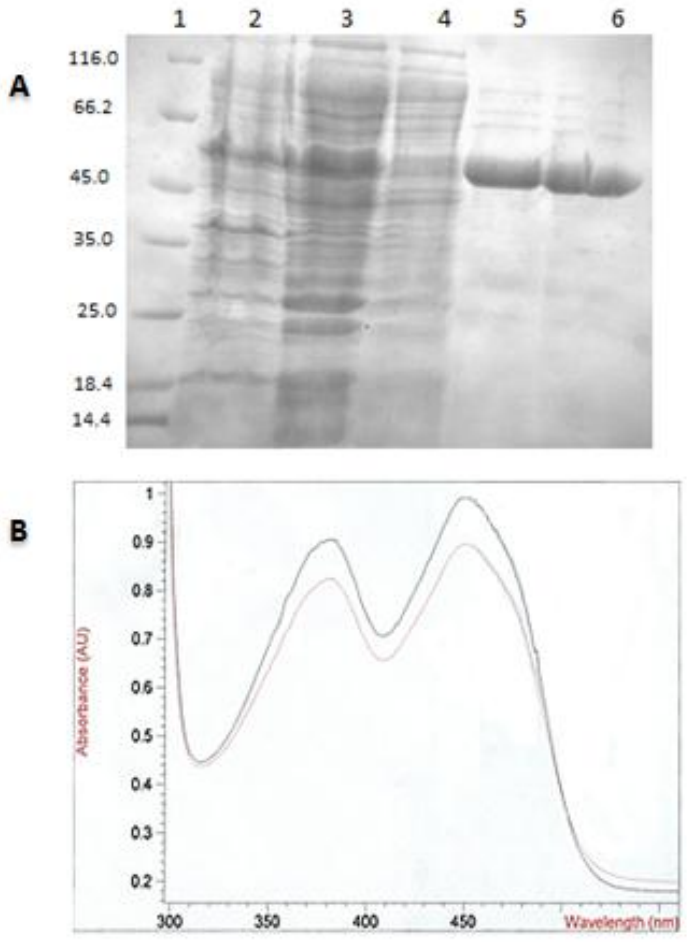


Figure 5. Summary of AfSidA purification. (A) SDS-PAGE gel summarizing whole process of purification of *Af*SidA: lane 1, molecular mass marker; lane 2, crude lysate; lane 3, clarified lysate; lane 4, flow through; lane 5, sample after column without His-Tag; lane 6, sample after column, with His-Tag. Lanes 5 and 6 are after the two chromatographic steps. (B) UV-Visible spectra of bound FAD in purified *Af*SidA, the red line is the *Af*SidA without His-Tag and the black one is the *Af*SidA with His-Tag.

The first chromatographic step resulted in the obtaining of the enzyme, corroborated by the gels a fraction of 57 kDa, in a more contaminated sample (Fig 6 A), for that reason another chromatographic step, consisting in performing a gradient with *buffer B* was done. A SDS-PAGE was used to evaluate the purity of the enzyme after the second chromatographic step (Fig. 6 B.), and according to the results, fractions were selected to be concentrated, dialyzed and stored.

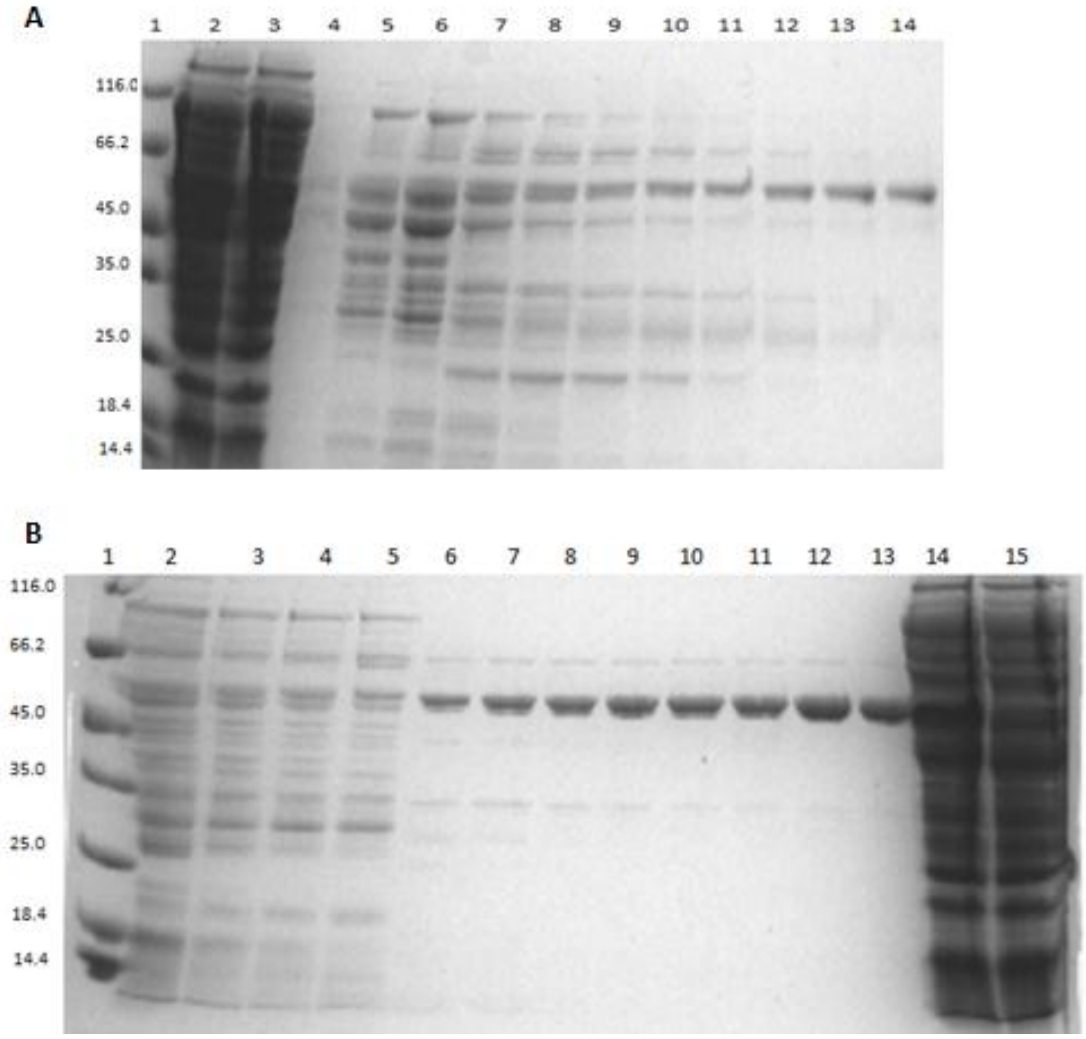


Figure 6. SDS-PAGE gel summarizing both chromatographic steps in the purification of *AfSidA*. A) No gradient, Lane 1, molecular mass marker; lane 2, crude lysate; lane 3, flow through; lane 4, hole; lanes 5-14 samples. B) using gradient. Lane 1, molecular mass marker; lanes 2-13 sample after column, eluted using a gradient of *buffer B*; lane 14, sample before column; lane 15, flow through. In this case, judging by the gel, the fractions from 6 to 13 were selected to previous concentration and dialysis steps.

5.2. Buffer System Selection.

Oxygen consumption rates were measured using different buffers at 100mM, to evaluate the functionality of the enzyme. The enzyme was shown to be functional in most of the buffers, giving the same rates of consumption (Table 1).

Table 1. Oxygen consumption rates reported for the different buffers tried, at different pHs.

Buffer, 100mM	**pH**	**Rate (*nmol/mL per min*)**	**Rate, min-1**
Potassium Phosphate	6.5	19.660	9.8300
MES	6.5	37.030	18.515
PIPES	6.5	28.400	14.200
Potassium Phosphate	7.5	63.81	31.905
HEPES	7.5	64.53	32.265
TRIS HCl	7.5	2.18	1.09
HEPES	8.5	44.60	22.3
CHES	8.5	39.60	19.8
Borate Buffer	8.5	18.66	9.33
Tricine	8.5	42.24	21.12
CAPS	8.5	45.86	22.93

As seen in the results, buffers Tris HCl and Borate are not suitable to carry out the posterior pH analysis with the *AfSidA* enzyme. Buffer tricine presented a good rate of consumption of oxygen, but when the rest of the pH where measured the resultant rate, was still the same (around 22 min^{-1}), thing that make it not appropriate for the assay. The buffer system selected consisted in buffer MES for a pH range from 5.5 to 6.5; potassium phosphate buffer for the range from 6.0 to 8.0 and finally buffer CHES to pHs from 8.5 to 10.0.

5.3. Enzyme Stability Assays

Enzyme stability assays were performed to ensure that the enzyme was stable in the selected buffers, over time. The oxygen consumption rates obtained were plotted (Fig. 7).

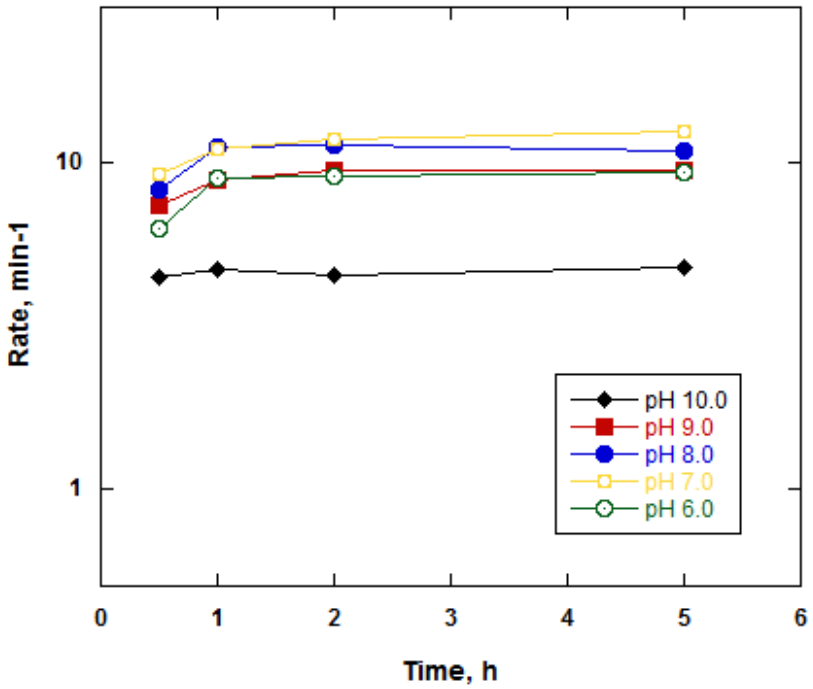


Figure 7. Rates (min^{-1}) obtained for the enzyme stability assay.

It can be appreciated in the figure that for the majority of pHs the enzyme maintains the same activity, but at the highest pH (10.0) the enzyme activity drops, although the activity does not vary.

5.4. NADH Control Assays

To evaluated the presence of possible enzyme uncoupling in the reaction, a NADH control assay was carried out. Using a concentration of 1µM of NADH, without substrate, the profile was determined, shown to be bell-shaped (Fig. 8), consistent with the involvement of two ionizable groups, one with an apparent pK_a value of 6.7 and the other with an apparent pK_a value of 9.5 (Table 4)

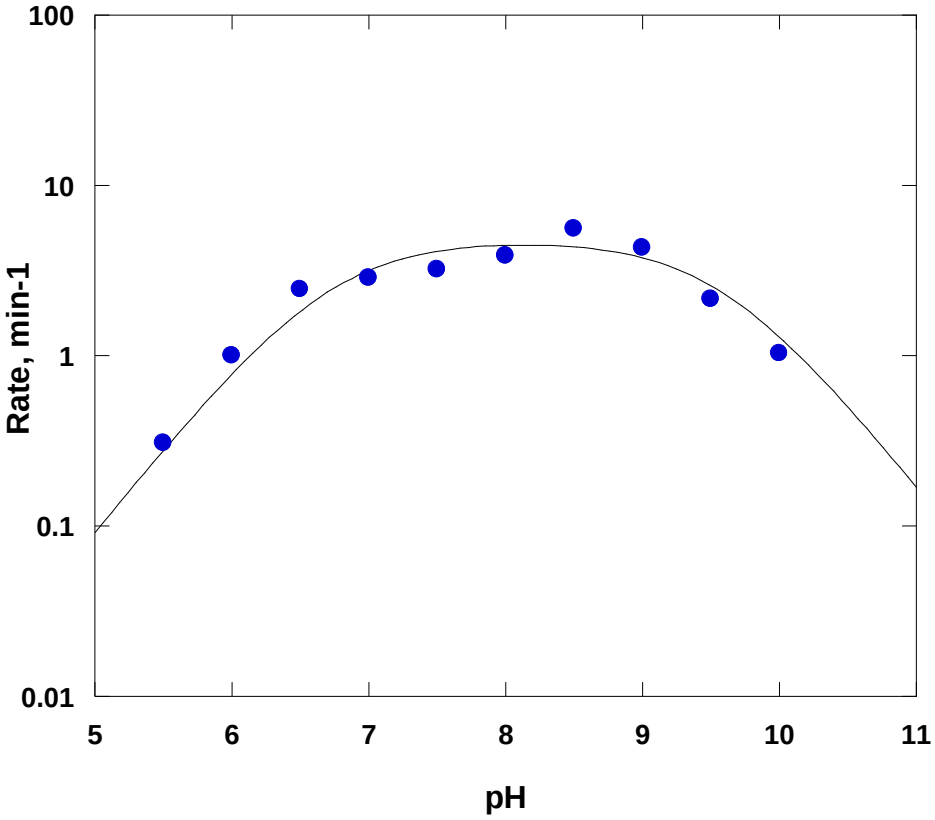


Figure 8. Reduce nicotinamide coenzyme pH profile curve determined by the oxygen consumption assay.

5.5. L-Ornithine pH Profile.

Oxygen consumption was measured, using the Oxygraph. Using a buffer system that permitted measure enzyme activity in pH from 5.5 to pH 10.0, oxygen consumption rates were determined, varying the concentration of substrate L-Ornithine. At the end, the rates obtained were plotted to obtain kinetic values k_{cat} and K_M (Table 2). Figures 9 to 18 show the saturation curve obtained in each pH evaluated.

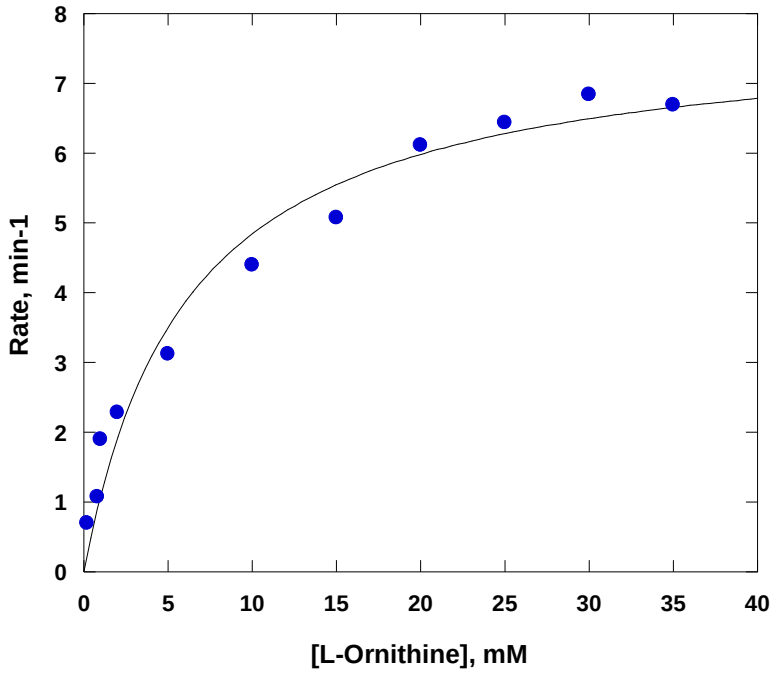


Figure 9. L-Ornithine saturation curve determined by oxygen consumption assay, at pH 5.5, using buffer MES 100mM.

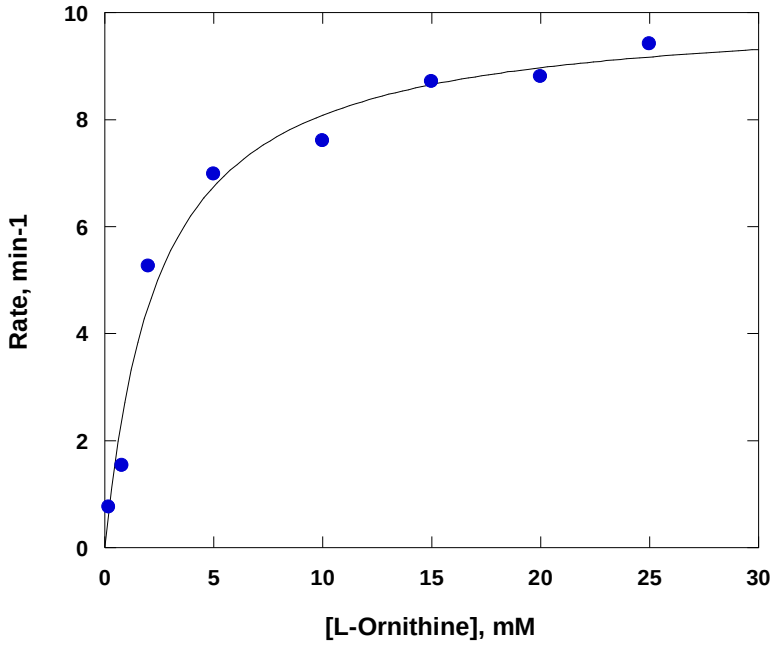


Figure 10. L-Ornithine saturation curve determined by oxygen consumption assay, at pH 6.0, using buffer MES 100mM.

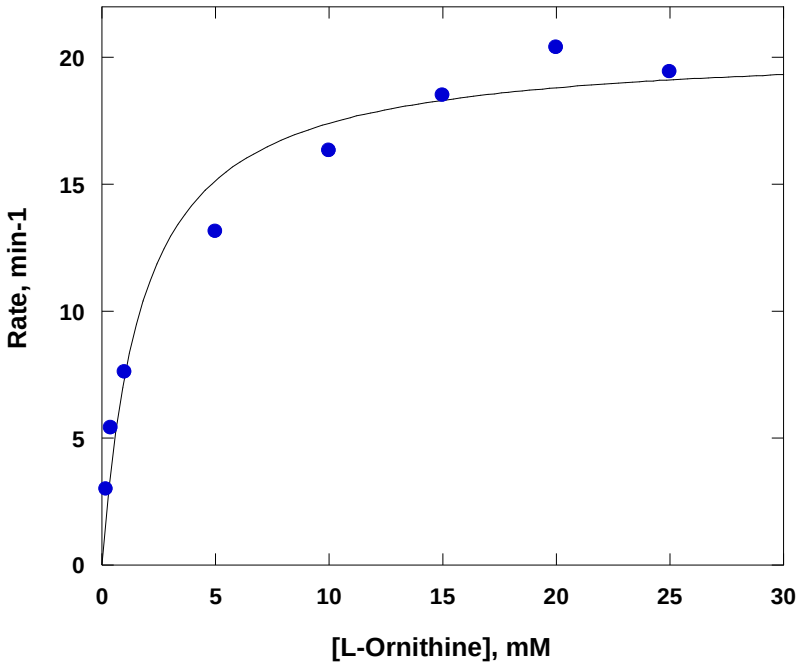


Figure 11. L-Ornithine saturation curve determined by oxygen consumption assay, at pH 6.5, using buffer MES 100mM.

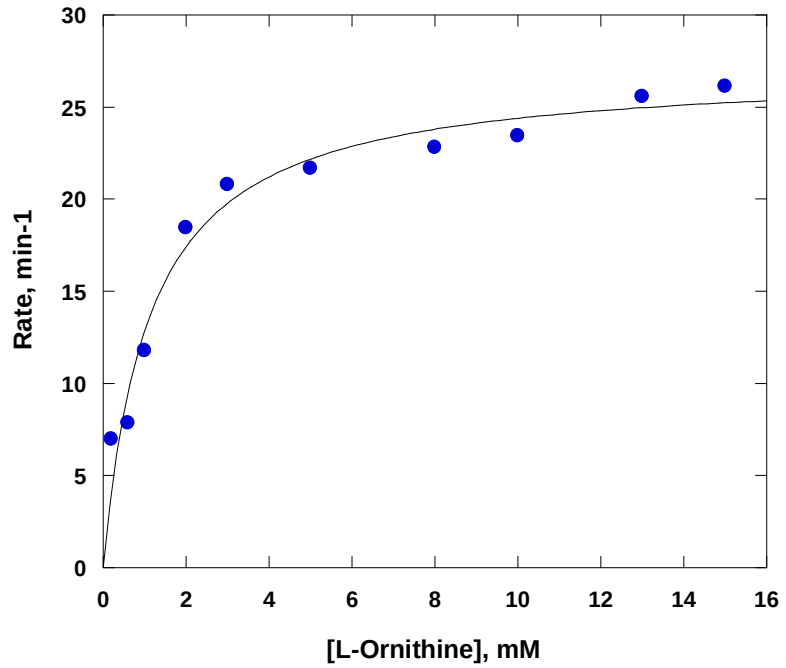


Figure 12. L-Ornithine saturation curve determined by oxygen consumption assay, at pH 7.0, using buffer Potassium Phosphate 100mM.

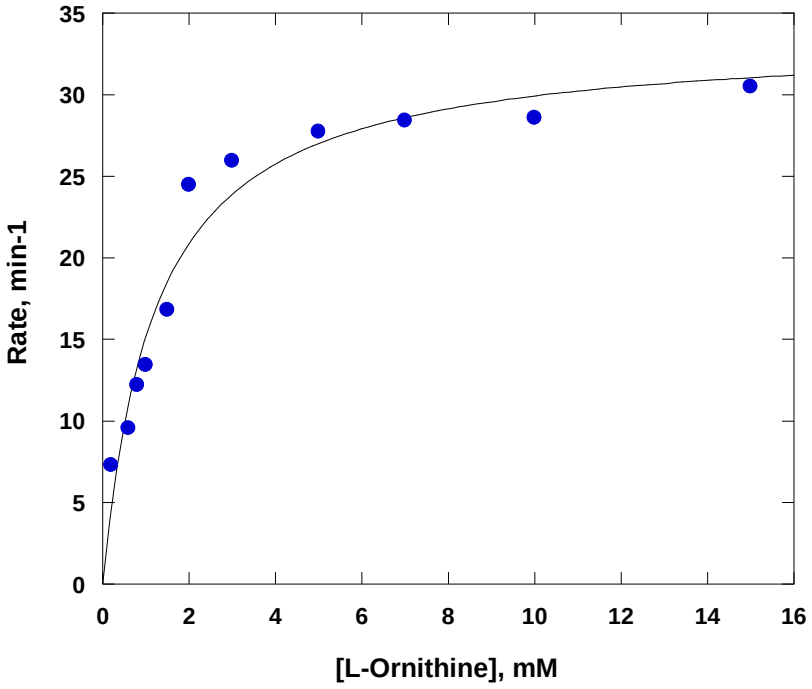


Figure 13. L-Ornithine saturation curve determined by oxygen consumption assay, at pH 7.5, using buffer Potassium phosphate 100mM.

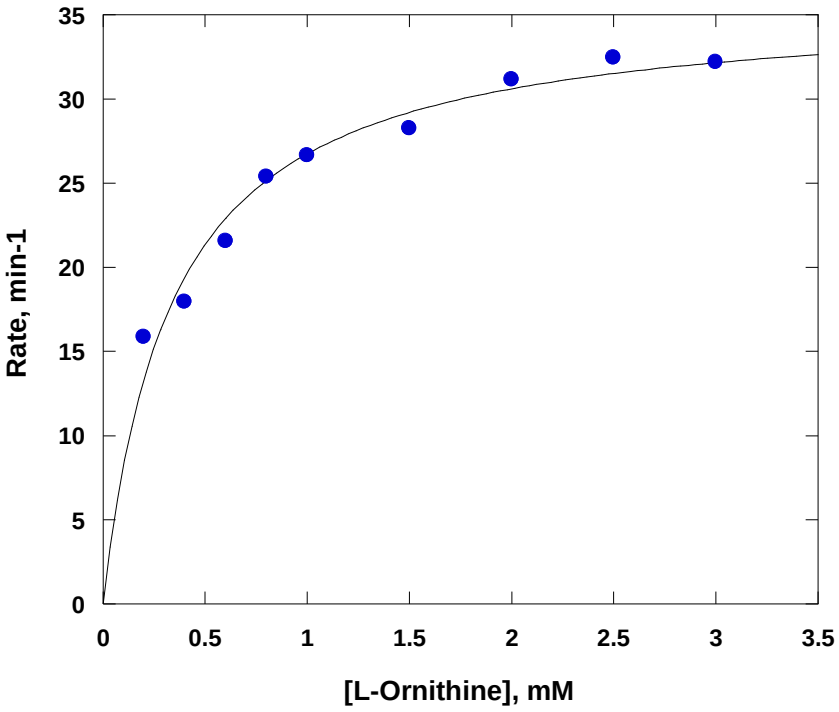


Figure 14. L-Ornithine saturation curve determined by oxygen consumption assay, at pH 8.0, using buffer Potassium phosphate 100mM.

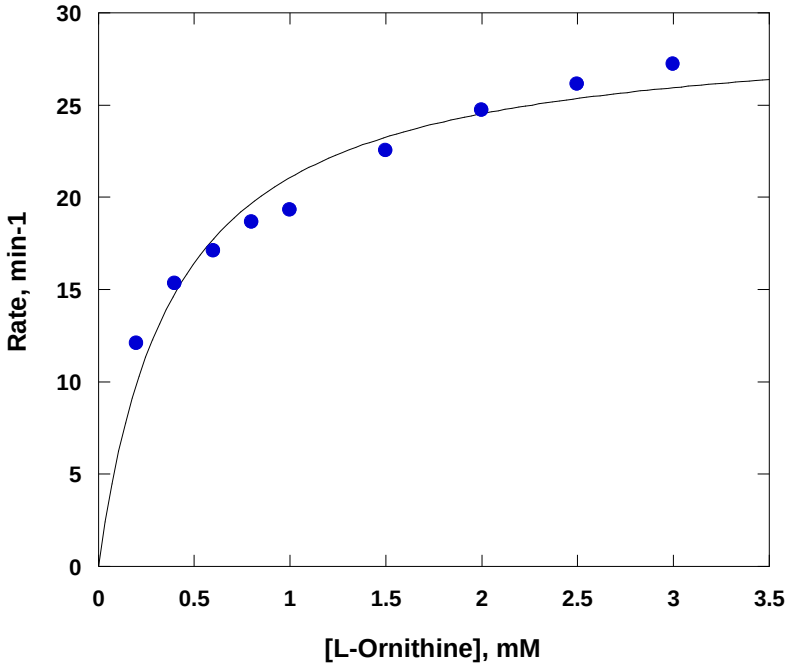


Figure 15. L-Ornithine saturation curve determined by oxygen consumption assay, at pH 8.5, using buffer CHES 100mM.

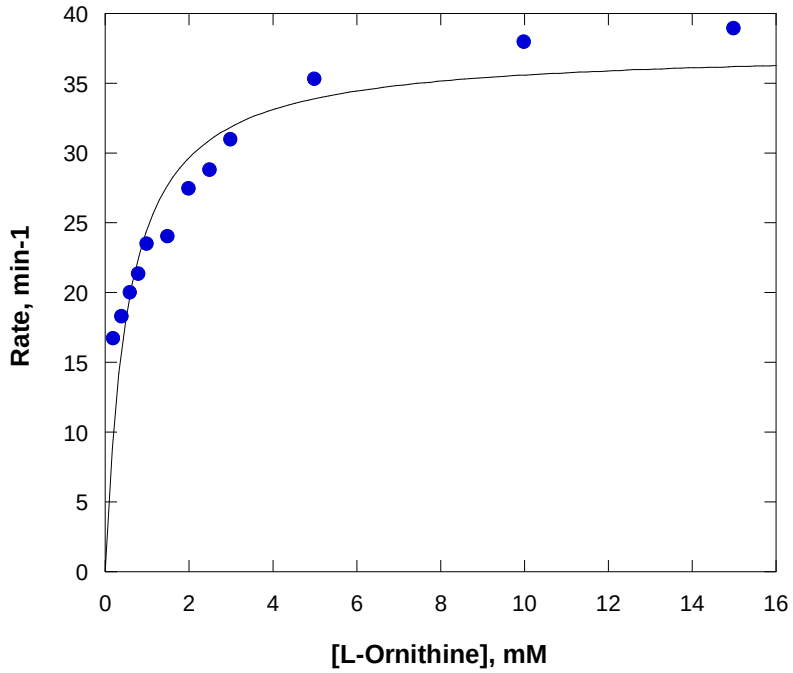


Figure 16. L-Ornithine saturation curve determined by oxygen consumption assay, at pH 9.0, using buffer CHES 100mM.

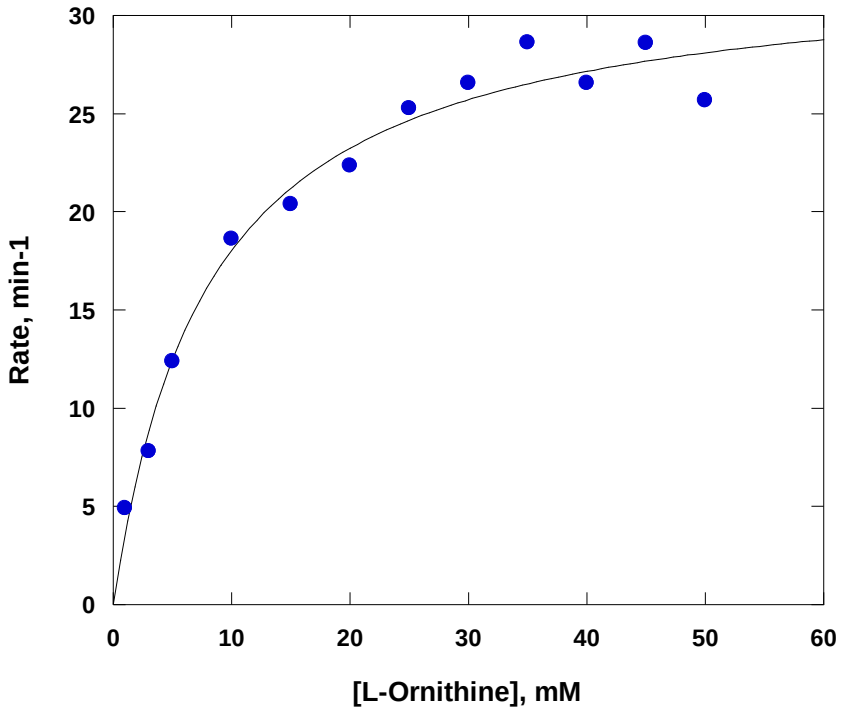


Figure 17. L-Ornithine saturation curve determined by oxygen consumption assay, at pH 9.5, using buffer CHES 100mM.

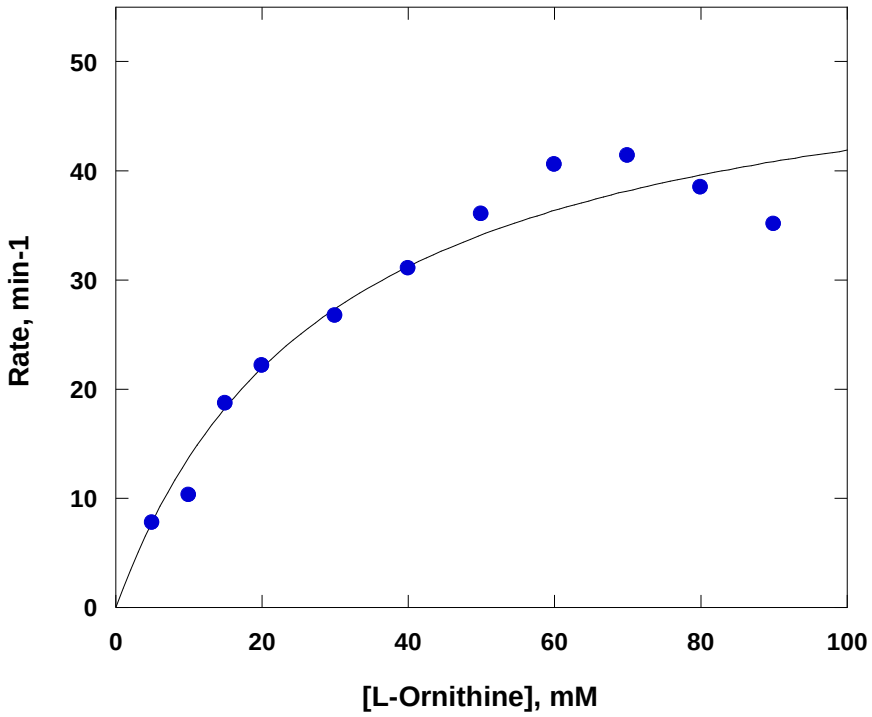


Figure 18. L-Ornithine saturation curve determined by oxygen consumption assay, at pH 10.0, using buffer CHES 100mM.

Table 2. Steady-State Kinetic Parameters Determined by Following the Rate of Oxygen Consumption, Using L-Ornithine.

pH	k_{cat}, min^{-1}	K_M, mM	k_{cat}/K_M, min^{-1} mM^{-1}
5.5	7.8 ± 0.5	6.2 ± 1.3	1.3 ± 0.2
6.0	10.1 ± 0.5	2.5 ± 0.5	4.1 ± 0.7
6.5	20.5 ± 1.0	1.7 ± 0.4	11.7 ± 2.5
7.0	27.1 ± 1.0	1.1 ± 0.2	24.4 ± 3.3
7.5	33.5 ± 1.5	1.2 ± 0.2	27.7 ± 3.5
8.0	35.8 ± 1.2	0.3 ± 0.04	105.8 ± 11.5
8.5	29.3 ± 1.3	0.4 ± 0.06	74.8 ± 9.6
9.0	37.5 ± 1.8	0.5 ± 0.1	71.4 ± 12.1
9.5	32.6 ± 1.2	8.1 ± 1.1	4.0 ± 0.4
10.0	54.2 ± 4.8	29.4 ± 6.8	1.8 ± 0.3

After determined the kinetic values k_{cat} and K_M, these data were plotted against pH (Fig. 19), and then fitted to the equations described in the methodology, depending on the behavior of the curve. Results obtained from the curve fitting described different pK_a values (Table 4).

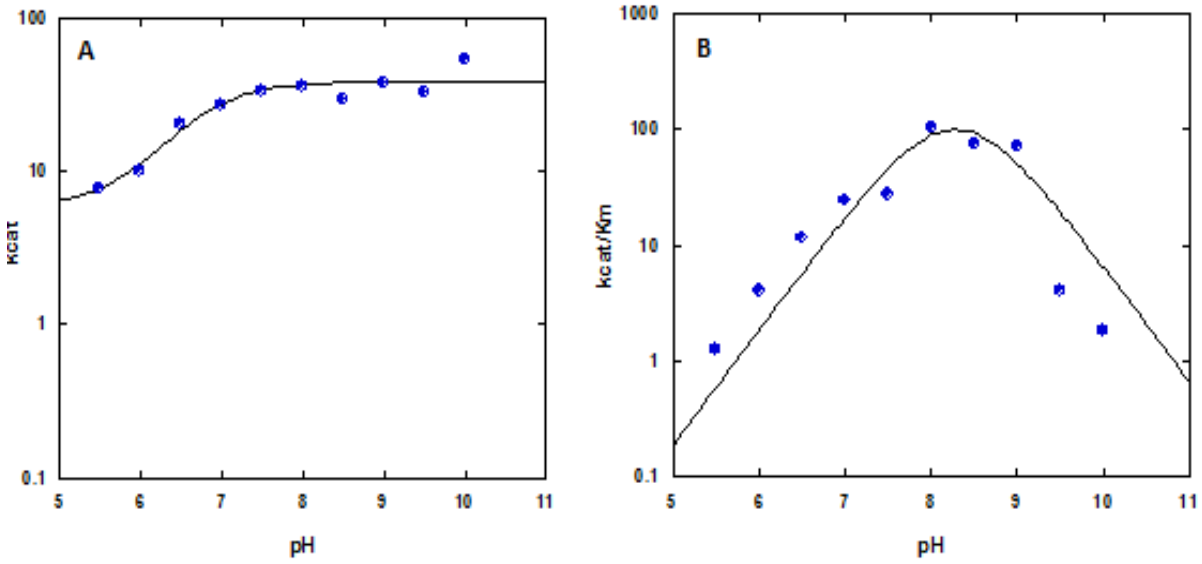


Figure 19. Effect of pH on *AfSidA* activity with L-Ornithine. The rate of oxygen consumption was measured in the presence of different ornithine concentrations, saturating concentration of NADH (1mM) and reaction initiated with 2µM of enzyme. A. Effect of the pH on the k_{cat} value. B. Effect of the pH on the k_{cat}/K_M value.

5.6. L-Lysine pH Profile

For this assay, again oxygen consumption was measured, with the help of a buffer system that permitted measure enzyme activity in pH from 5.5 to pH 10.0, and varying the concentration of substrate L-Lysine. At the end, the rates obtained were plotted to obtain kinetic values k_{cat} and K_M (Table 3). Figures 20 to 29 show the saturation curve obtained in each pH evaluated.

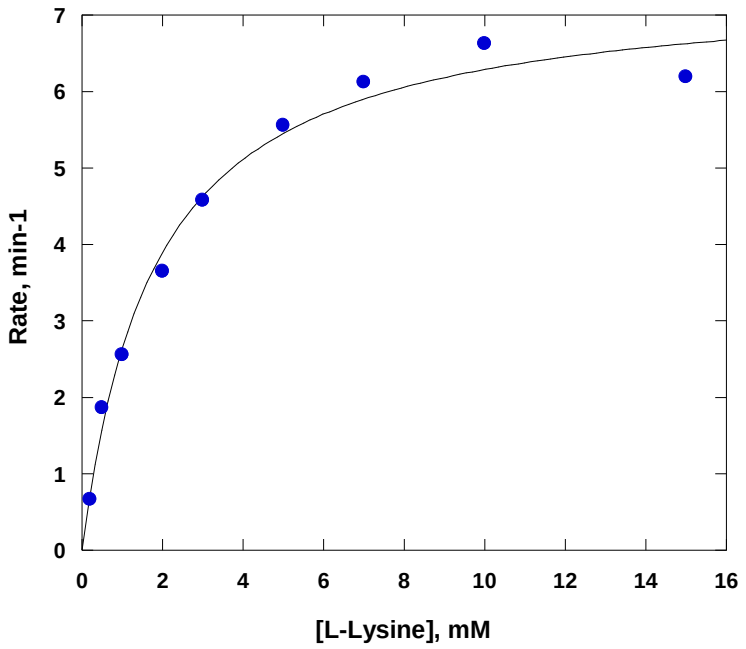


Figure 20. L-Lysine saturation curve determined by oxygen consumption assay, at pH 5.5, using buffer MES 100mM.

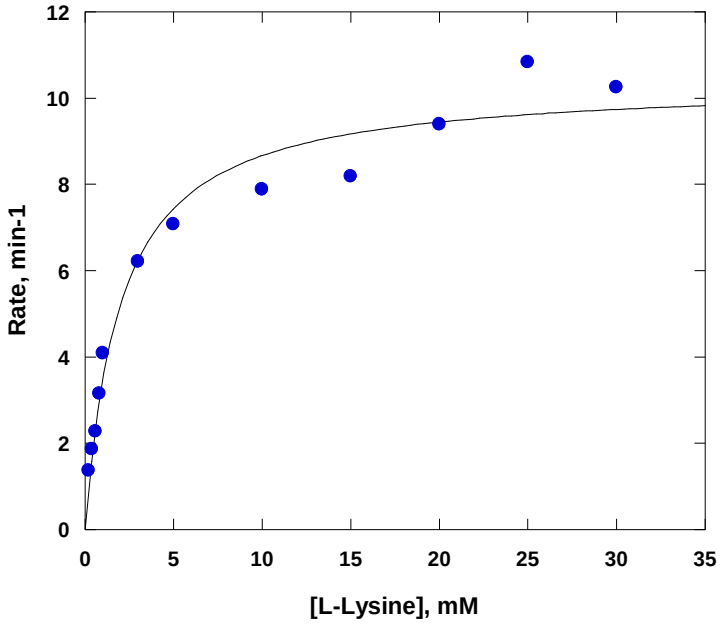


Figure 21. L-Lysine saturation curve determined by oxygen consumption assay, at pH 6.0.

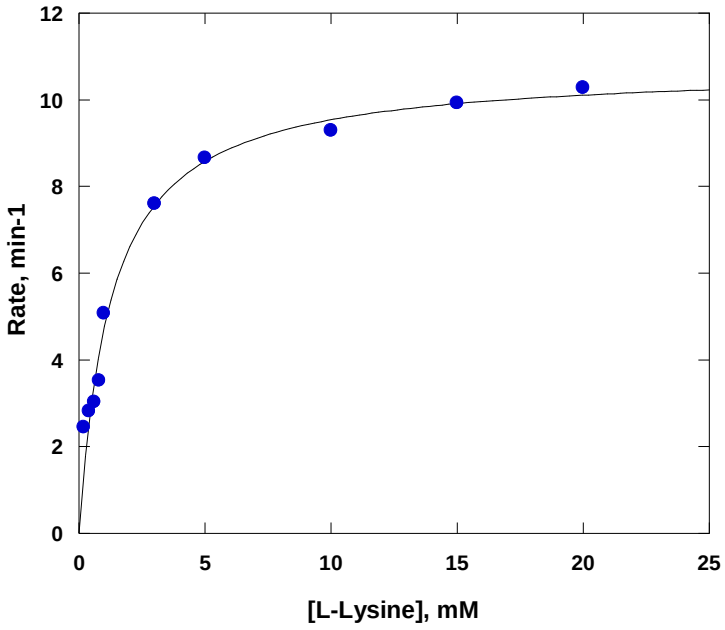


Figure 22. L-Lysine saturation curve determined by oxygen consumption assay, at pH 6.5

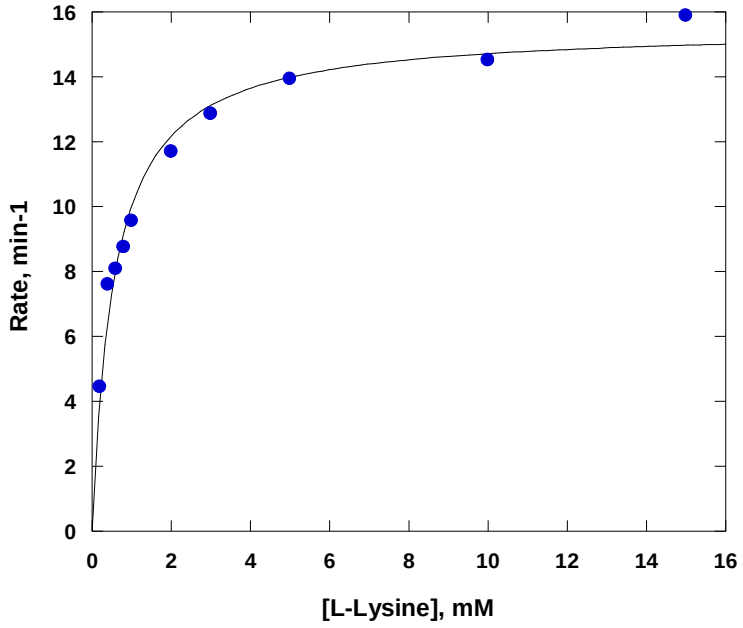


Figure 23. L-Lysine saturation curve determined by oxygen consumption assay, at pH 7.0.

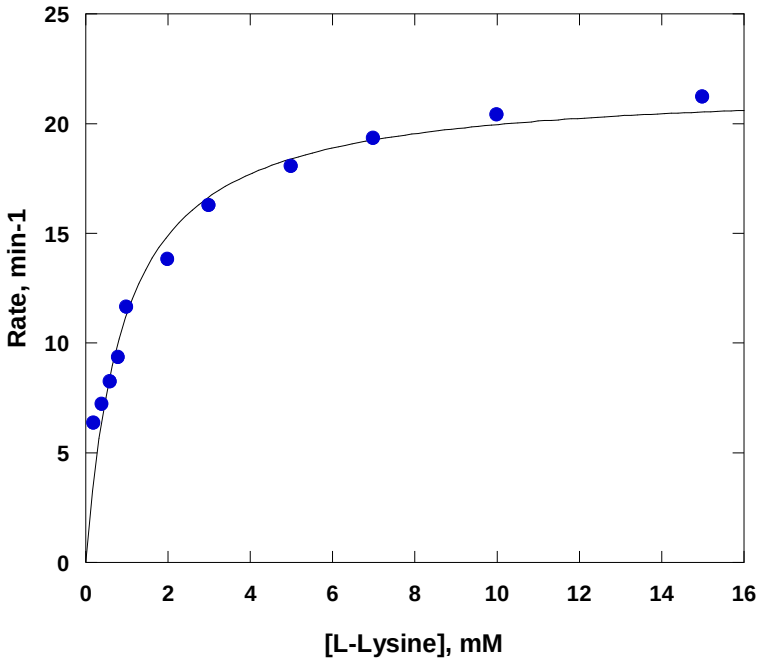


Figure 24. L-Lysine saturation curve determined by oxygen consumption assay, at pH 7.5.

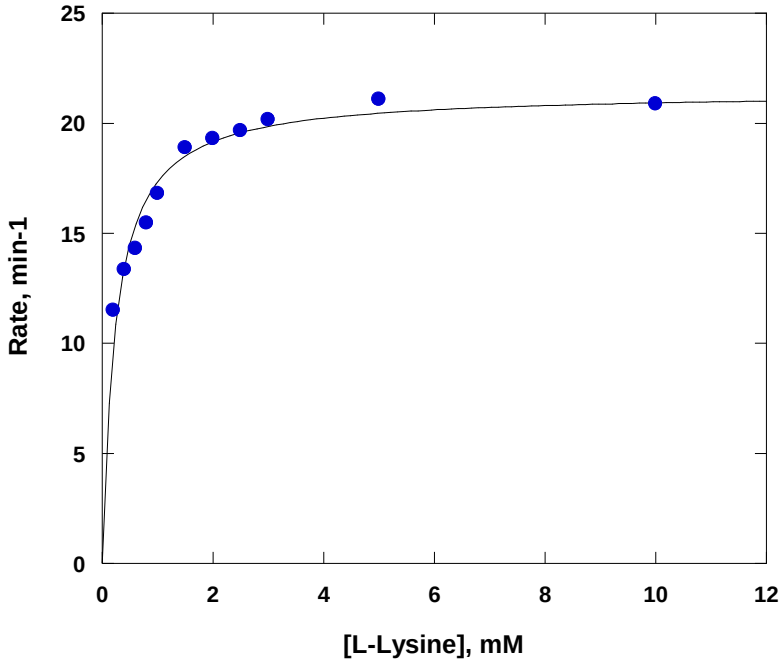


Figure 25. L-Lysine saturation curve determined by oxygen consumption assay, at pH 8.0

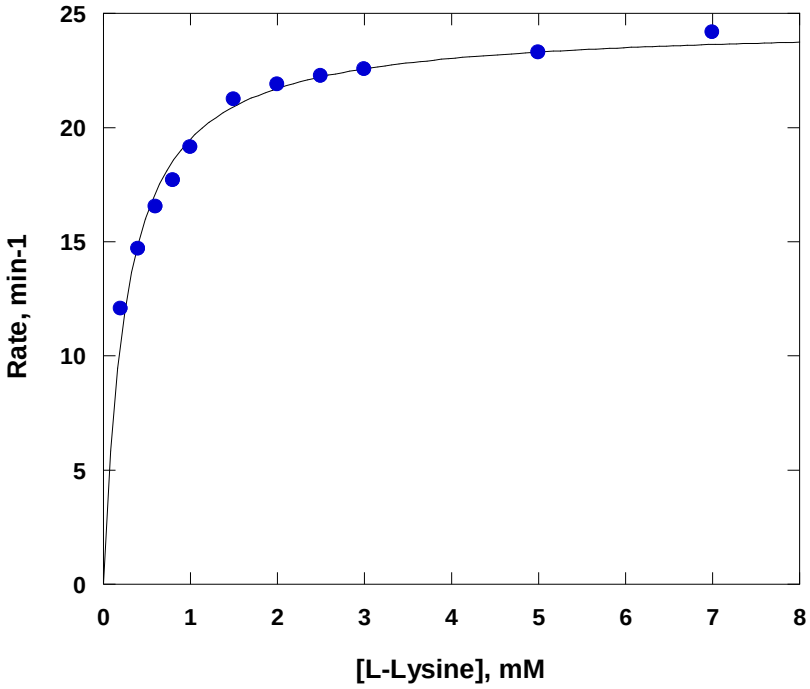


Figure 26. L-Lysine saturation curve determined by oxygen consumption assay, at pH 8.5

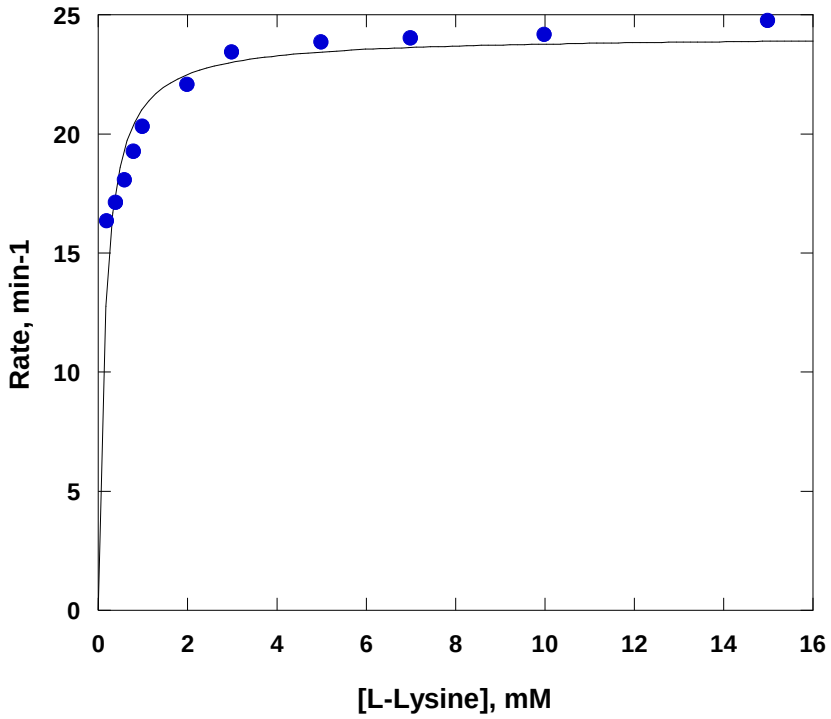


Figure 27. L-Lysine saturation curve determined by oxygen consumption assay, at pH 9.0

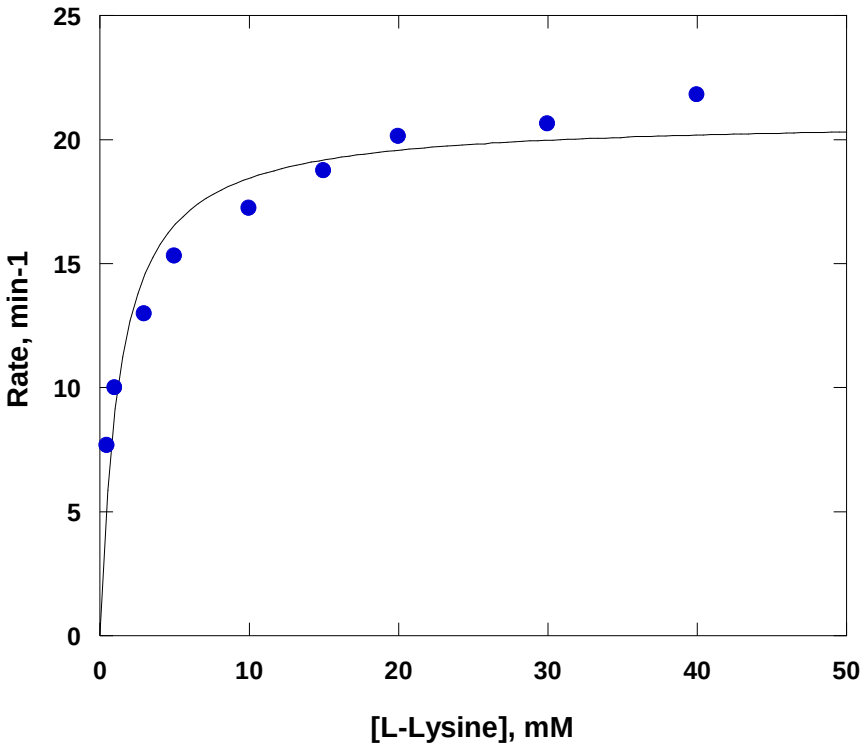


Figure 28. L-Lysine saturation curve determined by oxygen consumption assay, at pH 9.5

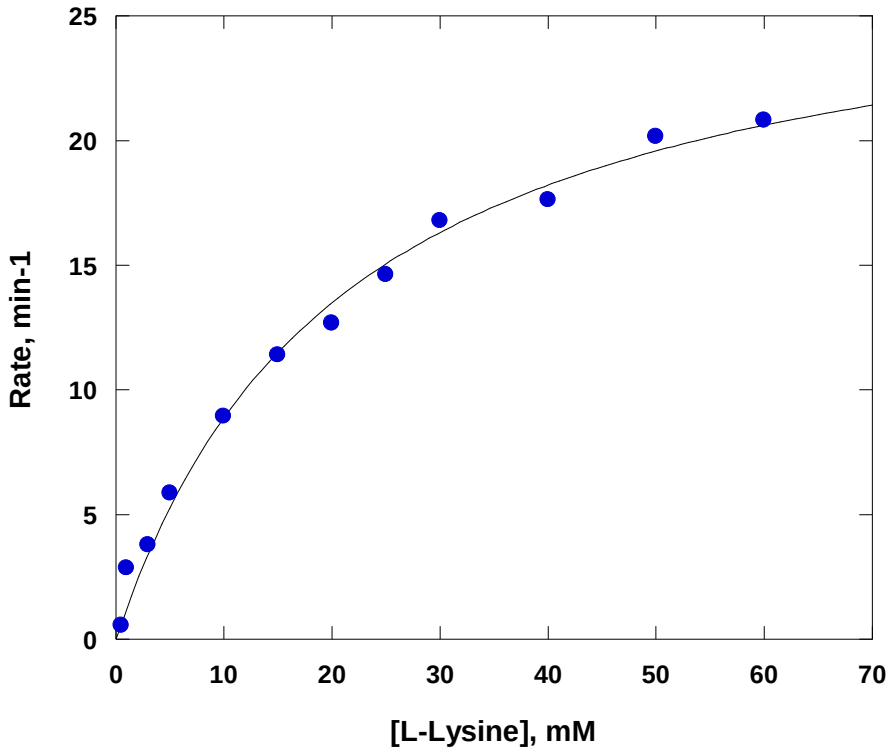


Figure 29. L-Lysine saturation curve determined by oxygen consumption assay, at pH 10.0

Table 3. Steady-State Kinetic Parameters Determined by Following the Rate of Oxygen Consumption, Using L-Lysine.

pH	k_{cat}, min-1	K_M, mM	k_{cat}/K_M, min-1 mM-1
5.5	7.4 ± 0.3	1.8 ± 0.2	4.1 ± 0.4
6.0	10.4 ± 0.2	1.9 ± 0.2	5.4 ± 0.4
6.5	10.7 ± 0.3	1.3 ± 0.1	8.5 ± 0.8
7.0	15.3 ± 0.3	1.01 ± 0.04	15.1 ± 2.05
7.5	21.8 ± 0.7	0.9 ± 0.1	23.6 ± 2.3
8.0	21.7 ± 0.5	0.2 ± 0.03	87.5 ± 11.01
8.5	24.5 ± 0.4	0.2 ± 0.02	95.3 ± 6.5
9.0	24.1 ± 0.5	0.1 ± 0.02	167.5 ± 24.2
9.5	20.8 ± 0.8	1.3 ± 0.3	16.1 ± 2.9
10.0	27.9 ± 1.4	21.5 ± 2.5	1.3 ± 0.09

After determined the kinetic values k_{cat} and K_M, these data were plotted against pH (Figure 30), and then fitted to the equations described in the methodology, depending on the behavior of the curve. Results obtained from the curve fitting described different pK_a values (Table 4)

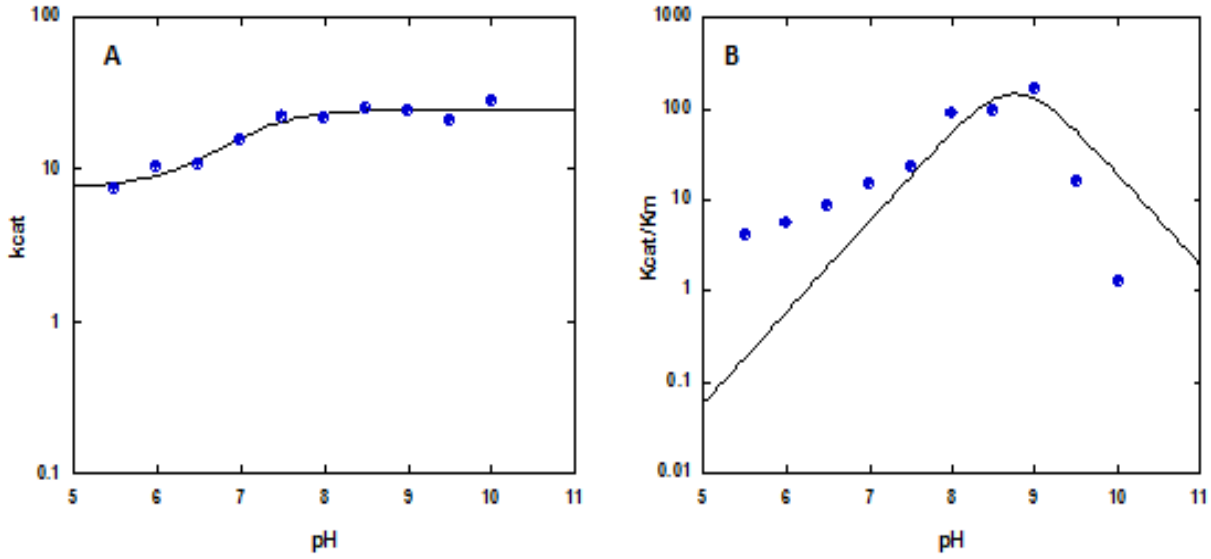


Figure 30. Effect of pH on AfSidA activity using L-Lysine. The rate of oxygen consumption was measured in the presence of different L-Lysine concentrations, saturating concentration of NADH (1mM) and reaction initiated with 2µM of enzyme. A. Effect of the pH on the k_{cat} value. B. Effect of the pH on the k_{cat}/K_M value.

Once the results were plotted, the different curves were fitted according the behavior displayed, using the equations shown in the methodology. A summary of the different pK_as obtained is represented in Table 4.

Table 4. pKa values for the different pH profiles determined for *N5*-ornithine hydroxylase (*Af*SidA)

Substrate	**Coenzime**	**Parameter**	**pKa**	**pKb**	**Equation**
L-Ornithine	NADH	k_{cat}	6.7 ± 0.4		1
		k_{cat}/K_M	8.1 ± 0.5	8.5 ± 0.5	2
L-Lysine	NADH	k_{cat}	7.5 ± 1.8		1
		k_{cat}/K_M	8.2 ± 2.3	9.3 ± 2.3	2
None	NADH	k_{cat}	6.7 ± 0.2	9.7 ± 0.2	2

6. DISCUSSION

The flavin dependent monooxygenases (FMOs) are a group of enzymes capable of catalyzing epoxidations, Baeyer-Villiger oxidations, and hydroxilations (Mayfield *et al*, 2010). Among the members of this family, the least studied are the N-hydroxilating monooxygenases (NMOs), which play an essential role in siderophore-mediated iron trafficking in many human pathogens.

AfSidA is a fungal flavoprotein monooxygenase that catalyzes the hydroxylation of the amino acid L-ornithine in order to obtain N^5-hydroxyornithine, which is an essential step in the biosynthesis of siderophores in *Aspergillus fumigatus*. The pH dependence of catalysis in this enzyme has not been yet study, which is why, is important the development of pH profiles to analyze and elucidate ionizations that control the enzyme activity that may permit posterior inhibitors studies and drug development.

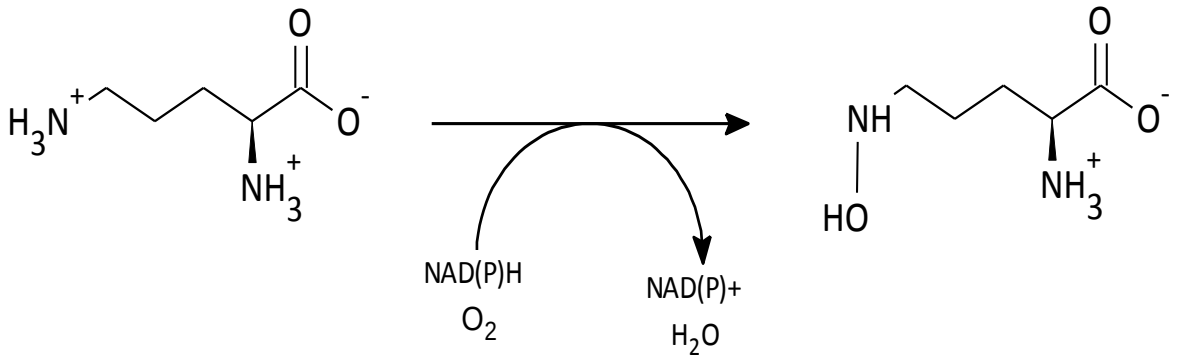
In general words, the enzymes are protein catalysts that speed up the rate of chemical reaction, without being used up in the process. They achieve their effect by temporarily binding to the substrate and, in doing so, lowering the activation energy needed to convert it to a product. The rate at which an enzyme works is influence by several factors, like the concentration of substrate molecules, because the more of them available, the quicker the enzyme molecules collide and bind with them.

The present of inhibitors is another factor, for example Chocklett and Sobrado (2010) observed that NADPH in concentrations above 1 mM may affect the enzyme *AfSidA* activity presenting uncoupling effect. And finally, other important aspect is the pH, because the conformation of a protein is influenced by pH and as enzyme activity is crucially dependent on its formation, its activity is likewise affected (Aehele, 2007).

The study of the rate at which an enzyme works is called enzyme kinetics and the best assay to do this type of evaluation is by initial rate experiments, in which an enzyme is mixed with large excess of the substrate, the enzyme-substrate intermediate builds up in a fast initial transient. Then the reaction achieves a steady-state kinetics in which enzyme-substrate intermediates remains approximately constant over time and the reaction rate changes relatively slowly. Rates are measured for a short period after the attainment of the quasi-steady state, typically by monitoring the accumulation of product or oxygen consumption with time.

The *AfSidA* active site is very specific for substrate, this is observed in Chocklett *et al* (2010), where they tried different compounds like L-arginine, L-cysteine, histamine and tryptamine, and for all these amino acids the oxygen consumption rate was not stimulated and lacked of the formation of the hydroxylated product. One possible cause can be the peptide groups and the side-chain amines are required for catalysis and presumably are important for substrate positioning and binding, so by shortening of the side-chain length resulted in no significant activity; otherwise, if the side-chain is extended by one methylene group (L-lysine) a significant NADH oxidation without the formation of the hydroxylated product is the result, indicating that the reaction was uncoupled. Meneely *et al* (2007) reported the same results of active site specificity of enzyme *PvdA* which is the ornithine hydroxylase of *Pseudomonas aeruginosa*.

As told before, the AfSidA enzyme permits the catalysis from L-ornithine to the hydroxylated form of the amino acid. The enzyme is described as a flavindependent monooxygenase (FMO) is shown that requires NADPH for activity (van Berkel *et al,* 2006) (Figure 31).



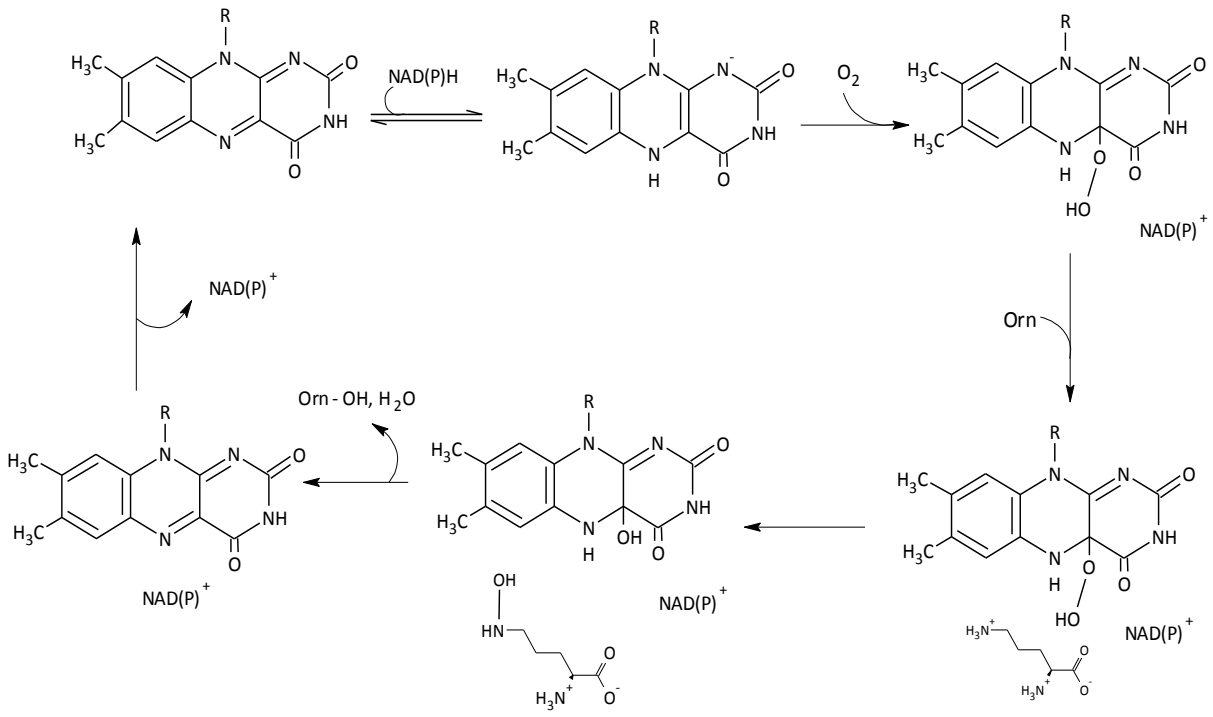
Source: Mayfield *et al*, 2010

Figure 31. Scheme of the reaction catalyzed by AfSidA, in which the substrate L-ornithine is hydroxilated by the flavin dependent enzyme.

It has been demonstrated the mechanism of this type of enzymes follows an ordered sequential cycle, in which the addition of O_2 and NAD(P)H precede the interaction of the substrate of the enzyme, liberating the oxygenated form of the substrate before releasing $NAD(P)^+$ or water (Figure 32).

The addition of substrate and posterior binding permits a rapid turnover by the enzyme, immediately forming the intermediate C4a-hydroperoxyxyflavin, which according to Ziegler (2002) the formation of this compound justifies the early liberation of the hydroxylated form of the substrate, because the last one is not important for the rest of the reaction.

The last product released is the $NAD(P)^+$, because it has been demonstrated that is a competitive inhibitor, more than NAD(P)H, so it stays glued to the flavin during the catalysis, being liberated just before returning to the reduce state of the flavin.



Source. Chocklett & Sobrado, 2011

Figure 32. General mechanism of oxygenation reactions catalyzed by flavoprotein monooxygenases, in this case N^5-ornithine hydroxylase from *Aspergillus fumigatus.*

6.1. AfSidA Expression and Purification.

The expression of the protein was carried out using *E. coli* strain BL21TI^R (DE3) and the plasmid pET-15b. The BL21 (DE3) bacteria are convenient for protein expression, by using the T7 promoter. The denomination DE3 specifies that the host is a lysogen of λ prophage DE3, and therefore carries a chromosomal copy of the T7 RNA polymerase gene under the control of the *lac*UV5 promoter, which carries the *lacI* gene in its chromosome, and this gene represses the expression of the T7 RNA polymerase is inducible by isopropyl-β-D-thiogalactoside (IPTG); and when added the IPTG induce the expression of the T7 RNA polymerase which, in turn, will transcribe the gene of interest.

A disadvantage of the use of IPTG for the induction is that this inducer is really expensive and toxic to human, so it is inappropriate for the industrial production of therapeutic proteins (Hyung-Kwon *et al*, 2004), so other option to induce the expression is by using an auto-induction solution, like 30*X*80155, that consists in 24% glycerol, 4.5% glucose (dextrose) and 15% lactose. The glycerol works as a late energy source, also supports growth about as well as glucose and does not prevent induction by lactose; the glucose functions as an early energy source and as a repressor, that prevent the uptake of lactose by lactose permease, the product of lacY. When glucose is depleted, lactose can be taken up by a small amount of lacY present in uninduced cells and converted to allolactose, the natural inducer, by b-galactosidase, the product of lacZ (Studier, 2005). On the other hand, the pET-15b plasmid used in the expression of the protein carries and N-terminal His-tag sequence, followed by a thrombin site and three cloning sites, also it has a region of ampicillin resistance, important in processes of purification and expression of proteins.

The cells were pre-inoculated in Luria-Bertani medium, which is the most common liquid medium used to grow bacteria and it is an excellent medium because is very efficient at stimulating growth and is suitable for many different organisms. It is considered a rich medium, because it contains peptides, peptones, vitamins and trace elements necessary to the proliferation of the microorganism. The cells were cultivated overnight in LB medium, and later were inoculated in Terrific Broth (TB), which supports a high cell density and, in the case of *E.coli*, maintains growth in the logarithmic phase for a long time.

After harvesting the cells, and lysed them, the purification was performed, by using the histidine tags present in the protein, this tags are widely used because they are very small and rarely obstruct with the function, activity, or structure of target proteins. For purifying the protein, the immobilized metal ion affinity chromatography (IMAC) technique was use, because is the most common method for cleansing histidine-tagged proteins. The IMAC chromatography media is charged with divalent metal ions, like nickel (Ni), which selectively retains histidine-tagged proteins and allow for the purification of insoluble histidine-tagged proteins from contaminants like inclusion bodies, cellular debris and other unwanted proteins.

The desired protein is retain in the Ni from the column, until imidazole is used to eluted the protein and finally permitting a successful purification that gives high yields of pure and active target protein. However, since many proteins have intrinsic histidine and/or cysteine amino acid residues, other nonspecific proteins bind to the IMAC media together with the target protein. To solve this particular problem it is necessary to optimize binding, wash, and elution conditions by varying the concentration of imidazole in these solutions. Increasing the concentration of imidazole in the binding and wash buffers generally decreases nonspecific binding, whereas lower concentrations give stronger affinity interaction. At the end, soluble active fractions of enzyme were obtained, with around 50% of FAD incorporated (Fig 5.B)

6.2. *Buffer System Selection.*

An exhaustive research was done, to select a series of possible buffers to realize the pH profile of the enzyme. The buffers selected are described in Table 1. Each of these buffers were tried by doing a measurement of oxygen consumption, using a constant concentration of substrate (15mM), NADH (1mM) and enzyme (2µM). Each quantification was performed in the overlapping region between buffers, and then by comparing the different results obtained. The control used was the rates obtained with Buffer Potassium phosphate at pH 7.5.

According to the results reported (Table 1), the buffers selected were MES, PIPES, HEPES, CHES, CAPS and Potassium phosphate, but after several assays, the list was reduced to MES, CHES and Potassium phosphate. The buffers TRIS-HCl, Tricine and Borate were not selected, first the TRIS-HCl buffer presented the lowest rates among all buffers, this because the presence of the chloride. One possibility is that the chloride may damage the enzyme, which is really delicate; also is has been reported that chloride can react with the oxygen present in the solution, lowering the total concentration of the molecule, impeding a good measure.

In the case of the tricine buffer, it gave a first good result, reporting consumption similar to the rest of the buffer, but after doing some other test using lower concentrations of substrate, the result kept being the same, which can be caused by a similarity of the structure of the buffer to the substrate. The buffer tricine comes from the union of TRIS and a derivate of glycine, which is an amino acid with similarities with the L-ornithine. Finally, the buffer borate reported consumption, but lower than the rest, possibly because the buffer reacted with the enzyme disrupting the system.

6.3. *pH dependence of k_{cat}*

The importance of developing pH profiles relies in that the enzymes are very sensitive to their environmental conditions, so much so that the rate of the reaction will vary depending on the conditions presented. For example, at extremes pHs, the native structure of the enzyme will be compromised, and the molecule will become inactive. Each enzyme has an optimal pH that favors the native conformation for maximum activity.

The pH effect results because of critical amino acids at the active site of the enzyme that participate in substrate binding and catalysis. The ionic or electric charge on the active site amino acids can enhance and stabilize interactions with the substrate. In addition, the ability of the substrate and enzyme to donate or receive an H is affected by pH.

According to the above, it was important to make sure that the enzyme was stable in the selected buffer system, so the stability was quantified. The measures were done for five hours of exposure of the enzyme in the different buffers. The results were plotted (Fig. 7) and it can be seen that the exposure of the enzyme in the buffer over time did not affect the activity, because the consumption of oxygen did not decreased.

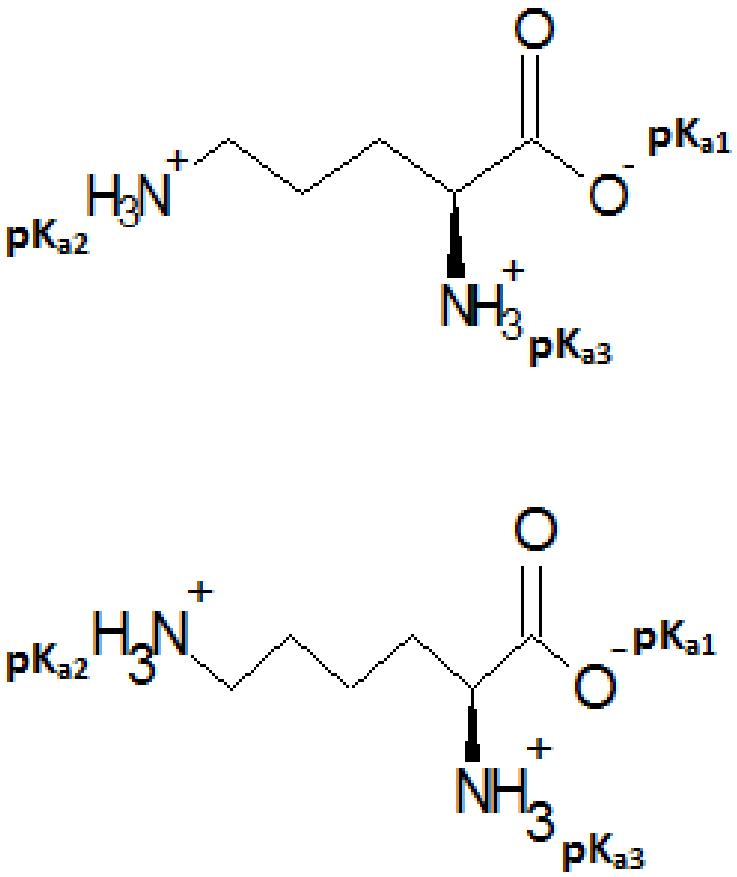
6.4. *Enzyme Stability Assays*

The k_{cat} is the catalytic constant of an enzyme, also referred to as the turnover number and represents the number of reactions catalysed per unit time by each active site. k_{cat} gives a direct measure of the catalytic production of product under optimum conditions (saturated enzyme). The units of k_{cat} are seconds^{-1} and the reciprocal of k_{cat} can be thought of as the time required by an enzyme molecule to "turn over" one substrate molecule. Alternatively, k_{cat} measures the number of substrate molecules turned over per enzyme molecule per second.

The pH dependence of the catalytic activity (k_{cat} vs pH) was determined using oxygen consumption in a pH range from 5.5 – 10.0 using a buffer system composed of potassium phosphate and a series of noncoordinating sulfonic acid buffers with overlapping pH ranges to allow for adjustments in catalytic rates due to buffer-dependent effects.

Figures 19 A and 30 A, show V_{max} versus pH data for the buffer system mentioned, the first one with L-ornithine as substrate and the second one with L-lysine. It is evident that the profiles are very similar, in both cases obtaining one pK_a value. The L-ornithine profile shows a pK_a value of 6.7 and the L-lysine profile, pK_a is 7.5. In both cases, the pK_a values represent the formation of active species, which can be connected to the dissociation constants of the substrates or the formation of an active specie during the reaction of the flavin with the NAD(P)H (Fig. 32).

According the literature, both amino acids have three pk_as (Fig. 33), pK_{a1}, is the lowest (2.18 and 1.71 in lysine and ornithine respectively), correspond to the carboxyl group. In case of L-ornithine pK_{a2} and pK_{a3} reported are 8.95 and 10.53; and in L-lysine 8.69 and 10.76, and in both cases, are related to the amine groups of the amino acids. According to the information above, the pKas reported in both profiles can be linked to the deprotonation of the amine group of both amino acids (Fig. 34).



Source. Kuo *et al*, 1985.

Figure 33. Scheme of the different pKa values presented in the amino acids used as substrate for the present analysis. The first one correspond to L-ornithine and the second one to L-lysine.

6.5. *pH dependence of k_{cat}/K_M*

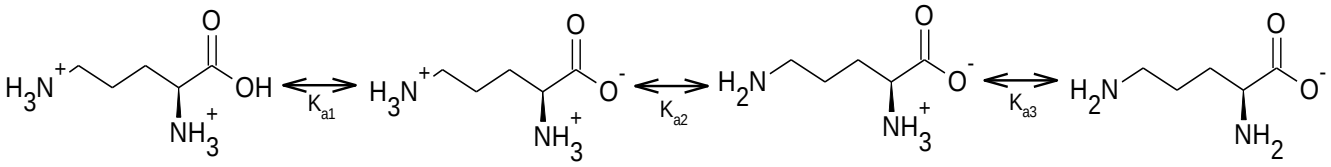
The K_M is defined as the concentration of reactant that gives half the maximum rate at saturating concentrations of all other reactants and with products maintained at zero concentration. For all the reactions that obey the kinetics described by Michaelis-Menten, K_M is a measure of the substrate concentration required for effective catalysis to occur. That is, an enzyme with a high K_M requires a higher substrate concentration to achieve a given reaction velocity than an enzyme with a low K_M. Units are given as concentration per time and most conveniently as millimolar per min.

On the other hand, the ratio k_{cat}/K_M is frequently described as a measure of the efficiency of the enzyme. This measure of efficiency is useful in determining whether the rate is limited by the creation of product or the amount of substrate in the environment. If the rate of efficiency is high, k_{cat} is much larger than K_M, thus the enzyme complex turns more enzyme-substrate into product than the enzyme encounters substrate; and if the k_{cat}/K_M ratio is low, the rate of product turnover is much lower than the substrate concentration, thus the enzyme and substrate have high affinity for each other.

In the case of the K_M, some problems happened, because the K_M values in some curves were too low, thing that make harder the determination of these values. For example, in both cases the basic pHs from 8.0 to 9.0 the K_M values were to low (Tables 2 and 3), making difficult the measurement of points below them (Figs. 16 and 27).

According to the information presented and the values of k_{cat}/K_M obtained, using both substrates, the K_M value is higher at pHs from 8.0 to 9.5, which means that in these pHs, the enzyme requires higher concentration of the substrate to accomplish a certain reaction velocity; counter to acid pHs (5.5-6.5) where the value is to low, what means that to attain the reaction velocity there is no need of using lots of substrate.

The concave-down pH profiles of apparent k_{cat}/K_M values for both profiles (Figs. 19B and 30B) indicate that two ionizable groups are involved not only on the formation of the enzyme-substrate complex, but also in the catalysis. The formation of the complex and the catalysis require the correct ionization states of the groups. The data fitting gave two basic dissociation constants for the enzymatic reaction with both substrates.



Source. Kuo *et al*, 1985.

Figure 34. The successive dissociation steps of ornithine. The values K_a1, K_a2 and K_a3 are the molecular dissociation constants of the L-ornithine.

The pH profile shows an increase to a broad maximum between pH 5.5 and 8 and the declines, becoming essentially inactive above pH 10. The profile implies that a deprotonation event with pK of 8.0 leads to the activation of the enzyme while further protonation step with pK value of 9.0 convert the active species to a less active state (Table 4).

The values 8.0 (L-orn) and 8.2 (L-lys) can be attributed to the dissociation that occur when the NAD(P)H reacts with the flavin of the enzyme, making it active (Figs. 32). And the higher pK_as can be to the dissociation constants of the substrates (Fig. 33 and 34) or to the formation of a protonated intermediate C4a-peroxyflavin, which pK_a value is 8.4, according to Sheng *et al* (2001).

6.6. *NADH Control Profile*

To evaluated the presence of a possible enzyme coupling in the reaction, a NADH control assay was performed. Using a concentration of 1µM of NADH, without substrate, the profile was determined, shown to be bell-shaped, consistent with the involvement of two ionizable groups, one with an apparent pK_a value of 6.7 corresponding to a deprotonation and the other with an apparent pK_a value of 9.5, related to a protonation step (Table 4), values similar to the ones obtained in the profiles using the substrates.

Previous studies revealed that the C4a-peroxyflavin intermediate can be formed and stabilized in the presence of an organic substrate, Chocklett and Sobrado (2010), showed that the enzyme reacts with the NADPH in absence of L-ornithine, also Pazmiño et al (2007) reported that the *CHMO* (cyclohexanone monooxygenase) forms the intermediate without the presence of any organic substrate; **t**his indicates that the substrate is not required to bound to the oxidized enzyme before de coenzyme binding and the hydride transfer, permitting the reaction of the enzyme with the molecular oxygen and a slow formation of the intermediate.

According to the information above and the pKs obtained, the first one is related to a deprotonation, caused by the reduction of the flavin previous to the reaction with oxygen and the second one represents a protonation that is provoked by the formation and stabilization of an intermediate, the C4a-peroxyflavin (Fig. 32). Also, it was shown that there is not an uncoupling effect presented by NADH, this because the rates obtained were lower that the rates presented in the different saturation curves.

7. CONCLUSIONS

After performing the protocol of expression and purification of protein, the enzyme *Af SidA* was successfully obtained, by using the vector pET15B and strains of E. coli BL21TIR. Fractions of soluble active protein were isolated, with 50-60% of FAD incorporated.

The enzyme was active and stable in a series of sulfonic buffers (MES, CHES, CAPS, HEPES), but not in a buffers like TRIS-HCl or borate, that can demonstrate the sensibility of the enzyme to external agents like chloride and borate. After the analysis a stable buffer system was chosen to the previous pH assays, the system consisted in buffers MES-Potassium phosphate-CHES, providing a good buffer capacity for the pH range from 5.5 to 10.0. Also it was demonstrated that the enzyme was active using both substrates L-ornithine and L-Lysine.

Two pH profiles of the enzyme using the primary substrate, L-Ornithine, where obtained. The k_{cat} profiles showed one pK_a value of 6.7. The substrate ornithine possess a dissociation constant for one of its amine group of 8.9, this pK_a can be associated to the one obtained in the results. In the case of the profile k_{cat}/K_M, two dissociation constants are shown, one resulting from a deprotonation, that can be also linked to the one reported for ornithine's amine group; the second one evidencing a protonation, that can be highly associated with the development of a protonated intermediate.

In case of L-Lysine, it is well known that this amino acids does not form product, only present a consumption of oxygen, but looking the results obtained, the pK_as resultants are very similar to the ones obtained using ornithine as substrate. In both profiles (k_{cat} and k_{cat}/K_M) the pK_as obtained are related to same things as the L-ornithine profile constants.

Using concentrations of NADH of 1mM resulted in no enzyme-coupling effect, because the values reported in the profile of NADH, contrary to the literature, where concentrations higher than 1mM resulted in uncoupling effect. Also, the reported pKs in the profile are too similar to the ones obtained using the substartes, which means the formation of an acive specie of the enzyme and the formation of a protonated intermediate.

8. RECOMMENDATIONS

As told before, the information of this particular enzyme is very limited, so the present work is one of the first efforts in getting to know the enzyme and some other specific things that surround it, so the only recommendation that can be made is the performance of more studies, for example, realize pH profiles with NADH and NADPH, to see the behavior of the saturation curve by varying the concentration of these two coenzymes, in aerobic conditions. Also, redoing the same profiles presented in this work, but including pHs above 10 and lower than 5.5, to be sure that the enzyme behaves pH independent or not.

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10. ANNEXES

10.1. *Bioinformatic Analysis*

Annex 1. Complete gene and complete product sequence of L-ornithine N^5-monooxygenase (*sidA*) of *Aspergillus fumigatus*.

Gene>

CTTCACTCTGCAAGTTCCTGACTTGCTTCCACCTGGAAAAATTACCTCCCCTTTGCCTTCCCCGCCTCAG
TTTTGACATCCTCCCGCCTCAAGTATAGGTACCTAATCTTCTTTTCTTTCTTTCTTGTACGATCTGATCT
ATGGAATCTGTTGAACGGAAGTCAGAATCGAGCTACTTGGGTATGCGCAACATGCAGCCCGAGCAGCGTC
TTTCTTTGGATCCTCCCCGCTTGAGGTCAACACCCCAGGATGAGCTTCATGATCTTCTGTGTGTTGGGTT
TGGACCCGCTTCCCTGGCCATTGCCATTGCTTTGCATGACGCTCTGGACCCTCGATTGAACAAGTCCGCT
TCCAATATCCATGCACAGCCTAAGATCTGCTTCCTGGAGCGCCAGAAGCAATTTGCGTGGCACTCGGGTA
TGCTGGTCCCCGGTTCCAAGATGCAGATCTCCTTCATCAAGGATCTCGCAACTCTCCGGGACCCCCGCAG
CAGTTTTACTTTTCTCAACTACCTCCACCAGAAGGGCCGTCTGATTCACTTCACTAACCTCAGCACCTTC
CTGCCGGCTCGGCTGGAGTTCGAGGACTACATGCGTTGGTGTGCGCAACAATTTTCGGATGTAGTGGCTT
ACGGGGAAGAGGTGGTCGAAGTGATTCCCGGGAAGTCTGATCCCAGCAGCTCGGTGGTTGACTTCTTCAC
TGTTCGGTCGCGCAACGTTGAGACGGGCGAGATCAGTGCCAGGAGGACCCGCAAGGTCGTTATTGCAATC
GGAGGCACTGCAAAGATGCCATCCGGACTGCCCCAGGATCCCCGGATTATACACTCGTCCAAGTACTGCA
CAACGCTGCCGGCCCTGTTGAAGGACAAGTCGAAGCCTTACAACATTGCCGTTCTGGGCAGTGGTCAGAG
TGCTGCGGAGATTTTCCATGACCTTCAAAAGAGATATCCCAACTCACGAACAACGCTGATTATGCGGGAT
TCTGCAATGCGGCCCAGTGACGACTCGCCTTTGTGAGCAGAGAATCAAGTAATCCATATTCTGCTTTCCG
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GATTGAGGAGATCTATAACGACATGTACCTGCAGAGGGTGAAGAACCCTGACGAAACTCAGTGGCAGCAT
CGCATCCTTCCGGAACGCAAGATCACACGGGTCGAGCACCATGGTCCTCAGAGTCGAATGCGCATCCACC
TCAAGTCATCCAAGCCCGAGTCGGAAGGCGCAGCAAACGACGTCAAAGAAACGCTGGAAGTCGACGCTCT
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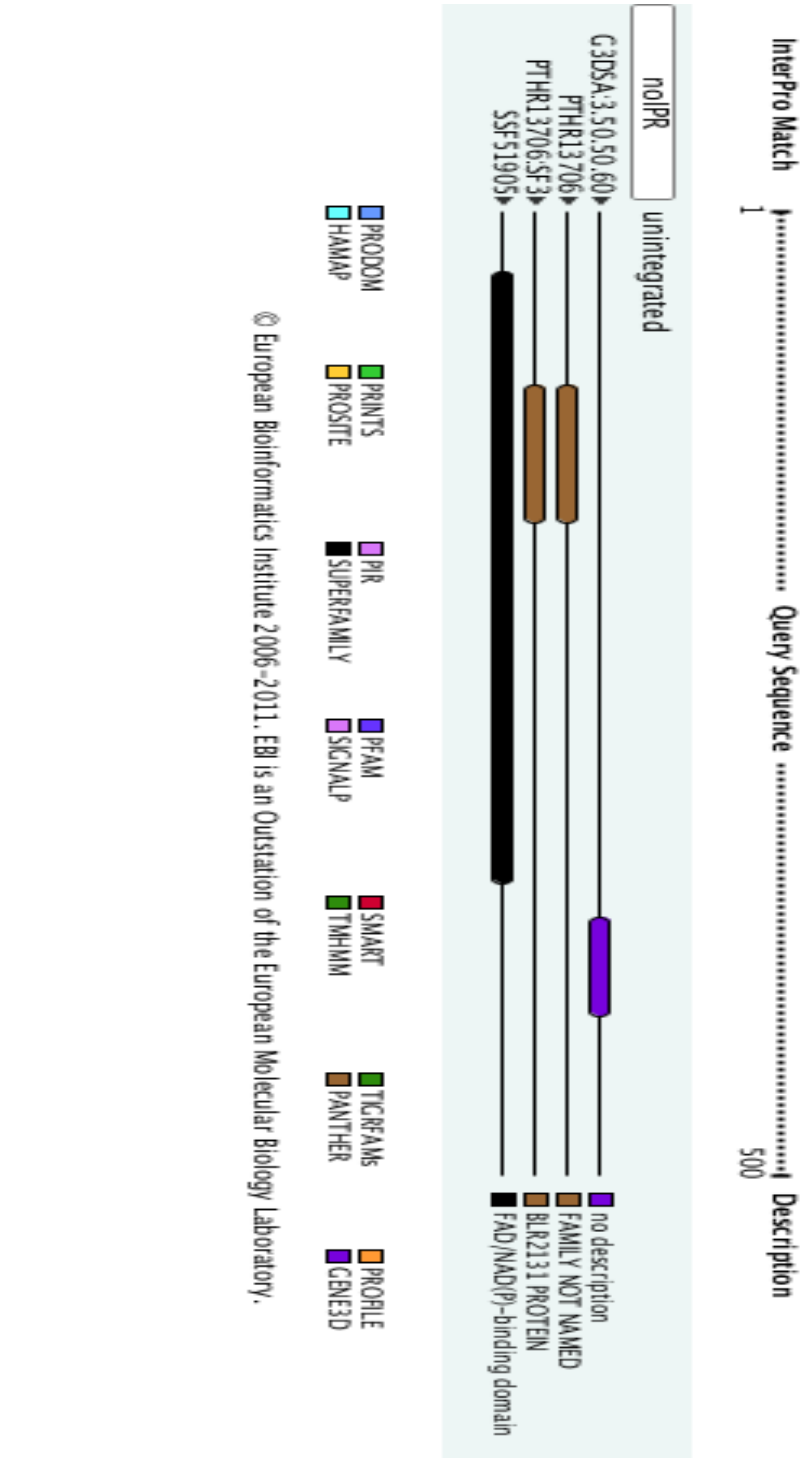
AAGCCGGCATCTGGCTTCAAGGTTGTAATGAGCGGACGCATGGCCTTAGTGACAGTTTGCTGTCGGTACT
GGCTGTTCGTGGTGGCGAGATGGTCCAATCGATTTTCGGAGAGCAGCTGGAAAGAGCGGCGGTACAGGGC
CACCAGCTACGAGCCATGCTGTAAGGGGCCCGAAAAAAGTGGAGGAAAAGCCGGATTGCATAGTTAGTTA
CTGTTTCAAGGCGTTGCGCTAAGCGAATGCCAAGGCCATTTCTCTGACAACACGATTGGAACTCCGGAAG
ACGGAGCAGACATGAGGGAACCTGTGAACACGATATCCGCCATTACTCTCCGACATTGATGGAATACCCA
TGTCGTGAATTTTTATTGATCTTCAATTGTGATTATTGACCATCATATACACTATTCTGGCGCAGTCAAA
AGATTTAGTTCATTCAGCGTTGT

Protein>

MESVERKSESSYLGMRNMQPEQRLSLDPPRLRSTPQDELHDLLCVGFGPASLAIAIALHDALDPRLNKSA
SNIHAQPKICFLERQKQFAWHSGMLVPGSKMQISFIKDLATLRDPRSSFTFLNYLHQKGRLIHFTNLSTF
LPARLEFEDYMRWCAQQFSDVVAYGEEVVEVIPGKSDPSSSVVDFFTVRSRNVETGEISARRTRKVVIAI
GGTAKMPSGLPQDPRIIHSSKYCTTLPALLKDKSKPYNIAVLGSGQSAAEIFHDLQKRYPNSRTTLIMRD
SAMRPSDDSPFVNEIFNPERVDKFYSQSAAERQRSLLADKATNYSVVRLELIEEIYNDMYLQRVKNPDET
QWQHRILPERKITRVEHHGPQSRMRIHLKSSKPESEGAANDVKETLEVDALMVATGYNRNAHERLLSKVQ
HLRPTGQDQWKPHRDYRVEMDPSKVSSEAGIWLQGCNERTHGLSDSLLSVLAVRGGEMVQSIFGEQLERA
AVQGHQLRAM

Annex 2. Domain studies of *AfSidA*



10.2. *Materials*

Annex 3. Materials and Equipment

- *E. coli* BL21TI^{R} DE3.
- pET15b plasmids
- LB Media
- TB Media
- Auto-induction Solutions
- Buffer A
- Buffer B
- Ampicillin
- Chloramphenicol
- DNase I
- RNase
- Lysozyme
- HisTrap Columns
- Bradford Reactive
- Liquid Nitrogen
- NADH
- NADPH
- L-Ornithine
- L-Lysine
- CHES
- MES
- CAPS
- HEPES
- PIPES
- MES
- TRIS
- Potassium Phosphate
- Borate

- Incubator Barnstead
- Incubator Innova 4230
- Autoclave AMSCO SCIENTIFIC SG-120
- Incubator Excella E25
- Sonicator
- Sovall RC-5B Refrigerated Centrifuge
- ÄKTAPrime Plus GE Healthcare
- AMICON 8200 – Milipore Concentrator
- Agilent 8453 Spectrophotometer
- Oxygraph Hansatech
- Fridges 4°C and -80°C

10.3. *Media and Reactive Recipes*

Annex 4. Media Recipes

Luria – Bertani Media

10.0 tryptone
10.0 g NaCl
5.0 yeast extract
Dilute to 1 L dH2O
Autoclave

Terrific Broth Media

12.0 g tryptone
24.0 g yeast extract
900 mL H2O
Autoclave

Annex 5. Reactives Recipies

Ampicillin (AMP) Stocks

Per 30 ml,

Add 3 g of AMP to 30 ml MQ H2O.
Manually mix.
Filter sterilize into large microfuge tube.
Pipet 250 ul for small tube stock
Pipet 1000 ul for large tube stock.
Store in freezer.

Cloramphenicol

Per 30 ml,

Add 1.02 g of CAM to 30 ml of EtOH.
Mix and dispense.

RNase and DNase Stocks:

Add 0.050 g RNase or DNase to 5 ml 10 Mm TRIS buffer (pH 8.2).
Dissolve in small beaker.
Pipet into 100 ul aliquots, in small tubes.
Store in freezer.

Lysozyme Stock:

Add 0.2 g Lysozyme into 20 ml 10 Mm TRIS Buffer
Dissolve in small beaker.
Pipet into 100 ul aliquots, in small tubes.
Store in freezer.

***Buffer A* 10X**

Reactive	Concentration	Quantity
HEPES	250 mM	65.070 g
NaCl	3 M	175.32 g
Imidazole	20 mM	13.616 g

For the preparation of Buffer A 1X, 200 ml from buffer A were diluted in 1800 ml of distilled water.

Buffer B

Reactive	Concentration	Quantity
HEPES	250 mM	65.070 g
NaCl	3 M	175.32 g
Imidazole	300 mM	20.424 g

Autoinduction Solution

Reactive	Quantity
MgSO4, 1M	2.4 mL
30X80155 Sol.	40 ml
Succinic Acid 15%, pH 6.2	30 ml
K Phosphate Buffer	100 ml

30*X*80155 Solution

In 1 L,

Reactive	Quantity
Glycerol	240 mL
Glucose (Dextrose)	4.5 g
Lactose	150 g

Annex 6. Buffers Recipes

Potassium Phosphate Buffer (100 Mm Phosphate, 200 ml)

- **0.2 M potassium phosphate, mono-potassium salt**: Dissolve 27.2 g KH2PO4 (MW=136.09) in 500 ml H2O and adjust to 1 liter with H2O.

- **0.2 M potassium phosphate, di-potassium salt**: Dissolve 34.8 g K2HPO4 (MW=174.18) in 500 ml H2O and adjust to 1 liter with H2O.

The table below gives the volumes in ml of these solutions and H2O that should be mixed together to obtain a 200 m solution of 100 mM K Phosphate with specific desired pH.

pH Table for Phosphate buffers*:

Desired pH	Mono-Salt	Di-Salt	H2O
6.0	87.7	12.3	100
6.5	68.5	31.5	100
7.0	39.0	61.0	100
7.5	16.0	84.0	100

*It is strongly recommended to check the final pH with a pH meter.

HEPES Buffer (100 mM)

pH	HEPES	NaCl	H_2O
7.5	11.915 g	3.095 g	450 ml
8.0	11.915 g	2.321 g	450 ml
8.5	11.915 g	1.864 g	450 ml

Final Volume: 500 ml c/u

CHES Buffer (100 mM)

pH	CHES	NaCl	H_2O
8.5	10.365 g	4.092 g	450 ml
9.0	10.365 g	3.502 g	450 ml
9.5	10.365 g	2.683 g	450 ml
10.0	10.365 g	2.05 g	450 ml

Final Volume: 500 ml c/u

CAPS Buffer (100 mM)

pH	CAPS	NaCl	H_2O
9.5	11.065 g	4.161 g	450 ml
10.0	11.065 g	3.648 g	450 ml

Final Volume: 500 ml c/u

MES Buffer (100 mM)

pH	MES	NaCl	H_2O
6.5	1.952 g	0.478 g	90 ml

Final Volume: 100 ml c/u

PIPES Buffer (100 mM)

pH	CAPS	NaCl	H_2O
6.5	3.024 g	----	90 ml

Final Volume: 100 ml

10.4. *General Information*

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Nombre del Proyecto: *"*pH effects on the reaction catalyzed by *Aspergillus fumigatus* Siderophore A (*Af*SidA)*"*

Profesor Asesor: MSc. Alejandro Hernández Soto

Horario de trabajo del estudiante: L-V 9am – 7pm

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