Ph.D. Thesis

On the Topic

"Studies on enzymes from *Leptospira interrogans* with special emphasis to Triosephosphate isomerase and putative L-amino acid oxidase"

Submitted to

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Under the Faculty of Allied Health and Basic Sciences

by

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Under the Guidance of

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Vaigundan D.

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List of Abbreviations used

TPI	-	Triosephosphate Isomerase
Li	-	*Leptospira interrogans*
LiTPI	-	*Leptospira interrogans* Triosephosphate Isomerase
Li-LAO	-	*Leptospira interrogans* full-length putative L-amino acid oxidase
Li-rLAO	-	*Leptospira interrogans* putative L-amino acid oxidase without signal sequence
EMJH	-	Ellinghausen, McCullough, Johnson and Harris Medium.
IPTG	-	Isopropyl β-d-1-thiogalactopyranoside
BSA	-	Bovine serum albumin
DTT	-	Dithiothreitol
EDTA	-	Ethylenediaminetetraacetic acid
PMSF	-	phenylmethylsulfonyl fluoride
EtBr	-	Ethidium bromide
bp	-	base pair
kb	-	kilobase
Da	-	dalton
kDa	-	kilodaltons
FAD	-	flavin adenine dinucleotide
$FADH_2$	-	flavin adenine dinucleotide reduced form
NADH	-	Nicotinamide adenine dinucleotide reduced form
NADPH form	-	Nicotinamide adenine dinucleotide phosphate reduced
FMN	-	Flavin mononucleotide
$KHCO_3$	-	Potassium bicarbonate
NaCl	-	Sodium chloride
$MgCl_2$	-	magnesium(II)chloride (anhydrous)
$MgCl_2 .6H_2O$	-	Magnesium Chloride, Hexahydrate
$ZnSO_4.7H_2O$	-	Zinc sulfate heptahydrate
$FeSO_4.7H_2O$	-	Ferrous sulfate heptahydrate
$CoCl_2\,2H_2O$	-	Cobalt(II) Chlorate Dihydrate
$CuCl_2$	-	Cupric chloride
$MnCl_2$	-	Manganese(II) chloride

$CaCl_2.2H_2O$	-	Calcium Chloride, Dihydrate
KH_2PO_4	-	Potassium phosphate monobasic
$Na_2HPO_4.\ 2H_2O$	-	Sodium phosphate dibasic dihydrate
$Na_2MoO_4.2H_2O$	-	Sodium molybdate
Na_2S	-	Sodium sulfide
PEG	-	Polyethylene glycol
m/z	-	mass to charge ratio
inten.	-	Intensity
mM	-	millimolar
µM	-	micromolar
mL	-	milliliter
mg	-	milligram
µL	-	microliter
OPD	-	o-Phenylenediamine
H_2SO_4	-	Sulfuric acid
Tris	-	2-Amino-2-hydroxymethyl-propane-1, 3-diol
HEPES	-	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
KI	-	Potassium iodide
Tris. HCl	-	2-Amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride
Gms	-	grams
LC-MS	-	Liquid chromatography–mass spectrometry
MS/MS	-	Tandem Mass Spectrometry
GnHCl	-	Guanidine Hydrochloride
SDS	-	Sodium Dodecyl sulfate
dNTPs	-	deoxy trinucleotides

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Abstract

Leptospirosis is an infectious disease that is prevalent in many parts of rural India. The definitive diagnosis of Leptospiral infections is limited by the very slow growth of the bacterium in urine culture from infected patients. The availability of PCR-based diagnosis is also limited. The work described in this thesis was undertaken to improve the methods of in-vitro growth of Leptospiral cultures and to investigate specific enzymes in Leptospira in order to provide a better understanding of the biochemistry of the organism. The availability of the full genome sequence for both pathogenic and non-pathogenic leptospira provides an opportunity to begin a systematic exploration of the metabolic enzymes of this organism. The work presented in this thesis will highlight studies to establish a new and better growth medium and to present the biochemical characterization of two Leptospiral enzymes - Triosephosphate isomerase and the putative L-amino acid oxidase. Before detailing the experimental work carried out, a brief introduction to leptospirosis and the causative organism is described in the first chapter.

Leptospira species are slow growers requiring a minimum of eight days for growth and positive identification. Their average generation time is about 12-18 hours. The second chapter describes various media available in the literature and the significance of the need for improved media for the growth of leptospira, before describing the various experimental attempts made to improve the growth of leptospires.

The third chapter describes the cloning, expression, purification, and biochemical characterization of Leptospiral Triosephosphate Isomerase (TPI). A brief account of Leptospiral metabolism precedes the presentation of the experiments. The fourth chapter describes the attempt that was made to clone and express the full-length *L. interrogans* LAO gene. The fifth chapter describes the biophysical and crystallographic characterization of the recombinant LAO that lacks N-terminus secretory sequences.

Chapter 1

Introduction

(Leptospira and Leptospirosis)

1.1 Introduction

Leptospirosis is an infectious disease which is prevalent in many parts of rural India. The definitive diagnosis of Leptospiral infections is limited by the very slow growth of the bacterium in urine culture from infected patients. The availability of PCR-based diagnosis is also limited. The work described in this thesis was undertaken to improve the methods of in-vitro growth of Leptospiral cultures and to investigate specific enzymes in Leptospira in order to provide a better understanding of the biochemistry of the organism. The availability of the full genome sequence for both pathogenic and non-pathogenic leptospira provides an opportunity to begin a systematic exploration of the metabolic enzymes of this organism. The work presented in this thesis will highlight studies to establish a new and better growth medium and to present the biochemical characterization of two Leptospiral enzymes - Triosephosphate isomerase and the putative L-amino acid oxidase. Before detailing the experimental work carried out, a brief introduction to leptospirosis and the causative organism is described in this chapter.

1.2 Leptospira

Weil described leptospirosis as severe jaundice in 1886; Weil's disease was characterised by renal failure and severe haemorrhage. Inada and Ido first identified Leptospires as the causative agent of leptospirosis in 1914 (Kobayashi Y., 2001). Leptospires are obligate aerobic motile tightly coiled spiral shape bacteria measuring 0.1 μm diameter and 6 - 20 μm length, with a helical amplitude of 0.1 to 0.15 μm, and the wavelength is ≈ 0.5 μm (Swain RHA. *et al.,* 1957; Faine S. *et al.,* 1999; Levett PN. 2001). These organisms have a double membrane structure in which the cytoplasmic membrane and peptidoglycan cell wall are closely associated and are overlaid by an outer membrane (Haake DA. *et al.,* 2001; Cullen PA. *et al.,* 2004). The ends of the bacteria are hooked. The bacterial mobility is by two flagella present in the periplasmic space (Trueba GA. *et al.,* 1992; Levett PN. 2001; Alder B. *et al.,* 2010). They grow in simple media enriched with vitamins B_1, B_{12}, long-chain fatty acids, and ammonium salts with an optimum temperature of 30 °C (Johnson RC. *et al.,* 1984).

1.3 Classification

The genus Leptospira is classified along with other Spirochaetes namely *Borrelia burgdorferi*, *Treponema pallidum,* and Brachyspira, Hyodysenteriae order Spirochaetales under a separate family Leptospiraceae. The *Leptospira* genus was divided into two species, *L. interrogans*, comprising all pathogenic strains, and *L. biflexa*, containing the saprophytic strains. Both *L. interrogans* and *L. biflexa* are divided into more than 230 serovars that are defined by agglutination after cross-absorption with homologous antigen (Dikken H. *et al.,* 1978; Kmety E. *et al.,* 1993*;* Levett PN., 2001).

In 1989 Leptospires were classified based on DNA-DNA hybridisation and the two previously described species (*L. interrogans* and *L. biflexa*) into 22 distinct genomospecies. The genus Leptospira is composed of 10 pathogenic species, 5 potentially pathogenic species (also called intermediate), and 7 saprophytic species. Though the genomospecies classification is correct it is not useful for clinical microbiologists; as both pathogenic and nonpathogenic serovars occur within the same species. It is incompatible with the serogroups classification system which has served clinicians and epidemiologists for many years (Levett PN., 2001; Alder B. *et al.,* 2010).

1.4 Genome of Leptospira

Leptospires are phylogenetically related to other spirochetes (Paster BJ. *et al.,* 1991). The genome of Leptospira is approximately 5,000 kb in size. The genome is comprised of two chromosomes, a 4,400-kb chromosome and a smaller 350-kb chromosome (Zuerner RL. *et al.,* 1991). Other plasmids have not been reported (Levett PN., 2001; Alder B. *et al.,* 2010).

The *Leptospira* genome is characterized by a G + C content of 35–41mol%, depending on species. The Leptospira Genome Project (2011) has generated significant whole genome information of at least 17 Leptospira species representing 8 pathogenic, 4 intermediate, and 5 saprophytic serovars (Ghazaei C. *et al.,* 2017). Leptospira encodes approximately 3654-3723 proteins (Mehrotra P. *et al.,* 2017), Out of these 3000 and odd proteins approximately about 600 coding sequences (CDS) are

unique to pathogenic Leptospira (Picardeau M. *et al.*, 2008). After the availability of genome sequences, many studies explored the cause of pathogenesis implicating various pathways, and genes that are essential for pathogenesis (Fouts DE. et al., 2016; Mehrotra P. *et al.,* 2017; Ghazaei C. 2018).

1.5 Habitat and Epidemiology

Leptospirosis has a worldwide distribution. All mammals appear to be susceptible to Leptospirosis. Wild and domestic animals are the primary hosts of the organism. Rodents are the major reservoir of infection. Humans are accidental hosts. The disease is transmitted via direct or indirect contact with urine containing virulent Leptospira species. Leptospira species do not multiply outside the host. In the environment, they can remain viable for a few too many weeks or months in contaminated soil and several weeks in cattle slurry. They can remain viable in water for several months. Farmers, veterinarians, sewer workers, septic tank cleaners, canal workers, miners are the occupational risk group to Leptospirosis. The highest risk is also associated with dairy farming as a major occupational risk factor throughout the world. The incidence of human infection is higher in the tropics (warm and humid) regions. Outbreaks have been related to heavy rainfall in various parts of the world. Incidence rates are underestimated due to the lack of awareness of the disease (WHO report 1999; Plank R. *et al.,* 2000; Bharti J. *et al.,* 2003).

Since the 1980s the disease has been reported from various states, during the monsoon months in mini epidemic proportions. Cases have been reported from Kerala, Tamil Nadu, Gujarat, Andamans, Karnataka, Maharashtra, Orissa, and Bihar. The true incidence of human leptospirosis in India is not known; however, there have been several studies examining rates. During periods of flooding, leptospirosis may cause severe outbreaks among individuals exposed to Leptospiral-contaminated waters. In addition, during harvest times, there can be outbreaks of leptospirosis due to increased contact with infected rat populations (Vijayachari P. *et al.,* 2008; Rao R. *et al.,* 2003).

1.6 Pathogenesis

The entry of Leptospires into the body is through small cuts, abrasions, via mucous membranes like conjunctiva, or wet skin. They circulate in the blood-stream, with the bacteremic phase lasting for up to 7 days. The molecular basis for virulence and mechanism by which *Leptospira sp.* cause the disease remain unknown, mainly due to the absence of genetic tools for the manipulation of *Leptospira (*Adler B. *et al.*, 2010).

1.7 Pathology

The clinical presentation of leptospirosis is biphasic with the acute or septicemic phase lasting about a week, followed by antibody production phase and excretion of the organism in the urine. Localization of Leptospires within the tissues during the immune phase represents the severe complications of leptospirosis (Levett PN., 2001; Adler B. *et al.*, 2010).

1.8 Anicteric Leptospirosis

The symptoms of leptospirosis are broad spectrum. The majority of infections caused by leptospires are either subclinical or of mild severity. Most of the cases present with a febrile illness of sudden onset. Other symptoms include chills, headache, myalgia affecting the lower back, thighs, and calves (often intense), abdominal pain, conjunctival suffusion, and less often a skin rash. The anicteric syndrome generally lasts for a week. The primary lesion of Leptospirosis is damaged endothelium of small blood vessels leading to localised ischemia in organs, resulting in renal tubular necrosis, hepatocellular and pulmonary damage, meningitis, myositis, and placentitis. Hemorrhages occur in severe cases as do jaundice, and frequently, platelet deficiency. There is usually mild granulocytosis and splenomegaly. Once circulating antibodies appear, leptospires are removed from the circulation and tissues by opsonophagocytosis. Tissue damage, even though it is severe, may be reversible and followed by complete repair. The headache is often severe, resembling that of dengue, with retro-orbital pain and photophobia (Kelley PW. 1998; Levett PN. 2001; Adler B. *et al.,* 2010). In anicteric disease, the erythrocyte sedimentation rate is elevated, and the white cell is also moderately elevated. Liver function tests of leptospirosis patients were shown to have slight elevation in aminotransferases,

bilirubin, and alkaline phosphatase in the absence of jaundice. Urinalysis shows proteinuria, pyuria, and often microscopic hematuria (Levett PN. 2001; Adler B. *et al.,* 2010).

1.9 Icteric Leptospirosis

Weil's disease represents the most severe presentation of Leptospirosis. The severe form of leptospirosis is characterized by the involvement of multiple organs. Leptospirosis is a common cause of acute renal failure (ARF). Conjunctival suffusion in the presence of scleral icterus is said to be pathogenomonic of Weil's disease. Serum amylase levels are raised significantly in association with ARF, but clinical symptoms of pancreatitis not present. In severe leptospirosis, a peripheral leukocytosis occurs with a shift to the left, whereas in dengue, atypical lymphocytes are commonly observed. Thrombocytopenia (platelet count of <100 × 109/liter) occurs in ≥50% of cases and is a significant predictor for the development of ARF. However, thrombocytopenia in leptospirosis is transient and does not result from disseminated intravascular coagulation. Renal function impairment is indicated by raised plasma creatinine levels. The degree of azotemia varies with the severity of the illness. In icteric leptospirosis, liver function tests generally show a significant rise in bilirubin, with lesser increases in transaminases and marginal increases in alkaline phosphatase levels. The increase in bilirubin and serum creatinine phosphokinase levels are generally out of proportion to the other liver function test values, in patients with ARF (Levett PN. 2001; Adler B. *et al.,* 2010).

1.10 Disease Treatment and Re-emergence of Leptospirosis

The clinical manifestations of leptospirosis are too variable and nonspecific to be diagnostically useful. Leptospirosis is diagnosed by using the Microscopic Agglutination Test (MAT). It is then confirmed by the isolation of the organisms. The disease can be treated efficiently with penicillin derivative antibiotics. Currently, leptospirosis is recognized as a globally re-emerging disease with a markedly increased number of cases and frequent outbreaks in South East Asia (Thailand, India, Malaysia, and Indonesia) and Latin America (Levett PN. 2001; Adler B. *et al.,* 2010).

1.11 Leptospiral proteins

Only about 22 classes of proteins have been studied from Leptospira sp. despite the clinical significance. These studies are listed in Table 1.1.

Table 1.1. Studies on Leptospiral proteins.

Protein	Outcome	Ref
ponA, pbpB	Characterized penicillin-binding proteins	Brenot A. *et al.*, 2001
CbiC	Crystal structure of putative precorrin isomerase CbiC determined	Xue Y. *et al.*, 2006
FNRs	Crystal structure of Ferredoxin-NADP(H) reductases (FNRs) determined. Enzymatic activity was shown to be similar to that of the plastidic enzymes and significantly different from the bacterial flavoenzymes.	Nascimento AS. *et al.*, 2007
Homoserine O-acetyltransferase	Crystal structure of Homoserine O-acetyltransferase determined at 2.2Å resolution using selenomethionyl single-wavelength anomalous diffraction method.	Wang M. *et al.*, 2007
SAM-dependent O-methyl transferase	Crystal structure of S-adenosylmethionine (SAM)-dependent O-methyltransferase in complex with S-adenosylhomocysteine was solved.	Hou X. *et al.*, 2007
LipL32	Demonstrated that fibronectin probably binds to LipL32 and postulates that Ca^{2+} binding to LipL32 might be important for Leptospira to interact with the host cell. Evaluated the LipL32 mutants' ability to interact with Ca^{2+} and with ECM glycoproteins.	Hauk P. *et al.*, 2009
Citramalate synthase	Demonstrated that the binding of isoleucine affects the binding of the substrate and coenzyme at the active site of citramalate synthase.	Zhang P. *et al.*, 2009
OmpA70	Demonstrated immunogenic and antigenic properties of that OmpA70, a putative outer membrane protein.	Fraga TR. *et al.*, 2010
LIC12922	Determined the crystal structure of a conserved hypothetical protein LIC12922 and postulated that LIC12922 is a periplasmic chaperone involved in OMPs biogenesis	Giuseppe PO. *et al.*, 2011
GroEL	Cloned and sequenced the gene encoding the immunodominant protein GroEL	Natarajaseenivasan K. *et al.*, 2011
Glucokinase	Identified ROK family of glucokinase in *Leptospira interrogans*	Zhang Q. *et al.*, 2011
LenA	Demonstrated that leptospiral endostatin-like	Verma A. *et*

	protein A (LenA) binds human plasminogen in a dose-dependent manner	*al.*, 2010
PLA	Hypothesized role of PLA in facilitating host tissue penetration and evading the immune system.	Vieira ML. *et al.*, 2012
Lsa30	Demonstrated that the role of Lsa30 in adhesion to the host and overcoming tissue barriers of host and escape the immune system.	Souza NM. *et al.*, 2012
Sph2	Demonstrate experimentally that Sph2 is an Mg (++)-dependent hemolysin with both sphingomyelinase and haemolytic activities	Narayanavari SA. *et al.*, 2012
Enolase	Demonstrated *L. interrogans* enolase has evolved to play a role in pathogen interaction with host molecules, which may contribute to the pathogenesis of leptospirosis.	Nogueira SV. *et al.*, 2013
LexA genes, recA, recN, dinP,	Demonstrated that *Leptospira interrogans* can tackle DNA damage due to environmental assaults.	Fonseca LS. *et al.*, 2013
CadD	Cloned CadD and demonstrated that it deaminates Camp.	Goble AM. *et al.*, 2013
LIC11360, LIC11009, and LIC11975	Characterized LIC11360, LIC11009, and LIC11975 and demonstrated that three proteins interact with laminin in a dose-dependent and saturable manner.	Siqueira GH. *et al.*, 2013
LruA	Identified the LruA gene product as a surface-exposed leptospiral virulence factor that contributes to leptospiral pathogenesis.	Zhang K. *et al.*, 2013
Proteases	Cloned and expressed 12 putative leptospiral proteases and showed that the elastinolytic activity may be important for leptospires-host interaction	Hashimoto VL. *et al.*, 2013
czcA	Identified czcA and its four subunit vaccine peptides would be ideal T-cell driven efficacious vaccine against leptospirosis	Umamaheswari *et al.*, 2012

A Memoranda on "Research needs in leptospirosis" published by Leptospira authoritarians in 1972 has suggested the following 16 different topics for research

(1) The ultrastructure of leptospires and the chemical nature of the various structural components;

(2) The immunological characteristics of antigenic components of leptospires;

(3) The development of culture media;

(4) The cross-protection afforded by natural infections and by vaccines;

(5) Immunoglobulin classes of leptospiral antibodies in man and various animals;

(6) The lysis of leptospires by factors in normal and immune sera;

(7) Metabolic processes, especially those of pathogenic strains;

(8) The lipolytic activities of strains;

(9) The genetics of leptospires;

(10) Improvements in immunofluorescence techniques and materials;

(11) The development and evaluation of serological tests, including screening tests for use with sera from domestic livestock and other animals;

(12) The roles of leptospiral and host factors in the pathogenesis and manifestations of leptospiral infection;

(13) The epidemiology of leptospirosis;

(14) The development of surveillance programmes;

(15) The preparation and assay of vaccines; and

(16) The effectiveness of new antibiotics.

Because of the organism's unusual metabolism and slow growth, even after 4 decades of these proposals, studies on Leptospira need enormous attention. Keeping the view of the above points, a modest attempt has been made to study Leptospira, with the following objectives

Objectives of the study

1) To test the effect of substrates for known membrane transport systems on the growth properties of *Leptospira interrogans.*

2) To clone, express, purify and analyze the kinetics of *L. interrogans* Triosephosphate isomerase.

3) To clone, express, purify and analyze the kinetics and substrate specificity of *L. interrogans* L-amino acid oxidase

4) To compare the kinetics and the substrate specificities of Triosephosphate isomerase and L- amino acid oxidase of *L. interrogans* with the respective human homologues.

Chapter 2

Improved growth medium of Leptospira

2.1 Introduction

Leptospira species are slow growers requiring a minimum of eight days for growth and positive identification. Their average generation time is about 12-18 hours (Staneck JL. *et al.,* 1973; Levett PN. 2001; Alder B. *et al.,* 2010). This chapter describes various media available in the literature and the significance of the need for improved media for the growth of leptospira, before describing the various experimental attempts made to improve the growth of leptospires.

Studies on the nutritional requirement of leptospira date back to the early 1930s. Various media developed for the growth of leptospira in the early years are listed in Table 2.1, 2.2, and 2.3. Leptospira show accelerated growth in the presence of amino acids, aspartic acid, arginine, glycine, glutamic acid, lysine, and tryptophan (Staneck JL. *et al.,* 1973; Greene MR., 1945; Grehardt MR. *et al.,* 1959). It shows reduced growth by the addition of arginine, glycine, and methionine (Johnson RC. *et al.,* 1963). It can grow on a protein-free medium containing inorganic salts, fatty acids, and vitamins (Fulton JD. *et al.,* 1956; Shenberg E. 1967). Bovine serum albumin, Tween 80, polysorbates, sodium acetate, beef extract, vitamin B_{12}, thiamine, ammonium chloride, and sodium pyruvate promote the growth of the nutritionally fastidious stereotypes of Leptospira (Stalheim OH. 1966; Johnson RC. *et al.,* 1973; Ellinghausen HC. *et al.,* 1976; Gonzalez Rodriguez A. *et al.,* 2006; Chideroli RT. *et al.,* 2017). The growth characteristics of the leptospira in various media are compared by (Ellinghausen HC. Jr. 1960). Leptospira species can be cultivated in leptospira Vanaporn Wuthiekanun (LVW) medium by using a Noble agar base supplemented with 10% rabbit serum, and an initial incubation at 30°C in 5% CO_2 for 2 days prior to continuous culture in the air at 30°C and it takes at least 5-8 days to confirm the presence of the organism (Wuthiekanun V. *et al.,* 2013).

The true incidence figures of human leptospirosis in India are not available. The risk of leptospirosis is aggravated and widespread during rain in spring and summer. During periods of flooding, Leptospirosis may cause severe outbreaks among individuals exposed to leptospiral-contaminated water. In addition, during harvest times, there can be outbreaks of leptospirosis due to increased contact with infected rat populations and their urine.

Table 2.1. Composition of Fletcher's Medium for growth of Leptospira

Fletcher's Media composition	Gms / Litre
Sodium Chloride	0.5
Peptone	0.3
Beef Extract	0.2
Rabbit Serum	80.0ml
Agar	1.5
Final pH 7.9 +/- 0.1 at 25°C	
Ingredients per litre of deionized water	
920ml of base media 80 ml sterile Leptospira Enrichment	

Table 2.2. Composition of Modified Korthof Medium for growth of Leptospira

Modified Korthof Media composition	
Ingredients	Gms / Litre
Peptic digest of animal tissue	0.800
Sodium chloride	1.400
Sodium bicarbonate	0.020
Potassium chloride	0.040
Calcium chloride	0.040
Monopotassium hydrogen phosphate	0.240
Disodium hydrogen phosphate	0.880
Final pH (at 25°C) 7.2±0.2	
100 ml sterile base, add sterile 8 ml inactivated blood serum 0.8 ml sterile hemoglobin solution	

Table 2.3. Composition of Stuart Leptospira Medium for growth of Leptospira

Stuart Leptospira Media	Gms / Litre
Sodium Chloride	1.93
Disodium hydrogen orthophosphate	0.66
Ammonium chloride	0.34
$MgCl_2 . 6H_2O$	0.19
Asparagine	0.13
KH_2PO_4	0.08
Glycerol	5.0ml
Distilled water	1000ml
Final pH (at 25°C) 7.2±0.2	
Autoclave and cool	
To 900ml media, 100ml of rabbit serum (inactivated at 56^0 C for 30 minutes) is added aseptically.	

It is estimated that 20% of all rats carry leptospira that can infect humans; this carrier rate may vary depending on the geographic area (Pappas G. *et al.,* 2008; Vijayachari P. *et al.,* 2008; Fischer RSB. *et al.,* 2017; Sambasiva RR. *et al.,* 2003). One of the important challenges in the diagnostics of leptospirosis disease is the culturing of the organism takes at least 5-8 days to confirm the presence of the organism.

Currently, the disease is diagnosed by Microscopic Agglutination Test (MAT), which is unreliable and not recommended. It is therefore necessary often to confirm the diagnosis by isolation of the organisms from blood and urine (Levett PN. 2001; Alder B. *et al.,* 2010). Many studies that use various advanced methods to detect leptospira like LigB-LAMP assay with pre-addition of dye, qRT-PCR, detection of leptospira using tapered optical fiber sensor, by latex agglutination test coated with recombinant LipL32 antigen, and real-time loop-mediated isothermal amplification (RealAmp) (Ali SA. *et al.,* 2017; Zainuddin NH. *et al.,* 2018; Esteves LM. *et al.,* 2018; Thongsukkaeng K. *et al.,* 2018; Monica NI. *et al.,* 2019).

Recent genomic studies of Leptospira provide a comprehensive analysis of the genes encoding regulatory system, signal transduction, and methyl-accepting chemotaxis proteins, reflecting the organism's ability to respond to diverse environmental stimuli (Ren SX. *et al.,* 2003; Jorge S. *et al.,* 2018; Nascimento AL. *et al.,* 2004a, 2004b). Genomic sequence analysis also reveals the presence of a competent transport system with 13 families of genes encoding for major transporters which are necessary for the long-term survival of the organism (Nascimento AL. *et al.,* 2004b). The proteomics studies provide insights into the mode of pathogenesis, host-pathogen interaction, and immune response by the host (Nally JE. *et al.,* 2011). In this study, various media were tested for the growth of Leptospira by incorporating combinations of nutrients for which transporters are present in the organism and the nutrients for which enzymes are differentially regulated in pathogen and the host reservoir, based on the genomic and proteomic information, respectively (Caspi R. *et al.,* 2016; Nally JE. *et al.,* 2011), mode of infection, and the host environment.

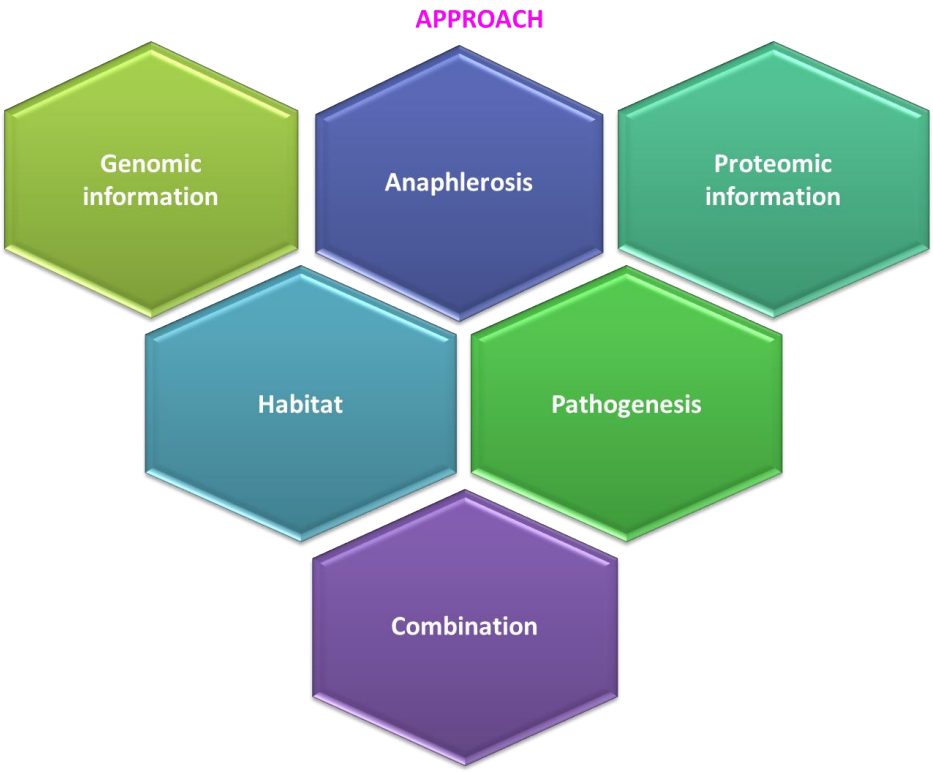


Figure 2.1 Approaches undertaken in the present studies.

As the outcome of this study is based on the wealth of information gained from these genomic and proteomics studies; knowledge of the mode of infection and the host environment (Fig. 2.1), we report M12 growth medium for the isolation and culture of Leptospira that shows visible growth in 40 h.

2.2 Materials

Di-sodium hydrogen phosphate dihydrate, ammonium chloride, glycerol, potassium dihydrogen phosphate, potassium chloride purchased from SDFCL, magnesium chloride, sodium acetate purchased from AMERSCO. Aspartic acid, sodium pyruvate, tryptone type-1, yeast extract, thiamine hydrochloride, ferrous sulfate heptahydrate, zinc sulfate heptahydrate, sodium chloride, bovine serum albumin, polysorbate-80, vitamin B_{12}, fetal calf serum, asparagine, leptospira Basal medium, leptospira enrichment medium are purchased from Hi-Media Laboratories Pvt. Ltd. Sodium bicarbonate was purchased from Sigma; glucose, and calcium chloride dihydrate purchased from Merck. N-acetyl glutamine was purchased from the National Biochemical Corporation.

2.3 Methods

2.3.1 Nutrient selection based on Genomic and Proteomics information

The presence of transporters in leptospira was analyzed from the Metacyc database (Caspi R. *et al.,* 2016) and the KEGG database. Accordingly, nutrients were chosen if there is a transporter that can take up nutrients into the cell (Table 2.2). From the proteomics studies available in the literature, the nutrients were chosen for which the host metabolic enzymes are downregulated during host-pathogen interaction.

2.3.2 Cultivation of Leptospira based on Genomic and Proteomic information

Stuart medium was prepared as per standard protocol; the modified Stuart medium was prepared by substituting aspartate instead of asparagine and excluding glycerol. The pH of both media was adjusted to 7.2 and autoclaved. To the cooled media, filter-sterilized 10% fetal bovine serum was added, and 4 mL of each medium was distributed in various 15-mL capped polypropylene tubes. The 100 mmol/L stocks of each amino acid (ornithine, lysine, arginine, and histidine), carbon source (100 mmol/L stock of sodium acetate and 100 mmol/L stock of fructose), and 100 mmol/L stock of *N*-acetyl aspartate were prepared separately, with the pH adjusted to 7.2, and filter-sterilized. Both the media were supplemented in various combinations, as follows: 1) histidine, arginine, lysine, and ornithine (amino acids); 2) fructose and acetate (carbon sources); 3) *N*-acetyl aspartate; 4) amino acids and *N*-acetyl aspartate; 5) carbon sources and N-acetyl aspartate; 6) amino acids and carbon sources; 7) amino acids, carbon sources, and *N*-acetyl aspartate; and 8) without any supplementation. Each combination amino acid consists of 100 µL of each amino acid (ornithine, lysine, arginine, and histidine); the combination of carbon sources consists of 100 µL of each of sodium acetate and fructose. Ellinghausen-McCullough-Johnson-Harris (EMJH) medium was prepared as per the manufacturer's instruction and used as a standard control. Apart from standard control, the EMJH medium was also supplemented with the individual nutrients and inoculated with pathogenic serovar. All the tubes were inoculated with either an equal quantity (approximately 10^4 Leptospires/mL) of the pathogenic serovar (*L. interrogans* serovar Pomona) or nonpathogenic serovar (*L. biflexa* serovar Patoc). Each of the media without inoculum

was kept as contamination control. The inoculated tubes along with the controls were incubated at 30 °C for 4 days. After the fourth day of inoculation, each of these tubes was analyzed under a Darkfield microscope and quantified with the standard counting (using a Petroff-Hausser counting chamber) protocol.

2.3.3 Secreted Proteins of Leptospira

Leptospira species were grown for the week in Modified Stuart Media containing Fetal Calf Serum. Spin down at 14000 rpm for 25 minutes at 20^0C. To the pellet, Modified Stuart Media containing 100mM different of nutrients was added. After incubating at 30^0C for 4 hours, cells were taken in different time points and cell lysate is made by adding Laemmle lysis buffer, and 10% SDS-PAGE was performed.

2.3.4 Growth Monitoring by Alamar Blue assay

In order to monitor the cell proliferation, the leptospires were grown in different media with a combination of nutrients for three days and on the third day, 100µL of leptospires were incubated with the Alamar Blue reagent (1mg of resazurin in 1mL of phosphate-buffered saline). After incubating with Alamar blue at 30°C for 3 hours, the Alamar blue reduced by the cells was measured by absorbance readings at 540nm (Page B. *et al.*, 1993).

2.3.5 Growth of Leptospira based on Habitat

Considering the natural habitat, mode of infection, and organelle in which Leptospira harbours, various media were prepared individually one after the other (not using any basal medium) that consisted of varying concentrations of urea, oxalic acid, succinic acid, and salt concentrations as shown in each column of Table 2.3. Each medium (each column of Table 2.3) was prepared individually, pH adjusted, autoclaved, and tested one after the other against the EMJH medium as a standard control. The EMJH medium was prepared as per the manufacturer's instruction and used as a standard control for each medium that was tested. After cooling, 4 mL of each medium was dispensed into the sterile 15-mL capped polypropylene tubes. To each tube, 10 µL of 1 of the 9 different serovars of (4 days old, cultured in EMJH medium) Leptospira—1) *L. biflexa* serovar Patoc, 2) *L. interrogans* serovar Hardjo, 3)

L. interrogans serovar Lai, 4) *L. interrogans* serovar Canicola, 5) *L. borgpetersenii* serovar Poi, 6) *L. interrogans* serovar Icterohaemorragiae, 7) *L. interrogans* serovar Australis, 8) *L. interrogans* serovar Pomona, and 9) *L. interrogans* serovar Autmanalis—was inoculated in duplicates and incubated at 30 °C. The medium without inoculum was used as contamination control (in duplicates). After 4 days of inoculation, each of incubated tubes was examined for visible growth of leptospires and also analyzed under a Darkfield microscope in comparison with standard EMJH medium qualitatively.

2.3.6 Holistic Approach to Growth of Leptospira

With an all-inclusive approach based on the mode of infection, transporters present, and replenishing the anaplerotic gaps, various media components were used for the growth of *Leptospira* (labeled M5–M12, M12Q, and M14; Table 2.4, Table 2.5). Each medium (as shown in each column of Table 2.4, Table 2.5) was prepared individually, pH adjusted, autoclaved, and tested one after the other against the EMJH medium as a standard control. The EMJH medium was prepared as per the manufacturer's instruction and used as a standard control. After bringing the media to room temperature, in a laminar flow hood, 4 mL of each medium was distributed into the various sterile 15-mL capped polypropylene tubes. To each tube, 10 μL of 1 of the 9 different serovars of (4 days old, cultured in EMJH medium) *Leptospira*—1) *L. biflexa* serovar Patoc, 2) *L. interrogans* serovar Hardjo, 3) *L. interrogans* serovar Lai, 4) *L. interrogans* serovar Canicola, 5) *L. borgpetersenii* serovar Poi, 6) *L. interrogans* serovar Icterohaemorragiae, 7) *L. interrogans* serovar Australis, 8) *L. interrogans* serovar Pomona, 9) *L. interrogans* serovar Autmanalis—was inoculated in duplicates and incubated at 30 °C. The medium without inoculum was used as contamination control (in duplicates). After 4 days of inoculation, each of incubated tubes was examined for visible growth of leptospires and also analyzed under a Darkfield microscope in comparison with a standard EMJH medium qualitatively. The M8 medium was further optimized for carbon source, nitrogen source, and salts (M8–M12, Table 2. 4). In order to compare the growth of leptospires in M12, M12Q, M14, and EMJH media, these media were prepared simultaneously on the same day and examined, and the study was repeated several times (Table 2.5).

2.3.7 PCR based detection of Leptospira

After 40 hours of inoculation, 15µL of each of the active culture media was taken separately and heat-inactivated at 95^{o}C for 20 min. The growth of nine serovars of leptospira was detected by PCR, using pathogenic serovars specific primers FP 5'GCAAGCATTACCGCTTGTGG3', RP 5'TGTTGGGGAAATCATACGAAC3' (Branger C. *et al.,* 2005). Briefly, 5µL of the heat-inactivated sample was mixed with 15µL of PCR reaction reagent, carried out by cyclic incubation at 94^{o}C for 15sec, 56^{o}C for 35sec, and 72^{o}C for 40 sec for 45 cycles, after initial denaturation of 94^{o}C for 5min. The PCR products were analyzed using a 1.2 % Agarose gel electrophoresis.

2.3.8 Clinical mimetic experiment

In order to mimic the culturing of leptospires from clinical samples; 4mL of M12 media were inoculated with 10µL of one of the nine serovars of leptospires in the presence of 100µL of a freshly collected urine sample from a normal healthy male volunteer and presence or absence of 100µL of 5-fluorouracil (500mg/10mL stock concentration) for selection and visualized after 40 hours of incubation at 30^{o}C.

2.4 Results

Initially, the Stuart medium was modified by various nutrients for which the transporters are known from the genomic information (Table 2.2).

The proteomic studies on the protein expression pattern in both pathogen and the carrier rat reservoirs of the organism indicate that the leptospira differentially regulates some of the host-specific enzymes that are involved in various processes (Nally JE. *et al.,* 2011). From these considerations, a working hypothesis could be developed that leptospira requires aspartate or N-acetyl aspartate (Fig. 2.2).

After the fourth day of inoculation, each of these tubes was analyzed under a Darkfield microscope (Fig. 2.3) and observations were documented upon supplementation of nutrients based on its genomic and proteomic data (Table 2.4). The nonpathogenic (*L. biflexa* serovar Patoc) serovar inoculated in tubes containing

various combinations of nutrients which are added to both Stuart medium and modified Stuart medium, were analyzed under a Darkfield microscope (Fig. 2.3 panel a and b).

Table 2.4. List of transporters, those take up the substrate inside the leptospira (Metacyc Database).

Substrate uptake: Compounds transported into the cytosol	***L. interrogans* serovars Copenhageni str. 2006007831**
Compounds transported into the cytosol	*(RS)*-Malate
	Bile acid
	Fatty acid
	Heme
	Sugar
	Ammonia
	Amino acid
	Chloride
	D-glucose
	Glutamate
	Proton (H^+)
	Potassium ion (K^+)
	L-alanine
	Magnesium ion (Mg^{2+})
	Sodium ion (Na^+)
	Phosphate
	Sodium sulfate
	Sucrose
	Sulfate
	Thiosulfate

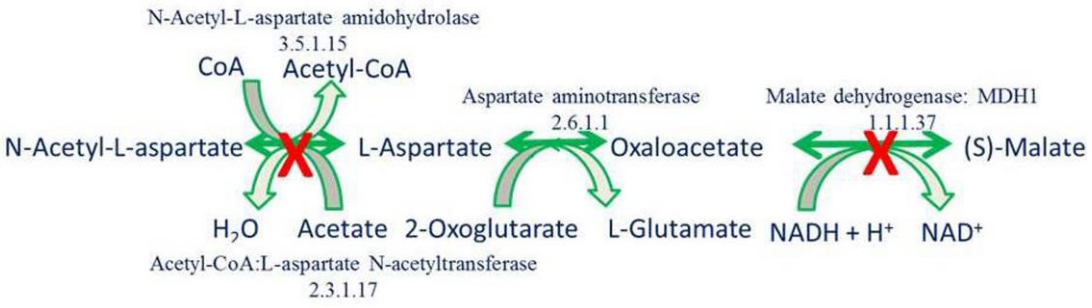


Figure 2.2. Host enzymes down-regulated in rats infected with leptospira

Likewise, the pathogenic (*L. interrogans* serovar Pomona) serovar containing tubes were also analyzed under a Darkfield microscope (Fig. 2.3 panel c and d). In comparison, the modified Stuart medium has given a better count, which is equivalent to that of the EMJH medium (Table 2.5). The EMJH medium was also modified to check the effect of individual components added (Fig. 2.3 panel e).

Table 2.5. Quantification of Non-pathogenic serovar (*L. biflexa* serovar Patoc) and Pathogenic serovar (*L. interrogans* or serovar Pomona) after the fourth day of inoculation; leptospira grown in EMJH medium, Stuart's medium, modified Stuart medium (Stuart medium devoid of glycerol and consisting of aspartate instead of asparagine); supplemented with a combination of nutrients.

Tube Name	Media	Strain inoculated	3rd day	4th day	
				count	observation
S1NP	Stuart	Non-pathogenic	-	450	
S2NP	Stuart	Non-pathogenic	-	500	
S3NP	Stuart	Non-pathogenic	-	450	
S4NP	Stuart	Non-pathogenic	-	200	
S5NP	Stuart	Non-pathogenic	-	450	Lengthy
S6NP	Stuart	Non-pathogenic	-	350	Agglutination
S7NP	Stuart	Non-pathogenic	-	615	
S8NP	Stuart	Non-pathogenic	-	240	
M1NP	Modified Stuart	Non-pathogenic	-	350	
M2NP	Modified Stuart	Non-pathogenic	-	350	Sluggish, uneven length
M3NP	Modified Stuart	Non-pathogenic	-	500	Agglutination
M4NP	Modified Stuart	Non-pathogenic	-	400	
M5NP	Modified Stuart	Non-pathogenic	-	550	
M6NP	Modified Stuart	Non-pathogenic	-	400	Agglutination
M7NP	Modified Stuart	Non-pathogenic		400	Agglutination
M8NP	Modified Stuart	Non-pathogenic	-	750	

S1P	Stuart	Pathogenic	80	80	
S2P	Stuart	Pathogenic	100	100	
S3P	Stuart	Pathogenic	110	100	
S4P	Stuart	Pathogenic	60	60	
S5P	Stuart	Pathogenic	160	180	Sluggish
S6P	Stuart	Pathogenic	80	200	Agglutination
S7P	Stuart	Pathogenic	90	100	Sluggish
S8P	Stuart	Pathogenic	120	120	
M1P	Modified Stuart	Pathogenic	80	80	
M2P	Modified Stuart	Pathogenic	200	200	
M3P	Modified Stuart	Pathogenic	180	200	
M4P	Modified Stuart	Pathogenic	110	100	
M5P	Modified Stuart	Pathogenic	210	200	Sluggish
M6P	Modified Stuart	Pathogenic	70	90	Sluggish
M7P	Modified Stuart	Pathogenic	180	100	Sluggish
M8P	Modified Stuart	Pathogenic	280	280	
EMJH His	EMJH	Pathogenic	-	100	
EMJH Ori	EMJH	Pathogenic	-	400	
EMJH Arg	EMJH	pathogenic	-	300	
EMJH Lys	EMJH	pathogenic	240	-	No growth
EMJH acetate	EMJH	Pathogenic	-	400	
EMJH N-acetyl-Asp	EMJH	Pathogenic	200	350	
EMJH fructose	EMJH	pathogenic	-	450	
EMJH	EMJH	pathogenic	280	430	
EMJH	EMJH	Non-pathogenic	-	800	

In these experiments, we observed slight morphological changes, reduction in motility, and aggregation or clumping (Table 2.3). The growth of leptospira upon supplementation to Stuart media or modified Stuart media was not convincing.

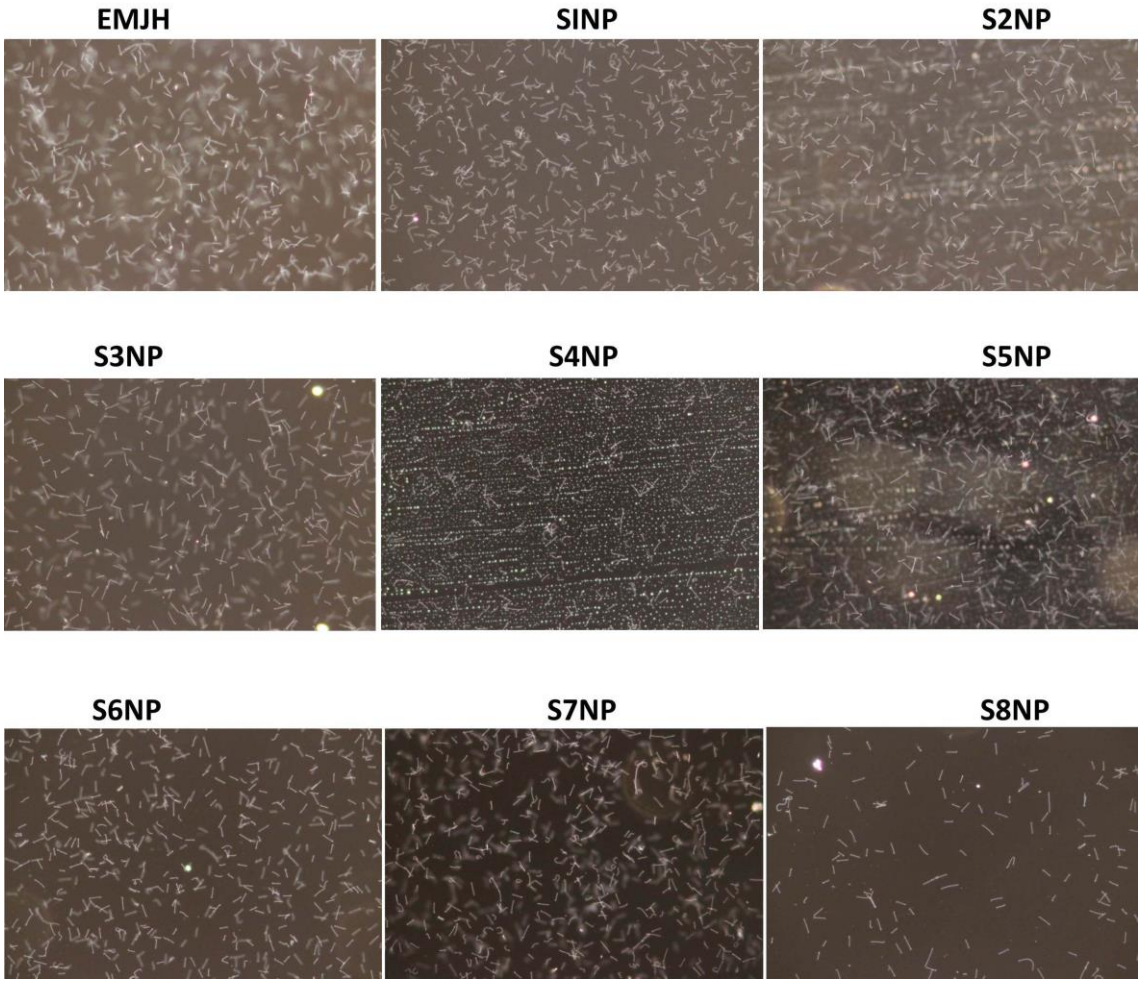


Figure 2.3a. Darkfield microscopy of fourth-day culture of *L. biflexa* serovar Patoc, Non-pathogenic (NP) organism inoculated in Stuart's (S) medium supplemented with various combinations of nutrients.

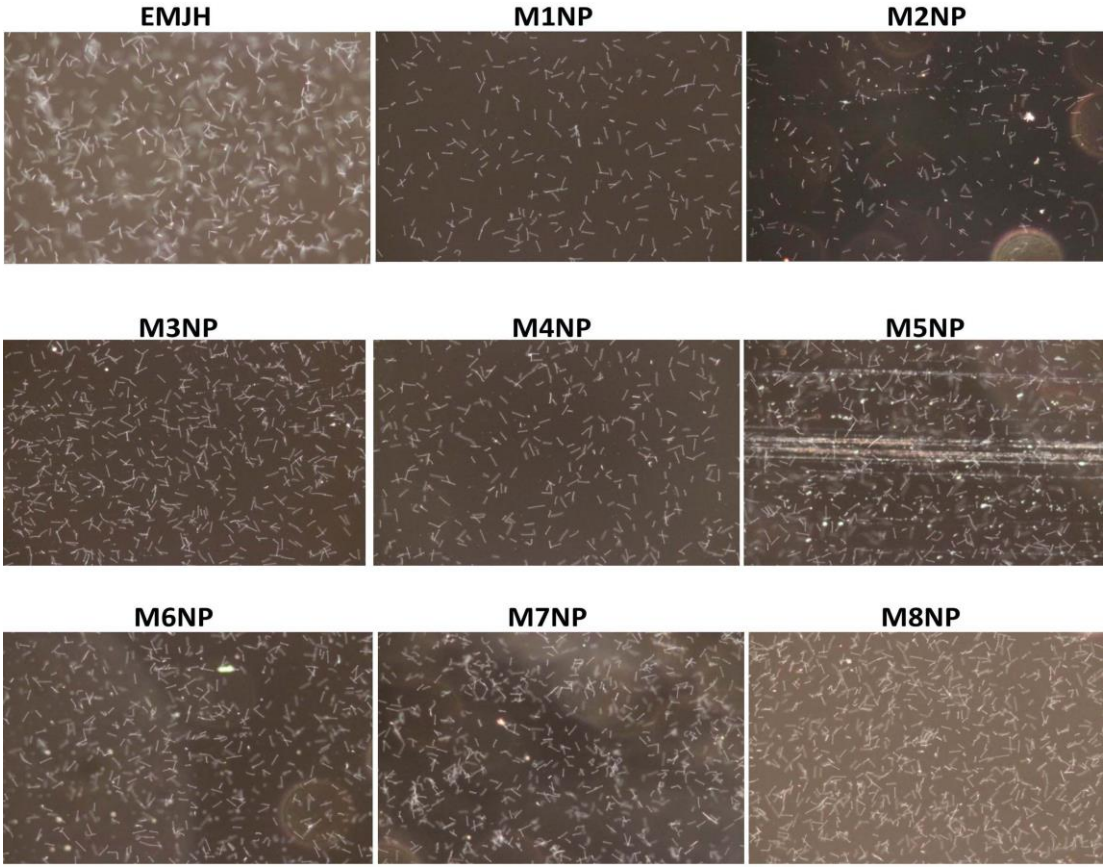


Figure 2.3b. Darkfield microscopy of fourth-day culture of *L. biflexa* serovar Patoc, Non-pathogenic (NP) organism inoculated in Modified (M) Stuart medium supplemented with various combinations of nutrients.

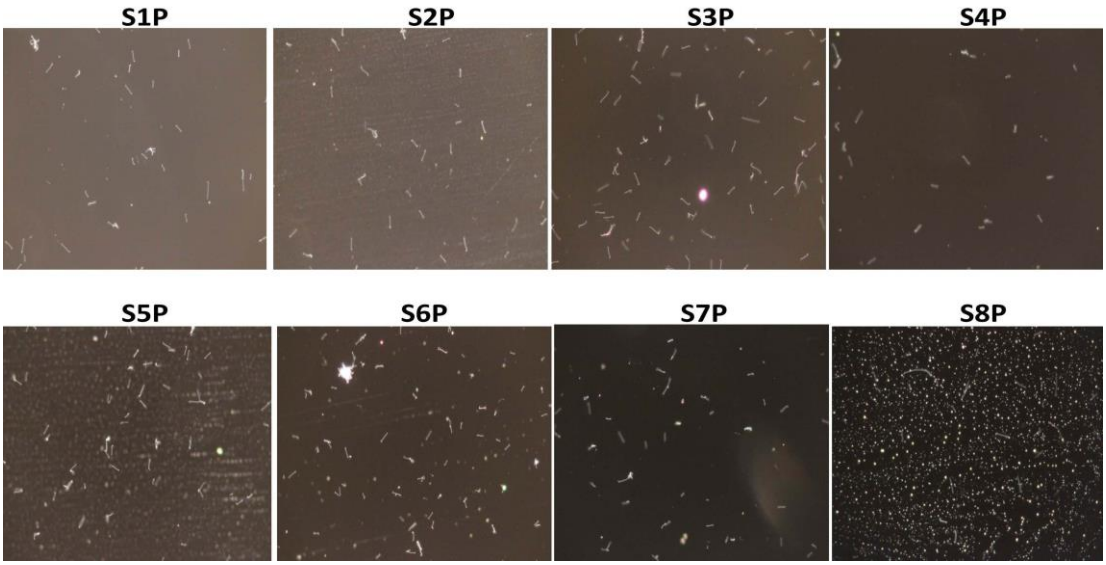


Figure 2.3c. Darkfield microscopy of fourth-day culture of *L. interrogans* serovar Pomona, Pathogenic (P) organism inoculated in Stuart's (S) medium supplemented with various combinations of nutrients.

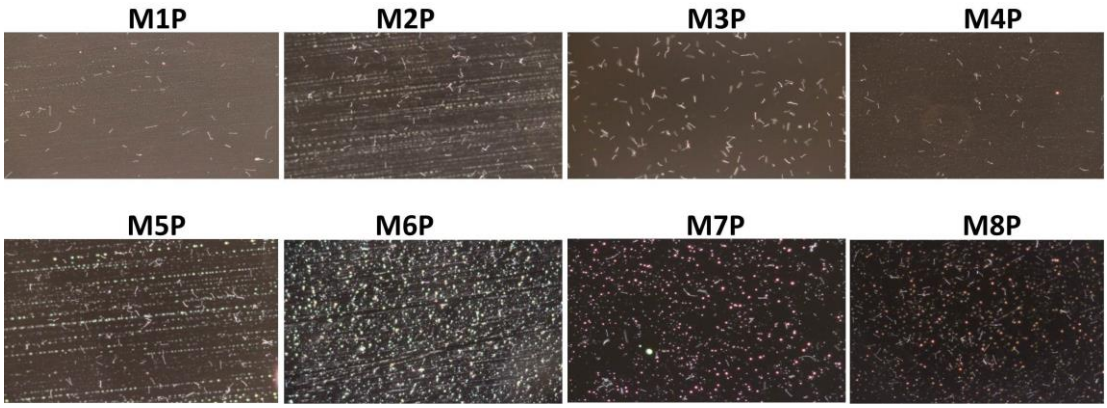


Figure 2.3d. Darkfield microscopy of fourth-day culture of *L. interrogans* serovar Pomona, Pathogenic (P) organism inoculated in Modified (M) Stuart medium supplemented with various combinations of nutrient.

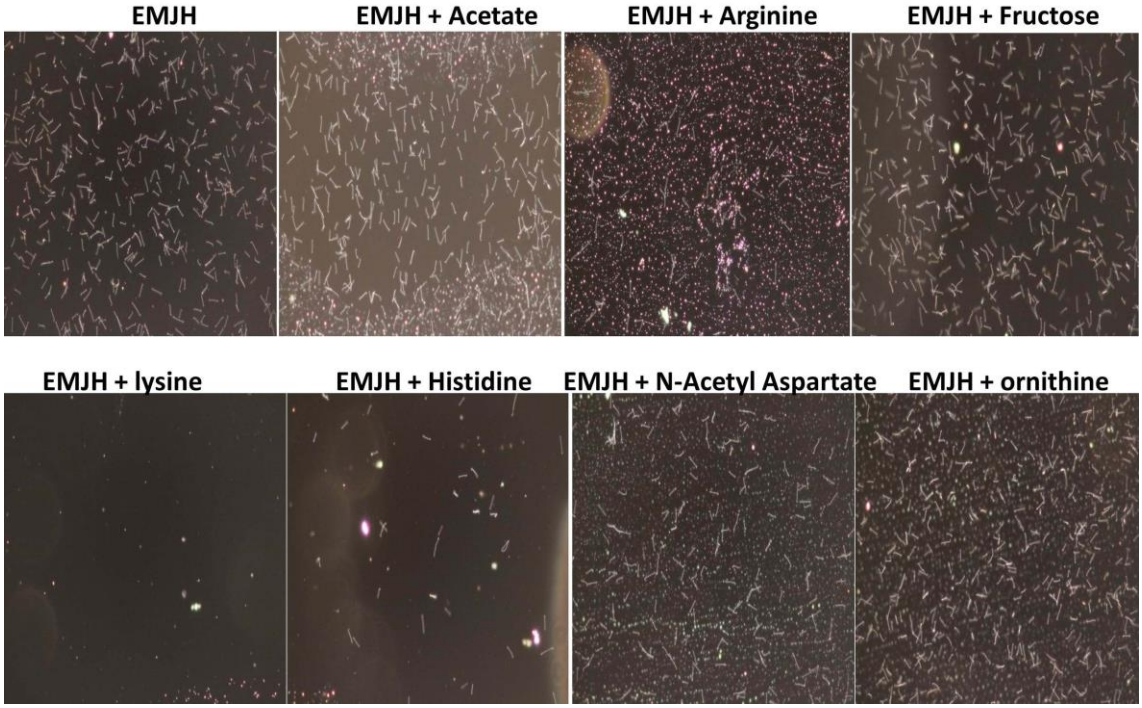


Figure 2.3e. Darkfield microscopy of fourth-day culture of *L. interrogans* serovar Pomona, a Pathogenic (P) organism inoculated in EMJH (E) medium supplemented with individual nutrients.

In order to check any variation in the secretory components of leptospira, after growing in the modified media the cells were incubated with various nutrients and after 4 hours of incubation at 30^0C, cells were taken in different time points, and spent media was subjected to SDS-PAGE.

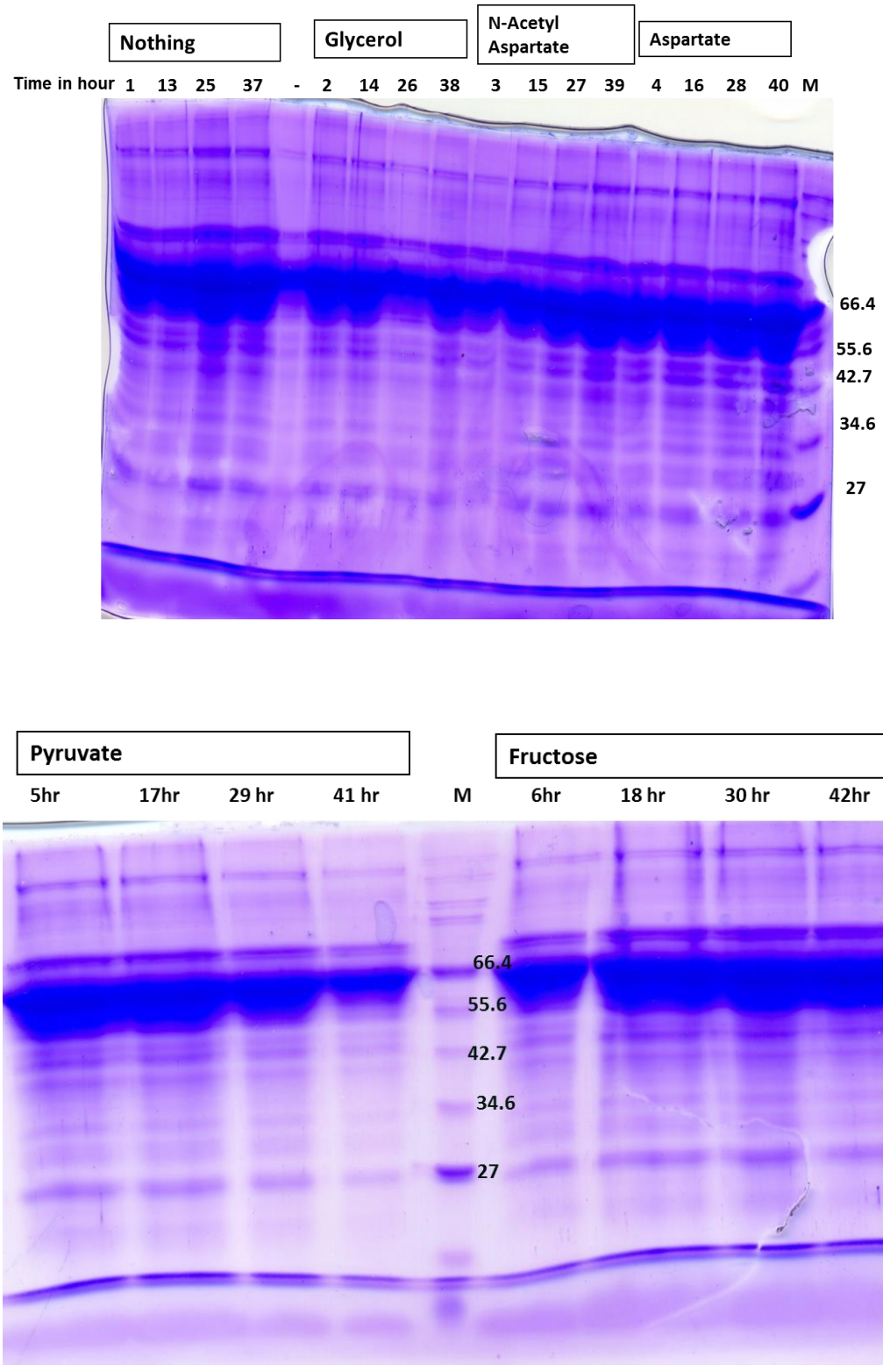


Figure 2.4. SDS PAGE of leptospira spent media monitored for a change in the secretory components upon supplementation of various nutrients in Modified Stuart Media.

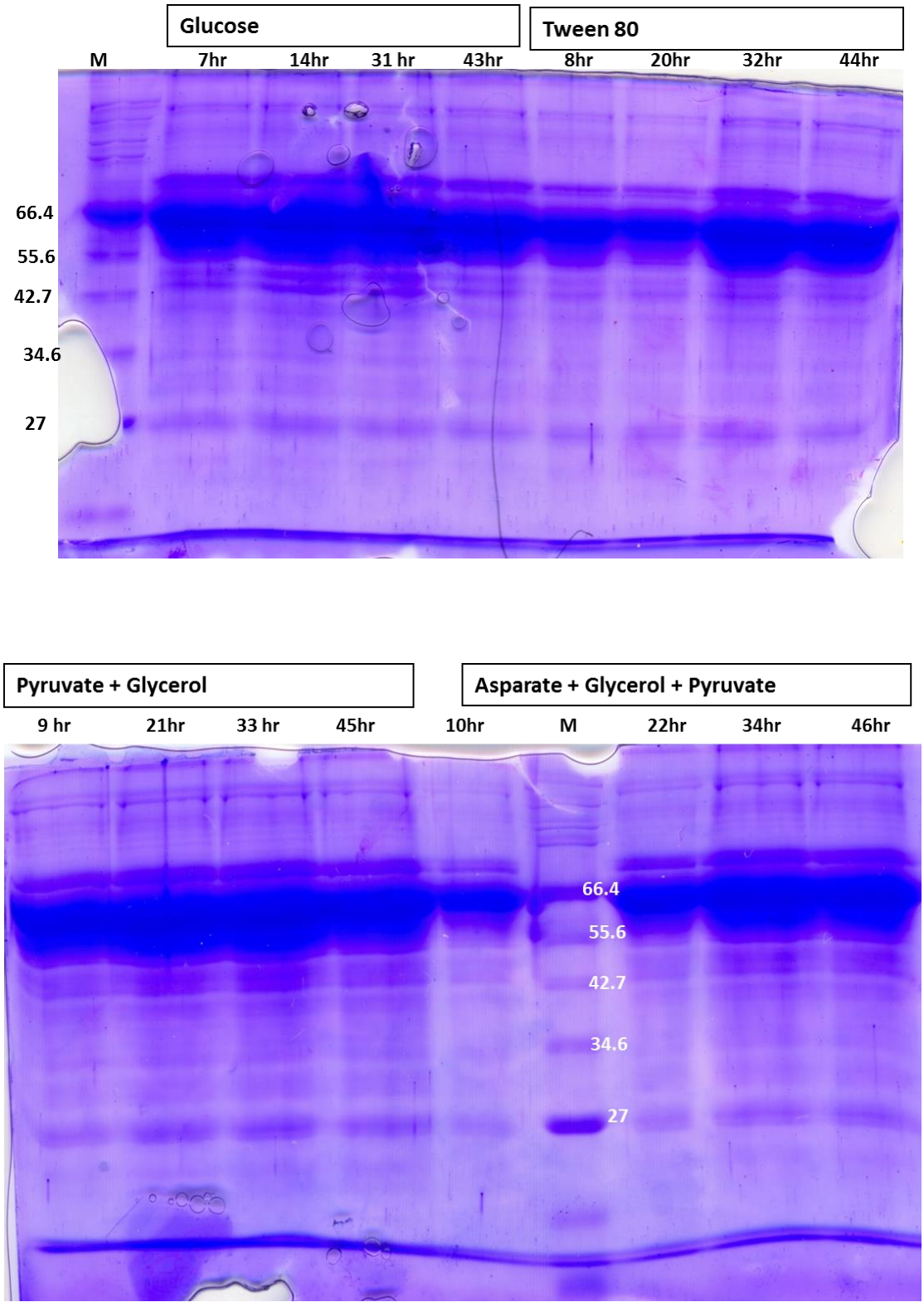


Figure 2.4. SDS PAGE of leptospira spent media monitored for a change in the secretory components upon supplementation of various nutrients in Modified Stuart Media.

The presence of fetal serum in the modified media interfered with the secretary component analysis experiment by SDS-PAGE. Most of the serum proteins were interfering significantly. In order to avoid the serum protein interference, the cell pellet was washed thoroughly several times with PBS. Washing of cell pellet with PBS several times did not overcome the serum protein interference (Fig 2.4).

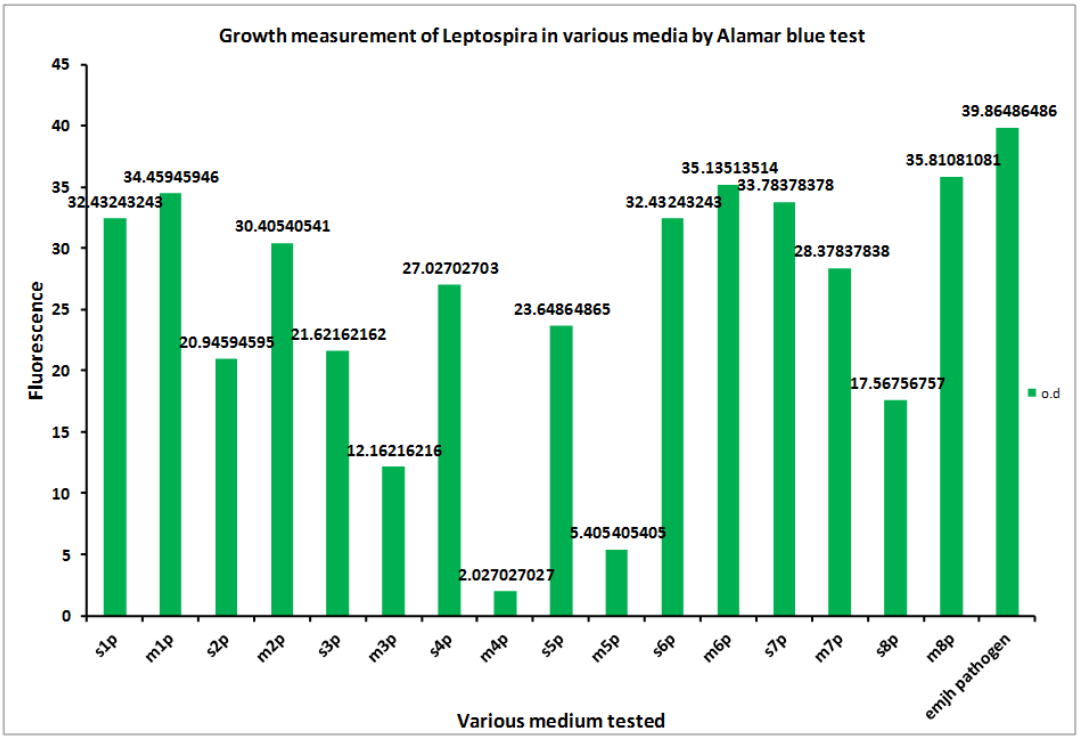


Figure 2.5. Alamar blue assay to assess the growth of leptospires.

The cell proliferation of the leptospires was monitored by Alamar blue reagent (resazurin dye, a water-soluble non-toxic dye, which yields a fluorescent signal and a colorimetric change when incubated with metabolically active cells). These experiments were not conclusive (Fig. 2.5). Additions of various components in combination lead to varying redox environments.

We reorganized the media compositions based on habitat and mode of pathogenesis, without depending on an already existing media as base media. Accordingly, various media were prepared by incorporating urea, oxalic acid, and succinic acid (Table 2.6). Nine serovars of leptospira were grown in each of these media with EMJH medium as standard. The use of urea, oxalic acid, and succinic acid

did not show any growth, but few organisms were detected compared to the standard EMJH medium.

Table 2.6. Media components (in mg) used for the growth of Leptospira based on host (Kidney) and natural environments habitat.

Components	**M1A**	**M1B**	**M1C**	**M1D**	**M3**	**M4A**	**M4B**	**M4C**
NH_4Cl	-	270	25	25	-	-	-	50
Urea	-	255	-	100	50	50	50	50
Aspartic acid	15	12	12	12	-	-	-	200
Sodium acetate	15	-	-	-	200	150	200	60
Sodium Pyruvate	100	300	50	50	60	-	60	200
Succinic acid	150	-	65	50	200	250	200	200
Oxalic acid	325	-	50	50	200	250	200	200
Glucose	-	300	-	100	200	100	200	-
Fructose	-	-	-	-	-	100	-	-
Glycerol	5mL	-	-	-	-	-	-	-
Benzoic acid	10	-	-	-	-	-	-	20
$KHCO_3$	10	25	-	-	20	-	20	-
NaCl	-	-	100	100	-	-	-	-
$MgCl_2$	10	-	5	5	-	-	-	10
$ZnSO_4.7H_2O$	-	-	10	10	-	-	10	20
$FeSO_4.7H_2O$	20	-	10	10	10	15	20	10
$CoCl_2\,2H_2O$	15	35	20	20	5	10	10	-
$CuCl_2$	-	-	-	10	-	-	-	-
$MnCl_2$	3-5	-	-	-	-	-	-	-
$CaCl_2.2H_2O$	-	-	10	10	-	-	-	-
KH_2PO_4	150	-	125	125	200	150	200	200
Na_2S	1	-	-	-	-	1	-	-
Final pH at 25^0C	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2
MilliQ water	100mL	100mL	100mL	100mL	100mL	100mL	100mL	100mL

Table 2.7. Media components (in mg) used for the growth of Leptospira based on the mode of infection, transporters present, and replenishing the anaplerotic gaps.

Components	M5	M6	M7	M8	M9	M10	M11	M12
NH_4Cl	25	85	25	15	15	15	15	15
Aspartic acid	12	133	133	40	40	40	15	15
Asparagine	10	-	-	-	-	-	-	-
Casein	-	-	-	-	140	100	100	100
Glucose	-	125	120	120	120	120	100	100
Sodium acetate	25	80	-	30	35	-	-	35
Sodium Pyruvate	50	50	50	60	-	60	6	6
Succinic acid	65	65	65	150	-	-	-	-
Oxalic acid	50	50	50	-	-	-	-	-
Benzoic acid	-	12	-	-	-	-	-	-
$KHCO_3$	15	250	250	-	-	-	-	-
$NaHCO_3$	-	-	-	100	100	100	40	40
Yeast extract	-	-	-	-	125	50	50	50
Thiamine HCl	-	2	2	2	2	2	2	2
NaCl	100	75	100	80	80	80	-	-
$MgCl_2$ (anhydrous)	-	5	5	17	17	10	15	15
$CaCl_2.2H_2O$	-	-	-	-	-	-	13	13
$CoCl_{2.}\ 2H_2O$	50	5	5	-	-	-	-	-
$CuCl_2$	7	4	5	-	-	-	-	-
$MnCl_2$	2	2	2	-	-	-	-	-
KCl	-	30	30	-	25	20	20	20
KH_2PO_4	125	-	-	-	-	24	10	10
$Na_2HPO_{4.}$ $2H_2O$	-	89	89	50	10	144	100	100
$Na_2MoO_4.2H_2O$	5	5	5	-	-	-	-	-
Na_2S	1	1	1	1	1	1	1	-
Final pH at 25^0C	7.2	7.2	7.2	7.2	7.2	7.2	7.2	7.2
MilliQ water	100mL	100mL	100mL	100mL	100mL	100mL	100mL	100mL

Notably, the medium in which urea was added, showed no growth or scant noticeable growth; when observed under a darkfield microscope, the organisms were viable in media with high concentrations of urea, even after four days of inoculation (data not shown). The media components were further optimized for filling the anaplerotic gaps (Table 2.7).

Table 2.8. Composition (in mg) of EMJH, M12, M12Q, and M14 media.

Components	EMJH	M12	M12Q	M14
$Na_2HPO_4.\ 2H_2O$	100	100	100	100
KH_2PO_4	30	10	10	10
Tryptone	-	100	100	100
Yeast Extract	-	50	50	50
KCl	-	20	20	10
Aspartic acid	-	15	15	5
Sodium Pyruvate	-	6	6	6
Glucose	-	100	100	100
$MgCl_2$ anhydrous	1	15	15	5
$CaCl_2.2H_2O$	1	13	13	3
NH_4Cl	25	15	15	7.5
Thiamine HCl	0.5	2	2	1
Sodium acetate	-	35	35	7.5
$NaHCO_3$	-	40	40	40
N-acetyl Glutamine	-	-	15	-
NaCl	100	-	-	-
$ZnSO_4.7\ H_2O$	0.4	-	-	-
$FeSO_4.\ 7H_2O$	5	-	-	-
$CuSO_4.\ 2H_2O$	0.03	-	-	-
BSA	1000	-	-	-
Polysorbate 80	1.25mL	-	-	-
100% Glycerol	0.5mL	-	-	-
Vitamin B_{12}	0.02	-	-	-
MilliQ water	100mL	100mL	100mL	100mL
Final pH at 25^0C	7.2	7.2	7.2	7.2

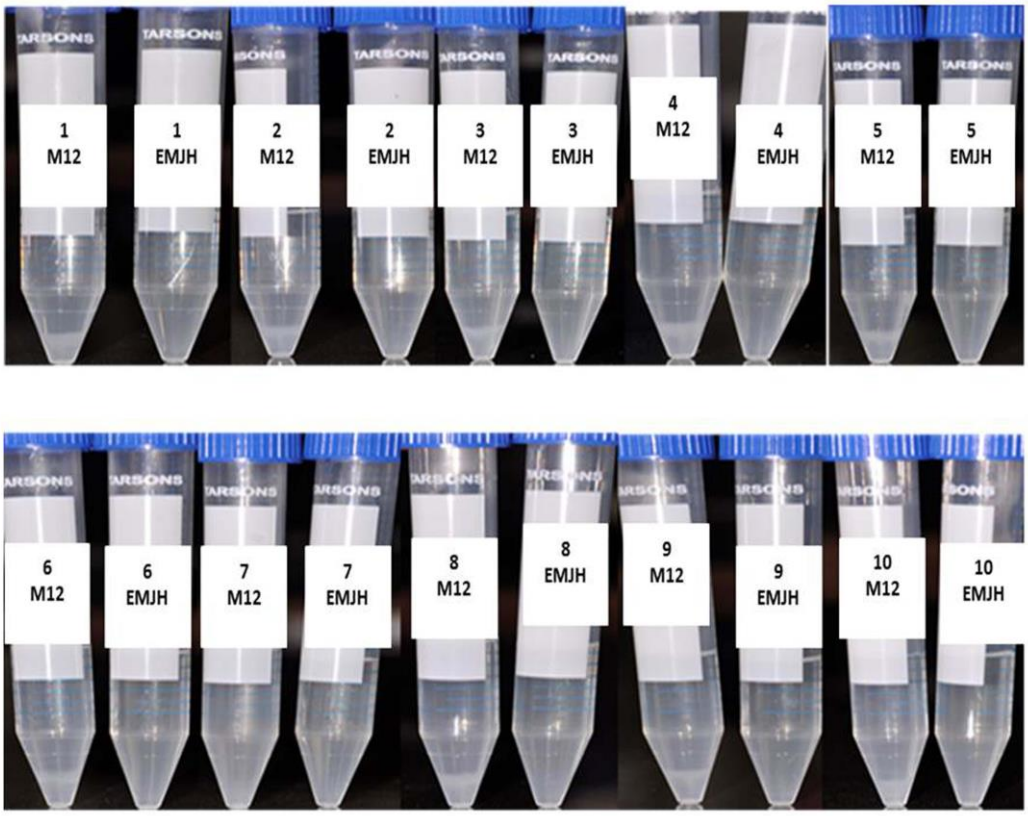
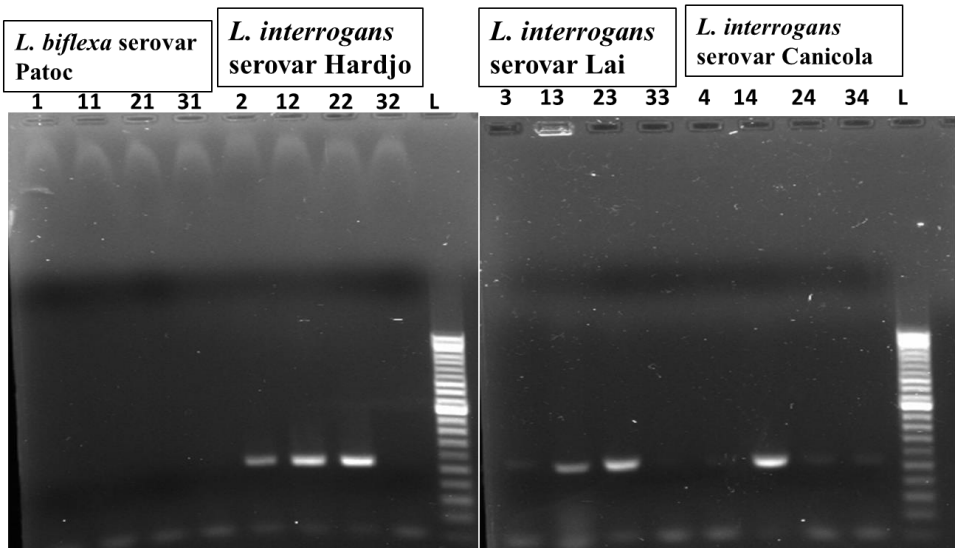
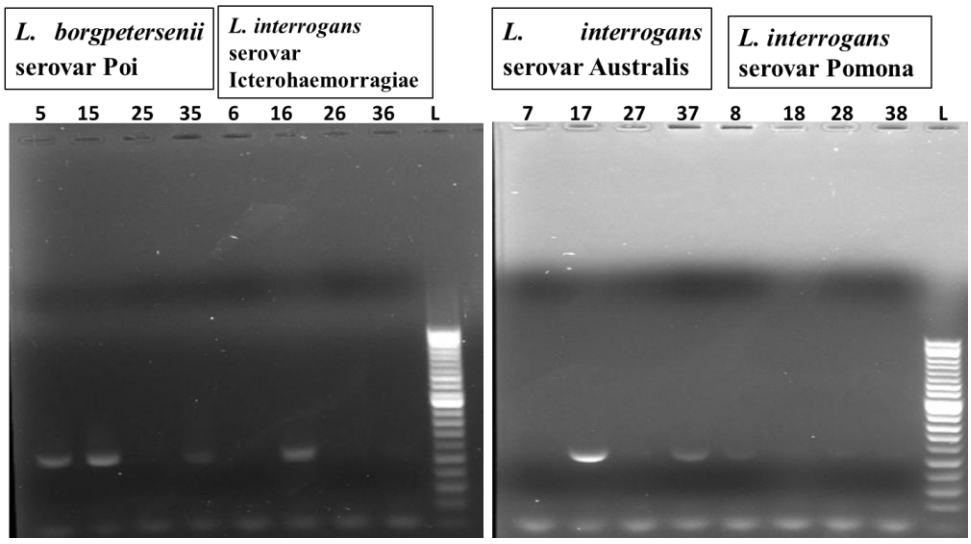


Figure 2.6. The Leptospira cultures in EMJH medium and M12 medium after 40 hours of inoculation. In the M12 medium ring-like white layer formed at the bottom neck of the tube. 1) *L. biflexa* serovar Patoc, 2) *L. interrogans* serovar Hardjo, 3) *L. interrogans* serovar Lai, 4*)* *L. interrogans* serovar Canicola, 5) *L. borgpetersenii* serovar Poi, 6) *L. interrogans* serovar Icterohaemorragiae, 7) *L. interrogans* serovar Australis, 8) *L. interrogans* serovar Pomona, 9) *L. interrogans* serovar Autmanalis and 10) uninoculated media control.

In the M8 medium, the organism showed better growth when examined under a darkfield microscope (data not shown). The M8 medium was further optimized by reducing the concentration of salts. In the M9 medium, the removal of the pyruvate and acetate affected the growth drastically. In the M10 medium re-introduction of pyruvate and removal of only sodium acetate did not produce a considerable effect. Though the initial concentrations of salts in the media M5 to M8 were fixed arbitrarily, the concentration of various metal ions namely, Na^+, K^+, Ca^{2+} was fixed to concentrations in serum (M12 and M12Q media; Table 2.8). In the M14 medium growth of the organisms was not satisfactory; the M12Q medium did not show any visible growth. In the M12 medium, all the nine serovars of leptospira showed visible growth of the ring at the bottom of the tube, after 40 hours of inoculation (Fig. 2.6).

We have also tested its growth in glass tubes, the 15ml polypropylene tubes seem to be better for visualization of the ring at the bottom of the tube.





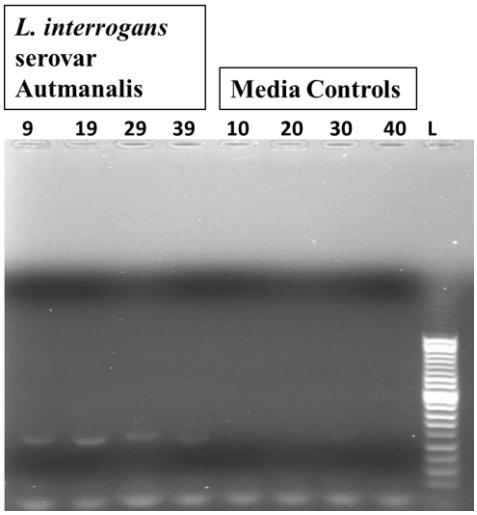


Figure 2.7. Detection Leptospira serovars by PCR amplification of the Hap1 gene (product size of 260 bp). Lane 1-10, 11-20, 21-30, and 31-40 represent the amplification of the Hap1 gene of various Leptospira serovar grown in EMJH, M12, M12Q, and M14 medium respectively. The 50bp ladder is labelled as 'L'.

The *L. biflexa* serovar Patoc (lanes 1, 11, 21, and 31) was used as cross-contamination control. Various serovars tested for the amplification of the Hap1 gene are
Lane 1, 11, 21, 31 = *L. biflexa* serovar Patoc
Lane 2, 12 22, 32= *L. interrogans* serovar Hardjo
Lane 3, 13, 23, 33 = *L. interrogans* serovar Lai
Lane 4, 14, 24, 34= *L. interrogans* serovar Canicola
Lane 5, 15, 25, 35= *L. borgpetersenii* serovar Poi
Lane 6, 16, 26, 36= *L. interrogans* serovar Icterohaemorragiae
Lane 7, 17, 27, 37= *L. interrogans* serovar Australis
Lane 8, 18, 28, 38= *L. interrogans* serovar Pomona
Lane 9, 19, 29, 39= *L. interrogans* serovar Autmanalis
Lane 10, 20, 30, 40= Media Control

Further, the growth of leptospira was confirmed by performing PCR (Fig. 2.7). The M12 medium showed better amplification of a 262bp segment of the Hap1 gene compared to other media EMJH, M12Q, and M14. The M12Q medium did not show any visible growth, but it showed a better amplification of the Hap1 gene compared to the EMJH medium. It is an interesting observation that even though the M12Q medium is identical to that of the M12 medium except for N-acetyl glutamine; it is yet unclear why the ring does not appear upon addition of N-acetyl-Glutamine to the medium. The removal of bicarbonate or sodium acetate or aspartate affected the growth of leptospira (data not shown). The M14 medium (with the reduction in the composition of media components from M12) is not ideal for the growth of leptospira.

2.5 Discussion

Stuart medium constitutes complex ingredients, it also has asparagine. It is a convenient medium to add or remove components for culture studies. The original rationale to use asparagine may be based on its conversion to aspartate by the asparaginase. However, the gene for the asparaginase enzyme is not present in the leptospira genome; thus the rationale for aspartate incorporation in the medium.

Initially, we used urea concentration broadly in the range of urine urea concentrations as organism harbours in the kidney. Later, we used urea concentration that is equivalent to that of normal serum urea concentrations or twice the serum urea concentrations in these media (Table 4) as the organism survives first in the blood. These experiments were not successful; the post facto analysis of the leptospira genome indicates the gene encoding urease enzyme was absent. The survival of these organisms in high concentration urea may be due to the presence of one or more amido-hydrolase that might show urease-like activity.

The rationale for the usage of oxalic acid and succinic acid was to replenish the anaplerotic gaps so that the organism will be driven towards nucleic acid biosynthesis and subsequently towards cell division. Further, the organism survives in the blood; we used the salts which will be equivalent in concentration to the serum composition. The organism has many ammonia transporters that take up ammonia into the cell (Caspi R. *et al.*, 2016). Hence we included ammonium chloride in the medium. Most importantly, it also needs carbon dioxide for its survival (wuthiekanun V. *et al.*, 2013), so we included bicarbonate in the medium. In fact reduction of bicarbonate does not show any visible growth in the M12 media (Fig. 2.8). Furthermore, the leptospires are actively motile organism; suggesting that the organism has to invest lots of its energy for mobility and the energy metabolism are not the limiting factor for its growth. We have included sodium acetate in the medium that can compensate the energy component without the need for the detoxifying effect of bovine serum albumin.

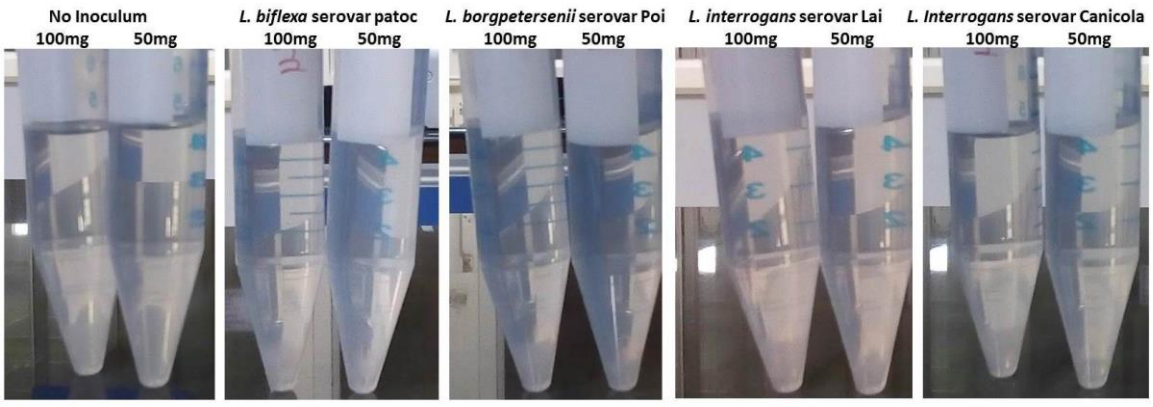


Figure 2.8. Showing dependence of bicarbonate by Leptospira.

The fact that the organism is resistant to 5-fluorouracil (Johnson RC. *et al.*, 1964), suggests that leptospires are solely dependent on the de-novo pyrimidine synthesis. Interestingly, the aspartate transcarbamylase enzyme (the first enzyme of the de-novo pyrimidine biosynthetic pathway) is secreted by Leptospira into the medium along with other exoproteins (Eshghi A. *et al.*, 2015). Decreasing the amount of bicarbonate in the M12 medium resulted in a drastic reduction in the growth of leptospira (data not shown); together with the fact that organism down-regulates aspartate-specific metabolic enzymes of the reservoir host. We hypothesize that leptospira's growth is enhanced by introducing supplements of aspartate and bicarbonate in the growth medium, which is necessary for the de-novo pyrimidine biosynthetic pathway.

In order to check the growth of leptospira serovar from urine samples in M12 medium; we mimicked the condition by inoculating with Leptospires in the presence and absence of urine obtained from healthy individuals and 5-fluorouracil used as a selective agent. After 40 hours of incubation at 30^0C, the tubes inoculated with leptospira showed a visible growth ring at the bottom of the tube; a visible growth ring with a fuzzy appearance was seen in tubes containing 5-fluorouracil and the tubes inoculated with only urine did not show any visible ring formation (Fig. 2.9).

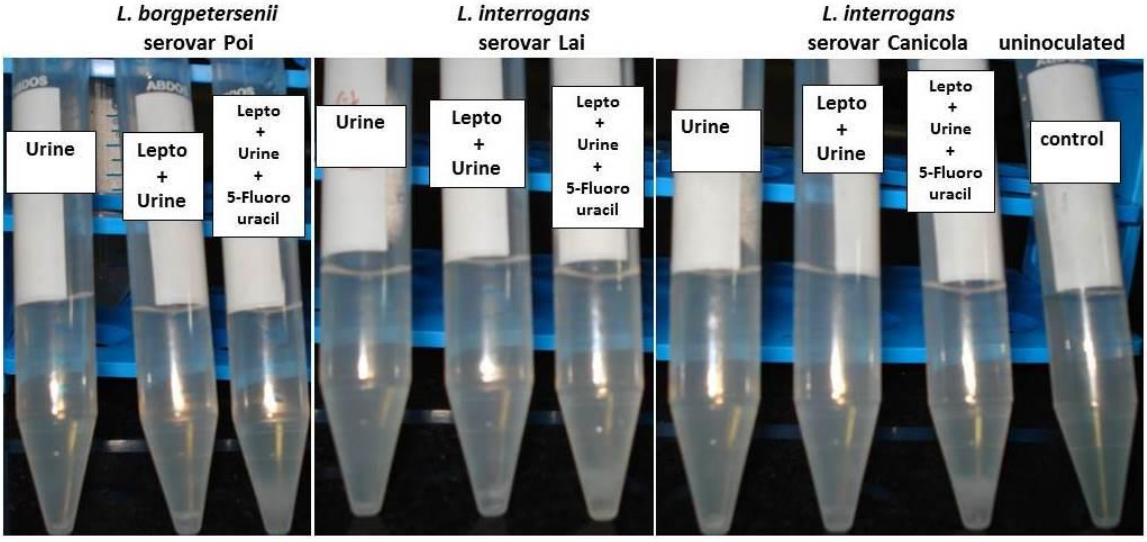


Figure 2.9. The growth of various Leptospira cultures in M12 media in the presence and absence of 5-Fluorouracil in urine after 40 hours.

The visible ring formation by leptospires is well documented in the literature; but after eight to nine days of initial inoculation in the semisolid medium (Levett PN., 2001). In principle, the formation of the ring is an indicator of the profuse growth of leptospires (Levett PN. 2001); it is still a challenging task in terms of diagnosis because it requires 4-6 weeks for confirmation of the disease by culture (Bhatia M. *et al.*, 2015). In our studies, we didn't include microscopic studies as the organism seems to be pleomorphic. In the present study, the M12 medium reported here shows visible growth (ring) of leptospira by 40 hours of inoculation, which might be helpful in the early diagnosis of Leptospirosis in the future.

2.6 Summary and Conclusion

We have developed an M12 medium for leptospira visible growth in 40 hours. The key optimization parameters are obtained from genomic and proteomic literature, the organism's natural habitat, and the mode of pathogenesis. This methodology of combining existing proteomics, genomics, natural habitat, and pathogenesis information can be used to other slow-growing pathogenic organisms that solely depend on the culture method as a gold standard for its disease diagnosis.

Chapter 3

Biochemical characterization of Triosephosphate isomerase from *Leptospira interrogans*

Triosephosphate isomerase

This chapter describes the cloning, expression, purification, and biochemical characterization of Leptospiral Triosephosphate Isomerase (TPI). A brief account of Leptospiral metabolism precedes the presentation of the experiments.

3.1 Introduction

The metabolism of *Leptospira interrogans* is an intriguing biochemical puzzle as leptospira does not use glucose as its major carbon and energy source. The importance of lipids as a source of energy for leptospires has been well established. Using a dense suspension of pathogenic leptospira strains it was demonstrated that oxygen uptake by whole cells could be detected and was stimulated by rabbit serum; the addition of amino acids and sugars did not affect respiration. Leptospires were shown to have a Qo_2 value of 12.5 with serum as substrate. The long-chain fatty acid along with albumin was shown to be stimulated the respiration of leptospira whereas only rabbit serum, amino acids, and sugars did not affect Leptospira respiration. Leptospira bacterium has catalase, lipase, oxidase, hemolysin, and transaminase. In general, Leptospira uses unusual metabolites (Tween80, Glycerol, and pyruvate) as the primary source of energy. It also has a functional TCA cycle, glyoxalate cycle, and electron transport chain and uses beta-oxidation of long-chain fatty acids as the primary carbon source (Baseman JB. *et al.,* 1969; Levett PN. 2001; Alder B. *et al.,* 2010).

The genome annotations, proteomic analysis (Ren SX. *et al.,* 2003*;* Nascimento AL. *et al.,* 2004; Picardeau M. *et al.,* 2008; Malmstrom J. *et al.,* 2009), and in vitro recombinant enzyme characterization (Zhang Q. *et al.,* 2011) have also identified functional glycolytic pathway enzymes (Ko AI. *et al.,* 2009). The genomic analysis revealed that amino acids and all nucleic acid biosynthesis pathways of the leptospira genus are complete in both pathogenic and non-pathogenic species except for few additional proteins involved in nitrogen, amino acid, and carbohydrate metabolism that are missing from pathogenic leptospires than their non-pathogenic counterparts.

From the published genome, we identified one such central metabolic enzyme of *Leptospira interrogans*, triosephosphate isomerase, which connects the fatty acid and glycerol metabolism to gluconeogenesis and TCA cycle via an anaplerotic route and provides intermediates essential for energy production and amino acid biosynthesis. Triosephosphate isomerase (TPI; EC: 5.3.1.1) interconverts dihydroxyacetone phosphate (DHAP) and D-glyceraldehyde-3-phosphate (D-GAP).

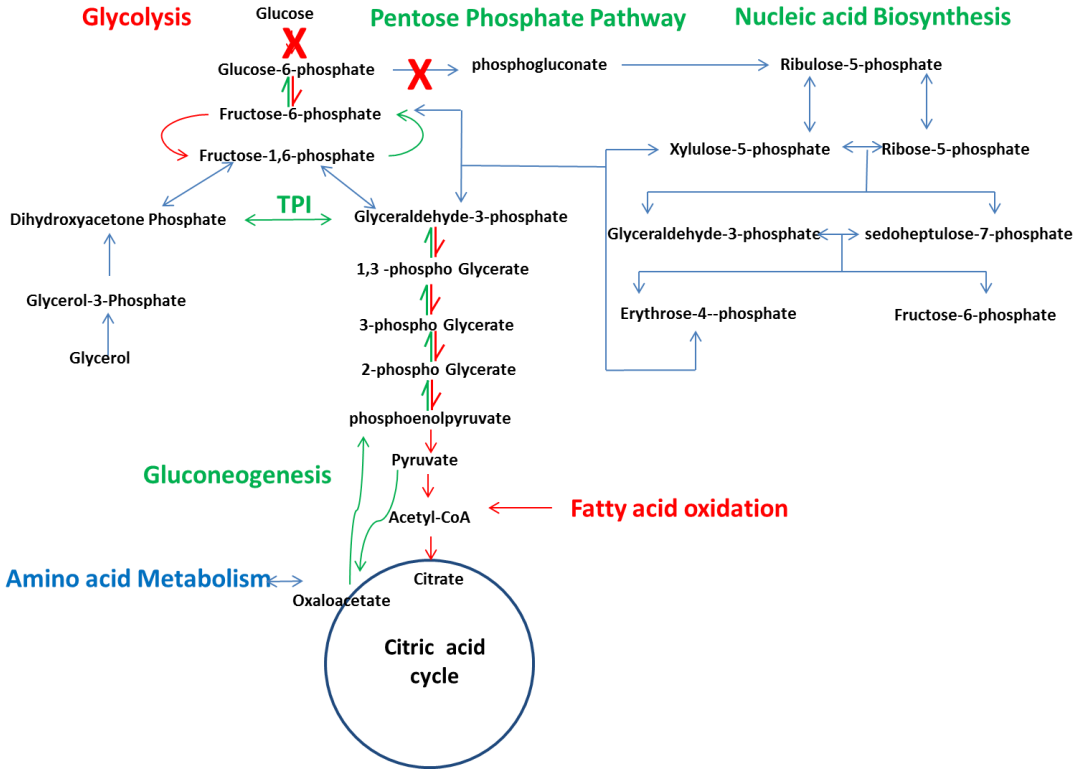


Figure 3.1. Proposed metabolic pathway of leptospira (reproduced from Baseman *et al.*, 1969).

TPI is a dimeric enzyme of identical subunits that is characterized by a high constitutive level of activity in all tissues of the higher organism. Its structural aspects are determined from several sources; kinetics, catalytic mechanism, and energetics of the reaction are well studied (Albery WJ. *et al.*, 1976; Alber T. *et al.*, 1981; Lolis E. *et al.*, 1990; Noble ME. *et al.*, 1993; Mande SC. *et al.*,1994; Delboni LF. *et al.*, 1995; Watanabe M. *et al.*, 1996; Harris TK. *et al.*, 1998; Maldonado E. *et al.*, 1998; Ationu A. *et al.*, 1999). TPI in Leptospira may also play an important role in the pentose phosphate shunt in the absence of Glucose-6-phosphate dehydrogenase.

It has been hypothesized the glycolytic enzyme may behave differently in Leptospira and have evolved to perform its function in the opposite direction of

glycolysis, such that resulting metabolites such as DHAP, GAP, and Fructose1,6-bisphosphate would be channelized towards the pentose phosphate pathway in order to synthesize adequate ribose sugars for DNA synthesis and reducing equivalents for the biosynthetic process (Baseman JB. *et al.*, 1969).

Further, Leptospira being a slow grower, TPI was found suitable enzyme to study to check the integrity of the central metabolic pathway of the organism. Presently, there are no studies on the enzymatic and physico-chemical properties of TPI from leptospira sp. In the present study, we cloned, expressed *Leptospiral interrogans* TPI (LiTPI) in *E. coli*, purified, and determined the biophysical, biochemical properties of LiTPI.

3.2 Materials

E.coli Strain DH5α, *E.coli* Strain AA200 (TPI null mutant), pTrc99A plasmid (Generous gift from Prof. Hemalatha Balaram), Tris, GnHCl, Urea, D-GAP, BSA, NADH, Dialysis membrane, EtBr, DTT, Agarose, SDS, Glycerol-3-phosphate dehydrogenase, PMSF, Primers from Sigma-Aldrich Co, India; 6x DNA loading dye, PR DNA Polymerase, dNTPs, protein markers, DNA ladder from Bangalore Genei, Bangalore, India; PHUSION DNA polymerase(Thermo scientific), NcoI endonuclease, BamHI endonuclease, T4 DNA ligase from NEB; NaCl, EDTA, Glycerol, Ammonium sulfate, Acrylamide, Bis-acrylamide, Bromophenol blue, Ammonium persulfate, TEMED, Glycine, HCl, from SDFCL.

3.3 Methods

3.3.1 Cloning of the LiTPI gene

Triosephosphate isomerase gene (TPI gene ID: LEP2GSC113_RS0110880) was amplified from cDNA obtained from five days old Ellinghausen–McCullough–Johnson–Harris (EMJH) culture of Leptospira interrogans (Li) serovar Icterohaemorrhagiae strain RGA (Regional Medical Research Centre, Indian Council for Medical Research, Port Blair, India; ATCC: 23581. Taxonomy ID: 1291351) by colony PCR (cell pellet from 30 µL of culture was resuspended in 60µL of autoclaved milli-Q water followed by heating at 95˚C for 20min. 15µL of the lysate thus obtained

was used as the template for 50µL PCR reaction mix). The PCR reaction was carried out using LiTPIFP and LiTPIRP (CATGCCATGGCTCGTAAGACAATTATTGCAGGAAACTGG and GCGGGATCCTTAGAAAAGTCCAGCGAAGGAACTG) (Sigma-Aldrich Co, India) as the forward and the reverse primers, inserting NcoI and BamHI endonuclease sites at the 5´ of the start and 3´ of the stop codons of the gene, respectively. To circumvent the problem of frame-shift in the gene caused due to the NcoI site at the 5' end, two extra nucleotides were inserted downstream of the endonuclease site (underlined in the primer). The extra codon (GCT) results in the insertion of Ala immediately after the N-terminal Met of the protein.

The PCR mixture contained, in a total volume of 25 µl: template DNA, 100 ng; mutagenic primer, 20 pmol; thermostable polymerase buffer (10X), 2.5 µl; dNTPs, 6 µl of a solution containing 2.5 mM of each dNTP; and polymerase 2 U. Following the hot-start PCR method, PR polymerase (Bangalore Genei, Bangalore, India) was added to the reaction mix after initial denaturation at 95˚C for 5 mins. Subsequently, 28 cycles of the steps, 1) 95˚C for 15 sec, 2) 55˚C for 20 sec, 3) for 90 sec with a final extension at 75˚C for 5 min yielded the amplified PCR product which was then purified (Qiagen Gel purification kit) and digested with Nco1 and BamH1 restriction endonucleases (NEB, India) for inserting the gene into the pTrc99A expression vector. The ligated product was transformed in DH5α cells and plated on LB agar containing 100 µg/mL ampicillin. 10 colonies were screened for a positive clone (with LiTPI insertion) by carrying out restriction digestion with Nco1 and BamH1 enzymes on the plasmid obtained from the respective colonies and confirmation by primer-specific PCR amplification, followed by confirmation with gene sequencing (final gene length 756 bp).

3.3.2 Protein expression, purification, and basic characterization

E. coli strain AA200 (TPI null mutant) was transformed with pTrc99A containing the recombinant LiTPI gene and grown in LB for 8-10 hours with 100 µg/ml ampicillin. The culture was used as 1% pre-inoculum in terrific broth (TB) culture grown at 37 °C. After induction with 0.3 mM IPTG (OD600nm 0.6-0.8), the culture was allowed to grow for another 16hrs at 30°C. Cells were harvested by centrifugation (20 min, 6K rpm, and 4˚C), resuspended in lysis buffer containing 20 mM Tris-HCl (pH 8.0), 1

mM EDTA, 1 mM PMSF, 2 mM DTT, and 10% glycerol and disrupted by sonication. After centrifugation (45 min, 12K rpm, 4 °C) and removal of the cell debris, the supernatant was fractionated with ammonium sulfate. The protein fraction containing TPI was precipitated between 35% and 65% ammonium sulfate saturation.

The precipitate was collected by centrifugation (30 min at 19,320 g at 4 °C) and re-suspended in buffer A (20mM Tris/HCl pH 8.0, 2 mM dithiothreitol, and 10% glycerol). Monitoring of each step was performed by SDS–PAGE analysis (12% polyacrylamide). The subsequent purification steps were performed at 4°C. The protein was dialyzed extensively against buffer A at 4 °C overnight, and purified using an anion exchange Q-Sepharose column (Amersham Biosciences, Uppsala, Sweden) eluted with a linear gradient of 0–1 M NaCl. The fractions containing the protein were pooled. The purified protein obtained was then extensively dialyzed overnight against buffer A at 4°C. Protein purity was checked by 12% SDS–PAGE and by LC-ESI mass spectroscopy. The molecular weight indicated in the figure is inclusive of an alanine inserted at the N-terminus of the protein.

3.3.3 Spectroscopic characterization of the native protein

Circular dichroism spectrum was recorded on JASCO-715 spectropolarimeter (JASCO technologies, Tokyo, Japan), Protein concentration ~20 μM. Fluorescence emission spectra were recorded on a HITACHI F2500 spectrofluorimeter. Protein samples were excited at 295 nm and the emission spectra were recorded from 300 nm to 450 nm. Excitation and emission bandpasses were kept as 5 nm and 10 nm, respectively. All the spectra were corrected by subtracting the signal from the buffer solution. Protein samples (final concentration ~5 μM) were prepared in 20 mM Tris HCl (pH 8).

3.3.4 Thermal denaturation

For thermal melting studies, LiTPI, (~20 μM final concentration in 20 mM Tris HCl, pH 8) was incubated at each temperature (20-85 °C with 5 °C temperature jumps) for 15 min for reaching equilibrium and three CD scans were performed at a scan speed of 10 nm*min−1. CD measurements were performed on JASCO-715 spectropolarimeter (JASCO technologies, Tokyo, Japan) equipped with a thermostat

cell holder controlled by a Peltier device. 1 mm path length cuvette, 2 nm bandpass was used. The HT voltage remained in the permissible range throughout all the scans. Change in the CD ellipticity at 220 nm (θ in mdeg) was plotted as a function of temperature.

3.3.5 Chaotrope mediated denaturation

Equilibrium unfolding was carried out by incubating LiTPI protein (6μM) with GdmHCl (0- 4 M) or urea (0- 8 M) solution freshly prepared in 20 mM Tris-HCl (pH 8.0), for 1 hr. Emission spectra (310- 450 nm) were recorded after exciting protein samples with 280 nm wavelength beam (5 nm bandpass filter) on a Hitachi F2500 spectrofluorimeter. Each titration was repeated thrice and the respective buffer spectrum was subtracted. The mean and standard deviation of emission wavelength maxima (λmax_{em}) at each concentration and intensity at 324 nm were plotted to follow unfolding causing the change in the protein intrinsic fluorescence. Data were fitted to the monophasic transition model using the Graphpad Prism (Version 5 for windows, Graphpad Software, San Diego, California, USA, www.graphpad.com). Spectra were corrected by subtracting the buffer signal.

3.3.6 Enzyme activity measurement

Enzyme activity was measured by a coupled enzyme assay method with modification of using BSA solution (final concentration ~20μg/mL) (Plaut B. *et al.*, 1972). TPI catalysed conversion of D-GAP to DHAP was monitored in the presence of the coupling enzyme, D-glycerophosphate dehydrogenase (GPDH). The reaction mixture contained (final volume 500μL) 100 mM TEA (pH 7.6), 5 mM EDTA, 0.5 mM NADH, and 20 mg/ml GPDH and GAP (for calculating the concentration it was assumed that the free acid solution contained D and L- GAP in equal concentration) to which TPI was added to initiate the reaction). Substrate concentrations were varied from 0.2 mM - 2 mM of D-GAP. The progress of the reaction was monitored at 25 ˚C by the decrease in absorbance of NADH at 340 nm ($\varepsilon 340nm$= 6220 $M^{-1}cm^{-1}$. Upon addition of BSA solution (final concentration ~20μg/mL) to the diluted enzyme solution as well as to the reaction mix, the initial rates showed a linear dependence on the enzyme concentration in the range studied (data not shown). The values for the kinetic parameters (Km, kcat) were determined by fitting the initial velocity data to

the Michaelis-Menten equation using Graphpad Prism (Version 5 for windows, Graphpad Software, San Diego, California, USA, www.graphpad.com). Protein concentration was determined by Bradford's assay and also by taking protein solution absorbance at 280 nm (theoretical value for ε280nm LiTPI = 17,000, assuming all Cys reduced). At least three independent measurements of all assays were performed and the error was found within 5% of the mean (This work was done by Vidhi Pareek, IISc).

3.3.7 Comparison of LiTPI with kinetic parameters of human TPI

Using BRENDA database the kinetic parameters of human TPI homologues were compared with the LiTPI.

3.4 Results

3.4.1 Cloning, expression, and purification of LiTPI

The triosephosphate isomerase gene was amplified from *Leptospira interrogans* cDNA and cloned in the expression vector pTrc99A as described in the Methods section (Fig. 3.2). The positive clones were confirmed by DNA sequencing. The DNA sequence confirmed clones were used for expression studies.

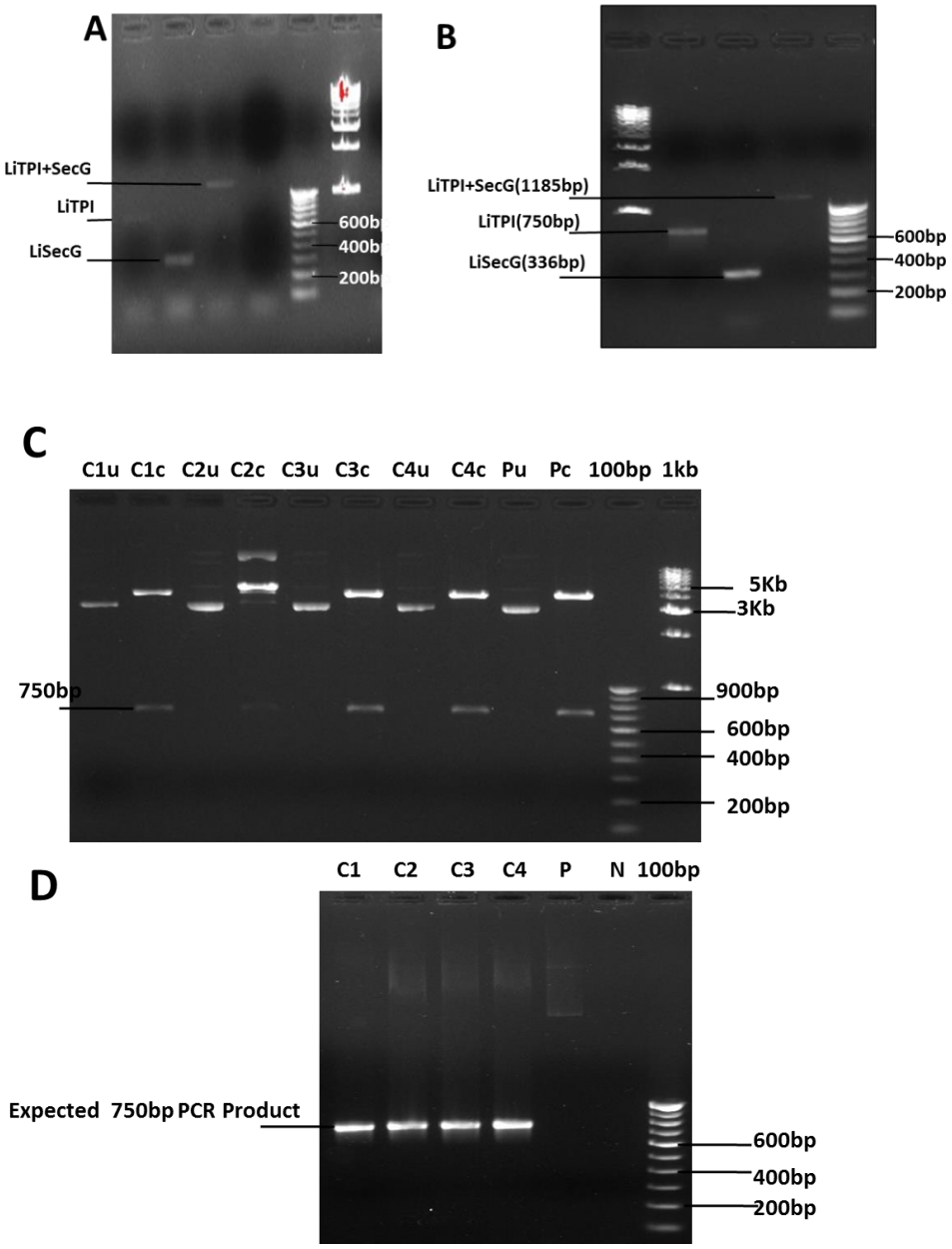


Figure 3.2. Cloning of *Leptospira interrogans* TPI. (A) PCR product of LiTPI, SecG, and TPI-SecG amplified by PR Polymerase. (B) Purified PCR product of LiTPI, SecG, and TPI-SecG. (C) Confirmation of LiTPI clones by restriction digestion with NcoI and BamH1. The clones without the addition of restriction enzymes are used as uncut controls to identify the 750bp pop out as the

confirmation of clones. (D) Confirmation of pTrc LiTPI Clones by LiTPI specific Primers.

E.coli AA200 strain (which is null mutants for expression of any endogenous TPI) was transformed with pTrc99A-LiTPI plasmid. Various clones are induced with 0.3mM IPTG and the expression of LiTPI in these clones, were monitored by comparing the induced cell lysates with uninduced cell lysate as controls (Fig. 3.3).

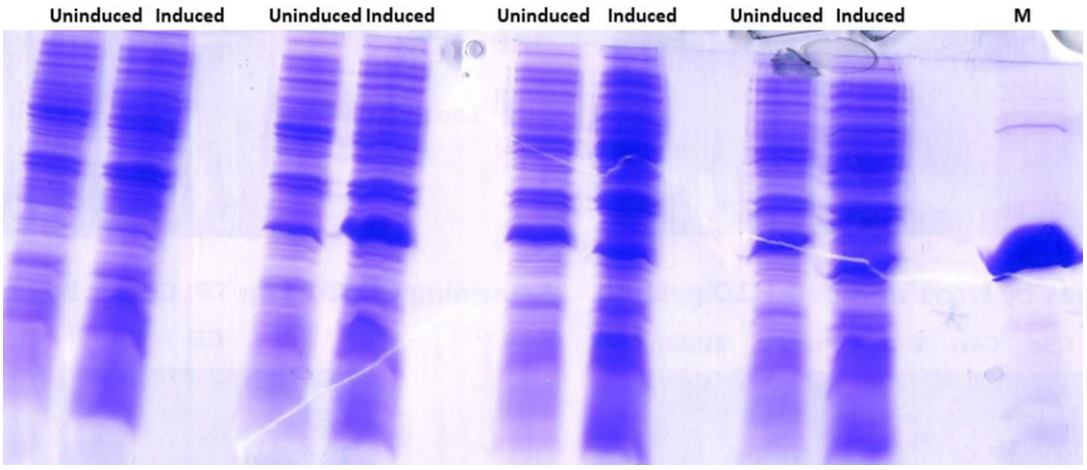


Figure 3.3. Expression of leptospira interrogans TPI gene in *E.coli* AA200 strain, shown on 12% SDS- PAGE gel. M- PfTPI as protein molecular weight marker.

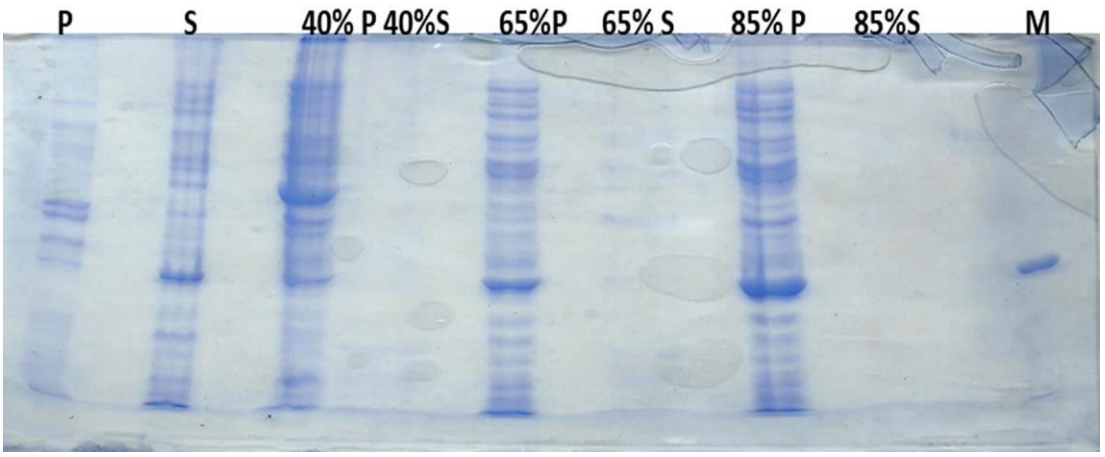


Figure 3.4. Protein purification of LiTPI was monitored on 12% SDS-PAGE. P- pellet, S- supernatant, M- PfTPI as protein molecular weight marker.

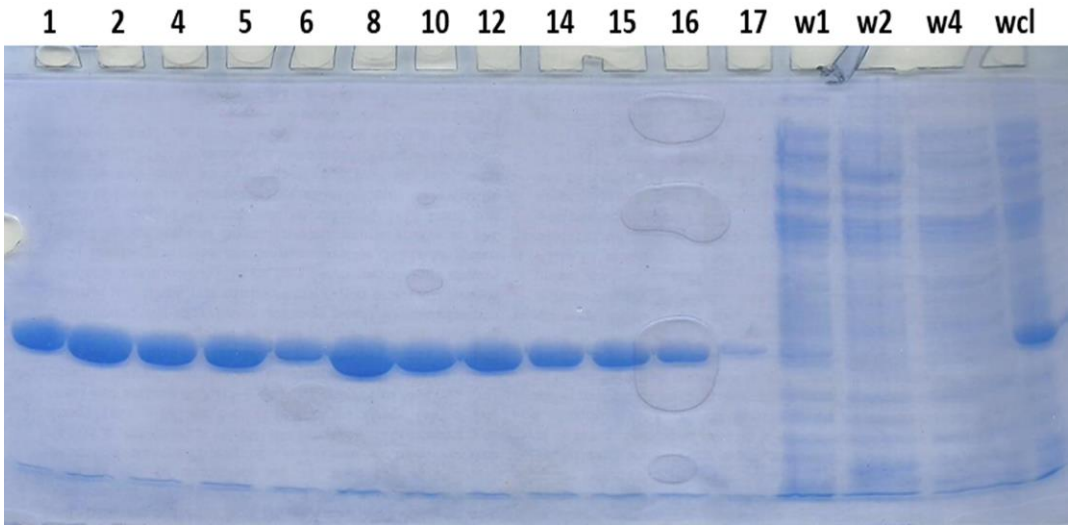


Figure 3.5. LiTPI fractions collected after Q-sepharose ion-exchange chromatography were monitored on 12% SDS-PAGE. w1- first wash, w2- second wash, w4- fourth wash, wcl- whole cell lysate.

The cell lysate was centrifuged and the supernatant was subjected to ammonium sulfate precipitation. Many impurities precipitated by 40% saturation. The 40% supernatant was further subjected to ammonium sulfate precipitation till 65% saturation. At 65% saturation majority of the TPI was precipitated (Fig. 3.4). The 65% pellet was resuspended in buffer A (20 mM Tris/HCl pH 8.0, 2 mM dithiothreitol, and 10% glycerol) and dialyzed against the same buffer to remove the ammonium sulfate salt.

The extensively dialyzed 65% pellet fraction was further loaded on to Q-sepharose column and eluted with a 0-1M sodium chloride linear gradient. The elution of proteins was monitored by a 280nm UV detector. The protein eluted by the salt gradient was collected in the 10ml fractions and all the fractions were analyzed for the presence of LiTPI by SDS-PAGE (Fig. 3.4). The fractions containing the LiTPI were pooled and dialyzed extensively against buffer A. After the final purification step, 95% pure protein was obtained (Fig. 3.5).

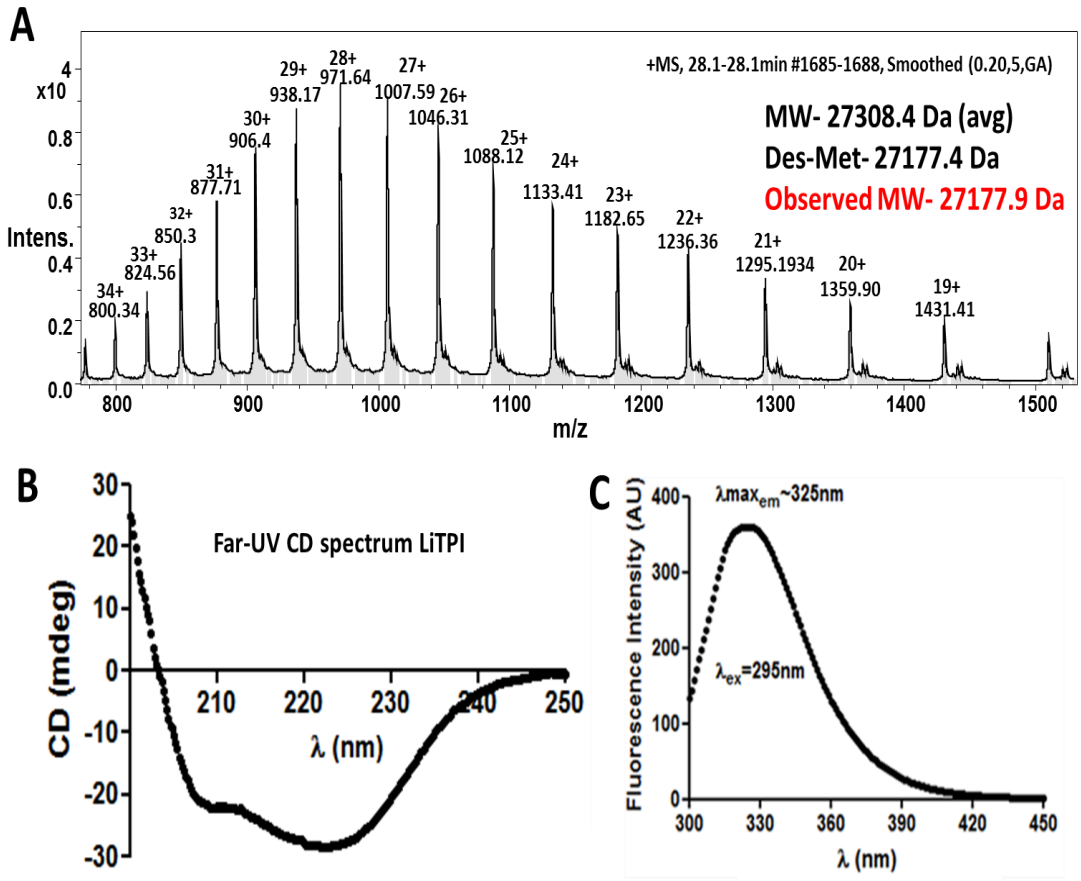


Figure 3.6. Biophysical characterisation of LiTPI (A) LC-ESI/MS of the purified LiTPI protein agrees with the expected mass. (B) Far-UV CD spectrum and (C) Tryptophan intrinsic fluorescence spectrum of the purified protein confirm a well-folded form in solution.

The LC/ESI-MS of the purified protein confirmed its exact mass with loss of the N-terminal methionine (observed mass: 27177.9 Da, Fig. 3.6A). The circular dichroism spectrum of protein showed a well-folded protein (Fig. 3.6B). The far-UV CD spectrum of LiTPI (from 200-250 nm) was similar to the reported spectra for TPIs from other organisms and characteristic of the canonical $(\beta/\alpha)_8$ fold. The spectrum shows one prominent negative band at ~208 nm and another very broad but more intense negative band at ~222 nm (likely including the contribution due to β-strands as well as helices). Additionally, aromatic side chains of Tyr, Phe, and Trp may contribute to CD intensities around 222 nm. Internal tryptophan fluorescence studies of purified protein showed a broad fluorescence emission band with λmax_{em} = 325 nm when excited at 295 nm wavelength light (Fig. 3.6C). LiTPI has two Trp (positions 11 and 172), four Tyr, and thirteen Phe residues.

3.4.2 Temperature dependence of stability

The thermal unfolding profile of LiTPI was determined following the change in the far-UV CD spectra of the LiTPI (Fig. 3.7). LiTPI (Tm of 46.5 °C) showed substantial precipitation between 40-50 °C. With the rising temperature, the first change observed was the loss of intensity around 220 nm followed by the loss of the helix signature peak around 208 nm resulting in a β-sheet dominated spectrum.

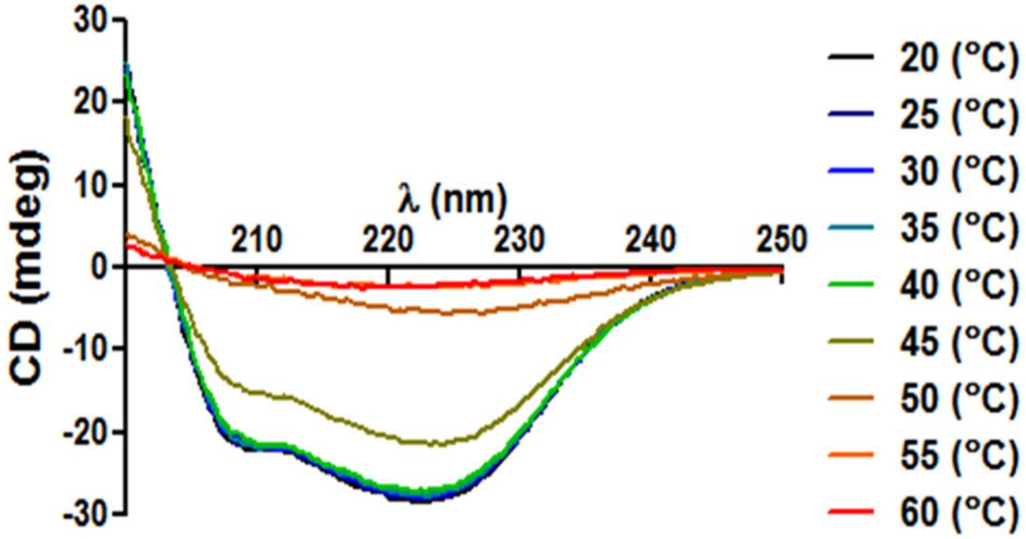


Figure 3.7. Temperature stability of LiTPI.

3.4.3 Chaotrope induced denaturation

Changes in intrinsic protein fluorescence intensity and emission wavelength maxima (λmax_{em}) were followed as a function of increasing chaotropic (urea and guanidine hydrochloride). There was a gradual decrease in fluorescence intensity and shift of the λmax_{em} as the chaotropic concentration was increased. The apparent mid-transition concentrations, Cm, for urea and GdmCl were 2.6 M and 0.8 M, respectively (Fig. 3.8 and Fig. 3.9). The λmax_{em} shows a substantial redshift from 324 to 348 nm between the native and the fully unfolded protein (characteristic of a change in Trp environment from buried to solvated).

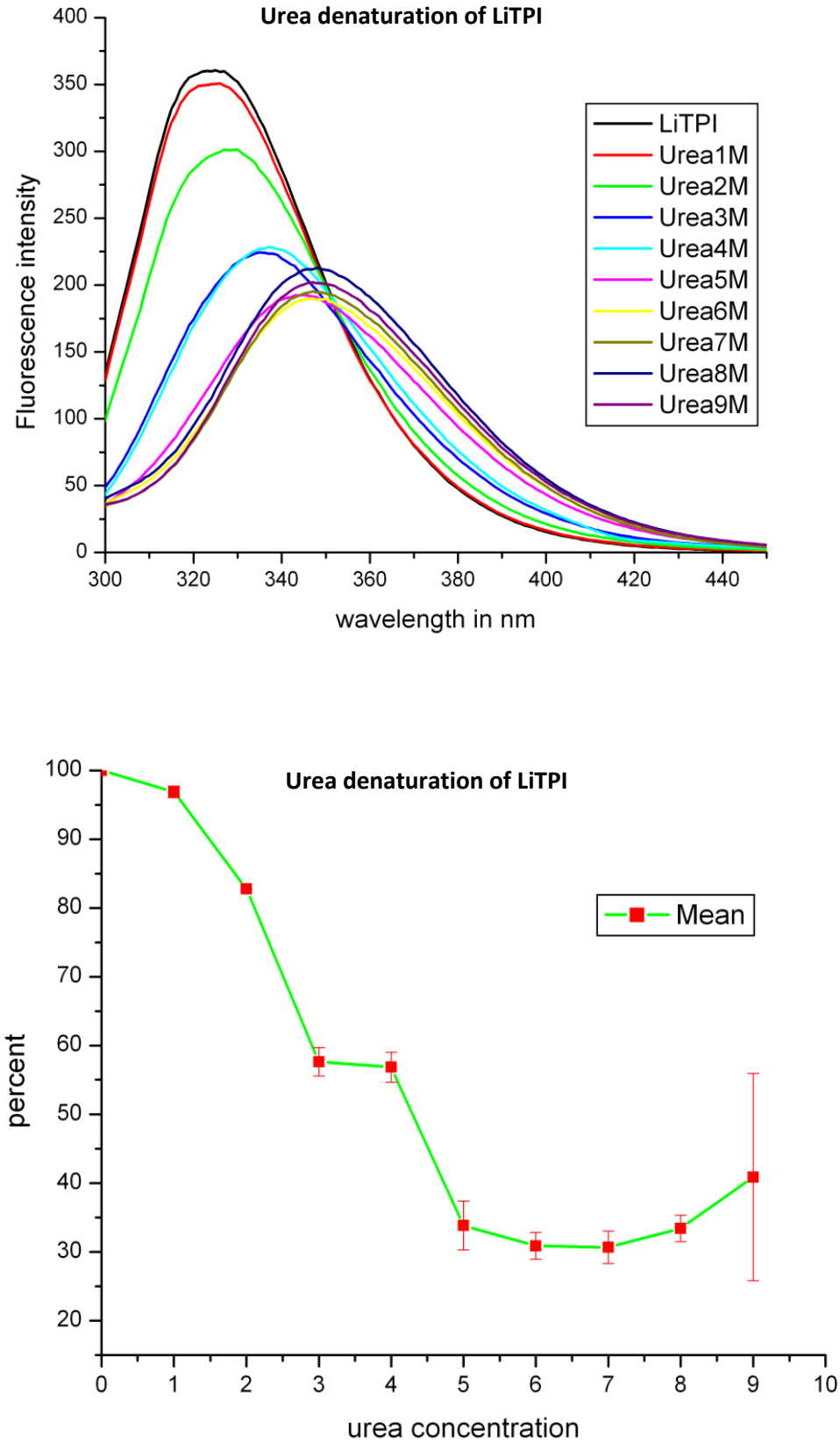


Figure 3.8. Urea denaturation by Fluorescence spectroscopy.

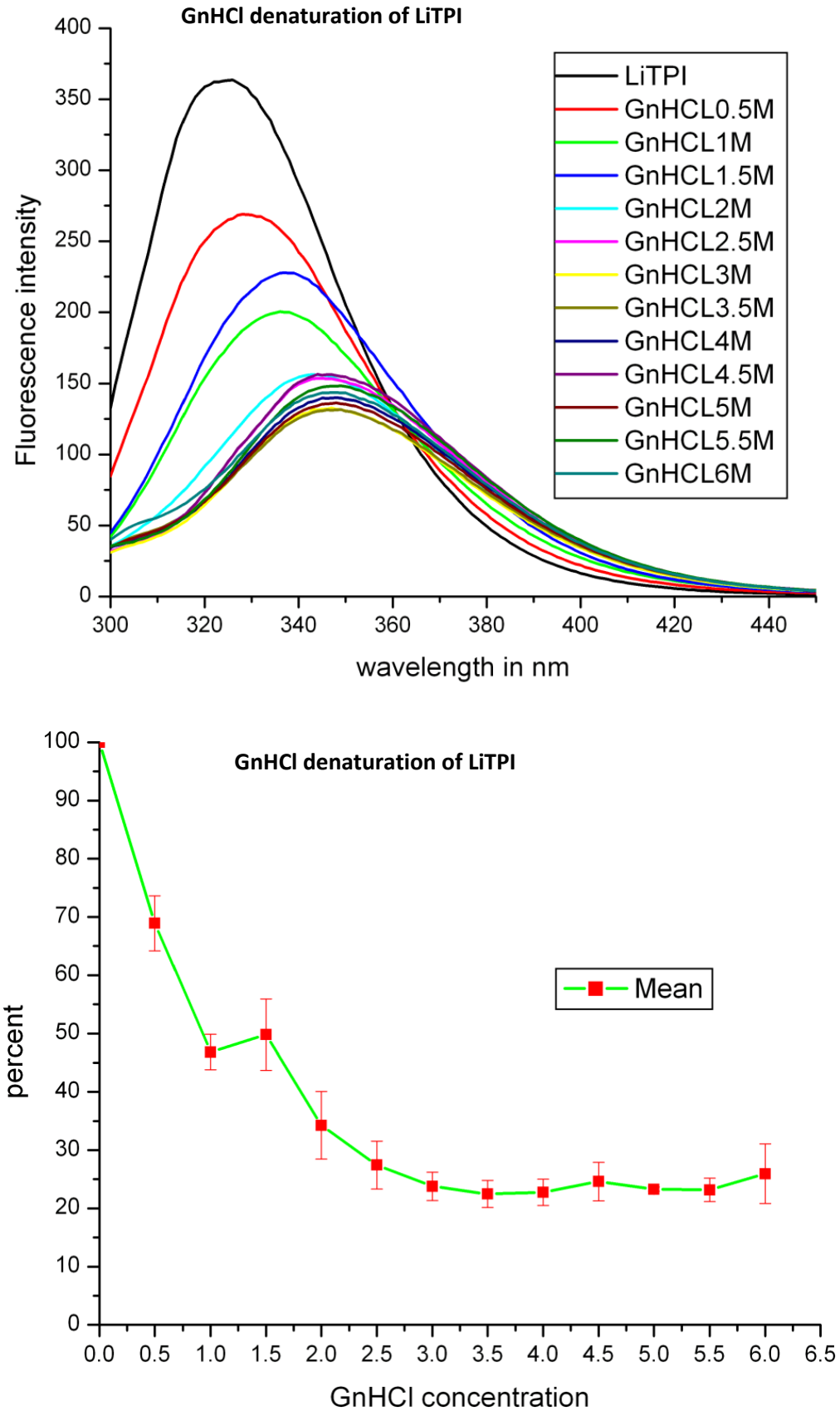
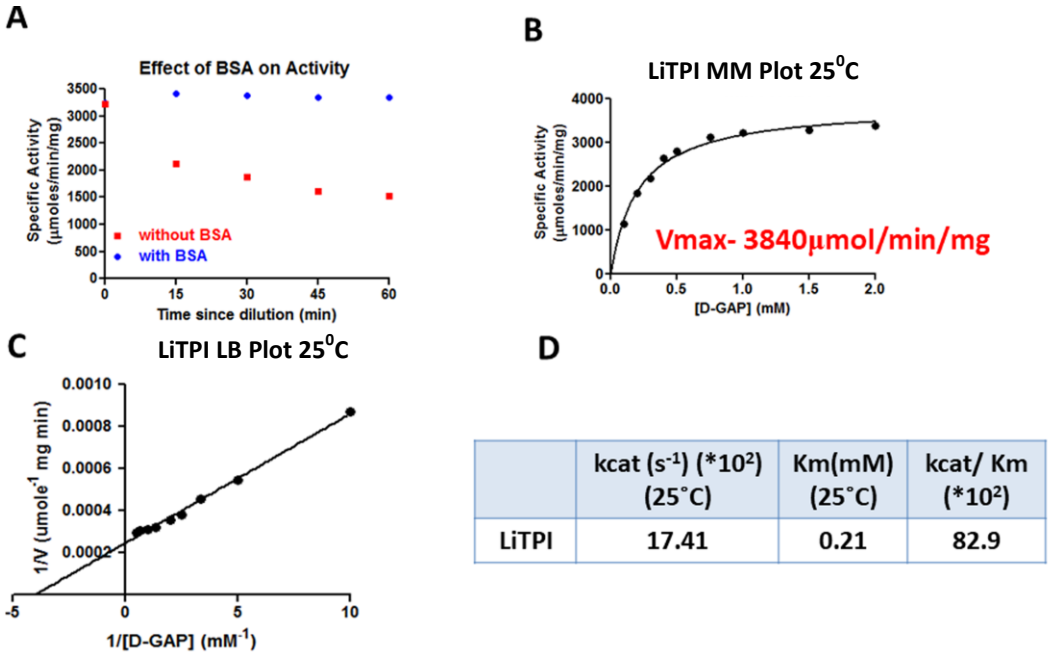


Figure 3.9. Guanidium hydrochloride denaturation by Fluorescence spectroscopy.

3.4.3 Kinetic characterization

To ascertain the functional identity of the cloned and purified protein, we determined the rate of isomerization of D-GAP to DHAP using a continuous, coupled enzyme assay (details in Experimental Procedures Section). We incubated the LiTPI protein with 1.5µM BSA in the dilution mix as well as the reaction mix (BSA alone showed no background activity, data not shown).



	kcat (s^{-1}) (*10^2) (25°C)	Km(mM) (25°C)	kcat/ Km (*10^2)
LiTPI	17.41	0.21	82.9

Figure 3.10. Biochemical characterization of LiTPI. The reduction in a specific activity, upon (A) incubation of diluted enzyme at 4 °C (assay was done at 25 °C). The activity was rescued by adding BSA to the enzyme solution and the assay mix (BSA alone showed no activity, data not shown). (B) The specific activity values were obtained by substrate (D-Glyceraldehyde-3-phosphate) titration. (C) LB plot from the experiment all experiments were repeated at least thrice and were reproducible. The graphs (A) (B) and (C) are one representative experiment. Assays reported in (B) were done with BSA in the reaction mix). (D) Catalytic parameters for triosephosphate isomerase (25 °C) from *Leptospira interrogans*.

With the same concentration of LiTPI as the earlier assays, in the presence of BSA, the specific activity remained constant up to 60 minutes (Fig. 3.10A and Fig. 3.10B). Using this method, D-GAP titration was performed at 25 °C and the specific activity values were fit to the Michaelis-Menten equation (Fig. 3.10D). The measured Km value for Leptospiral TPI when compared with human TPI isoforms (There are at

least two minor forms of triosephosphate isomerase apart from the major form); the Kinetic parameters of these three forms span from 0.2mM - 0.56mM for D-Glyceraldehyde-3-phosphate. The measured Km value for Leptospiral TPI is similar to that of human homologue.

3.5 Discussion

TPI is one of the most efficient enzymes known, enhancing proton transfer by a factor of 10^{10}, and a remarkable molecular catalyst, arguably a 'perfect enzyme' (Albery WJ. *et al.,* 1976; Harris TK. *et al.,*1998; Maldonado E. *et al.,* 1998). This makes it an ideal enzyme to study the adaptability to the given environment over evolution. In leptospira, it is encoded by an operon along with bacterial secretory pathway protein called SecG. Further, to understand the effect of peripheral residues on the function of TPI, we investigated the effect of temperature on activity and stability, and the effect of chaotropic on the stability of the three enzymes. Clearly, the temperature-dependent activity studies do not reflect on the stability of the enzyme, attributed to the diverse conformational sampling of active site residues before enzyme unfolding. Temperature-dependent stability studies shed light on the hydrophobic packing of the enzymes (Delboni LF. *et al.,* 1995). We have not performed the pH-dependent activity studies as the reaction involves a coupled enzyme assay. Therefore, the pH-dependence of the coupled enzyme will lead to complications. Instead, we carried out the Chaotrope induced denaturation studies that shed light on the hydrophilic and ionic interactions involved in the packing of the enzyme.

The 3-Carbon sugar phosphates - DHAP and GAP –that are interconverted by TPI participate in several metabolic pathways and undergo different reactions by being catalyzed by different enzymes. Thus, the equilibrium between GAP and DHAP is likely to dictate the metabolic flux in the direction of energy generation, amino acid metabolism, lipid metabolism (Schumperli M. *et al.,* 2007, van Heerden JH. *et al.,* 2015), and glyoxalate cycle (Sousa Silva M. *et al.,* 2013; Kalapos MP. *et al.,* 1999)

D-Glyceraldhehyde-3-phosphate $\rightleftharpoons$ Dihyroxyacetone phosphate

In general, it has been well documented that TPI prefers Glyceraldehyde-3-phosphate as its substrate over DHAP; but because of the action of downstream three glycolytic enzymes the TPI equilibrium shifts towards the formation of Glyceraldehyde-3-phosphate, so that the organism's glycolytic pathway is driven to meet the energy needs of an organism.

Baseman JP. *et al.*, 1969, predicted based on the investigations of the energy-yielding pathways in Leptospira, that 'in leptospira, the glycolysis might be operating in the opposite direction of glycolysis'. The presence of glyoxalate cycle and the absence of glucose-6-phosphate dehydrogenase, pyruvate dehydrogenase, pyruvate kinase, pyruvate oxidase, and hexokinase in leptospira mandate the organism to depend on Glyceraldheyde-3-phosphate and DHAP for ribose-5-phosphate production. Further, Leptospira, in order to operate pentose phosphate shunt pathway efficiently and compensate for high Km of its Glucokinase, TPI of leptospira might have evolved to work in low concentrations of DHAP and GAP, so that even because upstream enzyme's high Km values leading to the formation very few products, the overall pathway is operated efficiently and elegantly by adapting the downstream enzymes, so that the energy flow pathways are not affected.

It will be interesting to study other downstream enzymes of TPI from various pathways like Glycolysis, pentose phosphate pathway, fatty acid biosynthesis pathways to understand the direction of metabolic flux in the organism.

3.6 Summary and Conclusion

We cloned, expressed, and purified recombinant *Leptospira interrogans* triosephosphate isomerase (LiTPI) from AA200 (*E. coli* TPI null strain). The enzyme was kinetically characterised and thermal stability and Chaotropic stability were established. The availability of the pure enzyme permitted the growth of the diffraction quality crystal resulting in the determination of the 3D structure of Leptospiral TIM (Vidhi pareek, Ph.D. Thesis titled "Structure-Function Studies on Triosephosphate Isomerase: Some Old Questions, Some New Insights" available: **http://etd.iisc.ac.in/handle/2005/4064**).

Chapter 4

Cloning, expression, purification, and identification of purified Putative L-amino acid oxidase by Mass spectrometry

L-Amino acid oxidase

In this study, work has been undertaken to clone and express the Leptospiral L-amino acid oxidase (LAO) gene and to characterize biochemically and structurally the recombinant enzyme, assuming that the N-terminus signal sequence may facilitate protein secretion into the culture supernatant. A first attempt was made to clone and express the full-length *L. interrogans* LAO gene and the results are described in this chapter. The subsequent chapter describes the biophysical and crystallographic characterization of the Li-rLAO that lacks N-terminus secretory sequences.

4.1.1 Kidney and Leptospiral infection

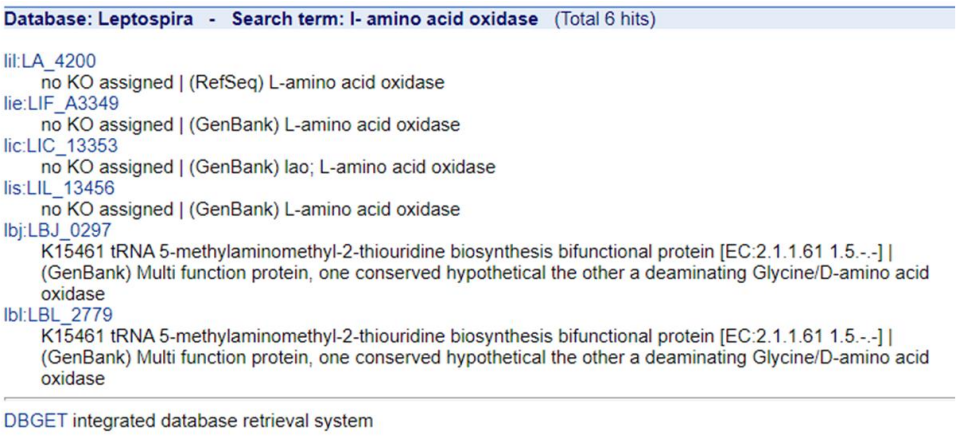
The kidney is the main target of *Leptospira sp*. in both acute and chronic infections. Acute kidney injury as a result of tubulointerstitial nephritis is an early and primary manifestation of systemic leptospirosis (Yang CW. *et al.,* 2001). Early *in vitro* observation has revealed that pathogenic, but not non-pathogenic Leptospira, attached to cultured renal epithelial cells, soon after adding to culture cells (Ballard SA. *et al.,* 1986). Ultrastructural study of the kidney after inoculation of *Leptospira sp*. in mice demonstrated that the entry route of *Leptospira species* is by penetration of the capillary lumen at day 2, followed by entrance into the interstitial tissue, elaborating edema and cellular infiltration at days 4–8. *Leptospira sp.* can be found in the proximal tubular cells on day 10 (Marshall RB. 1976; Morrison WI. 1976).

In most mammals, ≈99% of filtered amino acids are reabsorbed in the proximal tubule, which spares ≈70 g amino acids/d in a 70-kg person, Fractional excretions of most amino acids are between 0.2% and 2.5%, although this proportion may increase in various pathologic conditions ((Silbernagl S. 1983a, 1983b; Brosnan JT. 1986; Silbernagl S. 1988; Dantzler WH. *et al.,* 1988; Nakanishi T. *et al.*,1991; van de Poll MC. *et al.,* 2004).

In the human kidney, there is a general L-amino acid oxidase that catalyses the oxidative deamination of almost all amino acids, but this has low activity and is not important in terms of amino acid metabolism. In the kidney, compared to L-amino acid oxidase, D-amino acid oxidase has a relatively high activity, which is important for detoxification of D-amino acids derived from bacteria and a few other sources (Sacchi S. *et al.,* 2012; Pollegioni L. *et al.,* 2018).

4.1.2 Leptospiral LAO

The proximal tubule of the kidney is a rich source of amino acids. Leptospira find a safe haven here evading the immune system and utilising the rich nitrogenous source for survival. In order to harvest the rich source of amino acids available in the kidney, Leptospira may have evolved to produce enzymes for the utilisation of these amino acids. We speculated that in view of the relatively low levels of activity of L-amino acid oxidases (LAO) in the human kidney, pathogenic leptospira may produce a secreted L-amino acid oxidase enzyme, with consequent cellular uptake of the resulting alpha- ketoacids. Initial Kyoto Encyclopedia of Genes and Genomes (KEGG) database analysis revealed the presence of L-amino acid oxidase in the pathogenic strains of Leptospira (Fig. 4.1).



Database: Leptospira - Search term: l- amino acid oxidase (Total 6 hits)

lil:LA_4200
no KO assigned | (RefSeq) L-amino acid oxidase
lie:LIF_A3349
no KO assigned | (GenBank) L-amino acid oxidase
lic:LIC_13353
no KO assigned | (GenBank) lao; L-amino acid oxidase
lis:LIL_13456
no KO assigned | (GenBank) L-amino acid oxidase
lbj:LBJ_0297
K15461 tRNA 5-methylaminomethyl-2-thiouridine biosynthesis bifunctional protein [EC:2.1.1.61 1.5.-.-] | (GenBank) Multi function protein, one conserved hypothetical the other a deaminating Glycine/D-amino acid oxidase
lbl:LBL_2779
K15461 tRNA 5-methylaminomethyl-2-thiouridine biosynthesis bifunctional protein [EC:2.1.1.61 1.5.-.-] | (GenBank) Multi function protein, one conserved hypothetical the other a deaminating Glycine/D-amino acid oxidase

DBGET integrated database retrieval system

Source : https://www.genome.jp/kegg-bin/show_organism?category=Leptospira

Figure 4.1. KEGG database search results for amino acid oxidase in Leptospira genus.

A search of the NCBI database yielded a reference sequence (NCBI Ref. Sequence: WP_000778112.1 &NZ_LMAQ010000327.1; UniProt ID: IQ65_19870). In the NCBI database, the sequence was annotated as NAD(P)/FAD-dependent oxidoreductase, whereas in UniProt the same sequence was initially annotated as a putative *Leptospira interrogans* L-amino acid oxidase (Li-LAO).

>WP_000778112.1 NAD(P)/FAD-dependent oxidoreductase [Leptospira interrogans]

MKLSRSEFIKLGILTAAGISGLPGIKLSAQGTSSRKTVIVMGGGISGLYASYLLS
KTGIKVQLIEATDRLGGRIRTVTDVSGNFLDLGAEWIQAEHRTAKSLIRELGL
KTTDFEVQSDLFFGSYRKFGTWDISPKSQEILNKLVQMNSKINSTQQQELDRIS
FYNFLNYQGMSLEDLNILNFKYSLYYGDSLRSLSAQKVLSDLVNFPKYNTRV
EGGMETLTRALVSSLENTEIIFSDPVVSVSQGEGKVIVTTVSGKKIEGNACISTL
PANQLTTIQWDPELDKEKKLSALRIRYSRIYKTFLMLREAPWTRGSFSAYSDS
VAGFIYDAGTKINSEDKILGMISTGDRYDILASSTDAMKVEYIRLALESLGQGR
ELQVLRIQSSETSQSKFIPTGIATFPPGSYGSIISLLKPMDRIFFAGEHTAELNGT
VEGALASAIRAVNQV

This 447 residue polypeptide sequence contains an N-terminus putative signal peptide which may be cleaved after transmembrane transport. Proteomic analysis has provided evidence for secreted LAO in the cultures of leptospira (Eshghi A. *et al.*, 2015). There exists no experimental evidence in the literature regarding the biochemical characterization of the putative Li-LAO sequence.

The putative Li-LAO sequence displays relatively little homology to well-characterized LAO from other organisms with a sequence identity of only about 20 percent (Fig. 4.2).

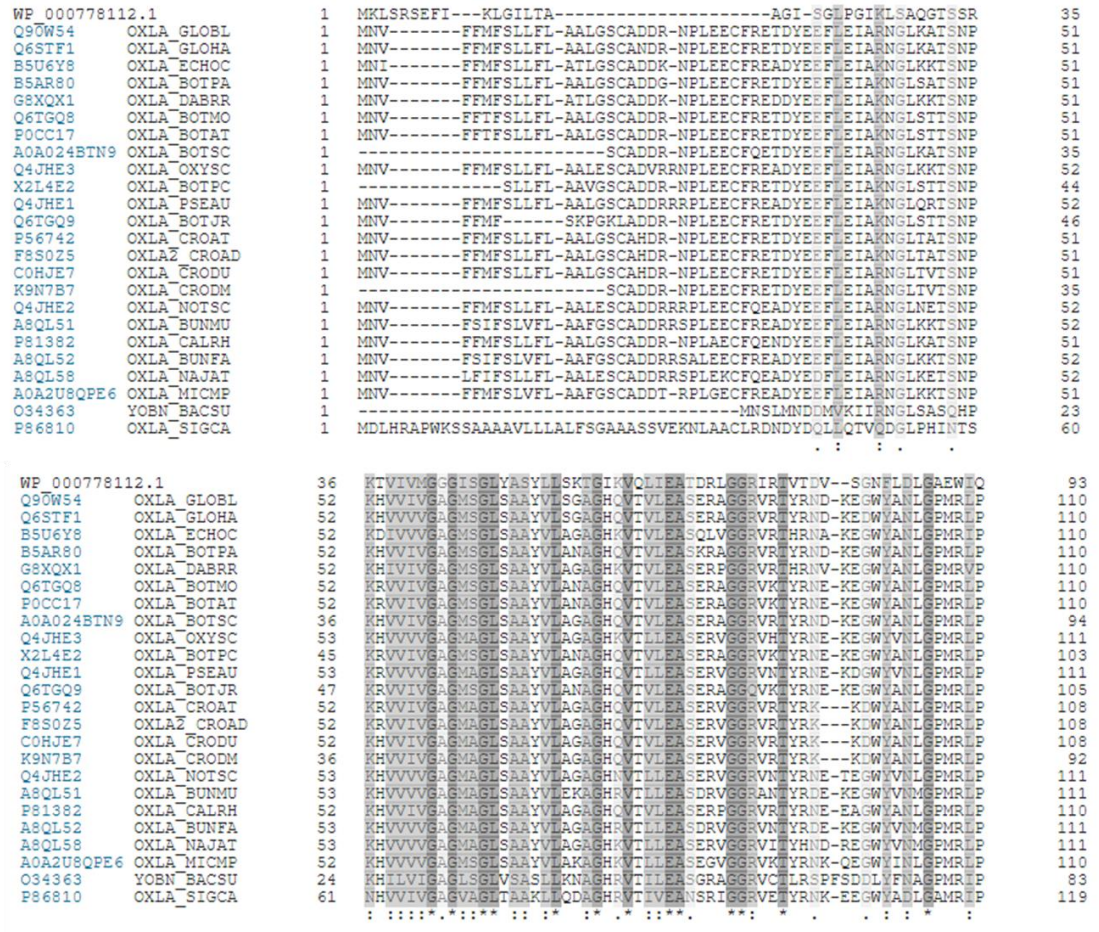


Figure 4.2. Sequence alignment of Leptospiral LAO with reviewed sequence from UniProt database.

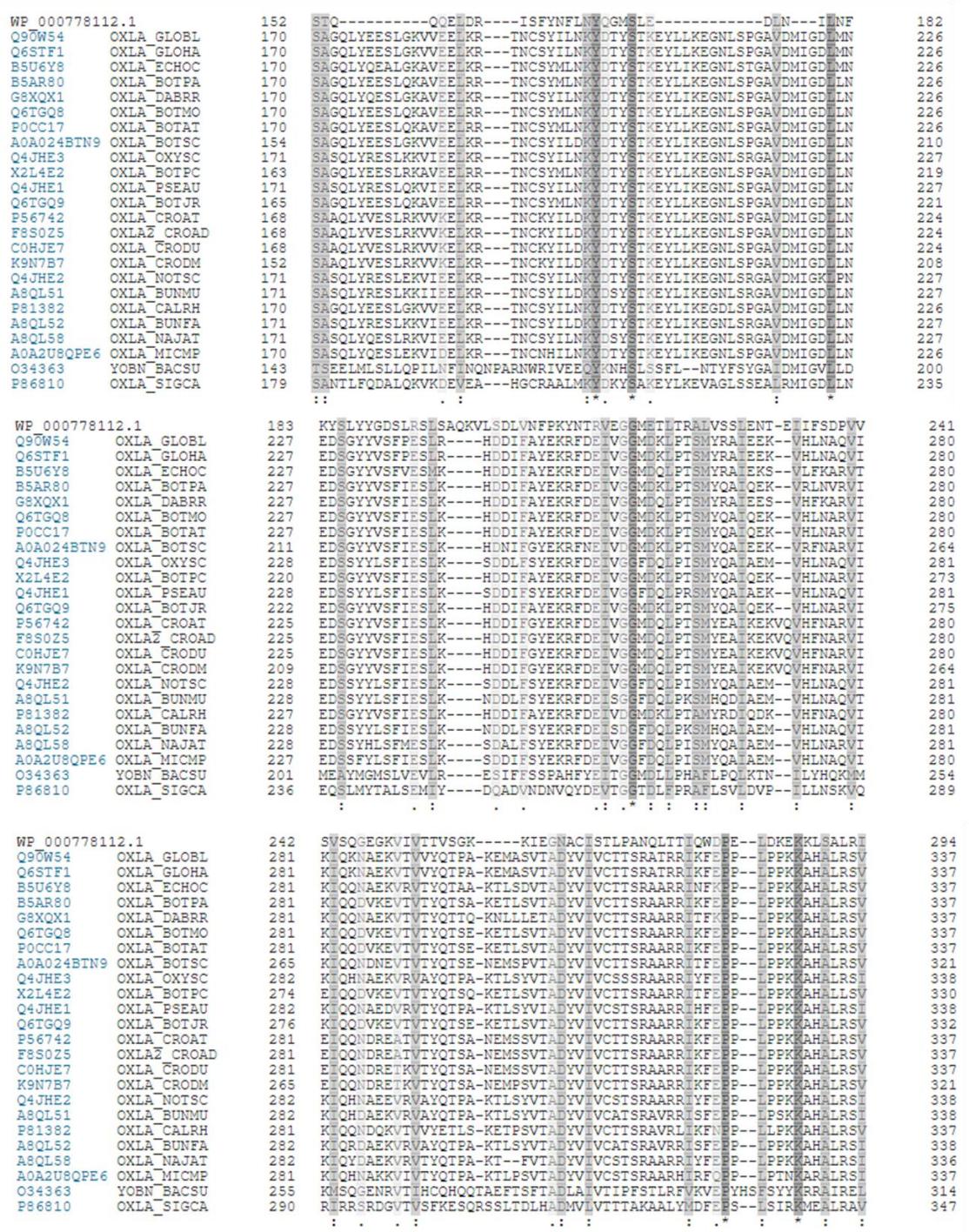


Figure 4.2. Sequence alignment of Leptospiral LAO with reviewed sequence from UniProt database.

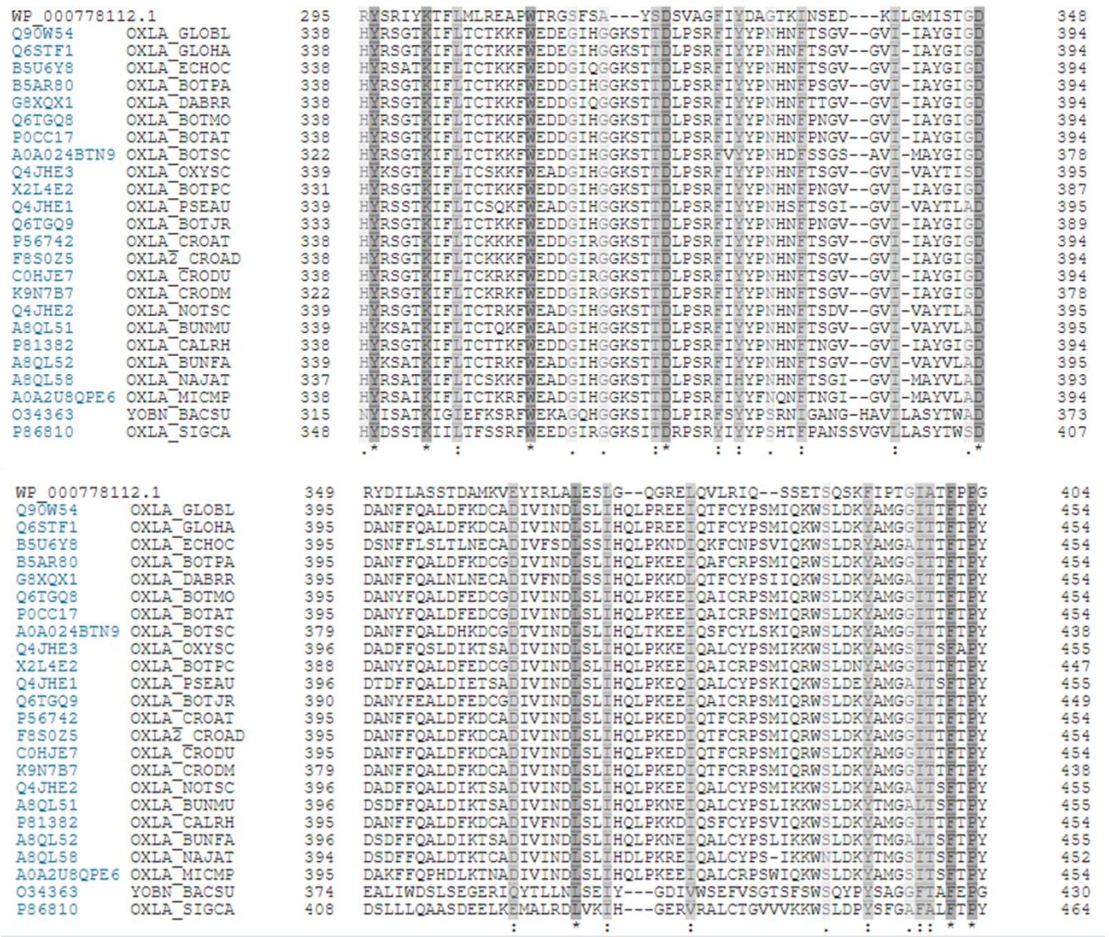


Figure 4.2. Sequence alignment of Leptospiral LAO with reviewed sequence from UniProt database.

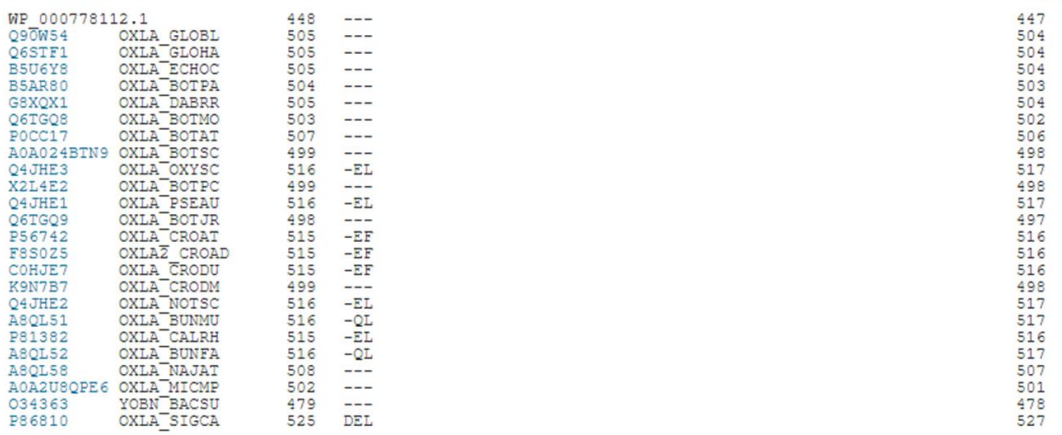


Figure 4.2. Sequence alignment of Leptospiral LAO with reviewed sequence from UniProt database.

Before detailing the experimental studies presented in this chapter a brief review of the properties of LAO from other organisms is presented.

4.1.3 Amino acid oxidase

Amino acid oxidases (AAOs) are enzymes that oxidize amino acids releasing ammonium and hydrogen peroxide. Amino acid oxidases can be classified based on the chirality of the amino acid used as substrate. 1) D-Amino acid oxidases (DAAOs or DAOs), EC 1.4.3.3, are flavoproteins showing strict specificity for D-amino acids (Pollegioni L. *et al.,* 2008; Pollegioni L. *et al.,* 20011; Campillo-Brocal JC. *et al.,* 2015). They catalyze the oxidative deamination of neutral and basic D-amino acids to give α-keto acids, ammonium, and hydrogen peroxide (Pollegioni L. *et al.,* 2008; Takahashi S. *et al.,* 2015). 2) L-Amino acid oxidases, EC 1.4.3.2, (commonly abbreviated as LAOs or LAAOs) are flavoenzymes that oxidize L-amino acids releasing the corresponding α-keto acid in addition to ammonium and hydrogen peroxide.

4.1.4 L- Amino acid oxidase

Zeller first described L-amino acid oxidase (LAO; EC 1.4.3.2) in 1944 (Zeller A. *et al.,* 1944; Hossain GS. *et al.,* 2014). The LAOs from animals are clustered into gastropod enzymes (that are evolved separately in the innate immune system) and into vertebrate enzymes (that include snake venoms, fishes, and mammals) (Hughes AL. *et*

al., 2010; Kasai K. *et al.,* 2015). The sea hare *Aplysia californica* LAO is the best-studied gastropod LAOs. It is synthesized in a separate gland and its substrates (Lys and Arg) are synthesized from a separate gland. The enzyme and the substrate are mixed at the time of the attack of the predator (Johnson PM. *et al.,* 2006; Ko KC. *et al.,* 2008; Ko KC. *et al.,* 2012). Snake venom LAOs (svLAOs) is best-studied vertebrate enzymes. svLAOs show substrate specificities with a preference towards hydrophobic amino acids such as L-Leu, L-Phe, and L-Met (Du XY. *et al.,* 2002; Calderon LA. *et al.,* 2014; Izidoro LF. *et al.,* 2014; Campillo-Brocal JC. *et al.,* 2015). The skin of the fish is a barrier preventing the infection by microorganisms (Kitani Y. *et al.,* 2008). In mammals, the interleukin 4-induced gene (IL4I1) codes for an L-amino acid oxidase with a strong preference for L-phenylalanine as the substrate in order to regulate the immune system (Boulland ML. *et al.,* 2007).

4.1.5 LAOs are Flavoproteins

svLAOs are usually homodimeric glycoproteins with non-covalently linked FAD (Flavin Adenine Dinucleotide) or FMN (Flavin Mononucleotide) cofactors with subunit molecular weights of around 50–70 kDa. In some flavoproteins, the FAD-binding can also be covalently linked. The purification of LAOs is in fact facilitated because of the presence of the pigment riboflavin that imparts the yellow coloration.
The subunits form a dimer by non-covalent interactions (Izidoro LF. *et al.,* 2014). svLAO can be found as acidic, neutral, and basic forms of the protein (Du *et al*., 2002). X-ray crystallographic studies confirm that svLAO is a functional dimer with each dimer having three domains: FAD-binding; substrate-binding; and a helical domain (Pawelek PD. *et al.,* 2000).

FAD binding motifs can be broadly classified into 15 different families. Amino acid oxidase belongs to the Glutathione reductase family. The FAD is bound by a Rossman fold variant motif consisting of β1α1β2α2β3 fold. The isoalloxazine ring of the FAD is essential for catalytic function, whereas the ribityl phosphate and the AMP moiety stabilize cofactor binding to protein residues. The flavin functions mainly in a redox capacity, by taking two electrons from one substrate and release them two at a time or one at a time to the second substrate or coenzyme. The protein moiety surrounding the cofactor controls many of the catalytic properties of the flavin

ring, such as the rate of accepting electrons, the pathway of electron flow within the flavin ring, and the flavin's oxidation-reduction potential (Mathews FS. 1991; Dym O. *et al.*, 2001).

The catalysis is proposed to follow one of two diverse mechanisms: in the first pathway the proton is transferred to the FAD cofactor from the α- carbon atom of the substrate, leaving a negative charge followed by a two-electron transfer which is also called a carbanion pathway; and in the second mechanism, an α hydrogen atom is transferred as a hydride ion carrying two electrons simultaneously by a hydride transfer pathway (Gaweska H. *et al.*, 2004).

In some cases, the activity also depends on the presence of other compounds in the protein apart from the flavin cofactor. In some cases, the enzymatic activity of a protein can be changed from L-amino acid oxidase to monooxygenase by changing the assay conditions (Matsui D. *et al.*, 2014). In many LAOs the actual enzymatic activity could be different from the one initially described (Tong H. et al., 2008; Zhou P. *et al.*, 2012).

4.1.6 Physiological functions

Numerous biological effects, such as antiparasitic, antimicrobial, apoptotic, cytotoxicity, edema, hemolysis, hemorrhage, inducing or inhibiting platelet aggregation, etc., have been reported for those enzymes (Ahn MY. *et al.*, 2000; Sakurai Y. *et al.*, 2001; Hanane-Fadila ZM. *et al.*, 2014; Vargas Muñoz LJ. *et al.*, 2014; Tan KK. *et al.*, 2018; Zainal Abidin SA. *et al.*, 2018). All these activities are by hydrogen peroxide generated by the basification of the medium due to ammonia accumulation and the deprivation of the amino acid (Puiffe M. *et al.*, 2013; Campillo-Brocal JC. *et al.*, 2015; Tan KK. *et al.*, 2018). LAOs can also be intracellular, membrane-bound, or extracellular. LAOs can be inactivated by decreasing the pH and vice versa (Wellner D. 1966), and inactivation by freezing is more pronounced between −20 and −30 °C (Curti B. *et al.*, 1968; Curti B. *et al.*, 1992).

4.1.7 Microbial LAOs

In contrast to snake venom LAO, very little is known about LAO from microorganisms. Although various LAO-coding sequences have been published revealing that LAO family members commonly have Flavin as a coenzyme and possess two conserved and characteristic sequence motifs, the "GG" motif (RxGGRxxS/T) and dinucleotide-binding (DMB) motif (β-strand/α-helix/β-strand). In recent years, many bacterial enzymes showing LAO activity have been reported (Hossain GS. *et al.,* 2014). The bacterial enzymes do not cluster into any defined groups (Campillo-Brocal JC. *et al.,* 2015). The enzymatic properties of LAOs from microbial sources are usually dissimilar with respect to the optimum pH, substrate specificity, and stability (Leese C. *et al.,* 2012; Liu L. *et al.,* 2013; Hossain GS. *et al.,* 2014). The LAO isolated from *Rhodococcus opacus* oxidizes 39 L-amino acids, including all of the 20 L-amino acids and their derivatives (Geueke B. et al., 2002).

The subsequent sections of this chapter detail the cloning, expression, and purification of full-length Leptospiral L-amino acid oxidase (Li-LAO).

4.2 Materials

E.coli Strain Top10, *E.coli* Strain DH5α *E.coli* Strain BL21(DE3), C41(Generous gift from Prof. Aravind pennmatsa), pET21b plasmid, 6x DNA loading dye, PR DNA Polymerase, dNTPs, protein markers, DNA ladder from Bangalore Genei, Bangalore, India; Pre-stained marker (Thermo scientific), NdeI endonuclease, XhoI endonuclease, T4 DNA ligase from NEB; Tris, GnHCl, Urea, BSA, FAD, NADH, Dialysis membrane, EtBr, DTT, Agarose, SDS, PMSF from Sigma; Primers from Eurofins, India; NaCl, EDTA, Glycerol, Ammonium sulfate, HCl, Acrylamide, Bis-acrylamide, Bromophenol blue, Ammonium persulfate, TEMED, Glycine from SDFCL, amino acid substrates L-Histidine, L-Alanine, L-Tryptophan, L-Methionine L-Glutamine, L-Asparagine, L-Glutamate, L-Aspartate, L-Serine, L-Arginine, L-Proline, L-Tyrosine from Sigma (Generous gift from Prof P. Balaram and Prof Jayanta Chatterjee) and L-Phenylalanine, L-Leucine, L-Isoleucine, L-valine, L-Threonine from SRL.

4.3 Methods

4.3.1 Cloning, protein expression, and purification of Li-LAO

Li-LAO gene was amplified from cDNA obtained from five days old Ellinghausen–McCullough–Johnson–Harris (EMJH) culture of *Leptospira interrogans* (Li) serovar Icterohaemorrhagiae strain Lai (Regional Medical Research Centre, Indian Council for Medical Research, Port Blair, India; ATCC: 23581. Taxonomy ID: 1291351) by colony PCR (cell pellet from 30 µL of culture was resuspended in 60µL of autoclaved milli-Q water followed by heating at 95˚C for 20min. 15µL of the lysate thus obtained was used as a template for 50µL PCR reaction mix). The PCR reaction was carried out using LAOIFP 5'GATAGCATATGAAACTAAGCAGATCGGAGTTTATCAAAC3' and LAORP 5'AATTCTCGAGTCAAACCTGATTGACCGCACGAATCG3' (Sigma-Aldrich Co, India) as the forward and the reverse primers, inserting NdeI and XhoI endonuclease sites at the 5´ of the start and 3´ of the stop codons of the gene, respectively.

The PCR mixture contained, in a total volume of 25 µl: template DNA, 100 ng; mutagenic primer, 20 pmol; thermostable polymerase buffer (10X), 2.5 µl; dNTPs, 6 µl of a solution containing 2.5 mM of each dNTP; and polymerase 2 U. Following the hot-start PCR method, PR polymerase (Bangalore Genei, Bangalore, India) was added to the reaction mix after initial denaturation at 95˚C for 5 mins. Subsequently, 28 cycles of the steps, 1) 95˚C for 15 sec, 2) 55˚C for 20 sec, 3) 68 ˚C for 90 sec with a final extension at 68˚C for 5 min yielded the amplified PCR product which was then purified (Qiagen Gel purification kit) and digested with Nde1 and XhoI restriction endonucleases (NEB, India) for inserting the gene into the pET21b expression vector. The ligated product was transformed into Top10 cells and plated on LB agar containing 100 µg/mL ampicillin. 10 colonies were screened for a positive clone (with Li-LAO insertion) by carrying out restriction digestion with Nde1 and XhoI enzymes on the plasmid obtained from the respective colonies and confirmation by primer-specific PCR amplification, followed by confirmation with gene sequencing (final gene length 1330 bp).

Expression of the Li-LAO gene was performed using the pET21b system. *E. coli* BL21(DE3) cells carrying the pET21b recombinant vector were grown at

35 °C in terrific broth containing 100 μg·mL^{-1} ampicillin. Cells were induced using 300 μM isopropyl-β-D-thiogalactopyranoside until they reached an optical density at 600 nm of 0.6–0.8, and were then harvested by centrifugation (15 min, 7245 ***g*** at 4 °C). Cells were resuspended in lysis buffer containing 20 mM Tris/HCl pH 7.5, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 2 mM dithiothreitol, and 10% glycerol, and disrupted using sonication. After centrifugation (45 min, 19 320 ***g*** at 4 °C), the protein fraction was subjected to Heparin column (Amersham Biosciences, Uppsala, Sweden) and eluted with a linear gradient of 0–1 M NaCl. Protein purity was checked by 10% SDS–PAGE.

E. coli strain C41 was transformed with pET21b containing the recombinant Li amino oxidase gene and grown in LB for 8-10 hrs with 100 μg/ml ampicillin. The culture was used as a 1% pre-inoculum in Louria Bertuni broth (LB) culture grown at 37 °C. After induction with 500 μM IPTG (OD600nm 0.6-0.8), the culture was allowed to grow for another 16hrs at 30 °C. Cells were harvested by centrifugation (20 min, 6K rpm, 4˚C), resuspended in lysis buffer containing 20 mM Tris-HCl (pH 6.8), 1 mM EDTA, 1 mM PMSF, 1 mM DTT, and 10% glycerol and disrupted by sonication (35% amplitude, 20sec off and 10sec on, 25 cycles). After centrifugation (45 min, 12K rpm, and 4°C) and removal of the cell debris, the supernatant was SP sepharose cation exchange chromatography. The column was equilibrated with 20mM Tris (pH 6.8), the protein was bound to the column with a flow rate of 0.5ml /minute and washed with 20 mM Tris-HCl (pH 6.8), the bound protein was eluted with a linear gradient containing buffer A (20 mM Tris-HCl, pH 6.8) and buffer B (20 mM Tris-HCl, pH 6.8 with 1M sodium chloride) with a flow rate of 1ml/ minute. The 10ml fractions are collected in a fraction collector.

Further, all the pooled fractions were concentrated in 30KDa cutoff centricon (4000 rpm, 4°C). The concentrated protein was subjected to gel filtration chromatography (Superdex-200, XK26 column, Amersham Biosciences), equilibrated with the 20 mM Tris-HCl (pH 6.8 with 100mM sodium chloride) (ÄKTA Basic 10 HPLC system, GE healthcare). Protein purity was checked on SDS-PAGE and LC-ESI/MS was done to determine the correct mass of the protein.

4.3.2 Mass spectrometry

Electrospray ionization mass spectra were recorded on maXis impact Q-TOF(BrukerDaltonics, Bremen, Germany) coupled to Agilent 1200 series online HPLC. The spectrometer was tuned by using a standard Agilent ESI Tune mix ranging from m/z 118 to 2721 in the positive ion mode. Data processing was done using the deconvolution module of the 'Data Analysis 4.1' software (BrukerDaltonics, Bremen, Germany) to detect the multiple charge states and obtain derived masses.

4.3.3 In-gel trypsin digestion and MS/MS

Li-LAO protein (20-10 ng per lane) was run on 10% SDS-PAGE (reducing) and stained with the Coomassie Brilliant Blue staining method. The bands corresponding to Li-LAO were cut out and processed for tryptic digestion using sequencing grade modified Trypsin (Promega Corporation) (Shevchenko *et al*., 2006). In brief, stained gel pieces were excised minced into $1mm^3$ pieces, and transferred into a sterile centrifuge tube. Gel pieces were washed with 500µl of wash buffer (50% acetonitrile, 50mM ammonium bicarbonate), till the coomassie dye is removed. De-stained gel pieces were dehydrated in 100% acetonitrile for 5 minutes and rehydrated in 150µl reduction solution (10mM DTT, 100mM ammonium bicarbonate) for 30 min at 56^0C. The reduction solution is discarded and incubated with 100µl of alkylating solution (50mM iodoacetamide, 100mM ammonium bicarbonate) for 30 minutes in dark at room temperature. Reduced and alkylated gel pieces are washed with wash solution and dehydrated with 100% acetonitrile for 5 minutes and completely dried at room temperature in a centrifugal evaporator. Gel pieces were rehydrated with 20µl of sequencing grade trypsin (20µg/ml) digestion solution and incubated overnight at 37ºC.

The digested peptides are extracted from the gel pieces with extraction solution (50% acetonitrile, 0.1% TFA), the extracted peptides from each sample were further concentrated in a vacuum concentrator and were subjected to MS-MS analysis.

4.3.4 Identification of cofactor

LAO protein was subjected to LC-MS in the positive mode to identify the bound cofactor. In order to find the nature of the interaction between protein and the

bound cofactor, the protein was dialyzed with a 3000-dalton cutoff and the dialyzed sample was subjected to LC-MS analysis.

Amino acid oxidase assay

The activity of LAO is measured as the amount of hydrogen peroxide formed which is in turn used by Horseradish peroxidase to oxidize o-Phenylenediamine to 2, 3-Diaminophenazine which is measured at 420nm. The Protein concentration was determined by Bradford's assay.

The assay was conducted in a 96-well microplate in triplicate with modification (Kishimoto M. *et al.*, 2001); 10 µl/well of enzyme solution (10µM-30 µM), 90µl/well of substrate solution were added to start the reaction. The standard reaction mixture contained 5 mM L-amino acid, 2 mM ortho-Phenylene Diamine (OPD), 0.81 U/ml Horseradish peroxidase (HRP), in a total volume of 100µl/well of 50mM borax–HCl buffer (pH 8.5). After incubation at 37°C for 60 min, the reaction was terminated by adding 50µl of 2M H_2SO_4. The absorbance of the reaction mixture was measured by a microplate reader at 492 nm, using 630 nm as a reference wavelength (492/630 nm).In the time-course experiment, absorbance was measured at 420/630 nm, instead of 492/630 nm, at appropriate time intervals without adding 2M H_2SO_4 to terminate the reaction.

4.4 Result

4.4.1 Cloning, expression, and purification of *Leptospira interrogans* L-amino acid oxidase gene

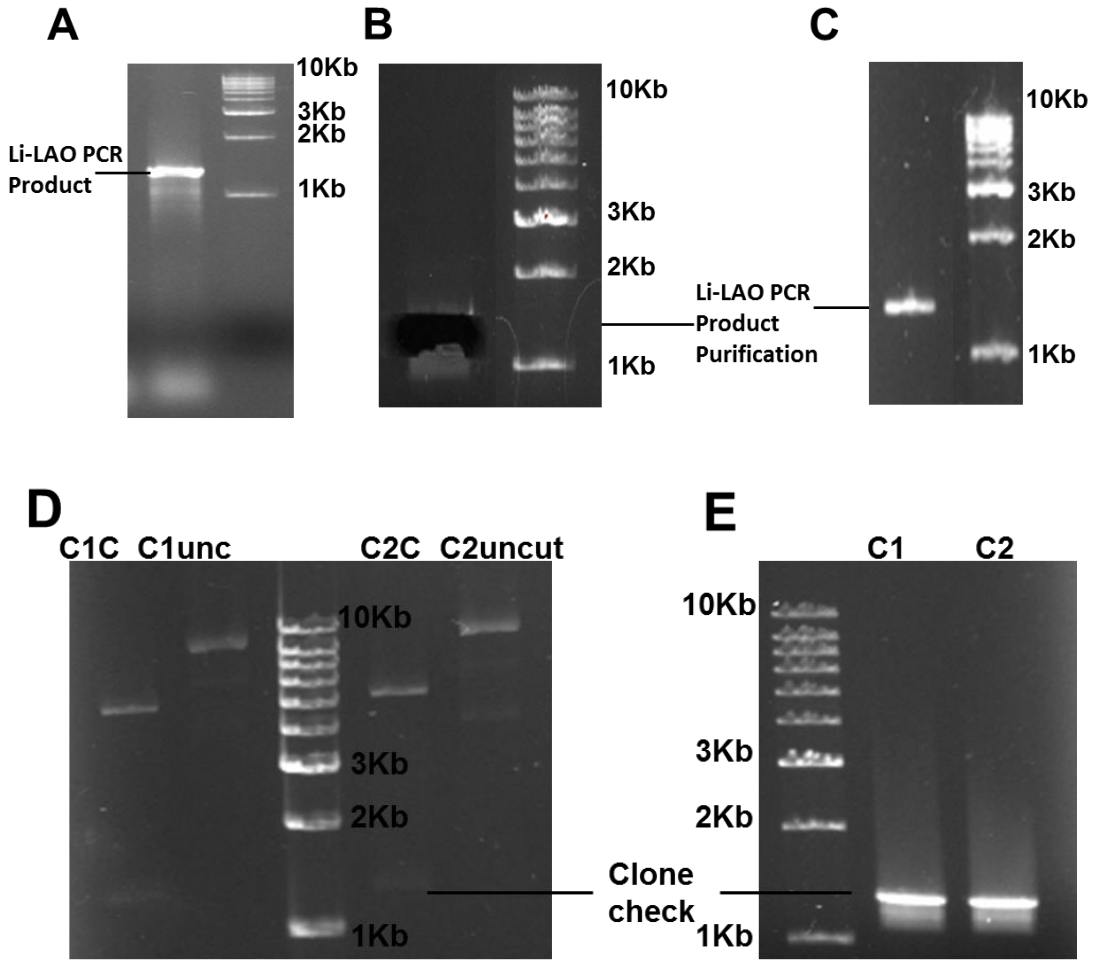


Figure 4.3. Agarose Gel showing cloning of *Leptospira interrogans* L-amino acid oxidase gene. (A) Purified PCR product of *Leptospira interrogans* L-amino acid oxidase gene. (B & C) Purification of Leptospira interrogans L-amino acid oxidase gene. (D) Screening of pET21bLAO clones by restriction digestion (Nde1 and XhoI Digestion). (E) Screening of pET21bLAO clones by gene-specific primer PCR.

Leptospira LAO gene was amplified with hi-fidelity enzyme and the 1330 basepair PCR product was gel purified and cloned in Nde1 and XhoI sites of pET21b vector. Clones were identified by restriction digestion and gene-specific primers (Fig. 4.3).

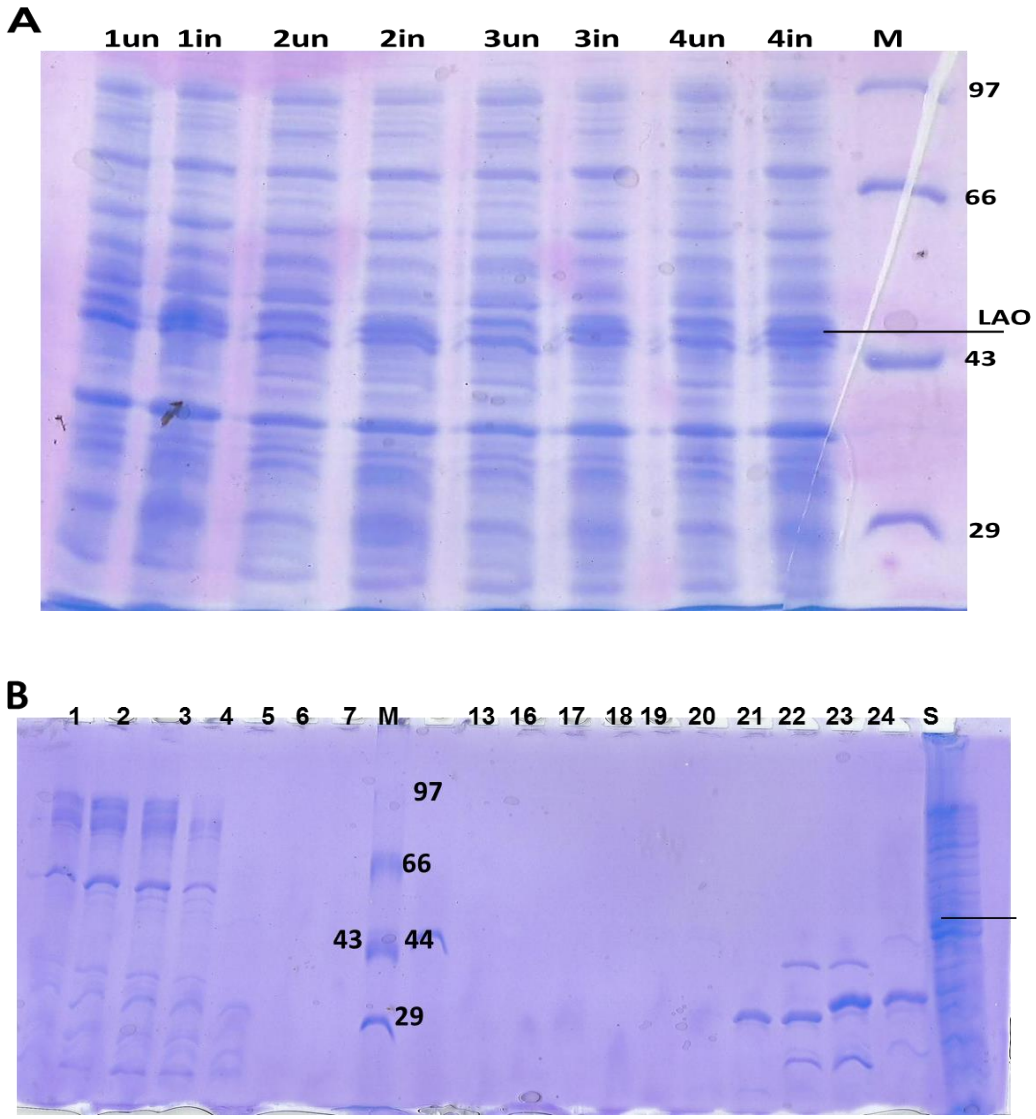


Figure 4.4. Expression of Li-LAO in *E.coli* BL21 (DE3) Strain (A) SDS-PAGE Gel showing Expression of Li-LAO in *E.coli* BL21 (DE3) Strain. (B) SDS-PAGE Gel showing Purification of Li-LAO using Heparin sulfate column.

One of the clones was chosen and the sequence of Li-LAO gene was sequenced in Genetic Analyzer using Bigdye terminator 3.1. The sequence of cloned Li-LAO sequence was compared with the reference sequence. Li-LAO gene was intact with few silent mutations (appendix 1).

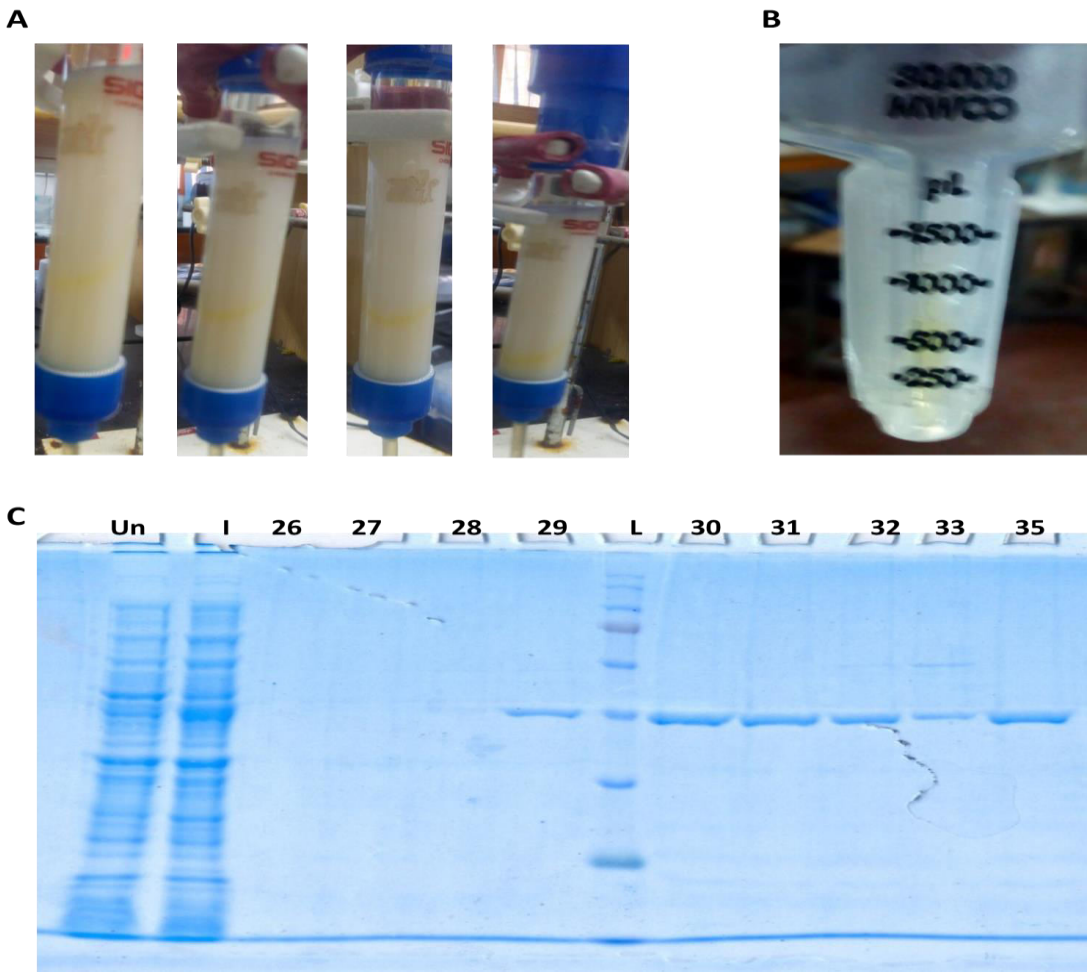
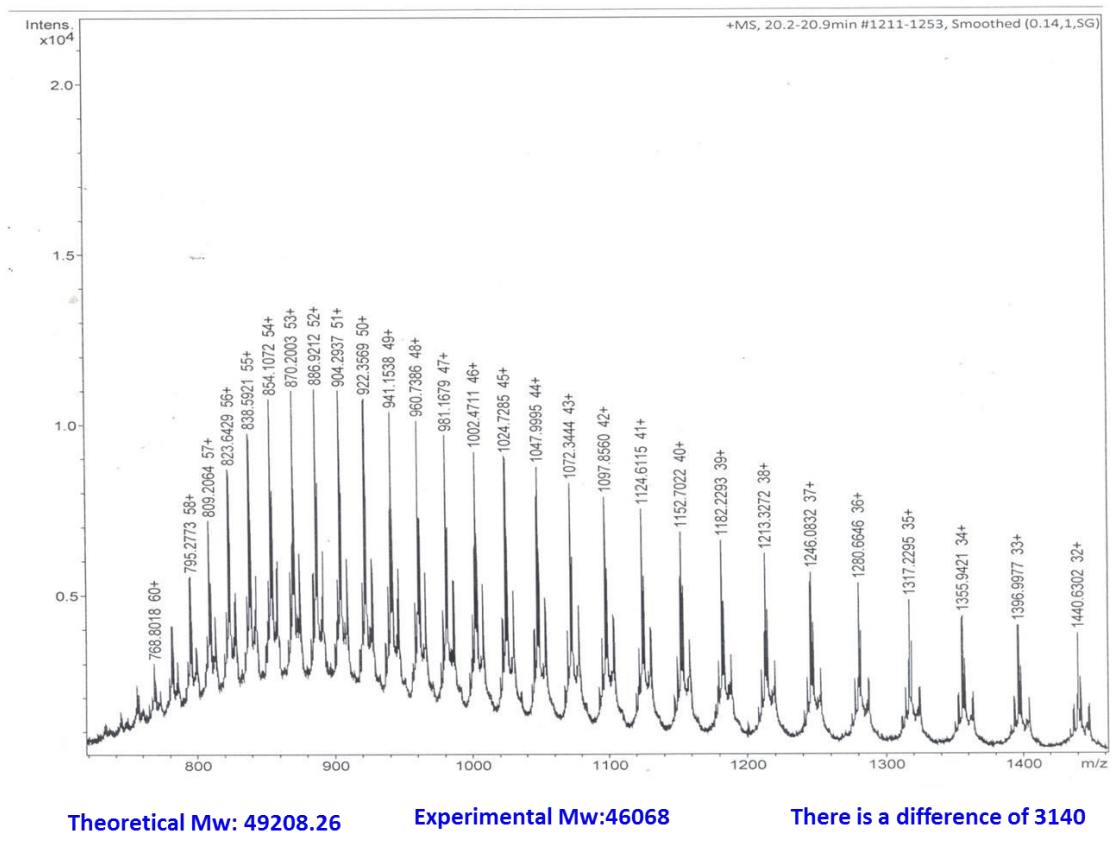


Figure 4.5. Li-LAO expression and purification from *E. coli* C41 strains (A) SP sepharose column purification of Li-LAO. (B) Concentration of Li-LAO by centricon. (C) 10% SDS PAGE showing the purified fractions of LAO by S200 gel filtration chromatography.

Cloned pET21bLAO vector was transformed in *E. coli* BL21 (DE3) strain expressed by inducing the log phase ($O.D_{600}$-0.6) culture with 0.5mM IPTG. The expression of Li-LAO in these clones was monitored by comparing the induced cell lysates with uninduced cell lysate as controls. The expressed protein was purified using a heparin sulfate column. The yield of the protein was very low (Fig. 4.4).

4.4.2 Mass spectrometry



Theoretical Mw: 49208.26 Experimental Mw:46068 There is a difference of 3140

Figure 4.6. LC-MS of Purified Li-LAO showing the mass difference between observed and expected mass.

Cloned pET21bLAO vector was transformed in *E. coli* C41 Strain expressed by inducing the log phase ($O.D_{600}$-0.6) culture with 0.5mM IPTG. The expressed protein was purified using an SP-Sepharose column. The protein fractions were pooled, concentrated, and subjected to gel filtration chromatography. Further, protein fractions were tested for purity on 10% SDS-PAGE (Fig. 4.5). The purified protein fractions were pooled.

The LC/ESI-MS of the purified protein showed the 3140dalton mass difference between observed and expected mass (Fig. 4.6). The mass difference of 3140 Dalton was an unexpected result. In order to rule out human error while handling the clones by mistake, the clone used for purification was re-sequenced. The DNA re-sequenced data of the clone was translated into protein sequence (Expasy) and compared with the reference sequence (Fig. 4.7). The cloned Li-LAO gene was intact.

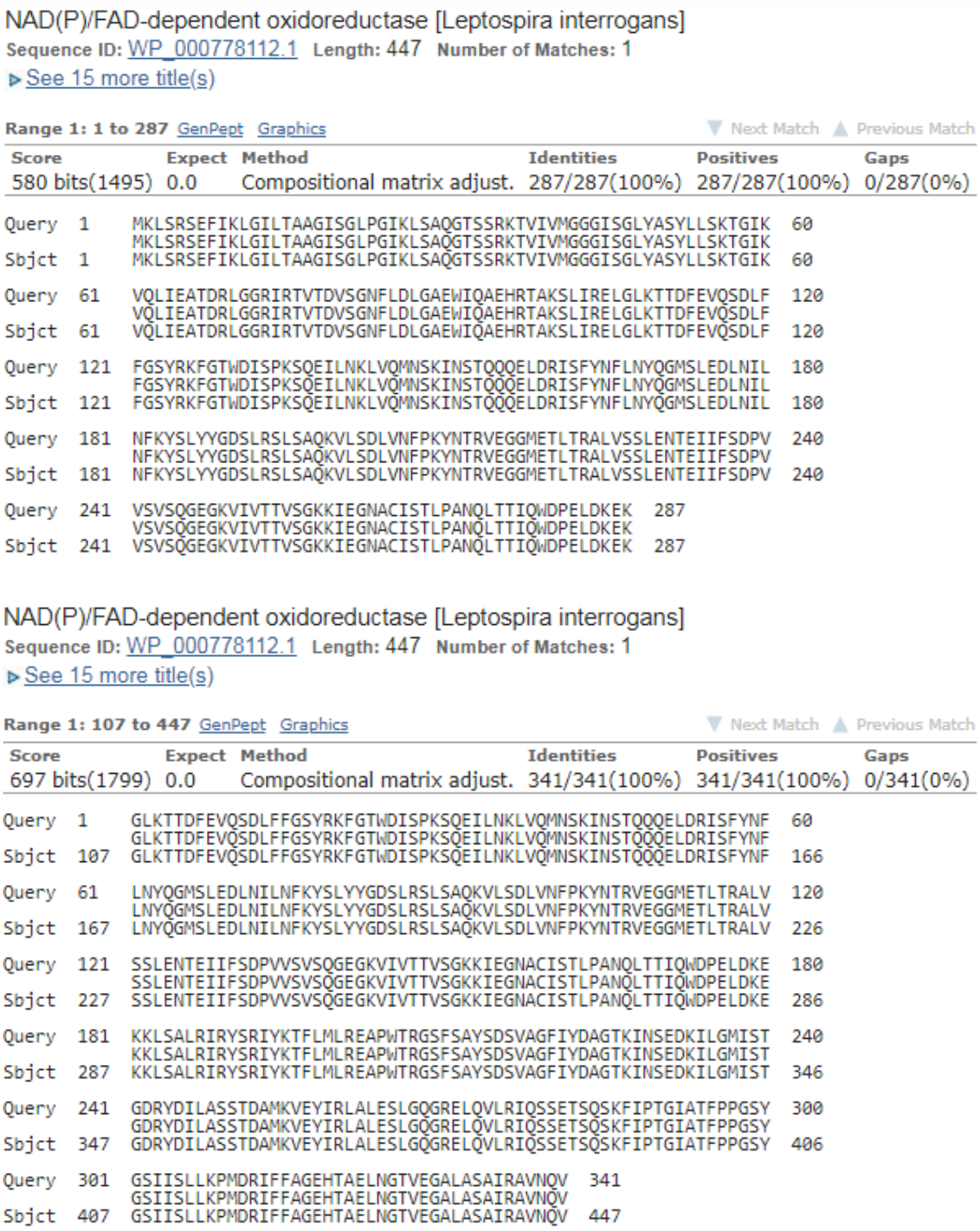


Figure 4.7. Alignment of re-sequenced data of Li-LAO clone.

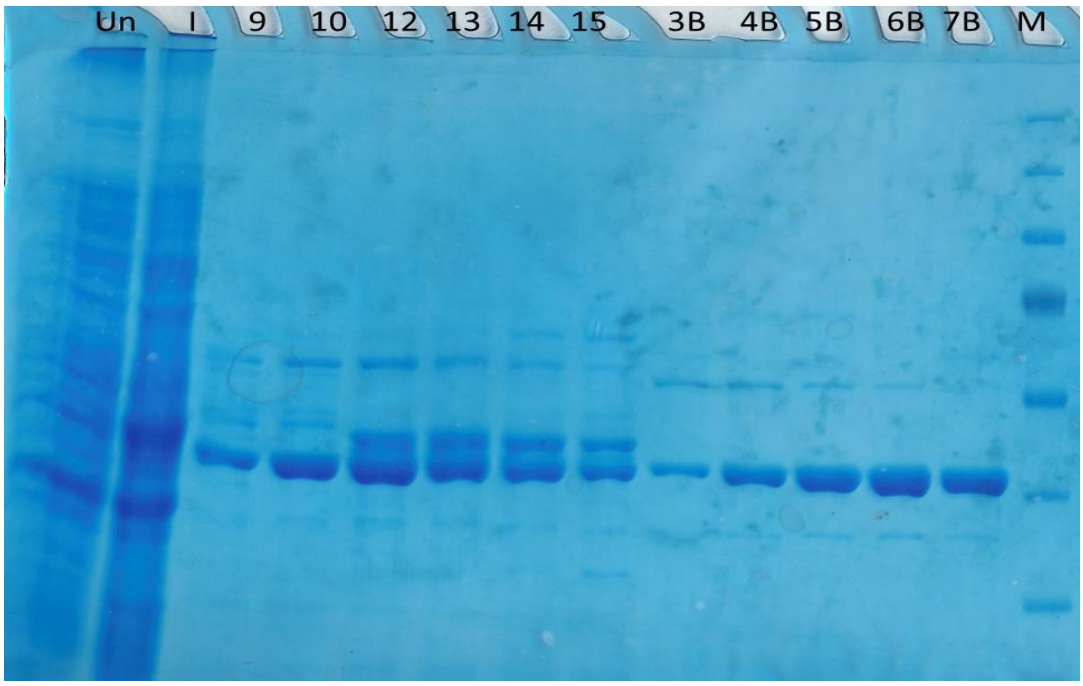
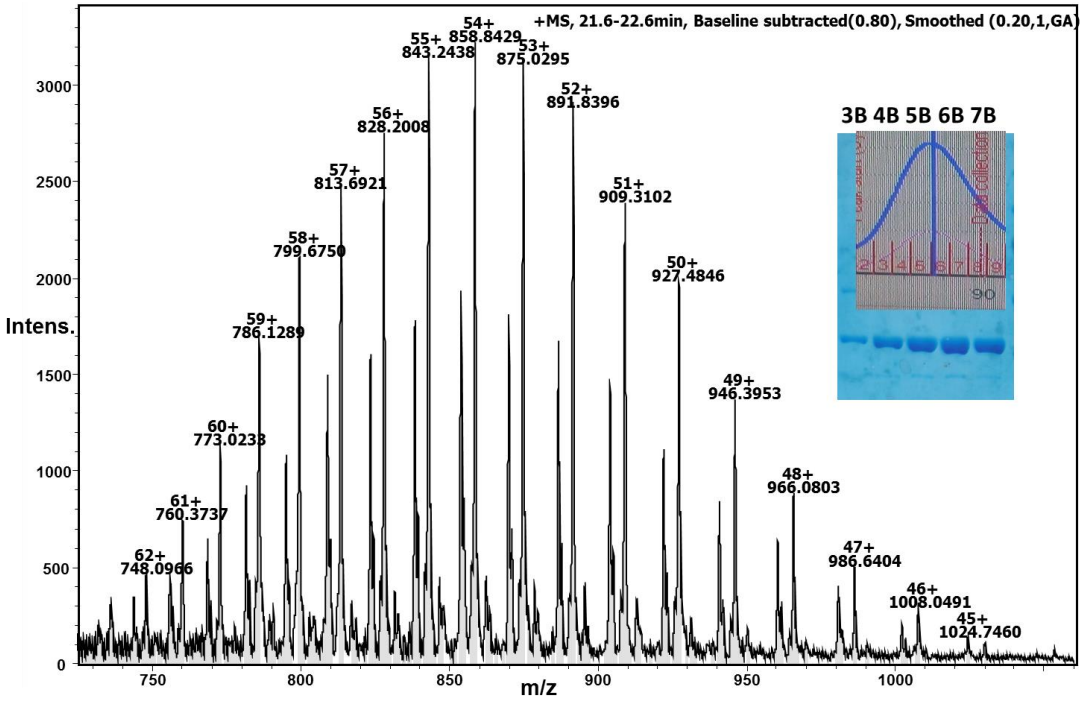


Figure 4.8. 10% SDS-PAGE of purified Li-LAO.

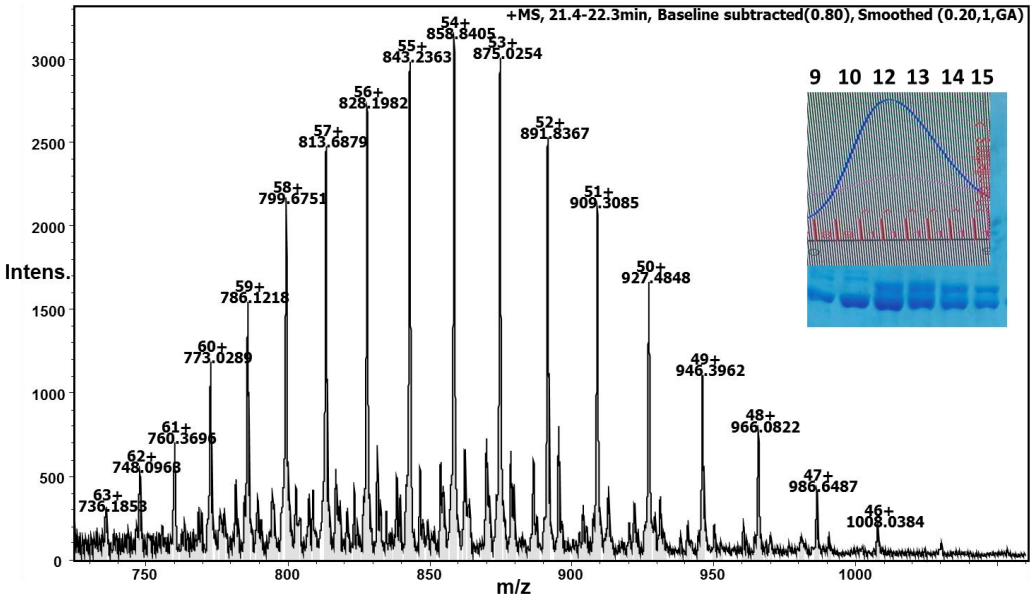
Further, the re-sequenced clone was transformed in *E. coli* C41 strain, and protein was purified (Fig. 4.8) and again subjected to mass spectrometric analysis. The purified protein showed the same mass difference of approximately 3000 daltons (Fig. 4.9).

4.4.3 In-gel trypsin digestion and MS/MS

In order to rule out the mistaken identity of any other host *E. coli* protein, the purified protein band was treated with trypsin, and the identity of the protein was established with mass spectrometric analysis of the extracted tryptic digested peptides from the gel band (Fig. 4.10 and 4.11). The cleavage of Li-LAO by trypsin was calculated by PAWS software (Fig. 4.12) and the observed mass of Li-LAO was backtracked to its sequence (Fig. 4.13).



Cmpt.	Mass	Abund.	Abd.[%]	Std.Dev.
A	46323.0054	32087	100.00	0.640526
B	46066.7564	19282	60.09	0.594566
C	46122.8924	8411	26.21	0.532155
D	46255.0447	3279	10.22	0.785106
E	46524.1421	4050	12.62	0.633684
F	68984.4779	2508	7.82	0.696323



Cmpt.	Mass	Abund.	Abd.[%]	Std.Dev.
A	46323.387	30102	100.00	0.593905
B	46524.2023	7157	23.78	0.858140
C	46066.1385	5924	19.68	0.594408
D	37837.8031	3844	12.77	0.668632
E	34275.0780	4466	14.83	0.810301
F	46120.9572	3713	12.33	0.724890

Figure 4.9. Mass spectrum of purified Li-LAO.

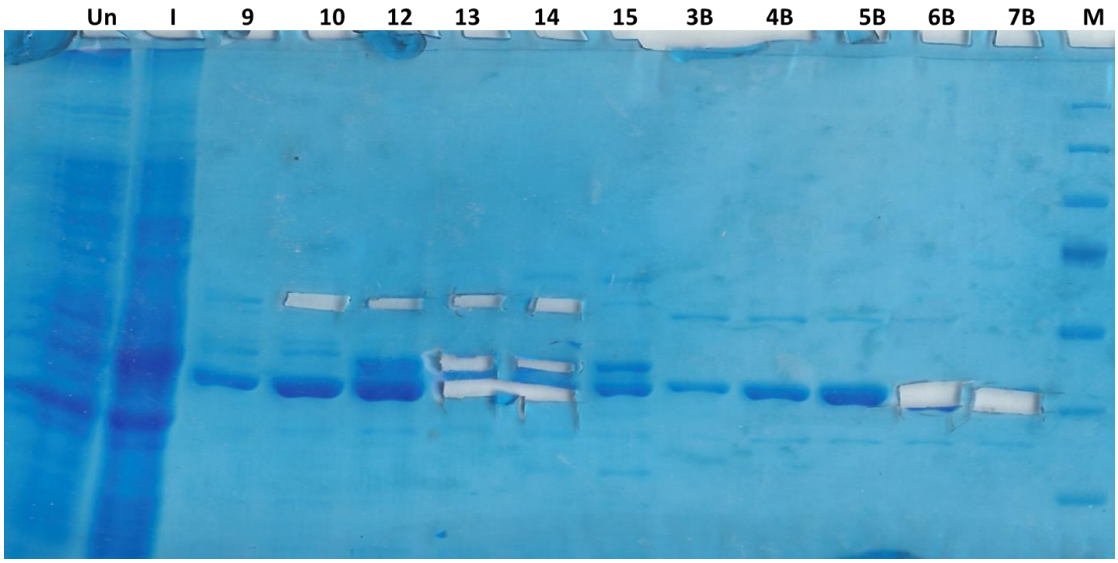


Figure 4.10. 10% SDS-PAGE showing cut gel bands that were subjected to the trypsin digestion followed by LC-MS

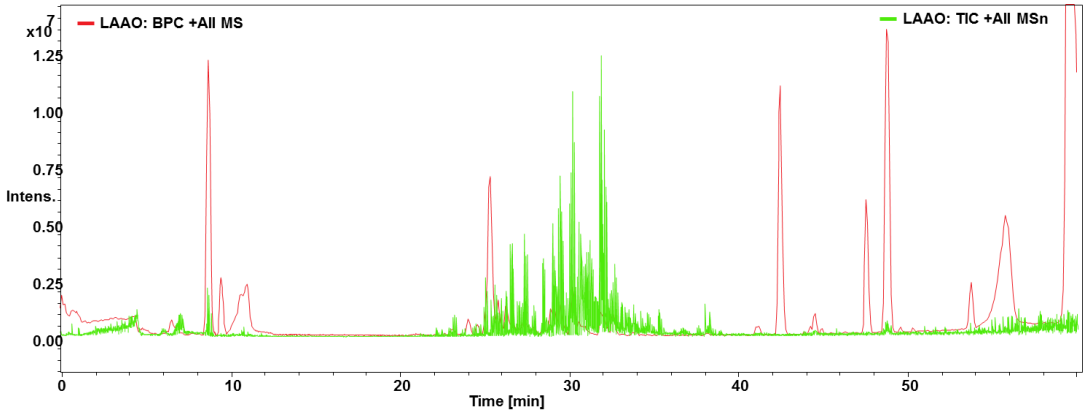


Figure 4.11. LC chromatogram of the tryptic digested Li-LAO peptides.

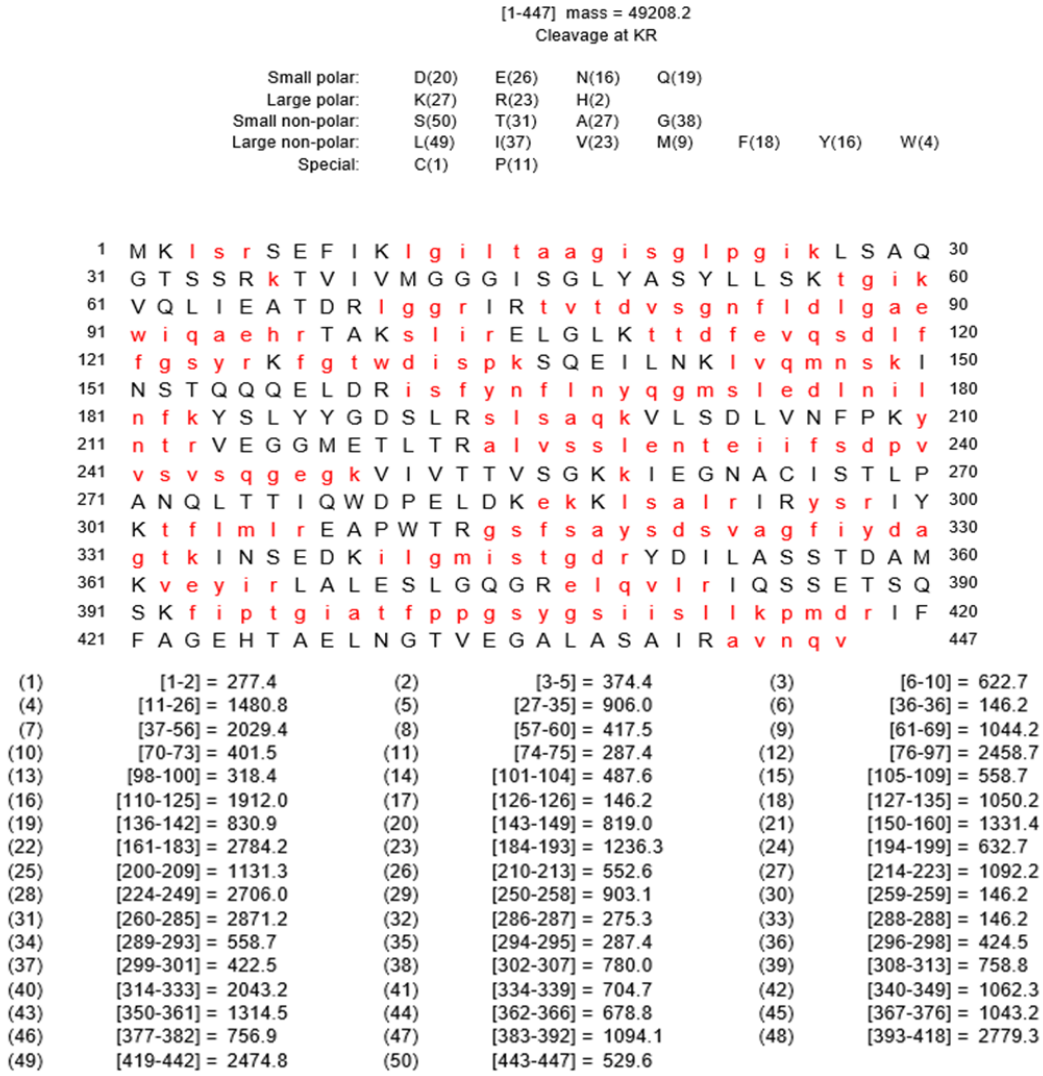


Figure 4.12. Theoretically calculated peptide fragments of Li-LAO upon trypsin digestion.

[1-419] mass = 46324.7
Cleavage at KR

Small polar:	D(20)	E(25)	N(16)	Q(19)			
Large polar:	K(24)	R(22)	H(2)				
Small non-polar:	S(46)	T(30)	A(25)	G(34)			
Large non-polar:	L(44)	I(33)	V(23)	M(8)	F(17)	Y(16)	W(4)
Special:	C(1)	P(10)					

```
  1  A Q G T S S R k T V I V M G G G I S G L Y A S Y L L S K t g  30
 31  i k V Q L I E A T D R l g g r I R t v t d v s g n f l d l g  60
 61  a e w i q a e h r T A K s l i r E L G L K t t d f e v q s d  90
 91  l f f g s y r K f g t w d i s p k S Q E I L N K l v q m n s  120
121  k I N S T Q Q Q E L D R i s f y n f l n y q g m s l e d l n  150
151  i l n f k Y S L Y Y G D S L R s l s a q k V L S D L V N F P  180
181  K y n t r V E G G M E T L T R a l v s s l e n t e i i f s d  210
211  p v v s v s q g e g k V I V T T V S G K k I E G N A C I S T  240
241  L P A N Q L T T I Q W D P E L D K e k K l s a l r I R y s r  270
271  I Y K t f l m l r E A P W T R g s f s a y s d s v a g f i y  300
301  d a g t k I N S E D K i l g m i s t g d r Y D I L A S S T D  330
331  A M K v e y i r L A L E S L G Q G R e l q v l r I Q S S E T  360
361  S Q S K f i p t g i a t f p p g s y g s i i s l l k p m d r  390
391  I F F A G E H T A E L N G T V E G A L A S A I R a v n q v  419
```

[1-416] mass = 46068.4
Cleavage at KR

Small polar:	D(20)	E(25)	N(16)	Q(18)			
Large polar:	K(24)	R(22)	H(2)				
Small non-polar:	S(46)	T(30)	A(24)	G(33)			
Large non-polar:	L(44)	I(33)	V(23)	M(8)	F(17)	Y(16)	W(4)
Special:	C(1)	P(10)					

```
  1  T S S R k T V I V M G G G I S G L Y A S Y L L S K t g i k V  30
 31  Q L I E A T D R l g g r I R t v t d v s g n f l d l g a e w  60
 61  i q a e h r T A K s l i r E L G L K t t d f e v q s d l f f  90
 91  g s y r K f g t w d i s p k S Q E I L N K l v q m n s k I N  120
121  S T Q Q Q E L D R i s f y n f l n y q g m s l e d l n i l n  150
151  f k Y S L Y Y G D S L R s l s a q k V L S D L V N F P K y n  180
181  t r V E G G M E T L T R a l v s s l e n t e i i f s d p v v  210
211  s v s q g e g k V I V T T V S G K k I E G N A C I S T L P A  240
241  N Q L T T I Q W D P E L D K e k K l s a l r I R y s r I Y K  270
271  t f l m l r E A P W T R g s f s a y s d s v a g f i y d a g  300
301  t k I N S E D K i l g m i s t g d r Y D I L A S S T D A M K  330
331  v e y i r L A L E S L G Q G R e l q v l r I Q S S E T S Q S  360
361  K f i p t g i a t f p p g s y g s i i s l l k p m d r I F F  390
391  A G E H T A E L N G T V E G A L A S A I R a v n q v  416
```

Figure 4.13. Li-LAO sequence, matching observed mass with the mass of the tryptic products (46323 and 46066 respectively).

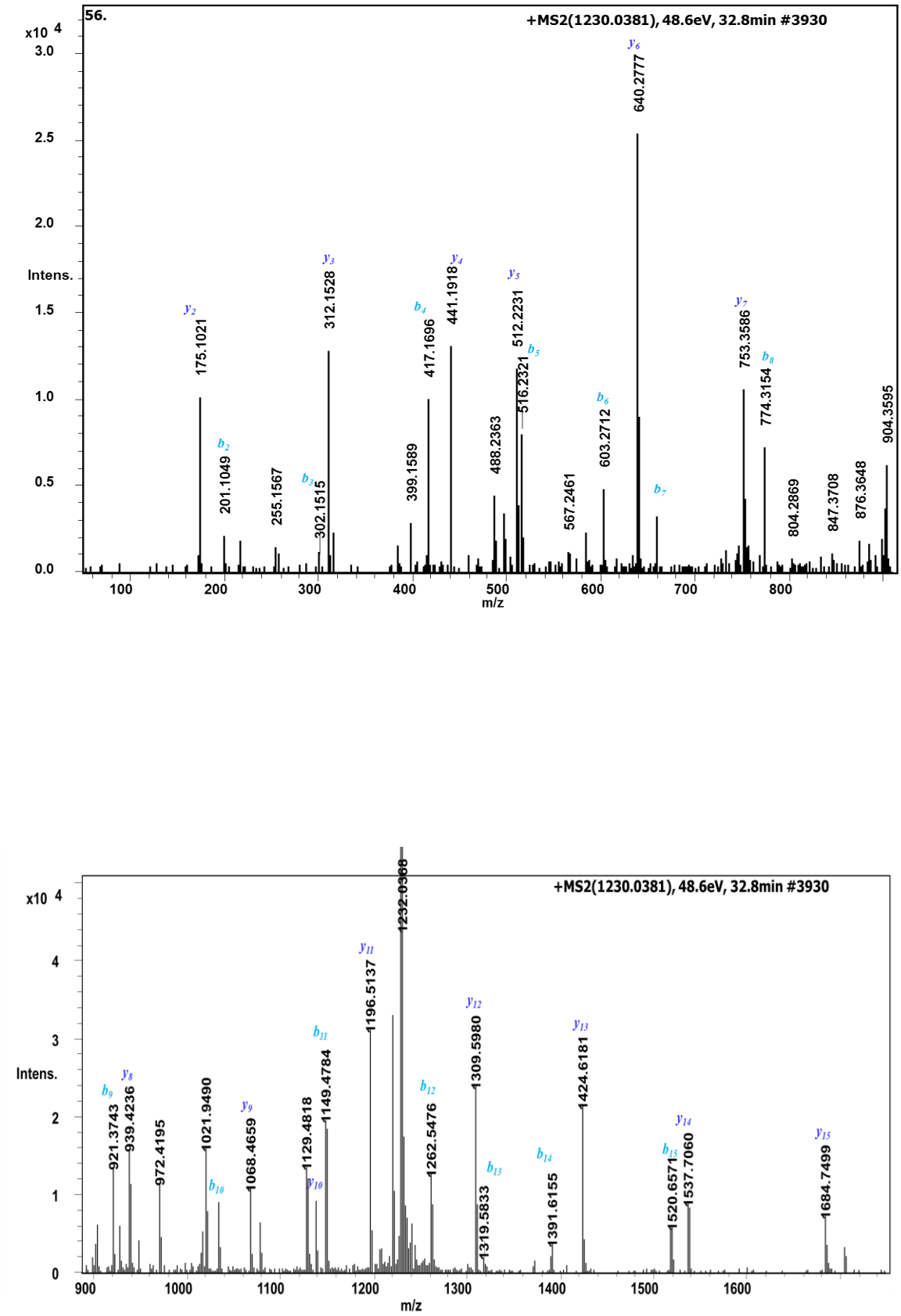
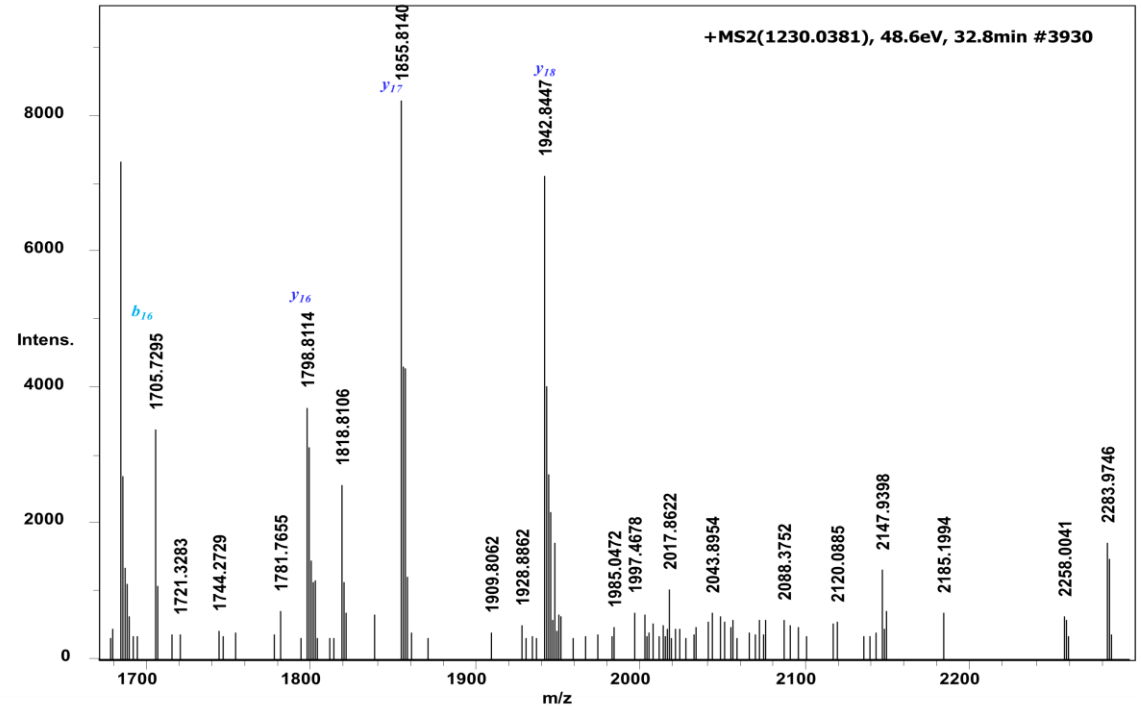


Figure 4.14. LC-MS spectral analysis of the tryptic digested product of Li-LAO gene.



TVTDVSGNFLDLGAEWIQAEHR

M = 2457.192

$[M+2H]^{2+}$ =1229.596

$[M+3H]^{3+}$ =820.064

RT =32.7min

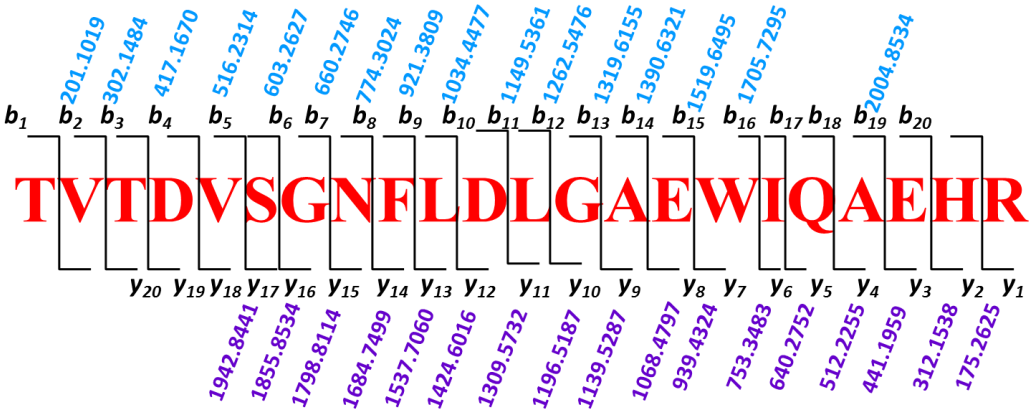
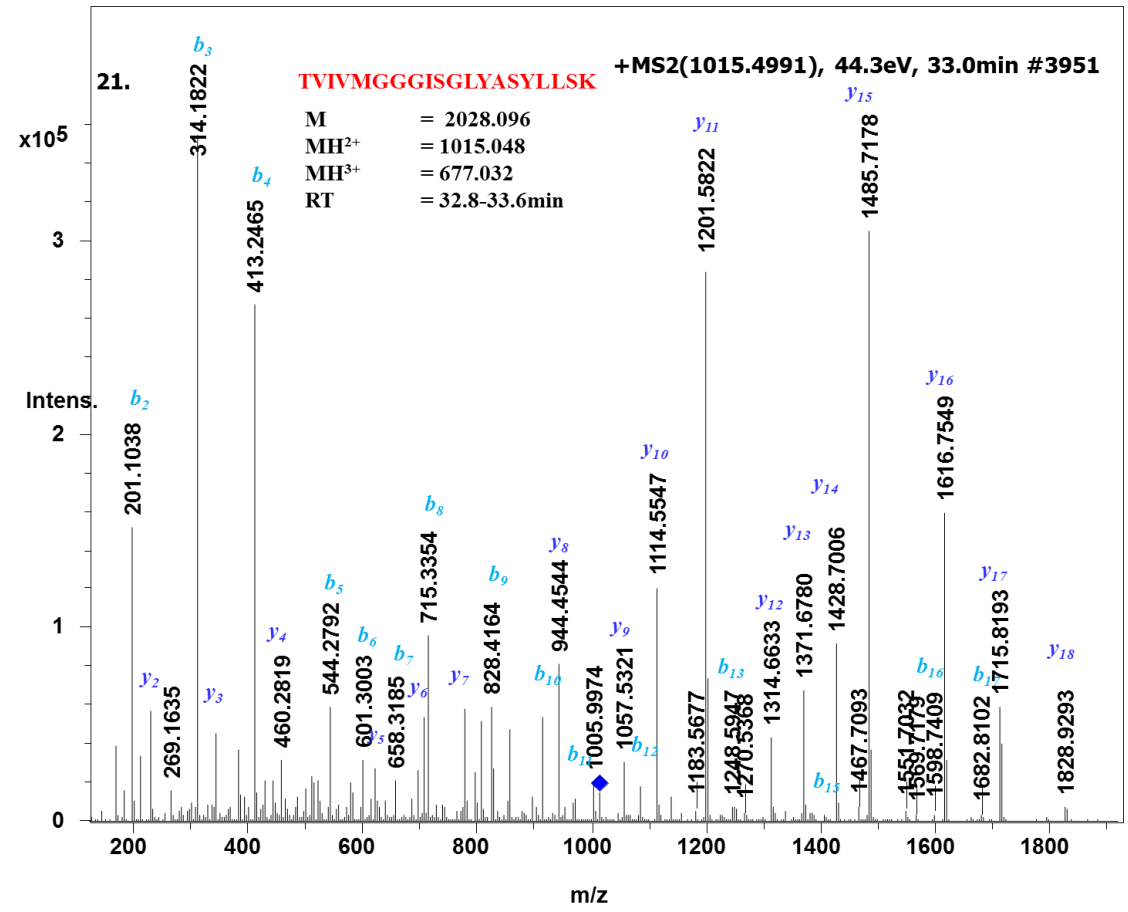


Figure 4.14. LC-MS spectral analysis of the tryptic digested product of Li-LAO gene.



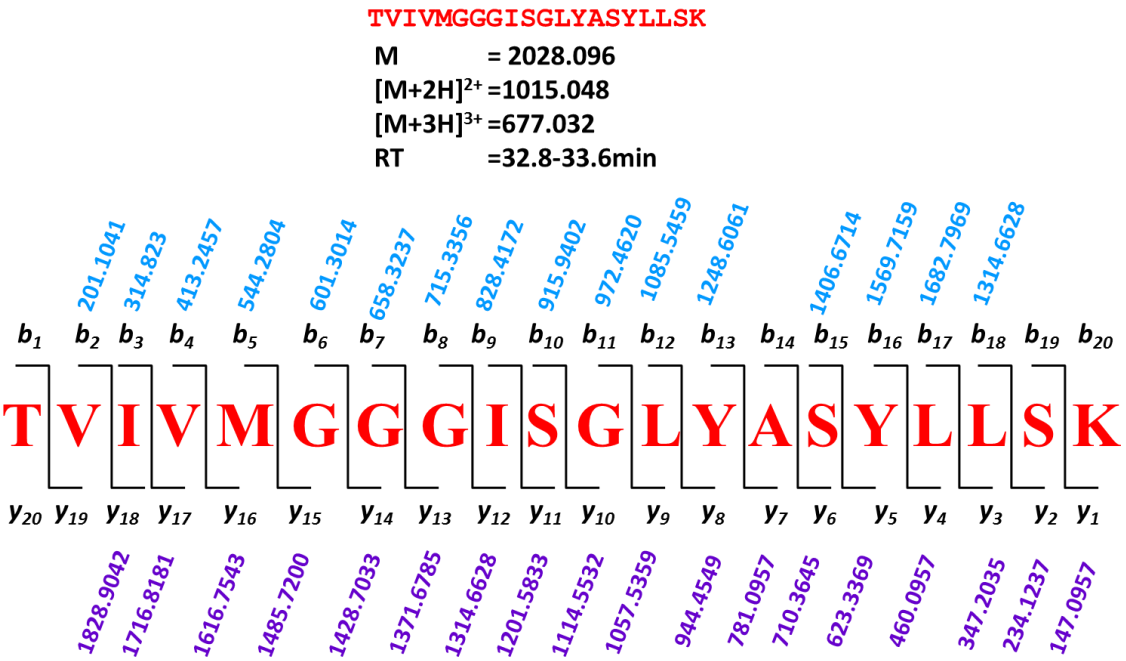


Figure 4.15. Representative LC-MS spectral analysis of the tryptic digested product of Li-LAO gene.

Table 4.1. Various fragments that are identified from LC-MS of the tryptic digested product of Li-LAO.

Observed	Mr.(expt)	Mr.(calc)	Peptide
522.7518	1043.4890	1043.5975	K.VkLIEATDR
531.7493	1061.4840	1061.5539	K.ILGMISTGDR.Y
546.7342	1091.4539	1091.5281	R.VEGGMETLTR.A
547.7319	1093.4492	1093.5251	R.IQSSETSQSK
566.2919	1130.5693	1130.6336	K.VLSDiVNFPK
618.7580	1235.5013	1235.5822	K.YSLYYGDSLR.S
653.3140	1304.6135	1304.6976	M.SLEDLNILNFK.Y
657.7721	1313.5296	1313.6173	R.YDILASSTDAMK.V
666.2882	1330.5619	1330.6477	K.INSTQQQELDR.I
583.5950	1747.7631	1747.8774	K.INSEDKILGMISTGDR.Y
874.8933	1747.7721	1747.8774	K.INSEDKILGMISTGDR.Y
637.9228	1910.7466	1910.8687	K.TTDFEVQSDLFFGSYR.K
956.3860	1910.7574	1910.8687	K.TTDFEVQSDLFFGSYR.K
658.9552	1973.8438	1973.9768	R.YDILASSTDAMKVEYIR.L
987.9342	1973.8538	1973.9768	R.YDILASSTDAMKVEYIR.L
676.9961	2027.9664	2028.0965	K.TVIVMGGGISGLYASYLLSK.T
1014.9986	2027.9826	2028.0965	K.TVIVMGGGISGLYASYLLSK.T
680.6166	2038.8280	2038.9636	K.TTDFEVQSDLFFGSYRK.F
681.6058	2041.7955	2041.9269	R.GSFSAYSDSVAGFIYDAGTK.I
1021.9135	2041.8123	2041.9269	R.GSFSAYSDSVAGFIYDAGTK.I
699.5802	2095.7187	2094.9898	K.GNFSAYLDSVAGFIYDAGTK.A
719.6910	2156.0512	2156.1915	R.KTVIVMGGGISGLYASYLLSK.T
1079.0396	2156.0647	2156.1915	R.KTVIVMGGGISGLYASYLLSK.T
786.6801	2357.0185	2357.1607	K.ILGMISTGDRYDILASSTDAMK.V
1179.5195	2357.0245	2357.1607	K.ILGMISTGDRYDILASSTDAMK.V
615.2655	2457.0328	2457.1925	R.TVTDVSGNFLDLGAEWIQAEHR.T
820.0251	2457.0535	2457.1925	R.TVTDVSGNFLDLGAEWIQAEHR.T
1229.5366	2457.0587	2457.1925	R.TVTDVSGNFLDLGAEWIQAEHR.T
619.2615	2473.0169	2473.2601	R.IFFAGEHTAELNGTVEGALASAIR.A
825.3766	2473.1079	2473.2601	R.IFFAGEHTAELNGTVEGALASAIR.A
1237.5684	2473.1221	2473.2601	R.IFFAGEHTAELNGTVEGALASAIR.A
677.0582	2704.2038	2704.3807	R.ALVSSLENTEIIFSDPVVSVSQGEGK.V
902.4130	2704.2171	2704.3807	R.ALVSSLENTEIIFSDPVVSVSQGEGK.V
1353.1258	2704.2370	2704.3807	R.ALVSSLENTEIIFSDPVVSVSQGEGK.V
695.3328	2777.3023	2777.4826	K.FIPTGIATFPPGSYGSIISLLKPMDR.I
556.4687	2777.3072	2777.4826	K.FIPTGIATFPPGSYGSIISLLKPMDR.I
926.7824	2777.3255	2777.4826	K.FIPTGIATFPPGSYGSIISLLKPMDR.I
1389.6709	2777.3273	2777.4826	K.FIPTGIATFPPGSYGSIISLLKPMDR.I
928.4104	2782.2094	2782.3676	R.ISFYNFLNYQGMSLEDLNILNFK.Y
995.7995	2984.3766	2984.5356	R.IFFAGEHTAELNGTVEGALASAIRAVNQV.-

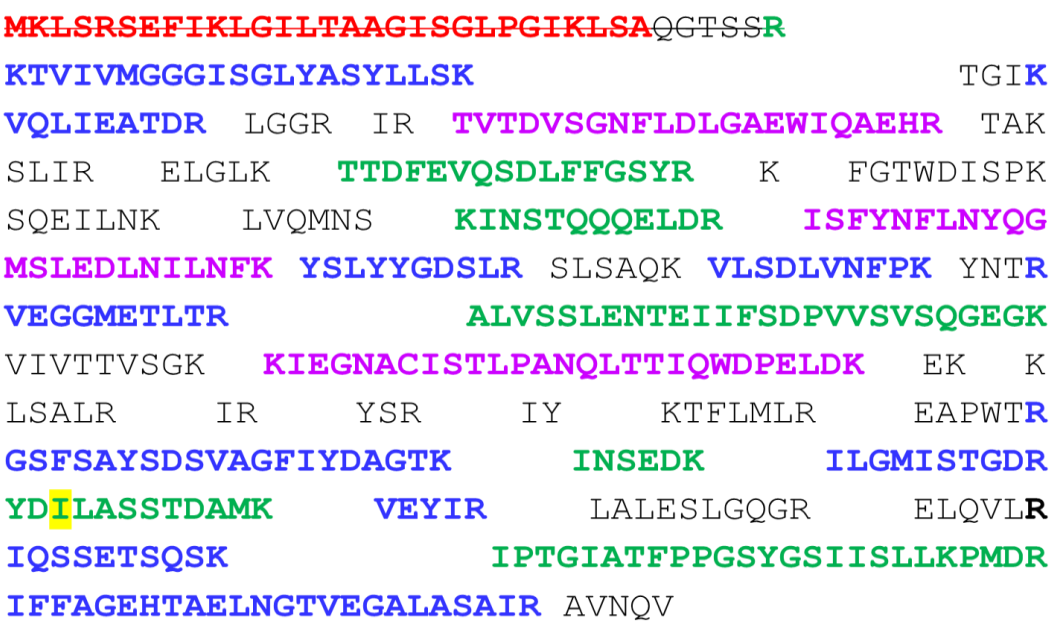
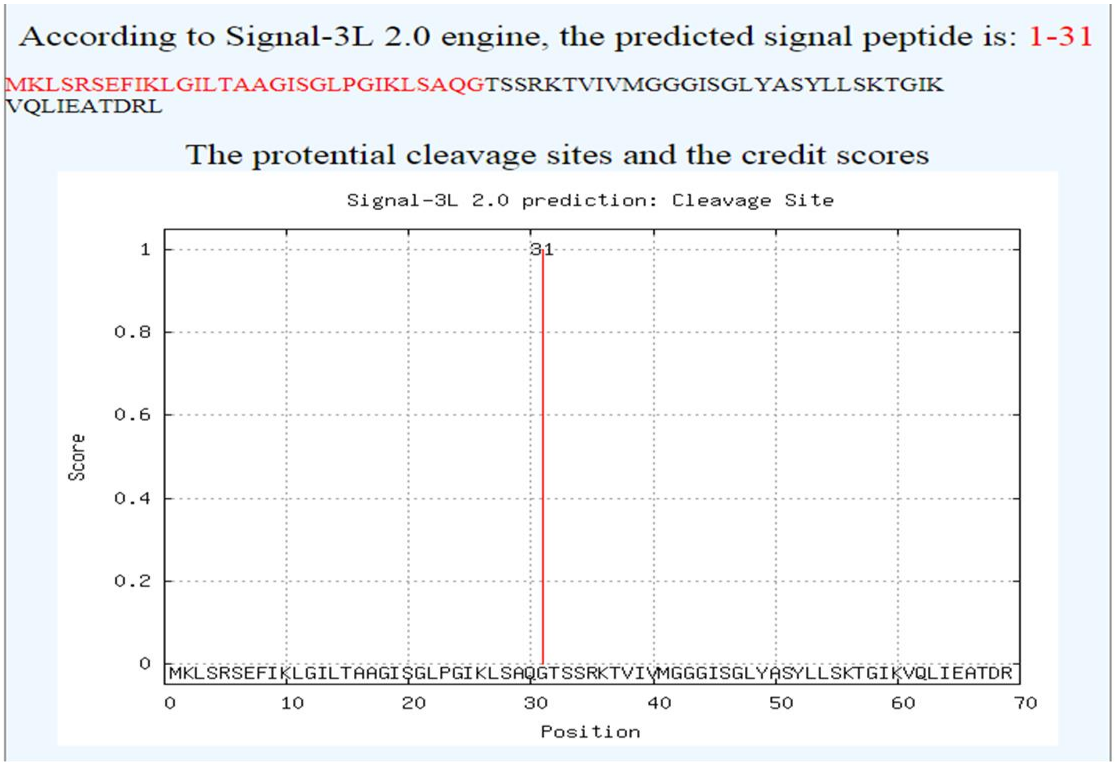


Figure 4.16. Pictorial representation of various fragments that are identified from LC-MS of the tryptic digested product of Li-LAO.

The tryptic digested peptide mass spectra analysis was done by interrogating mass spectra of peptides with that of various fragments of the Li-LAO sequences that can be generated (theoretically) upon trypsin digestion. Initially, this analysis was done manually for two peptides (Fig. 4.14 and 4.15). Later the data was analyzed by the mascot online server. Approximately 20 different fragments matched with the mass of Li-LAO sequences (Table 4.1) that covered the entire stretch of Li-LAO except for the initial 25-30 residues (Fig. 4.16).

Since the N-terminus of the protein was missing that can be attributed to some signal sequence. So the Li-LAO sequence was analyzed by various online signal peptide recognition servers. The Signal-BLAST server and Signal-3L 2.0 predicted 31 and 32 residue signal peptide for Li-LAO (Fig. 4.17) that matched with an observed mass of Li-LAO.




```
Query Sequence: >Unknown Sequence
MKLSRSEFIKLGILTAAGISGLPGIKLSAQGTSSRKTVIVMGGGISGLYASYLLSKTGIK...
--------------------------------------------------------------------------------
Significant Alignments:
Name                                                        Bitscore        SValue
--------------------------------------------------------------------------------
PHSS_DESBA (Signal Peptide)                                    29.3          100.0
DHSU_ALLVD (Signal Peptide)                                    29.3          100.0
TRMFO_FUSNN                                                    30.8            0.0
TRMFO_SORC5                                                    30.4            0.0
Y943_HELPY                                                     29.6            0.0
Y943_HELPJ                                                     29.6            0.0
OTEMO_PSEPU                                                    29.6            0.0
FIXC_AZOVI                                                     28.5            0.0
TRMFO_RHOPB                                                    28.1            0.0
PCPB_SPHCR                                                     27.7            0.0
--------------------------------------------------------------------------------
Insignificant Alignments:
--------------------------------------------------------------------------------
--------------------------------------------------------------------------------
Most Significant SValue:
>PHSS_DESBA (Cleavage Site after AA 32)
          Length = 60

 Score = 29.3 bits (64), Expect = 0.55
 Identities = 22/61 (36%), Positives = 29/61 (47%), Gaps = 11/61 (18%)

Query: 1  MKLSRSEFIKLGILTAAGISGLPGIKLSAQGTSSRKTVIVMGGGISGLYASYLLSKTGIK 60
          M LSR EF+KL     +AG++GL        PGI      +G       + V G G +G    S
Sbjct: 1  MSLSRREFVKL---CSAGVAGLGISQIYHPGIVHAMTEGAKKAPVIWVQGQGCTGCSVSL 57
                                        /\

Result: Signal Peptide, putative Cleavage Site after AA 32 (by similarity to PHSS_DESBA)
```

http://sigpep.services.came.sbg.ac.at/signalblast.html

Figure 4.17. Results of signal peptide identification online servers.

4.4.4 Identification of cofactor

After identification of Li-LAO, since it is a cofactor bound enzyme, before proceeding further, it was necessary to identify the bound cofactor.

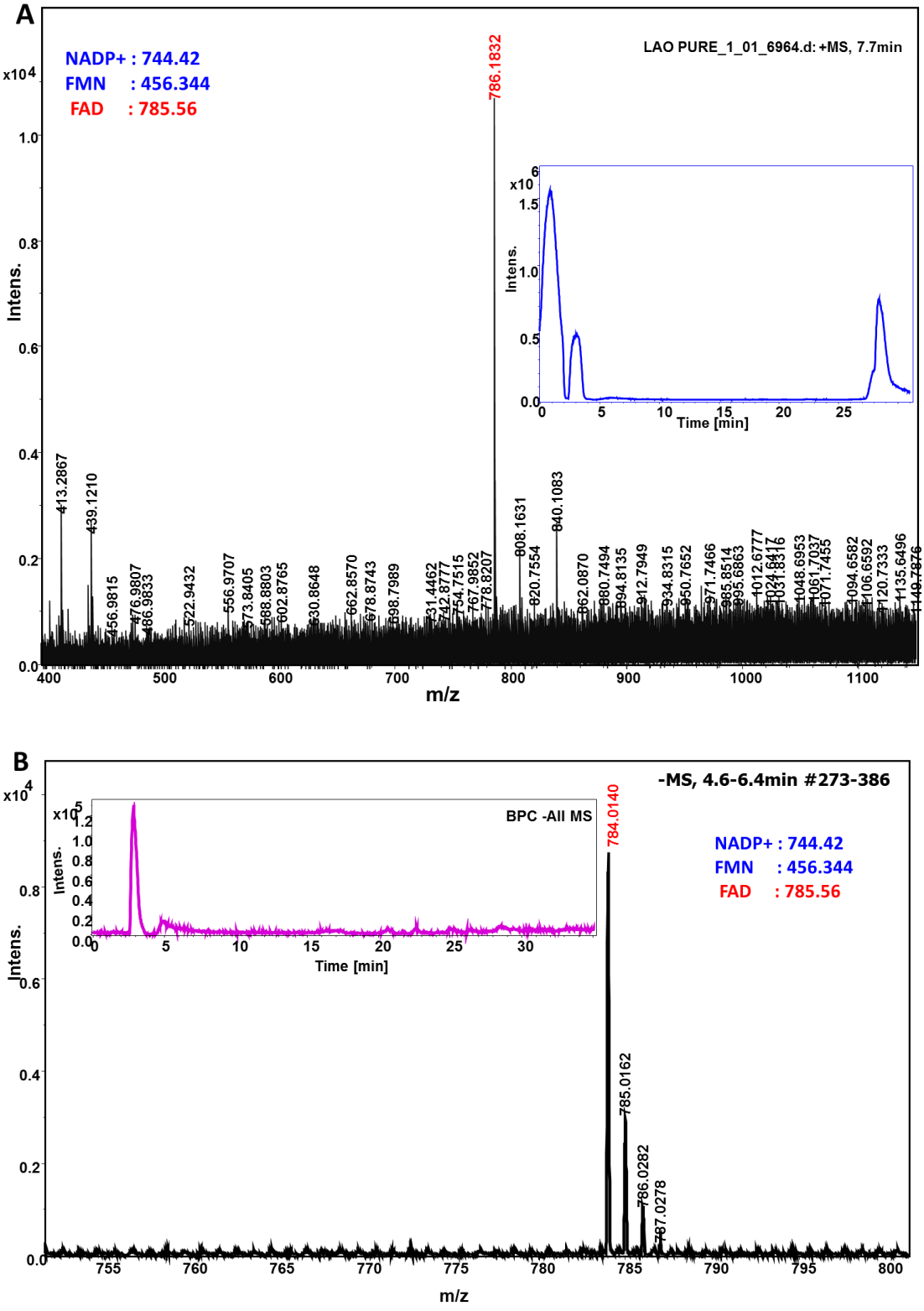


Figure 4.18. Identification of cofactor bound to Li-LAO by LC-MS (A) positive mode (B) negative mode.

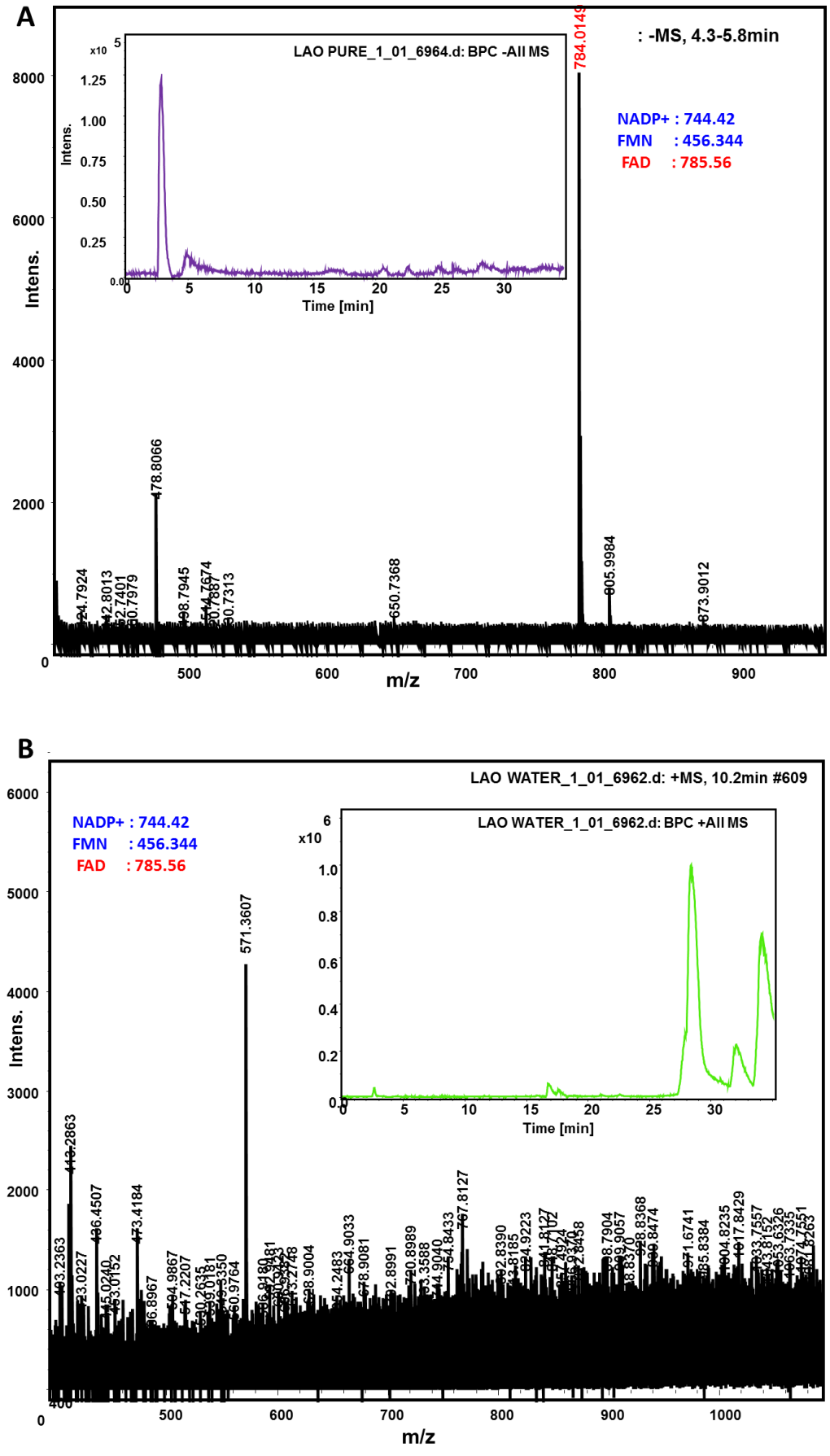


Figure 4.19. Identification of nature of cofactor binding in Li-LAO by LC-MS.

The Li-LAO mass spectrum was analyzed for the presence of FAD, FMN, and $NADP^+$ mass. The presence of FAD was established with an expected mass of 786 daltons. In order to reconfirm the identified species, the protein was analyzed in negative mode and reconfirmed bound cofactor of Li-LAO is FAD with the corresponding mass of 784 (Fig. 4.18). In order to establish the nature of the interaction between Li-LAO and bound FAD, Li-LAO was dialyzed against water and analyzed by LC-MS (negative mode). Weak ionic interaction between FAD and Li-LAO protein was confirmed by the absence of FAD upon dialysis compared to undialysed protein (Fig. 4.19).

LAO from Leptospira expressed and purified from *E. coli* C41 (Fig. 4.20) and assayed with various L-amino acid substrates, the activity of the purified enzyme was low (Fig. 4.21). The Li-LAO was getting truncated at N-terminus with cleavage of the first 28-31 residues.

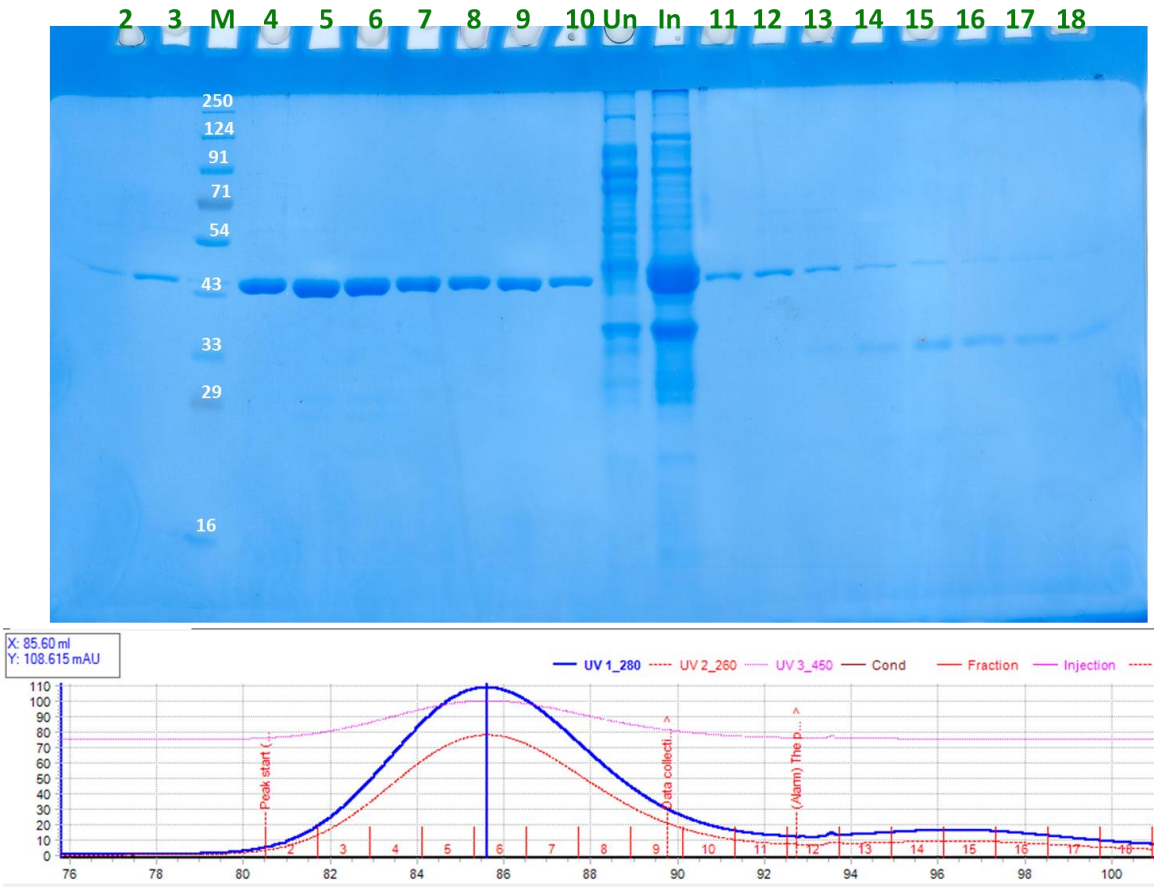


Figure 4.20. (A) SDS-PAGE of S-200 Gel filtration Chromatography fractions of Li- LAO. (B) Corresponding elution profile chromatogram of Li-LAO.

In order to reduce the proteolytic cleavage of expressed Li-LAO, the protein purification was carried out with a protease cocktail and purified enzyme was checked for the full-length protein, but we failed to get a full-length Li-LAO protein (Data not shown).

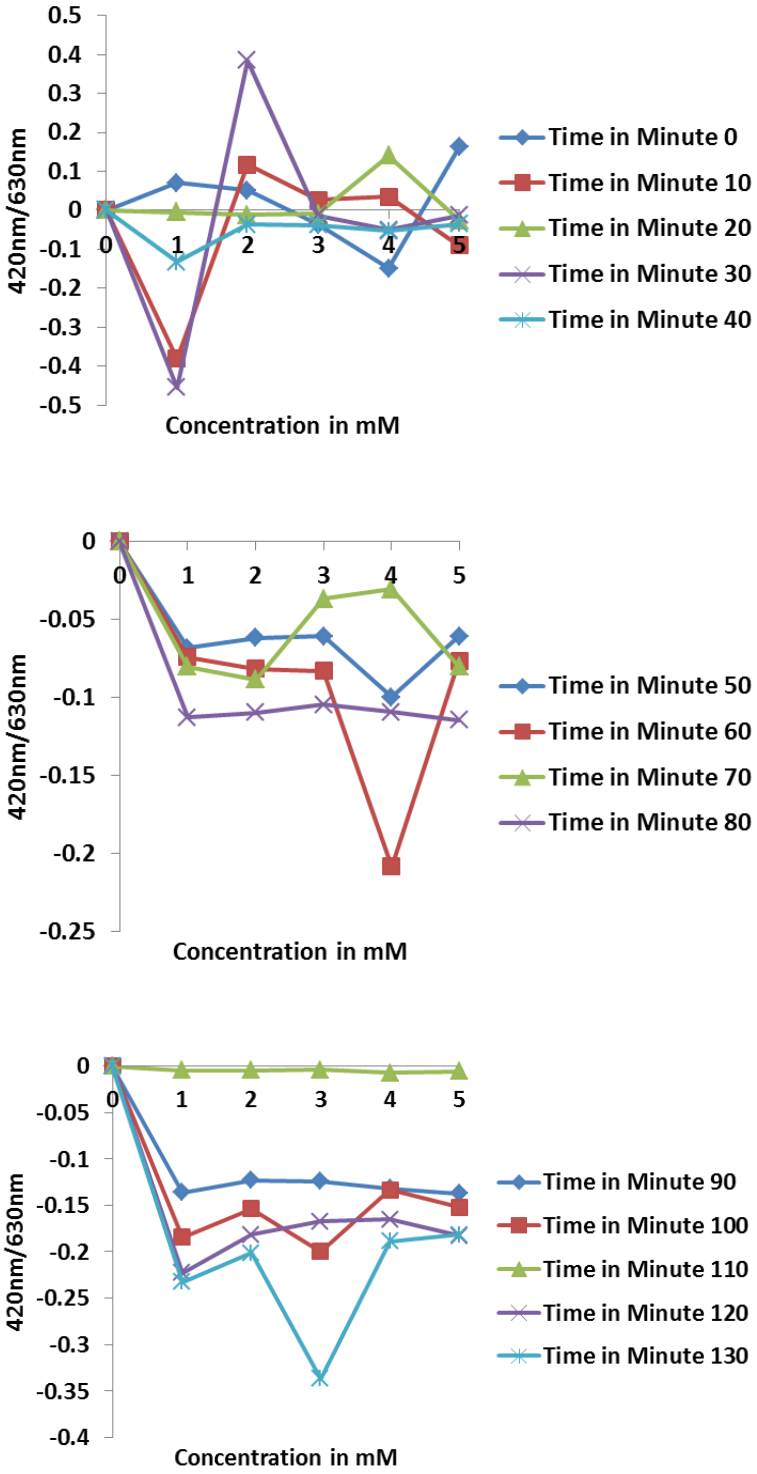


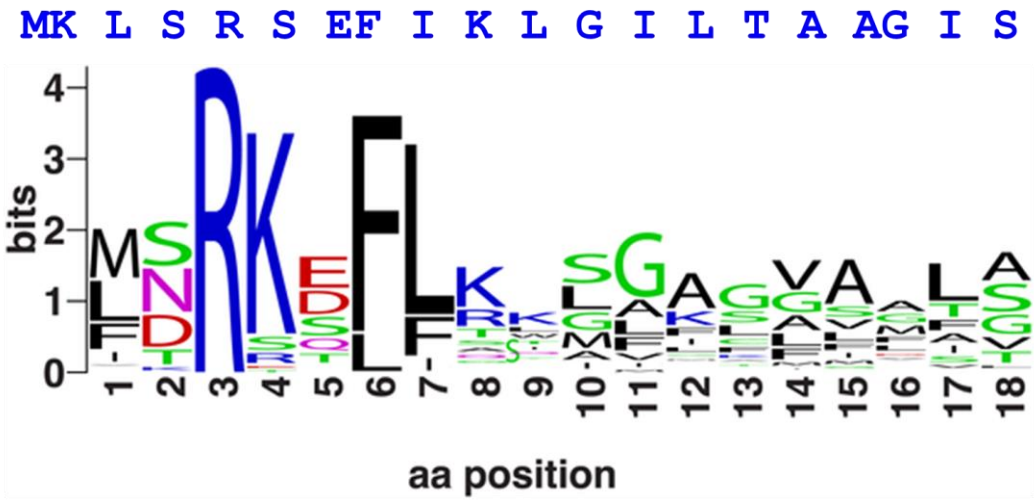
Figure 4.21. Representative Li-LAO assay result with an o-Phenylene diamine.

Further, the Li-LAO was found to be thrown out of the cell as an exoprotein (Eshghi A. *et al.*, 2015). So the recombinant protein was also searched in the spent media of *E. coli* strain expressing Li-LAO after induction that resulted in a very low quantity of protein not sufficient enough for purification and other biochemical characterization.

4.5 Discussion

The attempts described in this chapter to express the full-length Leptospiral LAO containing the putative secretory signal resulted in the isolation of two polypeptides which corresponds to deletion of 1-28 and 1-31 residues. It was also observed that relatively little protein was detectable in the culture supernatant with much of the extracted protein being obtained after cell disruption. Interestingly, despite the variable truncation (maybe the reason for low activity) presumably by membrane-bound signal peptidases, both truncated protein forms appeared to be properly folded and bound to the cofactor FAD without any supplementation. This was evident from the visible yellow color of the protein fractions, the identification of the FAD under the dissociative condition prevailing in the electrospray ionization source from the mass spectrometer.

In bacteria, a specific signal sequence referred to as TAT (Twin Arginine Translocation) at the N-terminus facilitates the transport and secretion of the fully folded protein across the membrane (Fouts DE. *et al.*, 2016).



TAT signal consensus sequence in Leptospira sp.

Figure 4.22. Comparison of TAT signal sequence with the N-terminal sequence of Leptospira L-amino acid oxidase.

Fig. 4.22 Shows the TAT signal consensus sequence of putative L-amino acid oxidase bears limited similarity. We, therefore, proceeded to clone and express the putative LAO / amine oxidase sequence from leptospira deleting sequence from 1-20 residues; this led to the isolation of a well-folded homogenous protein fully amenable for structural characterization. The results are discussed in the next chapter.

Chapter 5

Structural characterization of putative L-amino acid/amine oxidase

(Biophysical studies and structural characterization)

This chapter details the experimental characterization (Biophysical and spectroscopic characterizations) of the putative Leptospiral LAO (427 residues) protein, corresponding to residues 21 - 447, of the putative Leptospiral LAO sequence considered in Chapter 3 are described. Additionally, a summary of structural characterization of L-amino acid oxidases from other sources is provided as a background to the results described in this Chapter.

5.1 Analysis of Li-LAO sequence

The sequence alignment of Leptospiral putative LAO with the PDB database aligned with various amino acid oxidases most of them has an origin of the source from various snake venom and amine oxidases from human and rat. The list of the structure of LAOs available in the PDB database is given in Table 5.1. The representative of alignment using Clustalw-PBIL (Combet C. *et al.*, 2000; Thompson JD. *et al.*, 1994) of PDB database with L-amino acid oxidase is given in Fig. 5.1.

Table 5.1. List of L-amino acid oxidase structures available in PDB

PDB ID	Source	Chain Length	Ligand Name	Resolution	Mol. Weight	Residue Count	DOI
1F8R, 1F8S	*Calloselasma rhodostoma*	498	Citric Acid, FAD, Alpha-L-Fucose, N-Acetyl-D-Glucosamine	2.00	56299.30	1992, 3984	10.1093/emboj/19.16.4204
1REO, TDK, 1TDN, TDO	*Gloydius halys*	486	Citric Acid, FAD, N-Acetyl-D-Glucosamine,2-(Acetylamino)-2-Deoxy-A-D-Glucopyranose	2.31, 2.70, 2.70, 3.00	55207.20	486	10.1107/S0907444904000046
2IID	*Calloselasma rhodostoma*	498	FAD, Alpha-L-Fucose,N-Acetyl-D-Glucosamine,Phenylalanine	1.80	56299.30	1992	10.1016/j.jmb.2006.09.032
2JAE, 2JB, 2JB2, 2JB3	*Rhodococcus opacus*	489	FAD	1.25, 1.55, 1.45, 1.85	53412.00	978	10.1016/j.jmb.2006.11.071
3KVE	*Vipera ammodytes*	486	FAD, N-Acetyl-D-Glucosamine, Zinc Ion	2.57	55005.80	1944	
3WE0	Pseudomonas sp.	580	FAD	1.90	64516.90	1160	10.1016/j.fob.2014.02.002
4CNJ, 4CNK	*Streptococcus cristatus*	391	FAD, Glycerol, O-Methyl-Glycine, Sulfate Ion	2.70, 2.00	42734.80	1564	10.1042/BJ20140972
4E0V	*Bothrops jararacussu*	497	FAD	3.10	56358.50	994	10.1016/j.bbrc.2012.03.129

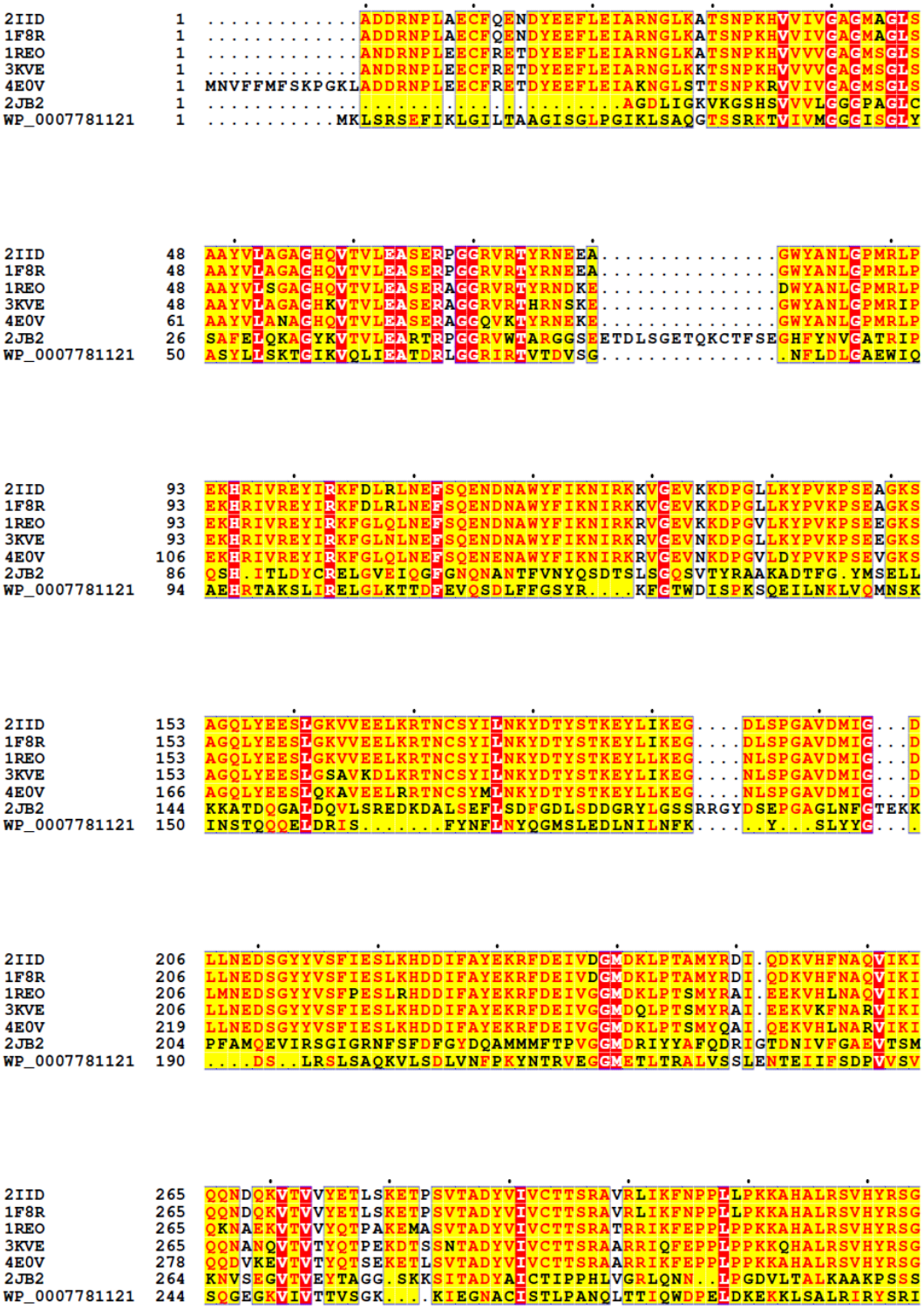


Figure 5.1. Sequence alignment of Li-LAO with sequences from PDB structure

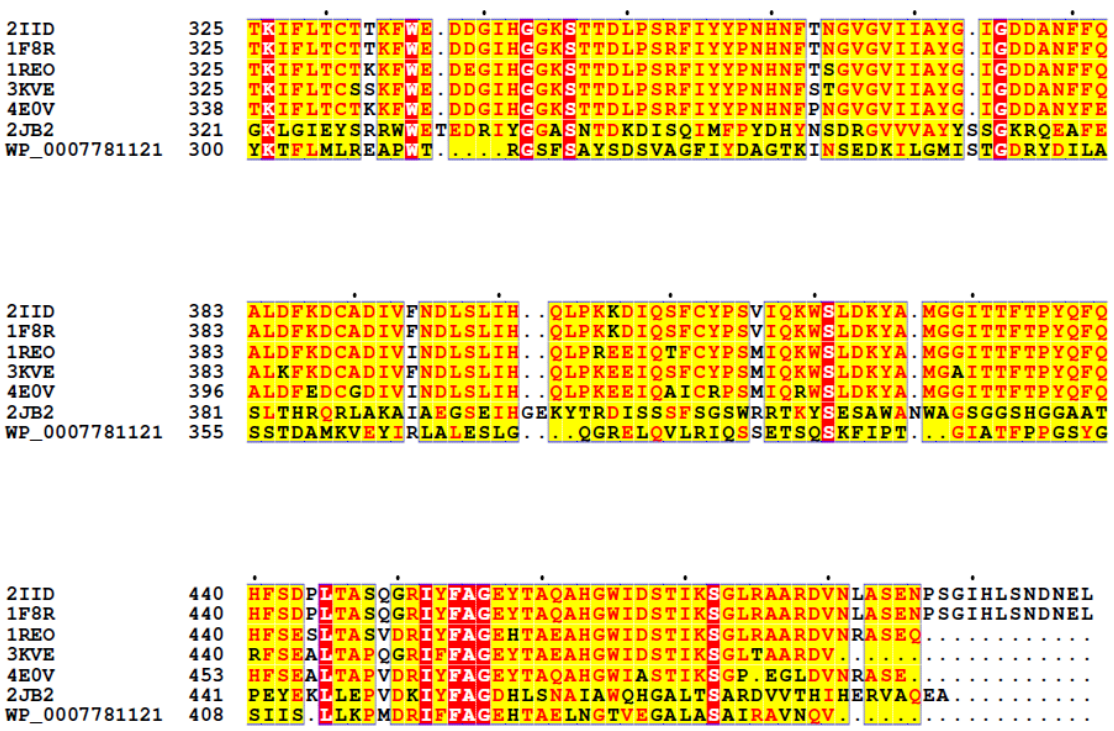


Figure 5.1. Sequence alignment of Li-LAO with sequences from PDB structure

Among the various L-amino acid oxidases, from snake venom and bacteria, the *Calloselasma rhodostoma* L-amino acid oxidase and *Rhodococcus opacus* L-amino acid oxidase are compared and well-studied (Pawelek PD. *et al.*, 2000; Faust A. *et al.*, 2007; Moustafa IM. *et al.*, 2006). Structural overlap of *Calloselasma rhodostoma* L-amino acid oxidase (PDBID: 2IID) and *Rhodococcus opacus* L-amino acid oxidase (PDBID: 2JB2) and residues that interact with FAD are mapped in Fig. 5.2. Overall both these enzymes show similar folds having a FAD-binding domain, a substrate-binding domain, and a helical domain. The *Calloselasma rhodostoma* LAO have shown to exhibit a long Y-shape which allows the substrate to interact with the enzyme in such a way that one portion of the input channel interacts with O_2, and the other is for product release occurs. In contrast in the case of Rhodococcus LAO there is no funnel-like structure to orient the trajectory of the substrate to the active site. The major structural difference lies in the helical domain. In fact, in various LAO the helical domain varies significantly. For example, in Rhodococcus, the helical domain forms an unusual dimerization mode which is not found in other members of the family.

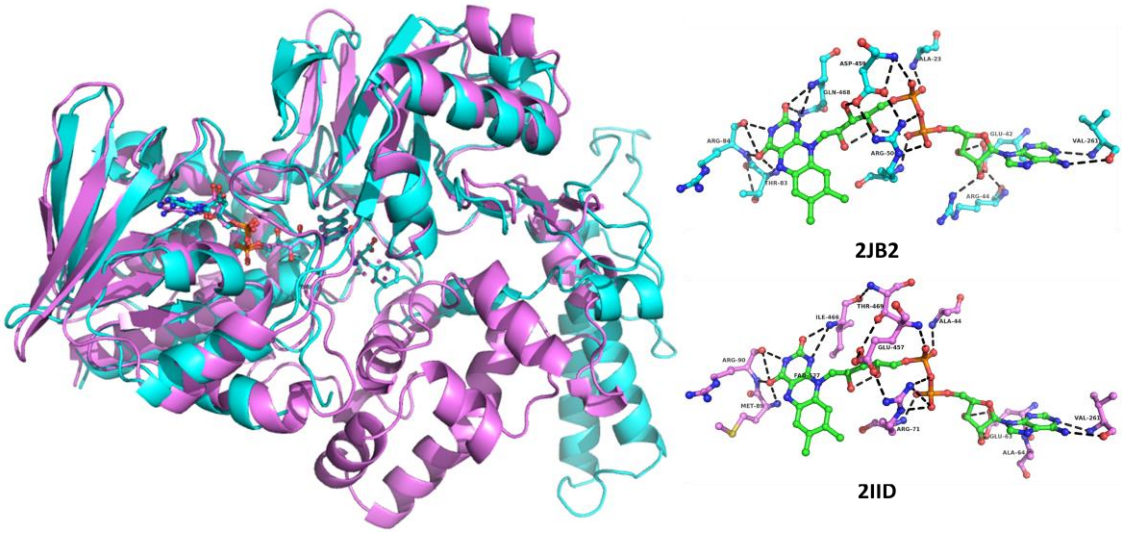


Figure 5.2 snake venom amino acid oxidase(2IID: pink) and Rhodococcus amino acid oxidase(2JB2: cyan) structural overlap and the conservation of FAD-binding residues.

With this substantial information of L- amino acid oxidase structural information the subsequence section will describe the experimental studies detailing the Leptospiral recombinant putative L-amino acid oxidase.

5.2 Materials

E.coli Strain Top10, *E.coli* Strain DH5α *E.coli* Strain BL21(DE3), C41(Generous gift from Prof. Aravind pennmatsa), pET21b plasmid, 6x DNA loading dye, dNTPs, protein markers, DNA ladder from Bangalore Genei, Bangalore, India; PHUSION DNA polymerase, Pre-stained marker (Thermo scientific), NdeI endonuclease, Xho1 endonuclease, Dpn1 from NEB; plasmid isolation kit (Qiagen), Tris, GnHCl, Urea, BSA, FAD, NADH, Dialysis membrane, EtBr, DTT, Agarose, SDS, PMSF from Sigma; Primers from Eurofins, India; NaCl, EDTA, Glycerol, Ammonium sulfate, HCl, Acrylamide, Bis-acrylamide, Bromophenol blue, Ammonium persulfate, TEMED, Glycine from SDFCL, amino acid substrates L-Histidine, L-Alanine, L-Tryptophan, L-Methionine L-Glutamine, L-Asparagine, L-Glutamate, L-Aspartate, L-Serine, L-Arginine, L-Proline, L-Tyrosine from Sigma (Generous gift from Prof P. Balaram and Prof Jayanta Chatterjee)and L-Phenylalanine, L-Leucine, L-Isoleucine, L-valine, L-Threonine from SRL. Amine substrates Dopamine and Spermine (sigma) from Prof. Aravind Pennmatsa lab, Spermidine (Sigma) from Prof. BG Lab, Histamine HCl from HiMedia, Putrescine and Tryptamine from Sigma (Prof. P. Balaram).

5.3 Methods

5.3.1 Cloning

DNA for Leptospiral recombinant LAO without 20-aminoacid signal peptide (Li-rLAO) was amplified by site-directed mutagenesis PCR using the primers Li-rLAO_Fp:ATGGGCCTTCCAGGAATAAAATTAAGTG and Li-rLAO_Rp:ATGTATATCTCCTTCTTAAAGTTAAAC and PHUSION DNA polymerase site-directed mutagenesis with the following condition of initial denaturation of 95°C for 30 sec, followed by 16 cycles of 95°C for 30 sec, 55°C for 1 minute, 68°C for 7 minutes and with the final extension of 68°C for 5 minutes from the plasmid pET21b plasmid containing full-length Li-LAO gene cloned between Nde1 and XhoI sites. The amplified PCR product was treated with Dpn1 for an hour and directly transformed into DH5α cells. The sequence of the clone was confirmed by DNA sequencing.

E. coli strain C41 was transformed with pET21b containing the recombinant LAO gene and grown in LB for 8-10 hrs with 100 µg/ml ampicillin. The culture was used as a 1% pre-inoculum in Louria Bertuni broth (LB) culture grown at 37 °C. After induction with 500 µM IPTG (OD_{600} 0.6-0.8), the culture was allowed to grow for another 16hrs at 30 °C. Cells were harvested by centrifugation (20 min, 6K rpm, 4˚C), resuspended in lysis buffer containing 20 mM Tris-HCl (pH 6.8), 1 mM EDTA, 1 mM PMSF, 1 mM DTT, and 10% glycerol (lysis buffer to bacterial culture volume was taken in the ratio of 1:20) and disrupted by sonication (35% amplitude, 20sec off and 10sec on, 25 cycles). After centrifugation (45 min, 12K rpm, 4 °C) and removal of the cell debris, the supernatant was subjected to an SP sepharose cation exchange chromatography column was equilibrated with 20mM Tris (pH 6.8) with the flow rate of 0.5ml /minute and washed with 20 mM Tris-HCl (pH 6.8), the bound protein was eluted with linear gradient containing 100ml of buffer A (20 mM Tris-HCl, pH 6.8) and 100ml of buffer B (20 mM Tris-HCl, pH 6.8 with 1M sodium chloride) with a flow rate of 1ml/ minute. The 10ml fractions are collected in a fraction collector.

Further, all the pooled fractions were concentrated by 30KDa cutoff centricon (3000 rpm, 44 °C). The concentrated protein was subjected to gel filtration chromatography (Superdex-200, XK26 column, Amersham Biosciences), equilibrated

with the 20 mM Tris-HCl (pH 6.8 with 100mM sodium chloride) (ÄKTA Basic 10 HPLC system, GE healthcare). Protein purity was checked by SDS-PAGE and LC-ESI/MS was done to determine the correct mass of the protein. The concentration of purified protein was estimated by the Bradford method.

5.3.2 Mass spectrometry

Electrospray ionization mass spectra were recorded on maXis impact Q-TOF(Bruker Daltonics, Bremen, Germany) coupled to Agilent 1200 series online HPLC. The spectrometer was tuned by using a standard Agilent ESI Tune mix ranging from m/z 118 to 2721 in the positive ion mode. Data processing was done using the deconvolution module of the 'Data Analysis 4.1' software (Bruker Daltonics, Bremen, Germany) to detect the multiple charge states and obtain derived masses.

5.3.3 In-gel trypsin digestion and MS/MS

Li-rLAO protein (20-10 ng per lane) was run in 10% SDS-PAGE (reducing) and stained with the Coomassie Brilliant Blue staining method. The bands corresponding to Li-rLAO were cut out and processed for tryptic digestion using sequencing grade modified Trypsin (Promega Corporation) (Shevchenko *et al*., 2006). In brief, Stained gel pieces were excised minced into $1mm^3$ pieces, and transferred into a sterile centrifuge tube. Gel pieces were washed with 500µl of wash buffer (50% acetonitrile, 50mM ammonium bicarbonate), till the coomassie dye is removed. De-stained gel pieces were dehydrated in 100% acetonitrile for 5 minutes and rehydrated in 150µl reduction solution (10mM DTT, 100mM ammonium bicarbonate) for 30 min at 56^0C. The reduction solution is discarded and incubated with 100µl of alkylating solution (50mM iodoacetamide, 100mM ammonium bicarbonate) for 30 minutes in dark at room temperature. Reduced and alkylated gel pieces are washed with wash solution and dehydrated with 100% acetonitrile for 5 minutes and completely dried at room temperature in a centrifugal evaporator. Gel pieces were rehydrated with 20µl of sequencing grade trypsin (20µg/ml) digestion solution and incubated overnight at $37C^0$. The digested peptides are extracted from the gel pieces with extraction solution (50% acetonitrile, 0.1% TFA), the extracted peptides from each sample were further concentrated in a vacuum concentrator and were subjected to MS-MS analysis.

5.3.4 UV-Visible Absorption spectrum of Li-rLAO

In order to obtain the UV-visible absorption profile of Li-rLAO, 285µM of Li-rLAO (in 20mM Tris pH6.8 and 100mM NaCl) was scanned between 200nm to 800nm with 20mM Tris pH6.8 and 100mM NaCl as blank.

5.3.5 Analytical Gel filtration

For determining the oligomeric state of Li-rLAO, analytical gel filtration chromatography was performed on a Superdex-200 (GE Healthcare) 10mm internal diameter x 300mm column. Gel filtration was done on a pre-equilibrated column with a flow rate of 0.2 mL/min, with Tris buffer (pH 6.8. 20 mM), 100 mM NaCl and absorbance at 220 nm and 280 nm was recorded to monitor the elution of protein. Injection volume was 500 µl with protein concentration ~140 uM. To remove any precipitate or particulate impurity, the protein was centrifuged at 14,000 rpm for 20 minutes at 4 °C before injection. Molecular weight standards Carbonic anhydrase(19kDa) and Alcohol dehydrogenase (150kDa), Albumin(66kDa), Apo-ferritin(443kDa), beta-amylase (200KDa), and Thyroglobulin(669kDa) were used to calibrate the column on a pre-equilibrated column with a flow rate of 0.2 mL/min, with Tris buffer (pH 7.2. 50 mM), 100 mM KCl.

5.3.6 Circular dichroism spectrum and Thermal denaturation

Circular dichroism spectrum was recorded on JASCO-715 spectropolarimeter (JASCO technologies, Tokyo, Japan), protein concentration ~90 µM. For thermal melting studies, Li-rLAO, (~90 µM final concentration in 20 mM Tris HCl, pH 6.8) was incubated at each temperature (25- 65 °C with 5 °C temperature jumps) for three CD scans were performed at a scan speed of 10 nm*min−1. CD measurements were performed on JASCO-715 spectropolarimeter (JASCO technologies, Tokyo, Japan) equipped with a thermostat cell holder controlled by a peltizer device. 1 mm path length cuvette, 2 nm bandpass was used. The HT voltage remained in the permissible range throughout all the scans. In order to find the temperature kinetics Li-rLAO, (~90 µM final concentration in 20 mM Tris HCl, pH 6.8) was monitored by CD measurements for every minute with a scanning speed of 50nm*min−1 with an interval of one minute with overall incubation of 30 minutes at 45°C, 50°C and 55°C

and change in the CD ellipticity at 220 nm (θ in mdeg) was plotted as a function of time at each of these specified temperatures.

5.3.7 Spectroscopic characterization

Fluorescence spectra of Li-rLAO were recorded on an F2500 fluorescence spectrophotometer (Hitachi Science and Technology, Japan), using a protein concentration of ~285 µM, with excitation wavelength 280 nm. Spectra were recorded in the range of 300-800 nm. For studying the effect of temperature on the tertiary structure of the protein, ~200 µM protein (buffer 20 mM Tris pH 6.8; 100 mM NaCl) was incubated at 37 °C and a spectrum was recorded with a cuvette path length of 1 cm and the bandpass used was 5 nm.

5.3.8 Sequence analysis of LAO

Sequence analysis was performed using BLASTP (Altschul SF. *et al.,* 1997) and PSI blast (Altschul SF. *et al.,* 1997), the motif search was performed in Pfam (Finn RD. *et al.,* 2014) and Conserved domain database (Marchler-Bauer A. *et al.,* 2013). Multiple sequence alignment with homologs was performed using the UniProt Knowledge database and UniProt Align ((Boratyn GM. *et al.,* 2013; UniProt. 2017).

5.3.9 Crystallization

The purified Li-rLAO protein was concentrated to ~14-16 mg/mL and was centrifuged at 14,000 rpm for 1hr at 4 °C prior to setting up for crystallization. The purified leptospira L-amino oxidase was screened for crystallization conditions of Hampton crystal screen kits using the under oil method. Crystals were obtained in the 95th condition of the Index screen in a crystallization drop containing 1µl of (~20mg/ml) protein and 1 µl of buffer condition (HR2-944-95 Containing 0.1 M Potassium thiocyanate, 30% w/v Polyethylene glycol monomethyl ether 2,000) at 298 K; micro-crystals appeared within 12 hours which were allowed to grow for a week to improve the crystal size and diffraction quality.

5.3.10 Data collection, structure solution, and refinement

For native crystal, diffraction data were collected on a cryo-loop, flash-frozen in liquid N_2, and the X-ray diffraction data was collected on Beamline XRD1/XRD2 at Elettra Synchrotron Trieste, Italy. Data were collected with 0.5-degree rotation. The native crystal diffracted up to 1.78 Å resolution. For anomalous signal, crystals were soaked with 1M potassium iodide for 15 minutes mounted on a cryo-loop, flash-frozen in liquid N_2, and the X-ray diffraction data was collected on Beamline XRD1/XRD2 at Elettra Synchrotron Trieste, Italy. Data were collected with 0.5-degree rotation. Data were collected twice for the same crystal; both data were merged with renaming files. The merged data were processed with iMOSFLM (Leslie AGW. *et al.,* 2007) and XIA2-DIALS (Winn MD. *et al.*,2011; Winter G. 2010; Winter, G. *et al.,* 2018; Evans PR. 2006, 2011; Evans PR. *et al.,* 2013), of the CCP4i2 suite of programs. The crystal soaked with (KI) diffracted to 2.38 Å resolution. The collected data of both the crystals were processed in the orthorhombic space groups $P2_12_12_1$. Iodine sites in the crystal were located using anomalous peak search and the final substructure was determined by Crank2.0 (Skubak P. *et al.,* 2013; Sheldrick GM. 2008; Schneider TR. *et al.,* 2002*;* Murshudov GN. *et al.,* 2011*;* Abrahams JP. *et al.,* 1996*;* Skubak P. *et al.,* 2010*;* Cowtan K. 2006, 2016) of the CCP4i2 package. The final built model (838 residues) was obtained by crank2 with a final R factor: 0.2735 and a Final Rfree factor: 0.3474. The asymmetric unit is compatible with 2 monomers and 39% water content. Manual model building and refinement of the structure were carried out using COOT v0.7.2.1(Emsley P. *et al.,* 2010) REFMAC (version 5.5.0109) (Kovalevskiy O. *et al.,* 2018; Murshudov GN. *et al.,* 1997, 1999, 2011; Nicholls RA. *et al.,* 2012; Vagin AA. *et al.,* 2004; Winn MD. *et al.,* 2003). Further redundant data were processed by molecular replacement software Molrep (Vagin A. *et al.,* 2008*;* Lebedev AA. *et al.,* 2008). The data set between 84.64Å and 1.78 Å were included throughout the refinement calculations. Five percent of the data were randomly chosen for free R-factor. The data collection and processing statistics and parameters after the final round of model building and refinement are provided (Tables 5.2).

5.3.11 Structural analysis

Solvent accessible area and interface residue analysis, accessible and buried surface area and $\Delta G_{dimer\ dissociation}$ were done by PISA server (Krissinel E. *et al.*, 2007; Krissinel E. 2010, 2012). The structural alignment was done by DALI (Holm L. *et al.*, 1995, 1996; Holm L. 2020; Hasegawa H. *et al.*, 2009). Ramachandran Map for the structures was generated using Procheck and Rampage. All the crystal structure analysis and figure preparation were done by Pymol program version 1.2r1.

5.3.12 Amino acid oxidase assay

The assay was conducted in a 96-well microplate in triplicate with modification (Kishimoto M. *et al.*, 2001); 10 µl/well of enzyme solution (10µM-30 µM), 90µl/well of substrate solution were added to start the reaction. The standard reaction mixture contained L-amino acid (initially 5 or 10 Mm, at later stages the concentration of substrate was reduced to 2.5mM), 2 mM ortho-Phenylene Diamine (OPD), and 0.81 U/ml Horseradish peroxidase (HRP), in a total volume of 100µl/well of 50mM borax–HCl buffer (pH 8.5). After incubation at 37°C for 60 min, the reaction was terminated by adding 50µl of 2M H_2SO_4. The absorbance of the reaction mixture was measured by a microplate reader at 492 nm, using 630 nm as a reference wavelength (492/630 nm). In the time-course experiment, absorbance was measured at 420/630 nm, instead of 492/630 nm, at appropriate time intervals without adding 2M H_2SO_4 to terminate the reaction.

The amino acid oxidase assay was also carried out at 30°C for 60 min at a varying concentration of amino acids. Further, the assay was also carried in a phased manner; only with buffered amino acid substrates incubated with the Li-rLAO for 45 min at 30°C followed by the addition of HRP and OPD incubation at 30°C for another 45 minutes, and measurements were made after 90 minutes.

5.3.13 Amine oxidase assay

The assay was conducted in a 96-well microplate in triplicate with modification (Holt A. *et al.*, 2006); the reaction was carried out in a mixture containing 50mM HEPES, 5mM KCl, 2mM $CaCl_2$, 1.4mM $MgCl_2$ 140mM NaCl pH7.4, 200 µM substrate, 500µM of 4-aminoantipyrene, 1mM Vanillic acid, $4Uml^{-1}$ of horseradish peroxidase and 10µM-30 µM of Li-rLAO for 30 minutes at 37^0C. The absorbance of the reaction mixture was measured by a microplate reader at 498 nm.

5.4 Results

5.4.1 Cloning

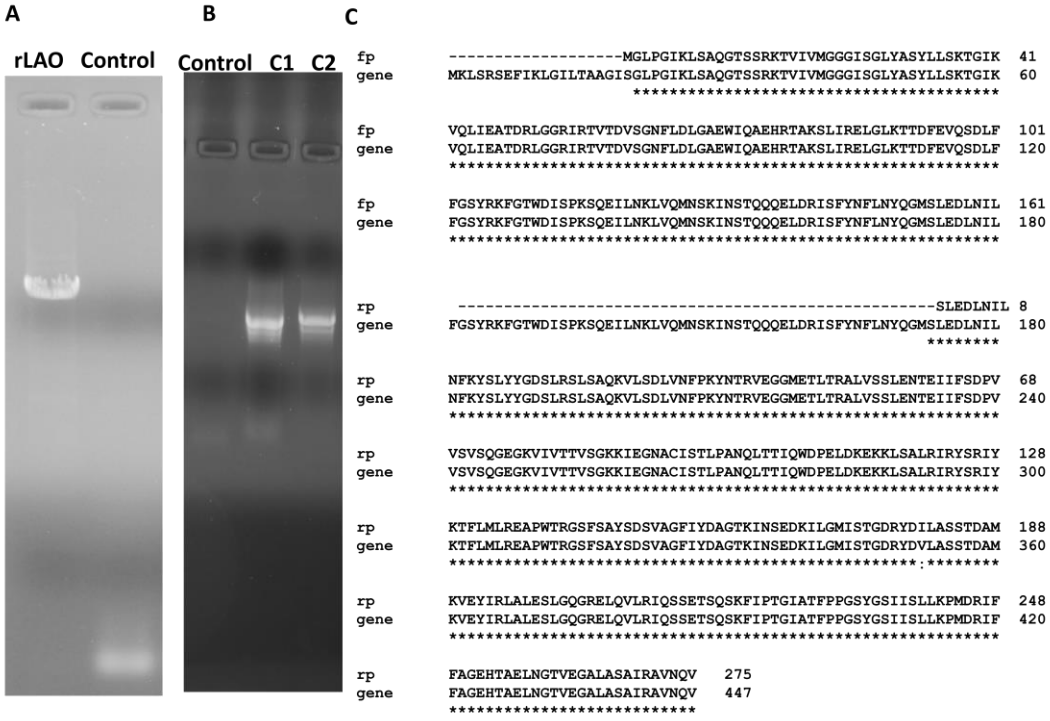


Figure 5.3. Cloning Li-rLAO. A) Site-directed mutagenesis PCR product of Li-rLAO on 0.8% agarose gel electrophoresis. B) Clone confirmation by PCR and C) Protein sequence alignment of clone obtained from DNA sequencing result (FP- sequence obtained by using T7 forward sequencing primer; RP- sequence obtained by using T7 reverse sequencing primer).

Li-rLAO was amplified by site-directed mutagenesis by the mega primer method as 7kb linear pET21bLAO with all the components except the Phusion DNA polymerase as a negative control. Both the PCR sample and control were treated with a Dpn1 restriction enzyme that specifically cuts the methylated parental strand DNA. The fraction of amplified PCR products post Dpn1 treatment loaded on 0.8% agarose gel (Fig. 5.3A) and 2µL of the sample and the control DNA was used to transform in DH5α cells. The plasmids were isolated from the positive clones using a Qiagen mini-prep plasmid isolation kit. The cloned plasmid DNA was checked for the proper insert by colony PCR (Fig. 5.3B) and further confirmed by DNA sequencing. The translated sequence of the clones was aligned with reference (Fig. 5.3C).

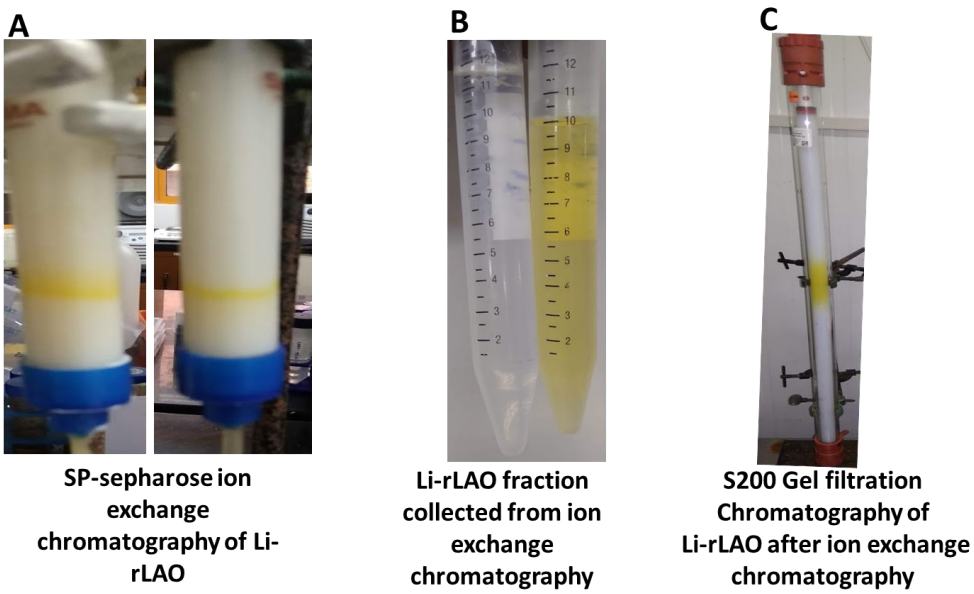
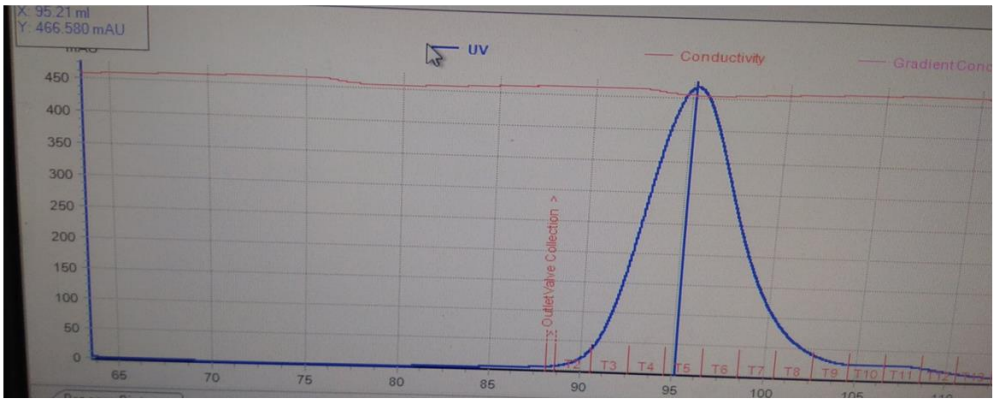


Figure 5.4. Purification of Li-rLAO. A) SP-sepharose ion-exchange chromatography showing protein (yellow color) bound to the column. B) Li-rLAO fraction collected from chromatography. C) S200 Gel filtration chromatography of Li-rLAO showing the yellow color of the protein.

Sequence confirmed Li-rLAO DNA (appendix 2) was transformed in *E.coli* C41 strains and selected in presence of ampicillin (final concentration of 100µg /mL). The positive clones were screened for expression of Li-rLAO protein (induced with 0.5mM IPTG) on 10% SDS-PAGE post-induction (Fig. 5.4A) by comparing the induced cell lysates with uninduced cell lysate as controls. The expressed protein was lysed by sonication, the cell debris was removed by centrifugation, and the supernatant obtained was loaded on to SP-sepharose column. The SP-sepharose-bound proteins were washed with 20mMTris, 100mM NaCl. Li-rLAO bound to the SP-sepharose column can be visualized easily by the presence of a yellow color ring in the column (Fig. 5.4B). The yellow-colored protein was easily collected compared with water (Fig. 5.4C). Upon molecular exclusion chromatography still, the yellow color of the protein can be easily monitored on the column (Fig. 5.4D).

A



B

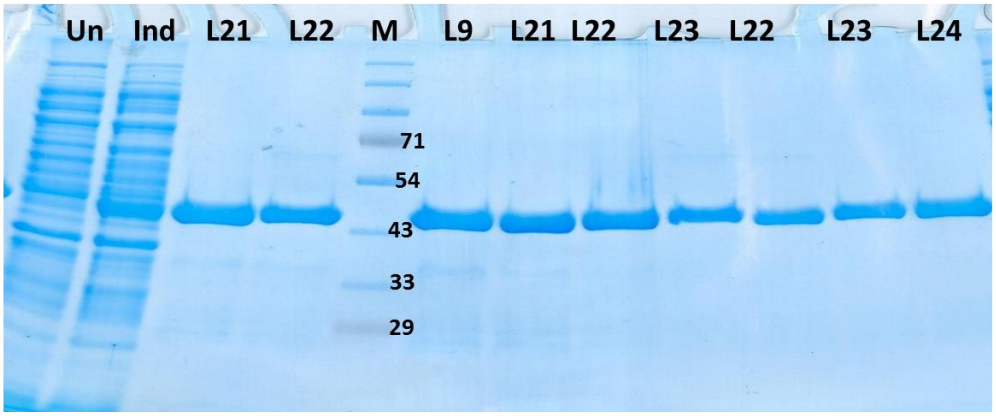


Figure 5.5. Purification of *Li*-rLAO. A) Representative S200 Gel filtration chromatography elution profile of Li-rLAO B) Representative SDS PAGE showing the purity of fractions of *Li*-rLAO, M represents marker(kDa); L21-L24 elution fractions from Gel filtration chromatography.

The purification of Li-rLAO was monitored by 280nm on Gel filtration chromatography (Fig. 5.5A) and collected as 2mL fractions. The obtained fractions were checked for purity on 10% SDS-PAGE (Fig. 5.5B).

5.4.2 UV-Visible Absorption spectrum of Li-rLAO

The purified protein was checked for UV-visible absorption spectra as the protein is bound by FAD (Fig. 5.6).

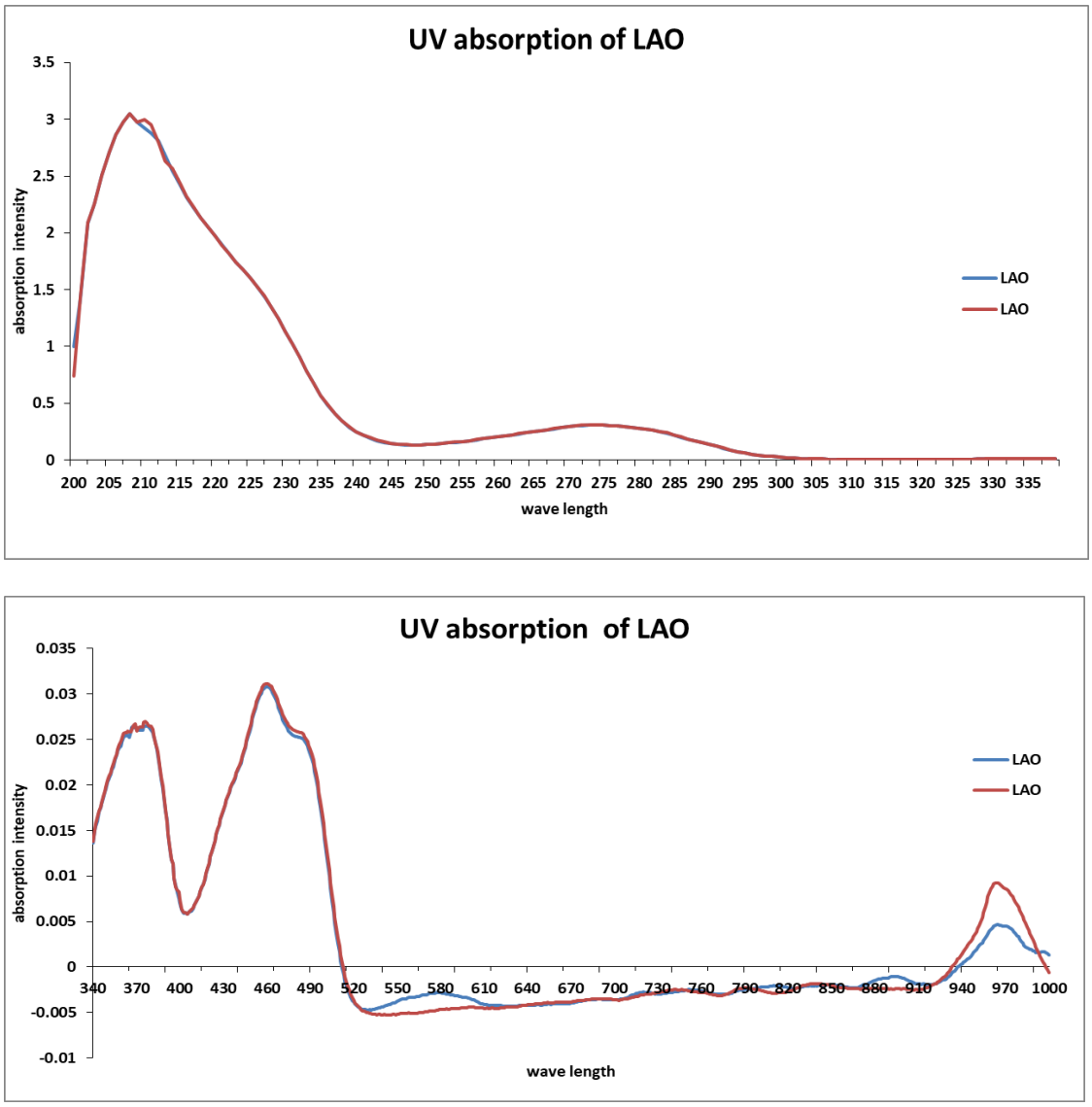


Figure 5.6. UV_Visible spectrum of Li-rLAO.

Clearly, the purified protein showed complex spectra with absorption at 284nm, 365 nm, 455nm, and 490 nm spanning over the entire region of 280 nm to 505 nm.

5.4.2 Mass spectrometry

The molecular mass of the purified protein was obtained from Q-TOF mass spectrometry. The protein showed a mass of 47090Da which is the mass 131Da less than the expected mass indicating the removal of N-terminal methionine in *E.coli* strain (Fig 5.7A). The protein was also subjected to mass spectrometry by negative mode and confirmed the presence of FAD (Fig. 5.7B).

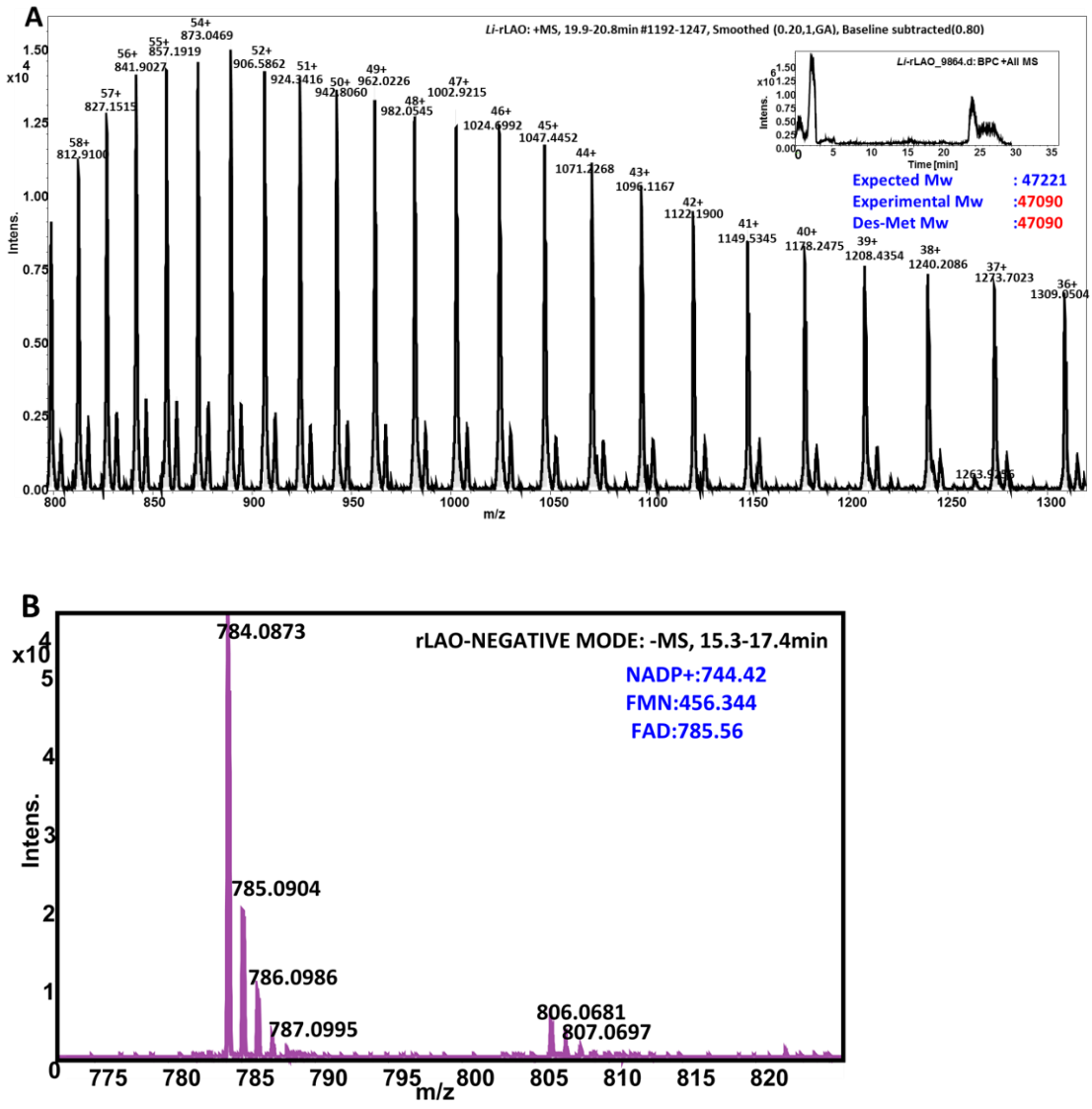
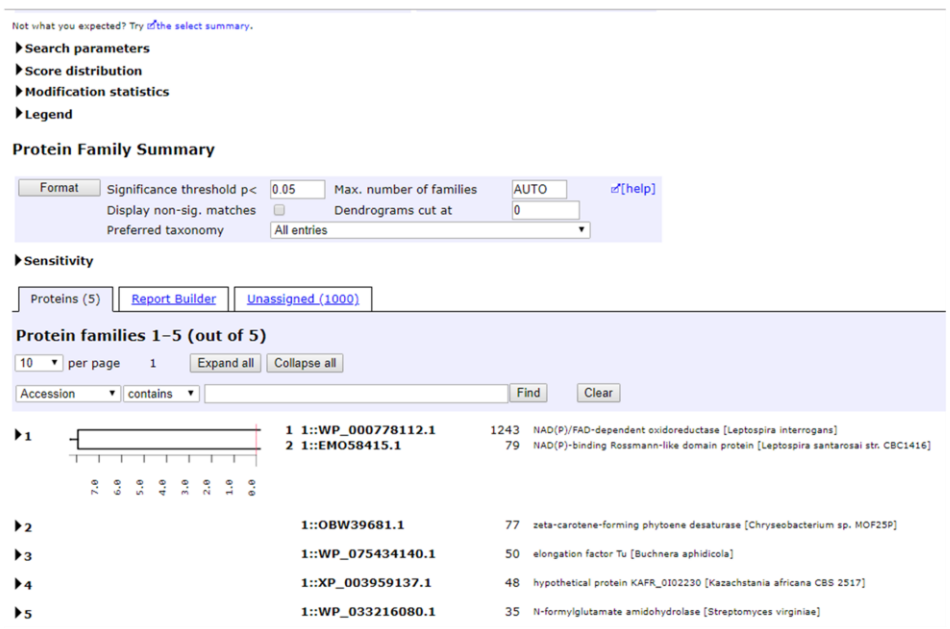


Figure 5.7. Mass spectrum of purified Li-rLAO protein A) showing an observed mass of 47090 Da which is equivalent to that of Des-Met mass B) Mass of FAD observed in purified Li-rLAO protein in negative mode.

A



B

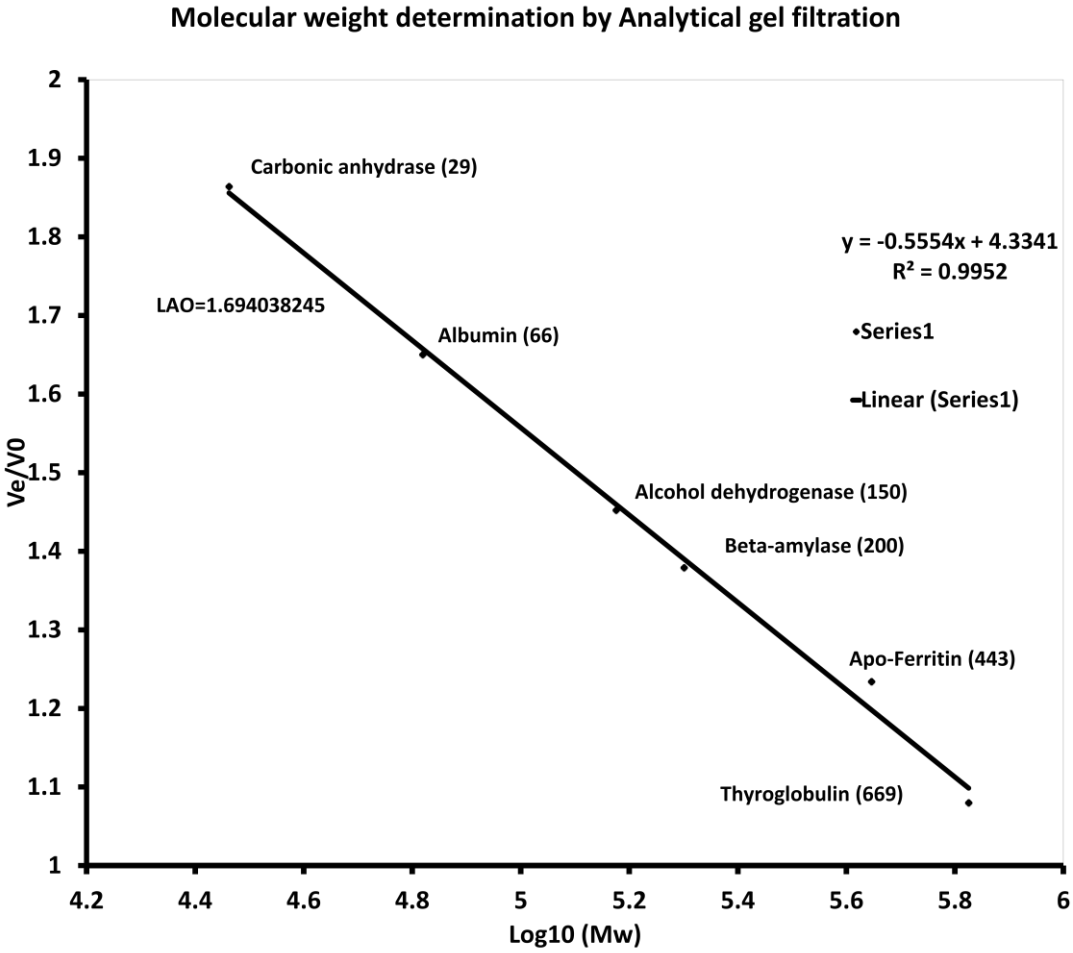
Query	Observed	Mr(expt)	Mr(calc)	Delta	Peptide
413	482.2401	962.4656	962.4781	-0.0125	K.LSAQGTSSR.K
484	560.2891	1118.5636	1118.5754	-0.0118	K.ILGMISTGDR.Y
522	1188.6393	1187.6321	1187.6550	-0.0230	K.VLSDiVNFPK
551	647.3008	1292.5870	1292.6037	-0.0167	K.YSLYYGDSLR.S
577	686.3166	1370.6186	1370.6387	-0.0201	R.YDILASSTDAMK.V
665	681.6413	2041.9021	2040.9065	0.9957	K.NFSAYSDSAAGFIYDAGTK.A
683	1043.5455	2085.0763	2085.1180	-0.0416	K.TVIVMGGGISGLYASYLLSK.T
694	1050.4617	2098.9089	2098.9484	-0.0395	R.GSFSAYSDSVAGFIYDAGTK.I
696	700.6457	2098.9153	2098.9484	-0.0331	R.GSFSAYSDSVAGFIYDAGTK.I
719	738.7326	2213.1759	2213.2130	-0.0371	R.KTVIVMGGGISGLYASYLLSK.T
791	1258.0897	2514.1648	2514.2139	-0.0491	R.TVTDVSGNFLDLGAEWIQAEHR.T
794	839.0647	2514.1723	2514.2139	-0.0416	R.TVTDVSGNFLDLGAEWIQAEHR.T
795	1266.1218	2530.2290	2530.2816	-0.0526	R.IFFAGEHTAELNGTVEGALASAIR.A
796	844.4185	2530.2336	2530.2816	-0.0480	R.IFFAGEHTAELNGTVEGALASAIR.A
800	633.5697	2530.2495	2530.2816	-0.0321	R.IFFAGEHTAELNGTVEGALASAIR.A
831	921.4563	2761.3470	2761.4022	-0.0552	R.ALVSSLENTEIIFSDPVVSVSQGEGK.V
871	1421.1739	2840.3333	2839.3891	0.9442	R.ISFYNFLNYQGMSLEDLNILNFK.Y
875	947.7867	2840.3383	2839.3891	0.9493	R.ISFYNFLNYQGMSLEDLNILNFK.Y
887	1019.1639	3054.4697	3054.5332	-0.0634	K.KIEGNACISTLPANQLTTIQWDPELDK.E
890	1062.1785	3183.5137	3183.5757	-0.0620	K.IEGNACISTLPANQLTTIQWDPELDKEK.K
891	796.8857	3183.5139	3183.5757	-0.0619	K.IEGNACISTLPANQLTTIQWDPELDKEK.K

Figure 5.8. Tryptic Digestion of Li-rLAO. A) Mascot software output results of the tryptic digest of purified Li-rLAO protein B) Table showing the various peptide fragments obtained from the digestion of the protein.

The identity of purified protein was confirmed by the tryptic digest of Li-rLAO subjected to Q-TOF mass spectrometry followed by data analysis by mascot software (Fig. 5.8A and 5.8B).

5.4.3 Analytical Gel filtration

Further, the oligomeric state of Li-rLAO was established as a monomer by analytical gel filtration column chromatography with the calibration of Molecular weight standards (Fig. 5.9).



	Mw	V_e	V_0	$\log_{10}$(Mw)	V_e/V_0
carbonic anhydrase	29000	16.57	8.89	4.462398	1.863892
albumin	66000	14.67	8.89	4.819544	1.650169
alochol dehydrogenase	150000	12.91	8.89	5.176091	1.452193
beta-amylase	200000	12.26	8.89	5.30103	1.379078
Apo-Ferritin	443000	10.97	8.89	5.646404	1.233971
Thyroglobulin	669000	9.6	8.89	5.825426	1.079865
LAO		15.06	8.89		1.6940382

Figure 5.9. Standard graph Molecular weight markers obtained from Analytical gel filtration column chromatography.

5.4.6 Circular dichroism spectrum and Thermal denaturation

The circular dichroism spectrum shows the protein is well-folded (Fig. 5.10).

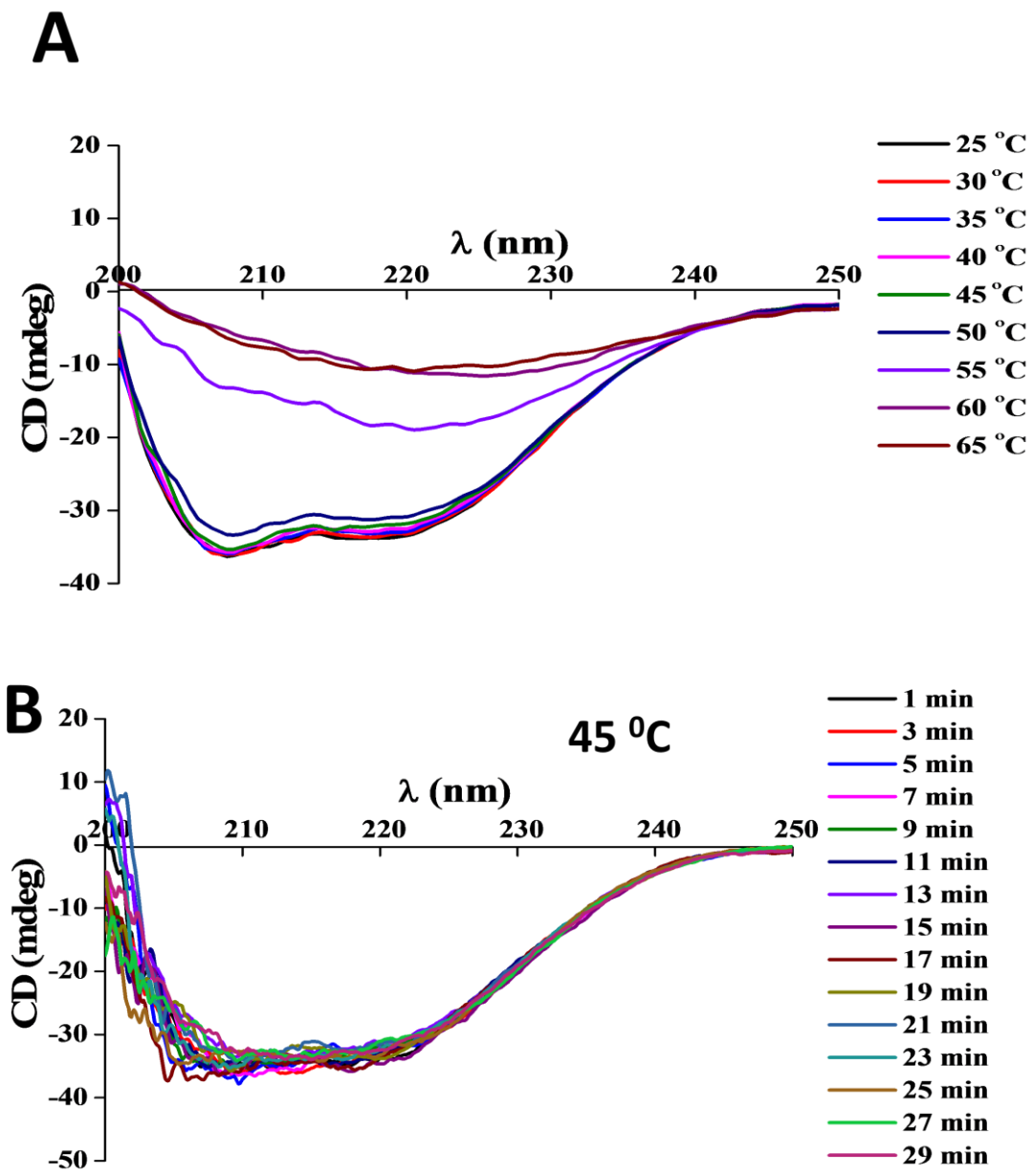


Figure 5.10. CD spectra and thermal melting curve of Li-rLAO.

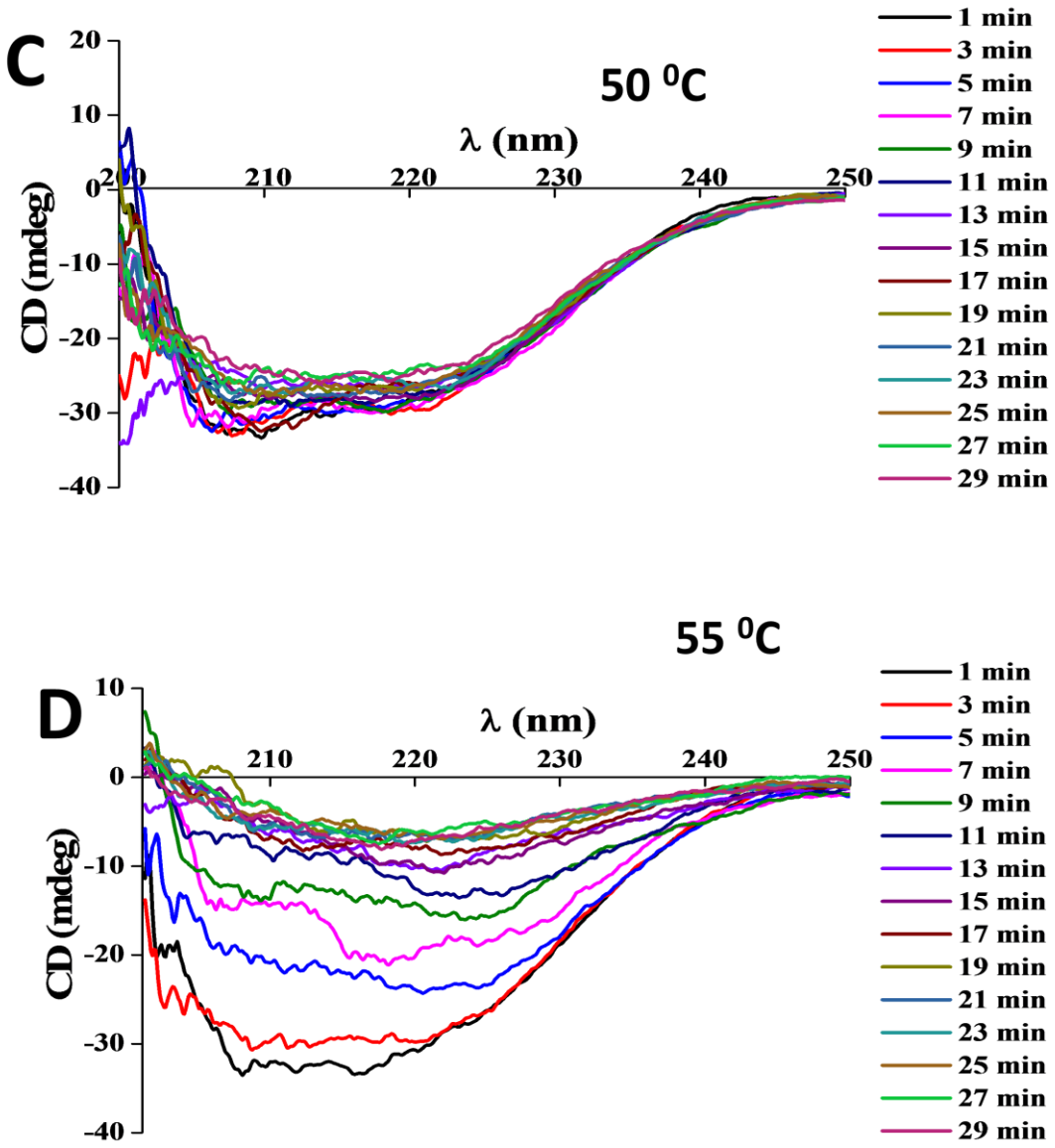


Figure 5.10. CD spectra and thermal melting curve of Li-rLAO.

The spectrum shows one prominent negative band at ~208 nm and another very broad but more intense negative band at ~222 nm (likely including the contribution due to β-strands as well as helices). Additionally, aromatic side chains of Tyr, Phe, and Trp may contribute to CD intensities around 222 nm.

The Thermal melting of Li-rLAO showed protein is stable till 50 ^{0}C. At 55 ^{0}C the protein showed sharp melting (Fig. 5.10A-D).

5.4.7 Spectroscopic characterization

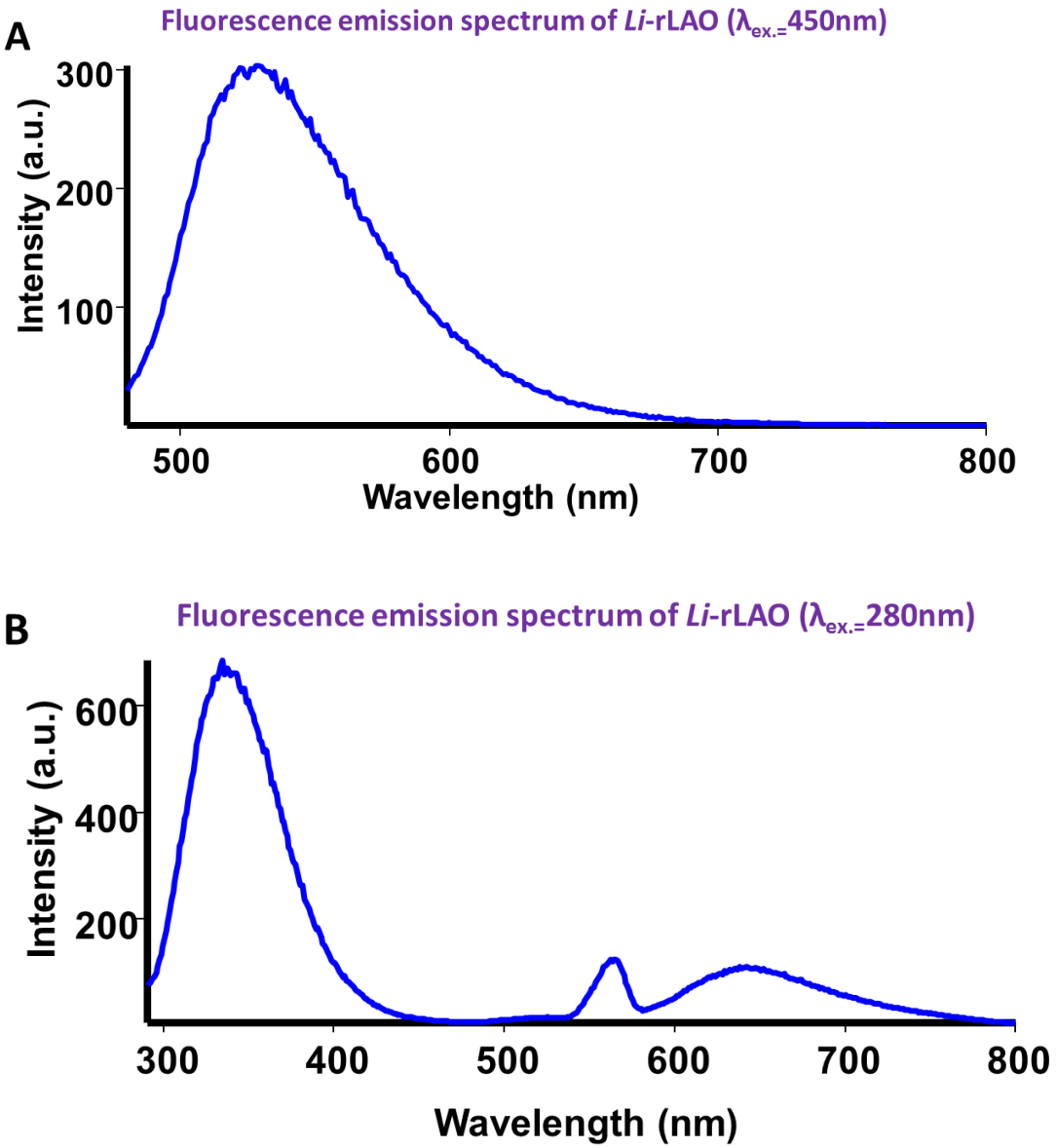


Figure 5.11. Fluorescence emission spectra of Li-rLAO.

The bound FAD showed a broad fluorescence emission band with λmax_{em} = 525 nm (Fig. 5.11A). The Internal tryptophan fluorescence studies of purified protein showed a broad fluorescence emission band with λmax_{em} = 330 nm when excited at 280 nm wavelength light and two more peaks at 560nm (might be corresponding to 280*2=560nm) and a peak at 640nm (Corresponding to $FAD^{\cdot-}$ radical) which needs further investigation (Fig. 5.11B).

5.4.8 Sequence analysis of LAO

FAD-binding protein [Sphingobacteriia bacterium]

Sequence ID: NBV12699.1 Length: 452 Number of Matches: 1

Range 1: 2 to 450 GenPept Graphics ▼ Next Match ▲ P

Score	Expect	Method	Identities	Positives	Gaps
213 bits(541)	4e-60	Compositional matrix adjust.	136/452(30%)	220/452(48%)	10/452(2%)

```
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            + R  FIK   + AAG    P     A G   +K VI++G G++GL A+Y L + G  V
Sbjct  2    VKRRSFIKTAGVIAAGTLLAPS---HAFGNRKKKRVIILGAGLAGLTAAYRLQQLGYGVS  58

Query  63   LIEATDRLGGRIRTVTDVSGNFL--DLGAEWIQAEHRTAKSLIRELGLKTTDFEVQSDLF  120
            ++E+  RLGGR+ T        L  ++G EWI + H   + L+ ++GLK  D   Q+ L
Sbjct  59   IVESKGRLGGRVLTYRPDPEQPLTVEMGGEWIGSSHHHMRKLVEDVGLKLVDHTFQNHLL  118

Query  121  F-GSYRKFGTWDISPKSQEILNKLVQMNSKINSTQQQELDRISFYNFLNYQGMSLEDLNI  179
            F   + + G WD+S K +  L  LV+  + +   Q+  +D+  ++ +L   G    DL++
Sbjct  119  FKAEHFRPGNWDLSDKGKASLRSLVEKYNSLKPDQRILIDKTDWWRYLRNSGFESRDLDL  178

Query  180  LNFKYSLYYGDSLRSLSAQKVLSDLVNFP---KYNTRVEGGMETLTRALVSSLENTEIIF  236
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            + PV  V Q  G V V T +G   E +  I  LP   +  I WDP    +KK +   ++Y
Sbjct  239  NQPVTRVRQNPGGVRVFTYNGTLFEADKVICALPVLAIRKIDWDPGFSDDKKEALNSLQY  298

Query  297  SRIYKTFLMLREAPWTRGSFSAYSDSVAGFIYDAGTKINSEDKILGMISTGDRYDILASS  356
            SRI KT  + +E  W    F   SD  A +IY A    +    +L   S GD   +L  +
Sbjct  299  SRITKTAFLFKERFWRDDQFGLLSDVHAHYIYHATRGQSGTHGVLMSHSIGDNAQVLGGA  358

Query  357  TDAMKVEYIRLALESLGQGRELQVLRIQSSETSQSKFIPTGIATFPPGSYGSIISLLK-P  415
            ++A +++ +  AL+ L    +  +L+  + + S+   +   +A + PG   S++  LK
Sbjct  359  SEAYRIKILNQALDPLFGPVDKSLLKQSTLDWSRDPIVMGAVALYGPGQVTSVMPELKQD  418

Query  416  MDRIFFAGEHTAELNGTVEGALASAIRAVNQV  447
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Sbjct  419  MHNVHFCGEHLGDWQGFMEGAVQSAYDVAEQI  450
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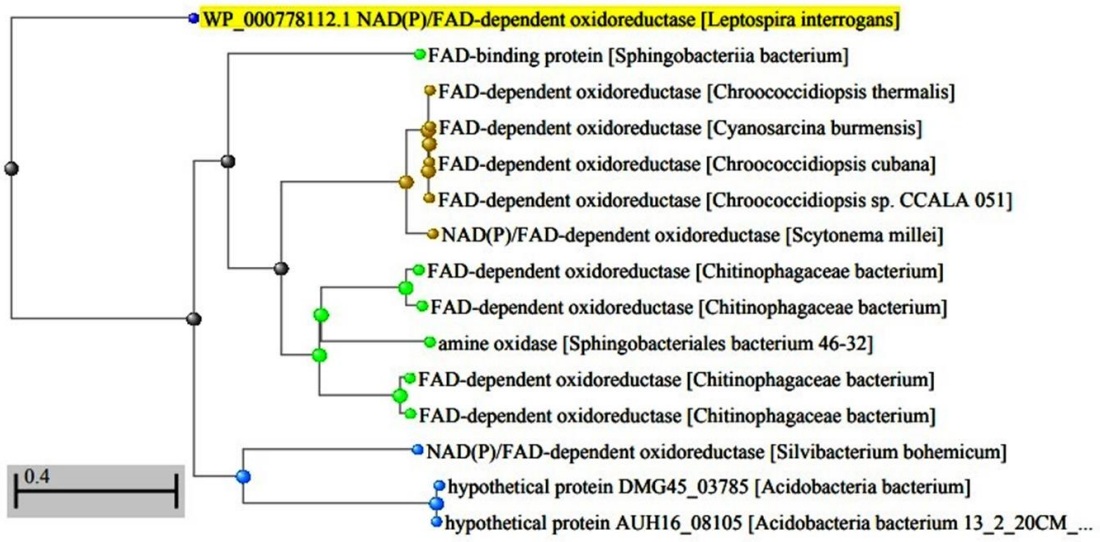
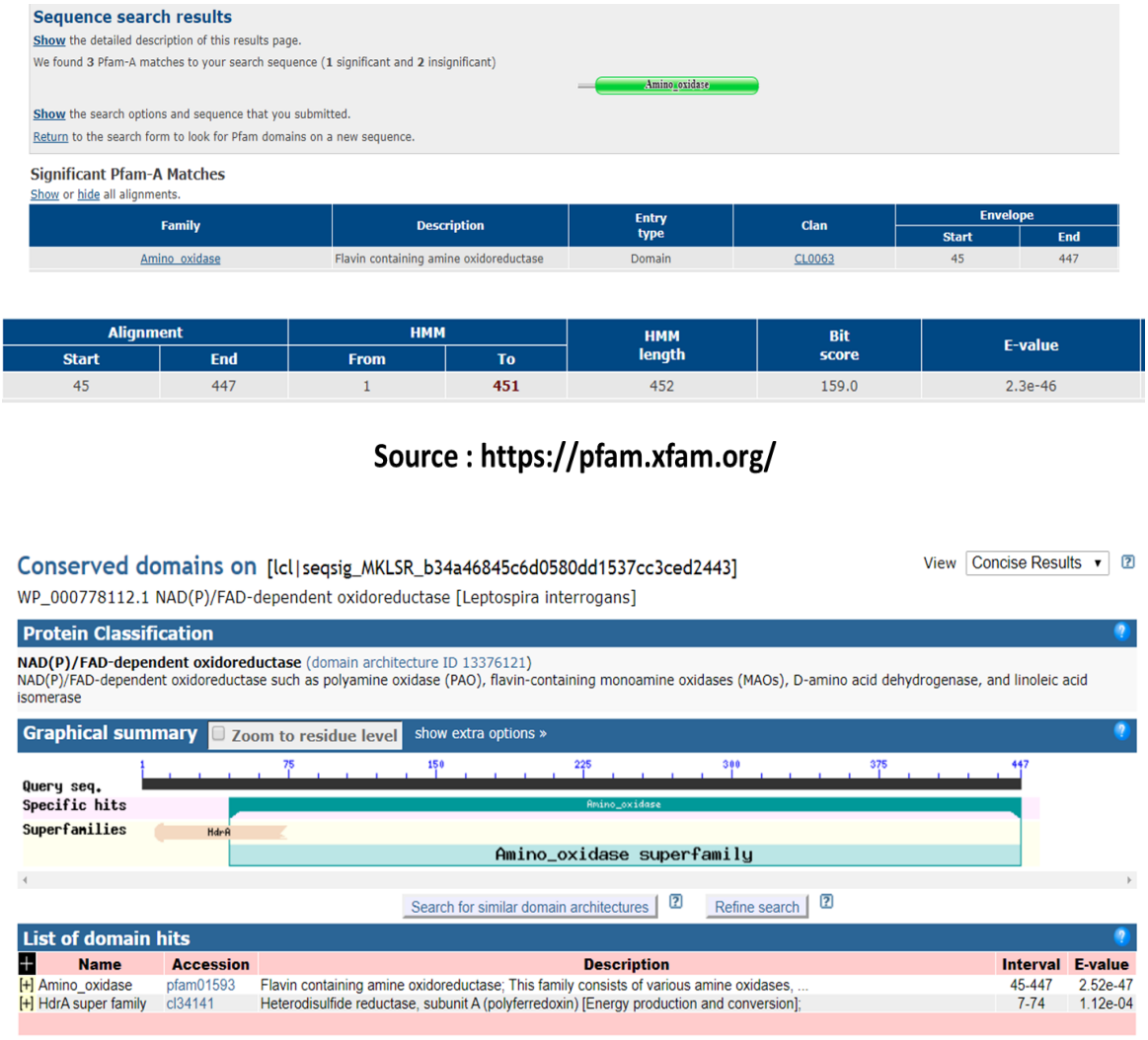


Figure 5.12. PSI-BLAST result of LAO. A. showing the best alignment. B. alignment evolutionary tree.



Sequence search results

Show the detailed description of this results page.

We found **3** Pfam-A matches to your search sequence (**1** significant and **2** insignificant)

Amino_oxidase

Show the search options and sequence that you submitted.

Return to the search form to look for Pfam domains on a new sequence.

Significant Pfam-A Matches

Show or hide all alignments.

Family	Description	Entry type	Clan	Envelope	
				Start	End
Amino_oxidase	Flavin containing amine oxidoreductase	Domain	CL0063	45	447

Alignment		HMM		HMM length	Bit score	E-value
Start	End	From	To			
45	447	1	451	452	159.0	2.3e-46

Source : https://pfam.xfam.org/

Conserved domains on [lcl|seqsig_MKLSR_b34a46845c6d0580dd1537cc3ced2443]

View Concise Results

WP_000778112.1 NAD(P)/FAD-dependent oxidoreductase [Leptospira interrogans]

Protein Classification

NAD(P)/FAD-dependent oxidoreductase (domain architecture ID 13376121)

NAD(P)/FAD-dependent oxidoreductase such as polyamine oxidase (PAO), flavin-containing monoamine oxidases (MAOs), D-amino acid dehydrogenase, and linoleic acid isomerase

Graphical summary

Zoom to residue level | show extra options »

Query seq. 1 75 150 225 300 375 447

Specific hits: Amino_oxidase

Superfamilies: HdrA, Amino_oxidase superfamily

Search for similar domain architectures | Refine search

List of domain hits

Name	Accession	Description	Interval	E-value
[+] Amino_oxidase	pfam01593	Flavin containing amine oxidoreductase; This family consists of various amine oxidases, ...	45-447	2.52e-47
[+] HdrA super family	cl34141	Heterodisulfide reductase, subunit A (polyferredoxin) [Energy production and conversion];	7-74	1.12e-04

Source : https://www.ncbi.nlm.nih.gov/cdd/

Figure 5.13. Pfam and CDD analysis of LAO.

The sequence alignment of LAO by PSI-BLAST indicated that it best aligned with FAD-dependent oxidoreductases from various bacteria (Fig. 5.12). The Pfam and conserved domain database identified LAO as an amino oxidase superfamily (Fig. 5.13).

5.4.9 Crystallization

The purified Li-rLAO was crystallized by the micro batch-method with a 1:1 ratio of 1µL of protein (20 mg/ml) with 1µL condition containing 0.1M potassium thiocyanate, 30%w/v polyethylene glycol monomethyl ether 2000. The crystals were over nucleated and to overcome this, the condition is diluted with 1 µL of water before mixing with the protein (Fig. 5.14). The crystals start appearing within 48 hours and left for at least one week for proper growth of the crystals.

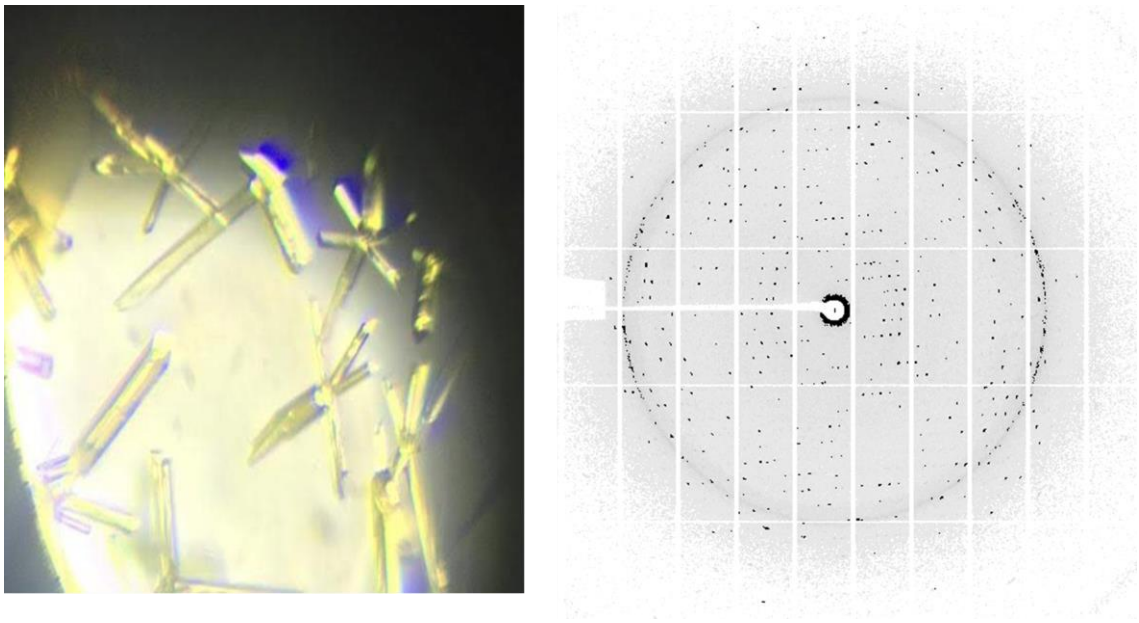


Figure 5.14. Crystals and representative diffraction pattern of Li-rLAO.

The diffraction data were used for structural determination by molecular replacement using Phaser, MrBUMP, MolREP, and automated structure determination server AUTORICKSHAW (Panjikar S. *et al*., 2005, 2009) but none of the molecular replacement programs was successful in building the model. Further, the data was also used in the Marathon MR (Hatti K. *et al*., 2017) which uses various domains available in the domain database; even this was not successful in building a model.

5.4.10 Data collection, structure solution, and refinement

So, the obtained crystals were soaked with various heavy metals salts. Crystals were stable upon soaking with platinum hexachloride and potassium iodide. The crystals, soaked with potassium iodide gave a weak anomalous signal but that was not

sufficient to determine the phase information. The potassium iodide soaked crystal data with redundant data was used by CRANK2.0.It identified 9 iodide atoms and using SHELX it built an initial model with 829 residues (R=27.35; Rfree=34.74). This model was refined several times by REFMAC and COOT. Data with 1.78 Aº resolution was further processed using the initial model by PHASER and refined several times. Structure determinations and refinements were done following standard crystallographic protocols, taking advantage of the good quality of the diffraction data which produced excellent electron density maps. The refinement statistics are given in the table (Table 5.2)

Table 5. 2. Crystal parameters and refinement statistics.

Parameters	Li-rLAO
Wavelength (Å)	1.07
Resolution limit (Å)	69.96-1.78 (1.81-1.78)
Unit cell dimension (Å)	51.76, 106.23, 139.92 (α=β=γ =90)
Space group	P 2_1 2_1 2_1
Rmerge (%)	11.8(39.0)
No. of unique reflections	74369
No. of molecules in the asymmetric unit	2
Completeness (%)	99.1(99.8)
Multiplicity	11.3(12.1)
Mean((I)/sd(I))	14.3(5.9)
Rcryst/Rfree (%)	17.7/22.3
RMSD bond length (Å)	0.0147
RMSD bond angle (˚)	1.834
Average B-factors (Å2)	
Protein	17.18
Water	25.08
Ligands	9.14
Ramachandran statistics (%)	
Favored region	94.2
Allowed region	5.55
Outliers	0.25

5.4.11 Structural analysis

The Leptospiral protein has been shown to exists as a monomer by analytical gel filtration, although the structure of the recombinant protein in the crystal forms approximately symmetrical crystallographic dimers (Fig. 5.15A), with the quasi dimer axes being non-coincident with any of three P2 axes inherent in the $p2_12_12_1$ crystal. The unit cell contains 16 individual polypeptide chains (Fig. 5.15B) with an asymmetric unit consisting of 2 chains. Superposition of the chains A and B yield an RMSD of 0.316 Å.

Both the subunits showed a clear electron density map for a single FAD molecule in each subunit (Fig. 5.16A). The crystals packs as $AB[FAD]_2$ with a surface area of 30850 $Å^2$ with a buried surface area of 6470 $Å^2$. Each subunit has a single cis peptide between Asp280, Pro281, and Glu282 (Fig. 5.16B). All protein residues have well-defined ordered conformations. Ramachandran plot illustrates 92 % of the residues were in the favoured region and 7.9 % of the residues were in the allowed region (Fig. 5.17). The electron density maps were missing for the initial 14 residues of the A chain and 15 residues in the B chain. Electron density map was missing for several residue's side chains in chain A: Ser34, Arg35, Lys36, Arg97, Lys109, Lys126, Glu138, Lys142, Lys209, Glu230, Asn231, Glu233, Lys249, Lys258, Lys39, Thr275, Gln374, Thr276, Gln379, and chain B: Lys36, Lys126, Glu138, Glu230, Glu247, Glu337, Gln374, Arg376, Glu377, Met416, Glu428, Arg428, and Val447. Atomic superposition indicates that the conformations of the crystallographically independent protein subunits are highly conserved across different crystal forms as shown by root-mean-square deviations in the range of 0.306 Å (using C-alpha atoms) (Fig. 5.18A). The electron density map between the second C atom of FAD containing C=O forms a hydrogen bond network with His96 fits a water molecule in one chain but in the other chain, its electron density is bigger than the regular water electron density map (Fig. 5.18B).

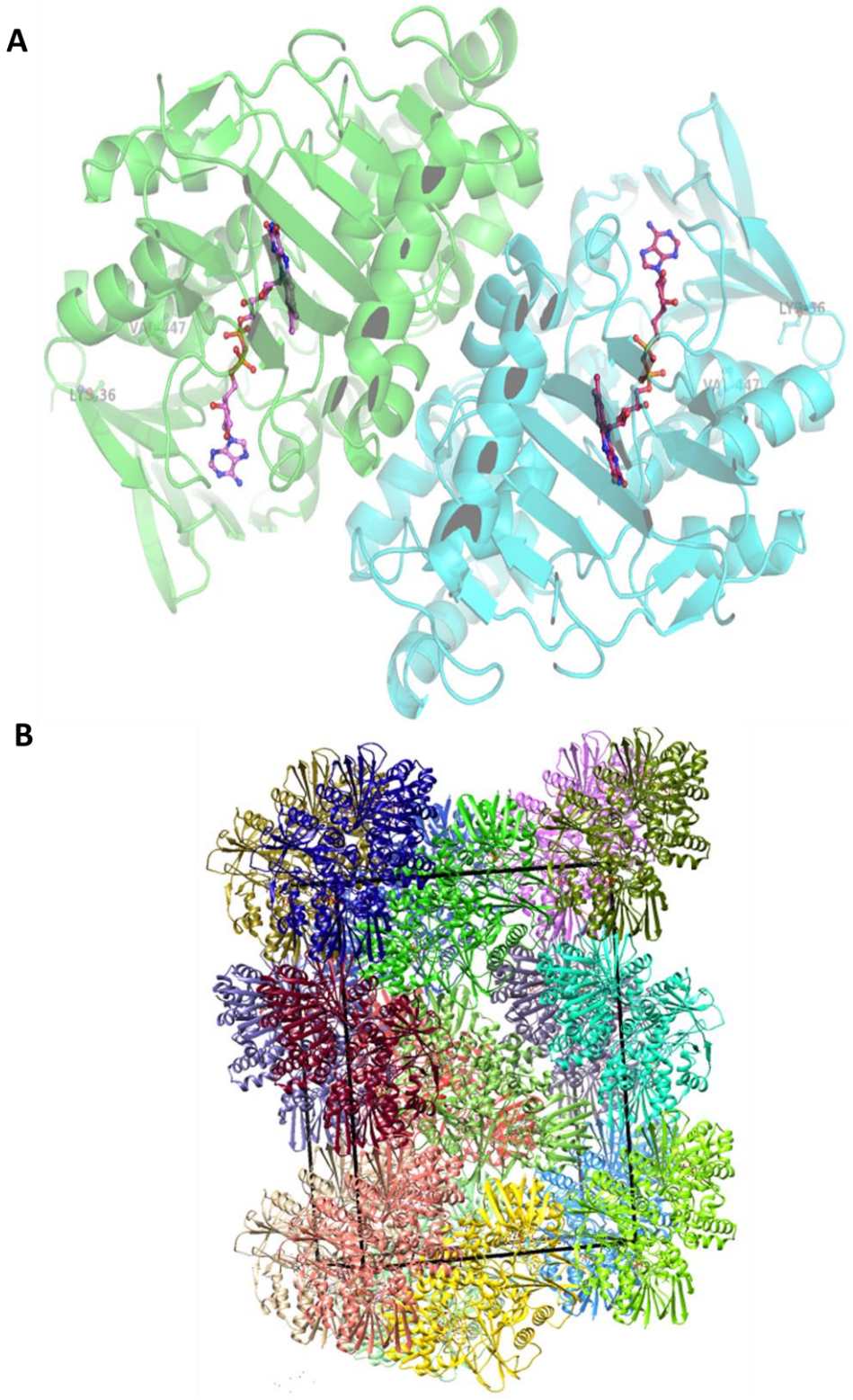


Figure 5.15. A) Dimer of Li-rLAO with bound FAD. B) Unit cell (P_{212121}) of Li-rLAO filled.

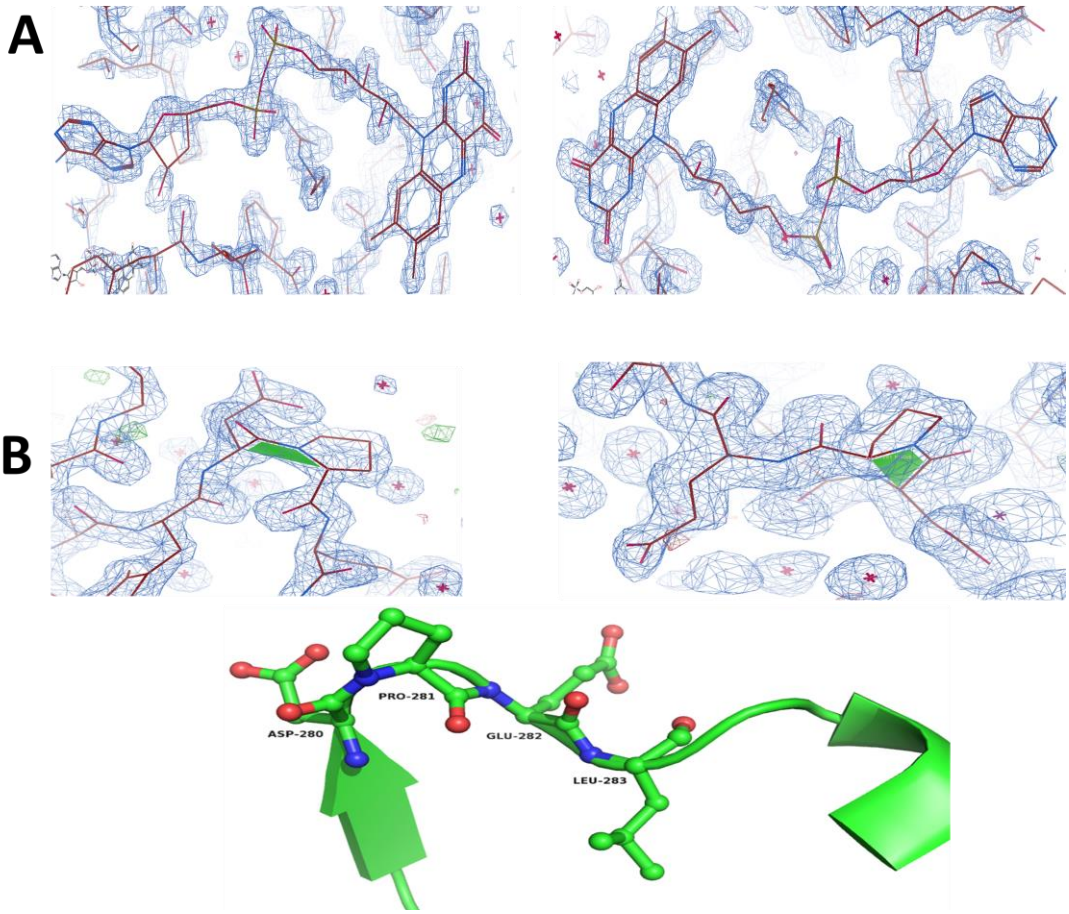


Figure 5.16. The special feature of the Li-rLAO. A) Electron density map of FAD bound to Li-rLAO in both the monomers. B) Li-rLAO structure showing cis-peptide at Asp280 and Pro281 in both the monomers.

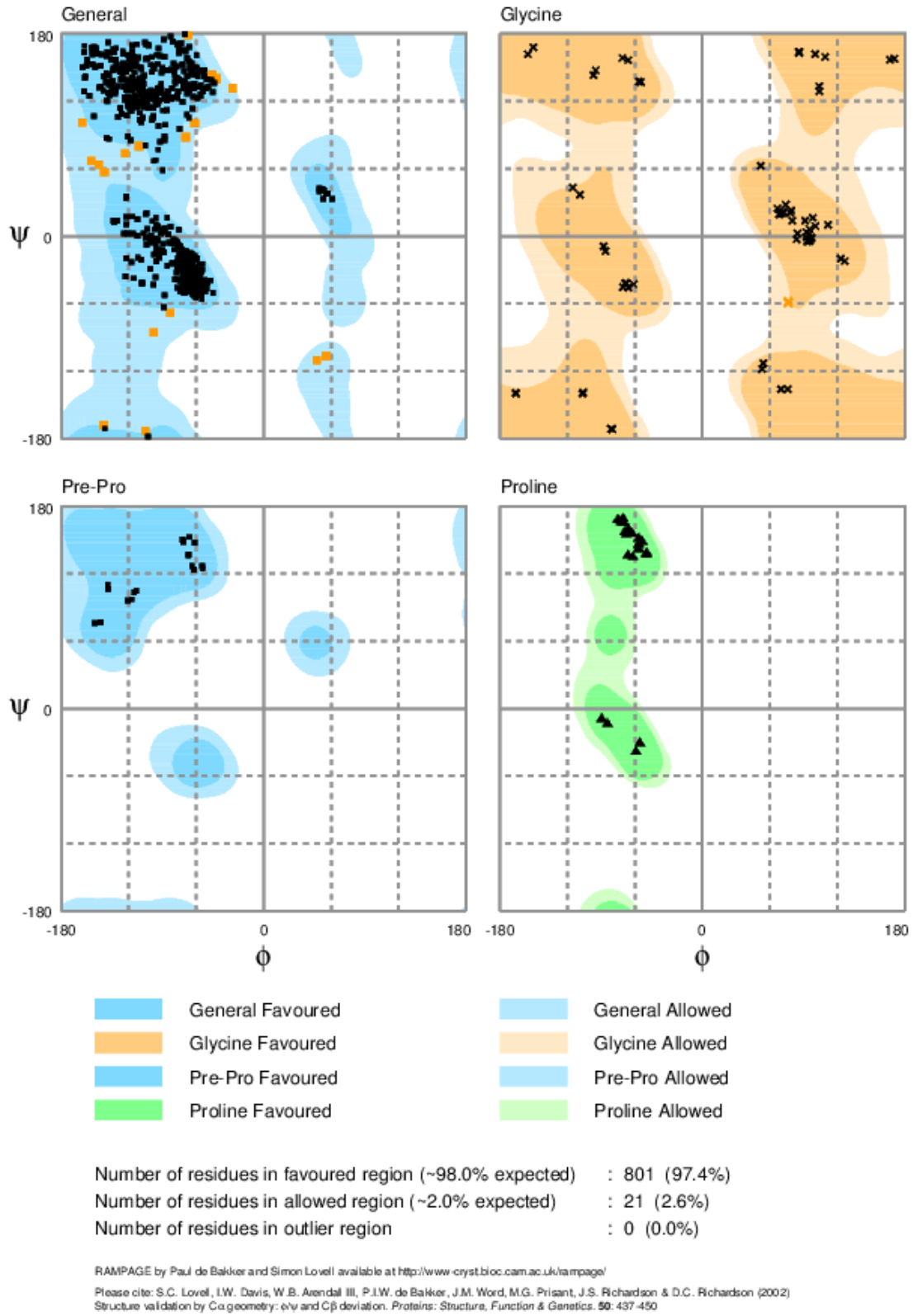


Figure 5.17. Ramachandran plot of Li-rLAO from RAMPAGE.

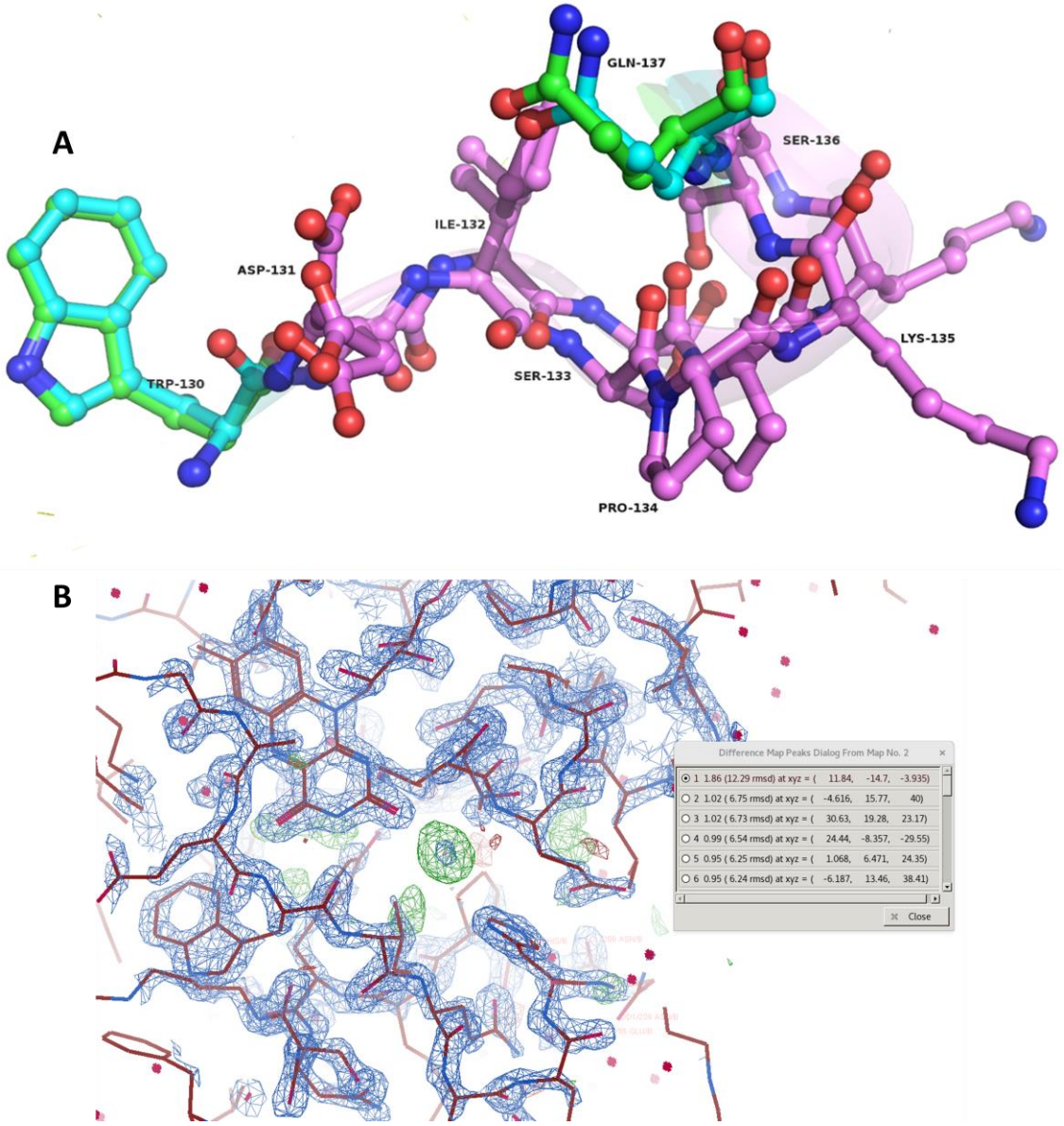


Figure 5.18. A) The overlap of the main chain of two subunits of Li-rLAO showing minor variation. B) Electron density map that lies between FAD and His96 in the B chain.

The Li-rLAO structure contains 15 helices, 19 beta-sheets, and 33 loop regions (Table 5.3 and Fig. 5.19). The overall structure of Li-rLAO is similar to those of Putrescine oxidase from *Rhodococcus erythropolis* with the highest Z score of 39.8 and 2.9 RMSD; human and rat MAO-A, which represent the closest structural homologues present in PDB. Atomic superposition yield root-mean-square deviations of 2.3 and 2.4 Å with 22% and 23% sequence identities, respectively (Table 5.4).

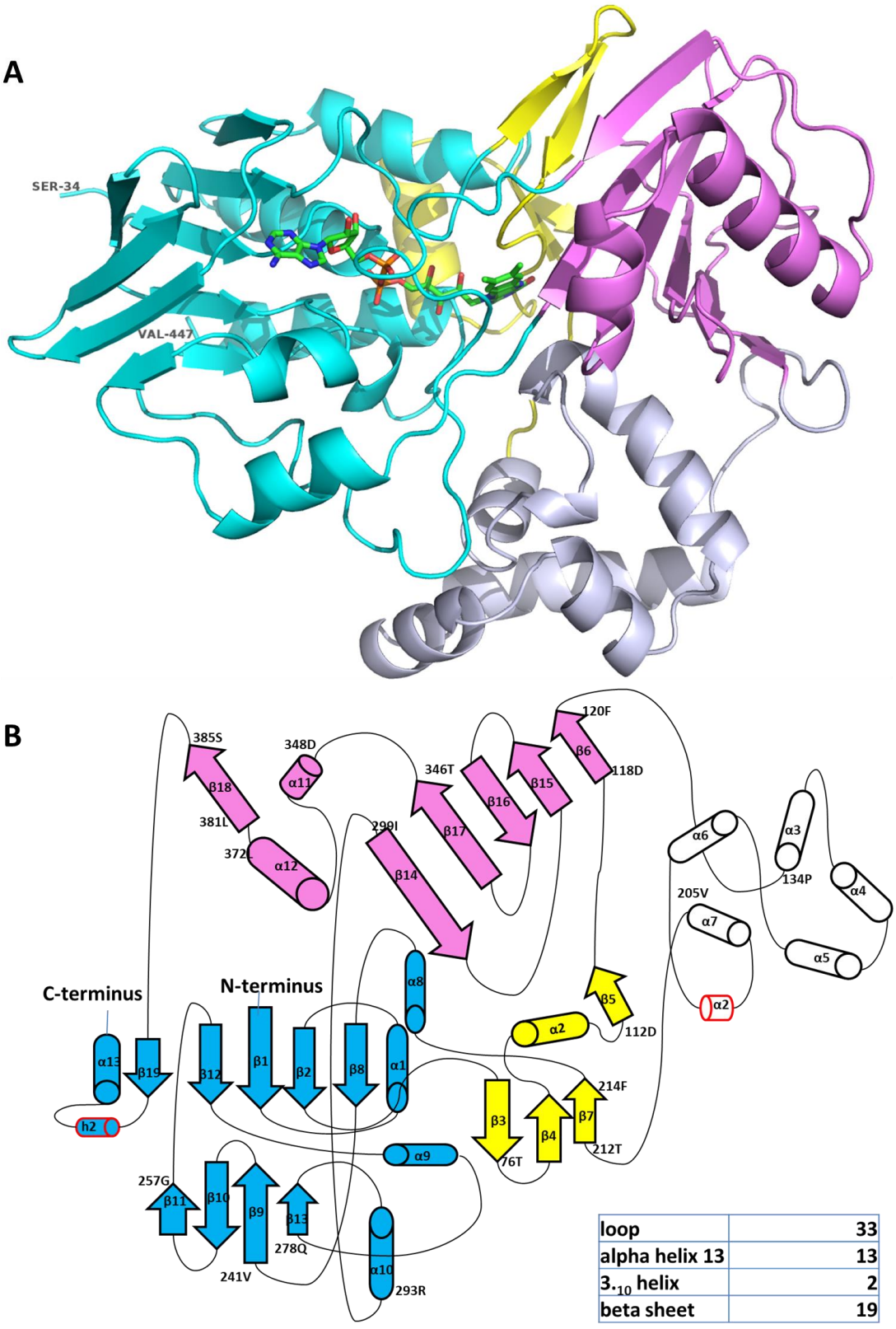


Figure 5.19: Various domains present in Li-rLAO and its cartoon representation.

Table 5.3. The residues number with various domains formation.

Alpha Helix	Residue No.	beta sheet	Residue No.	$3._{10}$ Helix	Residue No.
1	45I-57T	1	37T-41M	1	192L-194S
2	97K-106L	2	60K-65E	2	424E-426T
3	134P-149K	3	76T-79D		
4	152S-160R	4	82G-86D		
5	163F-170Q	5	110T-112D		
6	174L-188Y	6	118D-120F		
7	199K-205V	7	212T-214V		
8	218M-228S	8	232T-236F		
9	271A-276T	9	241V-246G		
10	285K-293R	10	249K-254T		
11	348D-355S	11	254T-257G		
12	358D-372L	12	264A-267S		
13	426T-433V	13	278Q-280D		
		14	299I-306L		
		15	317S-319Y		
		16	326F-329D		
		17	339K-346T		
		18	381L-385S		
		19	419I-421F		

Table 5.4. Dali output showing structurally similar proteins to Li-rLAO.

```
# Query: s001A
# No:  Chain   Z    rmsd lali nres  %id PDB  Description
   1:  2yg3-B 39.8  2.7  406   450   21   MOLECULE: PUTRESCINE OXIDASE;
   2:  6cr0-A 39.6  3.0  391   430   22   MOLECULE: (S)-6-HYDROXYNICOTINE OXIDASE;
   3:  2z5x-A 39.5  2.3  409   513   22   MOLECULE: AMINE OXIDASE [FLAVIN-CONTAINING] A;
   4:  4i58-A 39.4  2.6  404   451   20   MOLECULE: CYCLOHEXYLAMINE OXIDASE;
   5:  1o5w-C 38.9  2.4  409   512   23   MOLECULE: AMINE OXIDASE [FLAVIN-CONTAINING] A;
   6:  3ng7-X 38.5  2.9  387   427   21   MOLECULE: 6-HYDROXY-L-NICOTINE OXIDASE;
   7:  3rha-A 38.3  2.7  404   459   20   MOLECULE: PUTRESCINE OXIDASE;
   8:  6c71-B 38.2  2.8  392   440   21   MOLECULE: AMINE OXIDASE;
   9:  6fvz-A 38.1  2.4  410   500   21   MOLECULE: AMINE OXIDASE [FLAVIN-CONTAINING] B;
  10:  3kve-A 37.4  2.7  406   484   24   MOLECULE: L-AMINO ACID OXIDASE;
  11:  5ts5-A 37.2  2.7  405   483   22   MOLECULE: AMINE OXIDASE;
  12:  5z2g-A 37.1  2.7  403   477   22   MOLECULE: L-AMINO ACID OXIDASE;
  13:  2iid-A 36.9  2.8  402   484   22   MOLECULE: L-AMINO-ACID OXIDASE;
  14:  1tdk-A 36.7  2.8  402   486   24   MOLECULE: L-AMINO ACID OXIDASE;
  15:  2dw4-A 36.7  3.1  391   634   21   MOLECULE: LYSINE-SPECIFIC HISTONE DEMETHYLASE 1;
  16:  2jb2-A 36.4  3.7  354   479   23   MOLECULE: L-AMINO ACID OXIDASE;
```

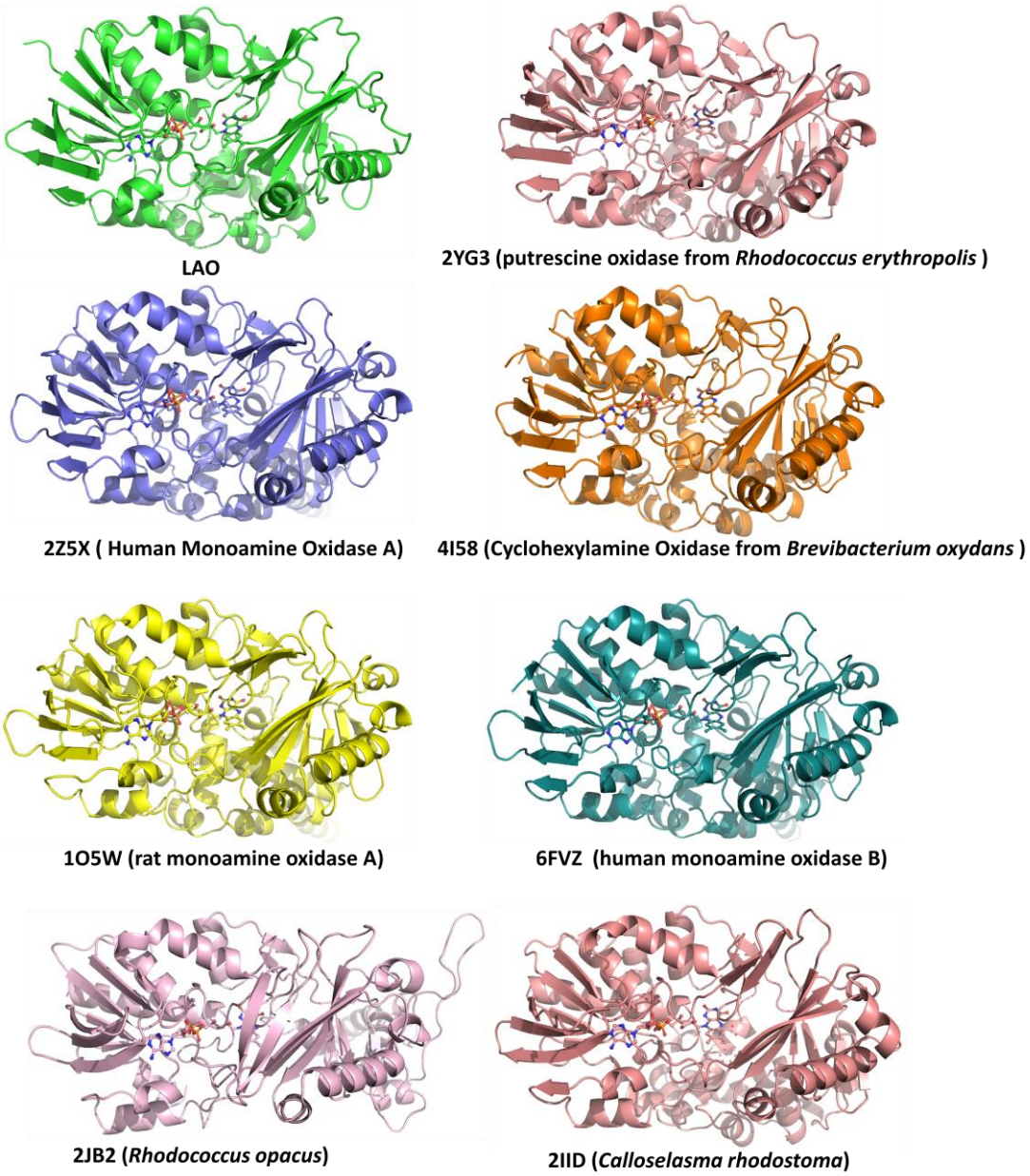


Figure 5.20. Structural organization of FAD-dependent oxidase showing maximum structural similarity with Li-rLAO.

Overall structures of FAD-dependent oxidase are similar (Fig. 5.20) with other species of FAD-dependent oxidase. Li-rLAO like other flavoproteins also contains a common FAD-binding fold, consisting of a four-stranded parallel beta-sheet sandwiched between a three-stranded antiparallel beta-sheet and an alpha-helix. The FAD is held by strong hydrogen bond networks (Fig. 5.21). The N1 atom of adenine moiety of FAD forms a hydrogen bond with a conserved Val240 and Pro239 backbone. In some flavin-containing oxidases, this proline residue is not conserved; The N3 atom forms a hydrogen bond with Ala66 and Glu65 backbone and interestingly Glu65 carboxyl group forms a hydrogen bond with ribofuranose ring –OH groups attached to adenine moiety. When this Proline is absent, the FAD is held further by hydrogen bonding with Arginine residues with ribofuranose OH groups apart from the conserved Glutamate residue hydrogen bonds. The N6 atom of adenine also forms a hydrogen bond with Gln273. The phosphate moiety attached to the ribofuranose forms a hydrogen bond with a conserved Arg73 side chain and its backbone NH. The phosphate attached to the ribityl moiety forms hydrogen bonds with the backbone NH of Ser46 and its side-chain OH group. In some of the flavin enzymes, this serine is substituted with threonine. Further, a conserved Gly423 also forms a hydrogen bond with the phosphate moiety. The ribityl moiety forms internal hydrogen bonds with its own OH groups and also with conserved water molecules surrounding it. The isoalloxazine ring of FAD is held by hydrogen bonding of backbones and not by any other polar side chain groups (Fig. 5.22). This is important for the evolution of FAD-dependent enzymes, oxidizing various substrates. Interestingly the 4^{th} carbon atom containing the C=O group of isoalloxazine ring forms a hydrogen bond with the backbone of a residue that is essential for holding the incoming substrates for oxidation either by hydrogen bonds or hydrophobic interactions. Apart from the FAD-binding domain, a C-terminal beta-sheet domain-containing, an alpha helix sandwiched by five antiparallel beta-sheets on one side and a single beta-sheet on the other side; a helical domain has 6 helices. The helical region forms the uniqueness of various FAD-containing proteins.

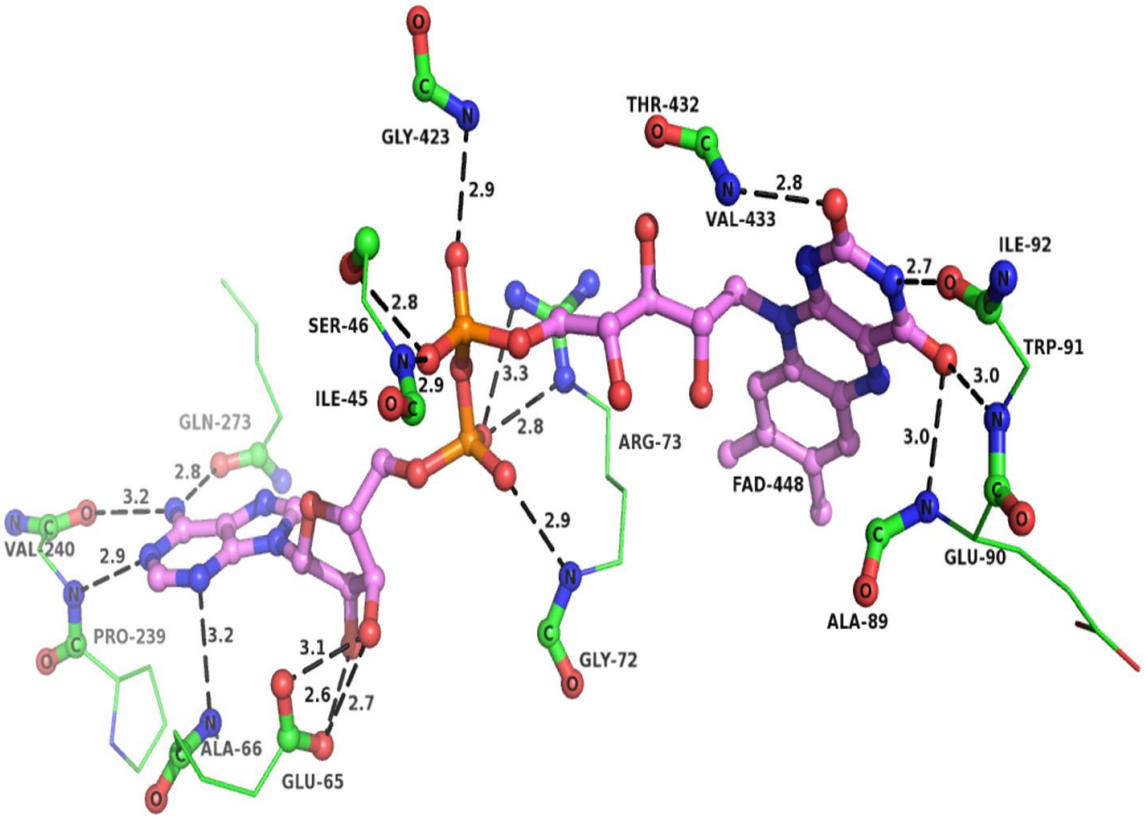


Figure 5.21. FAD interacting residues of Li-rLAO.

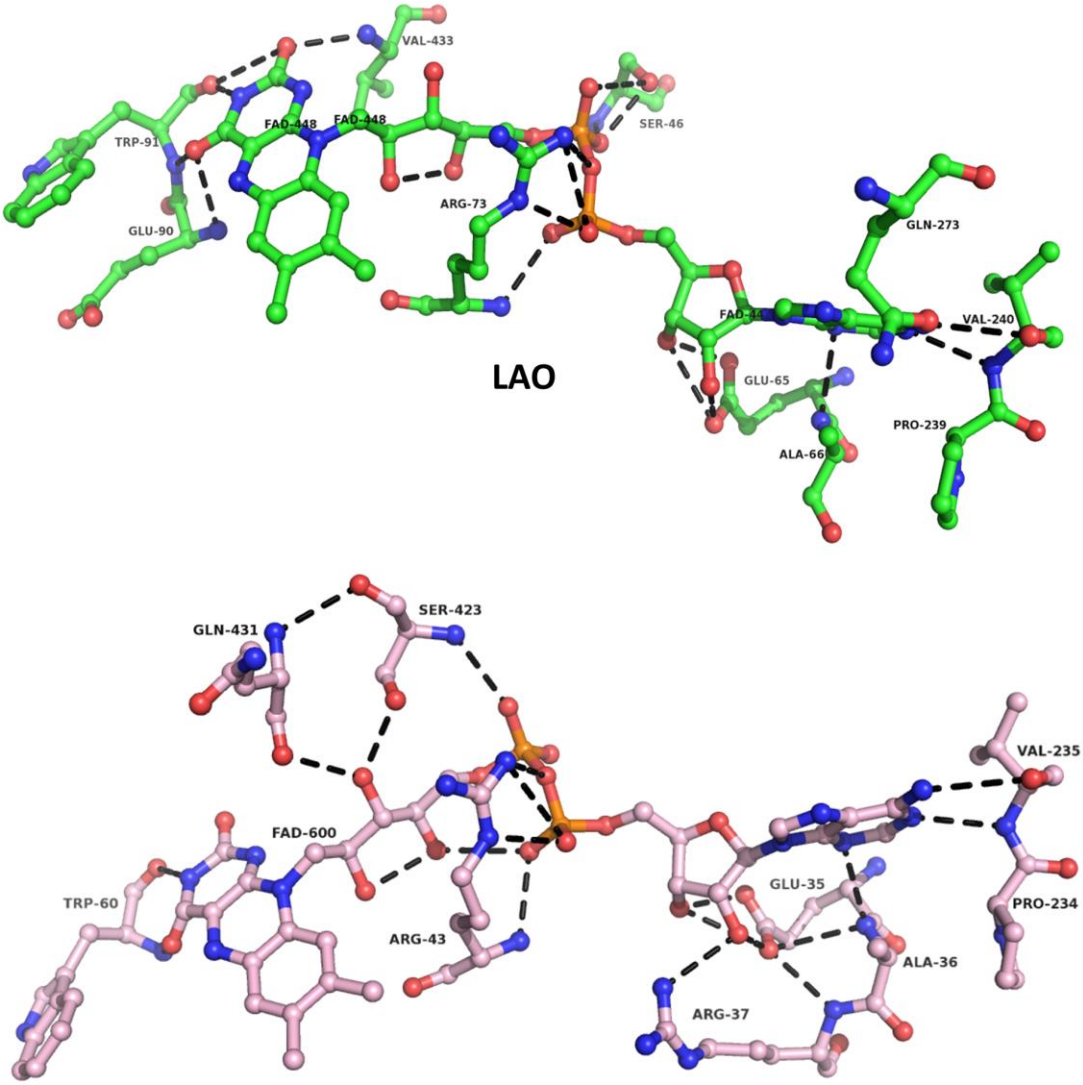
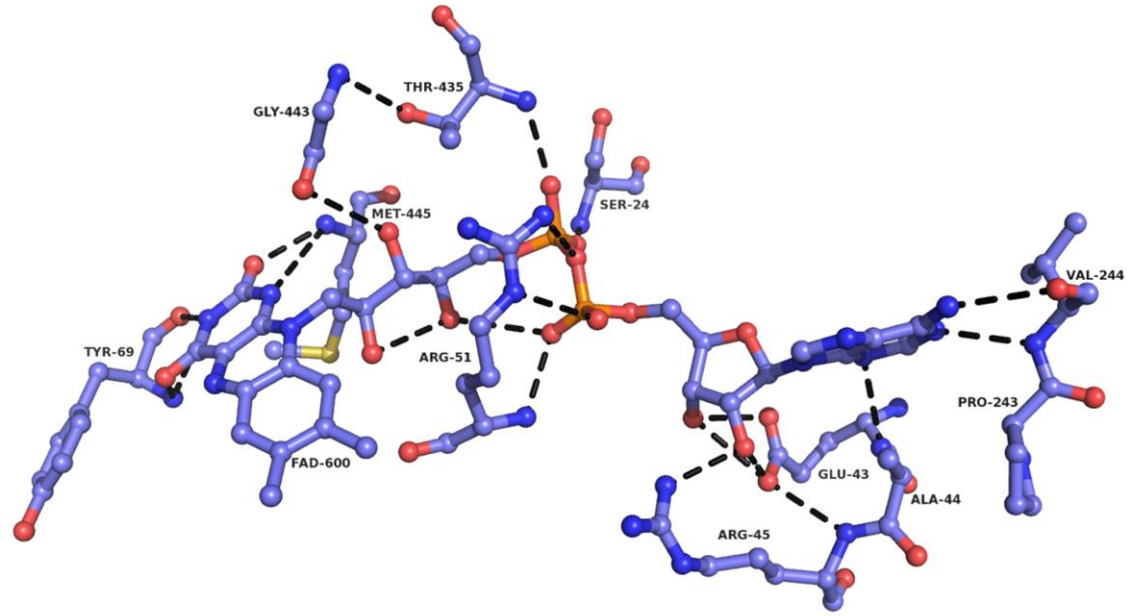
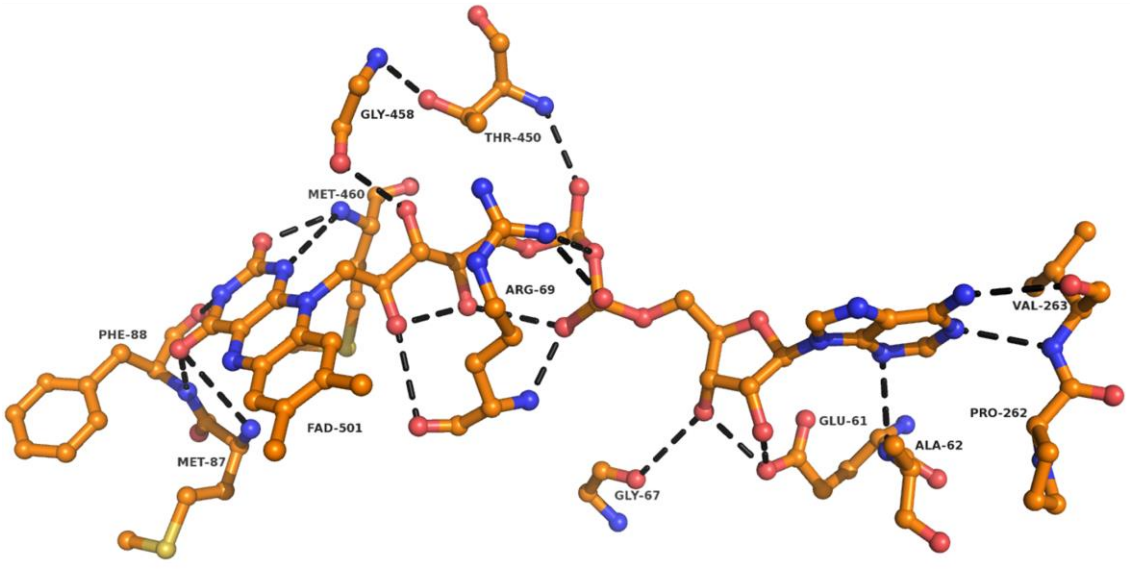


Figure 5.22. FAD binding motifs in various oxidases.

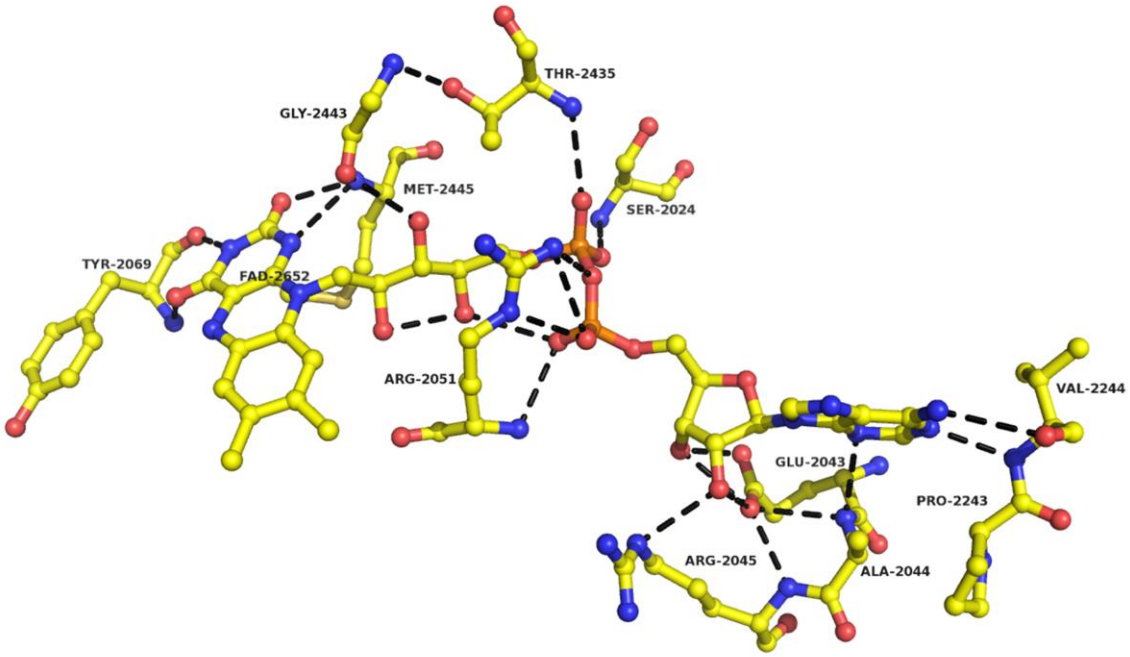


2Z5X (Human Monoamine Oxidase A)

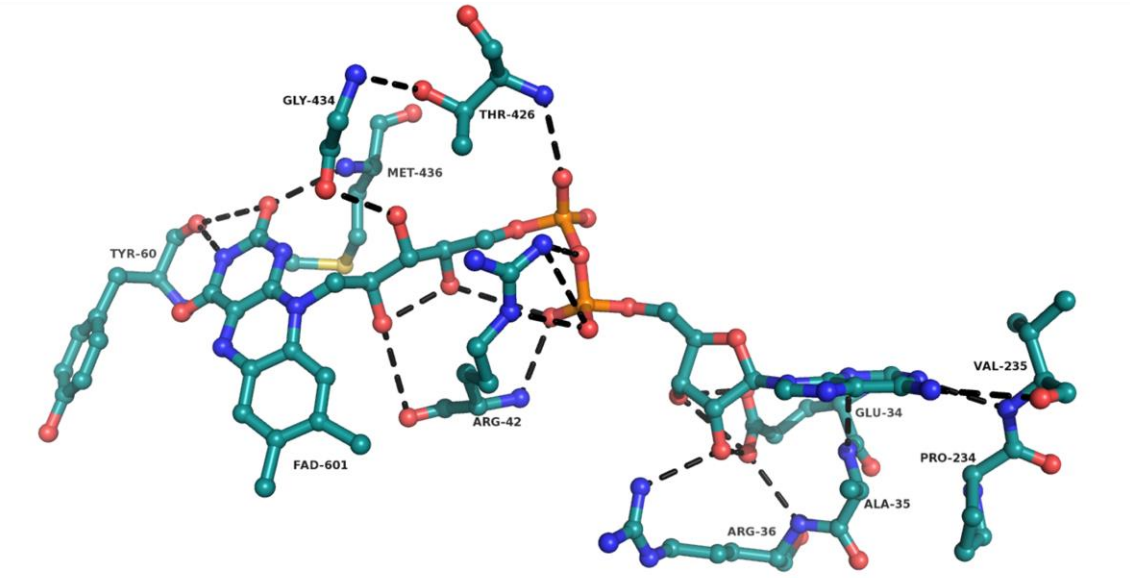


4I58 (Cyclohexylamine Oxidase from *Brevibacterium oxydans*)

Figure 5.22. FAD binding motifs in various oxidases.



1O5W (rat monoamine oxidase A)



6FVZ (human monoamine oxidase B)

Figure 5.22. FAD binding motifs in various oxidases.

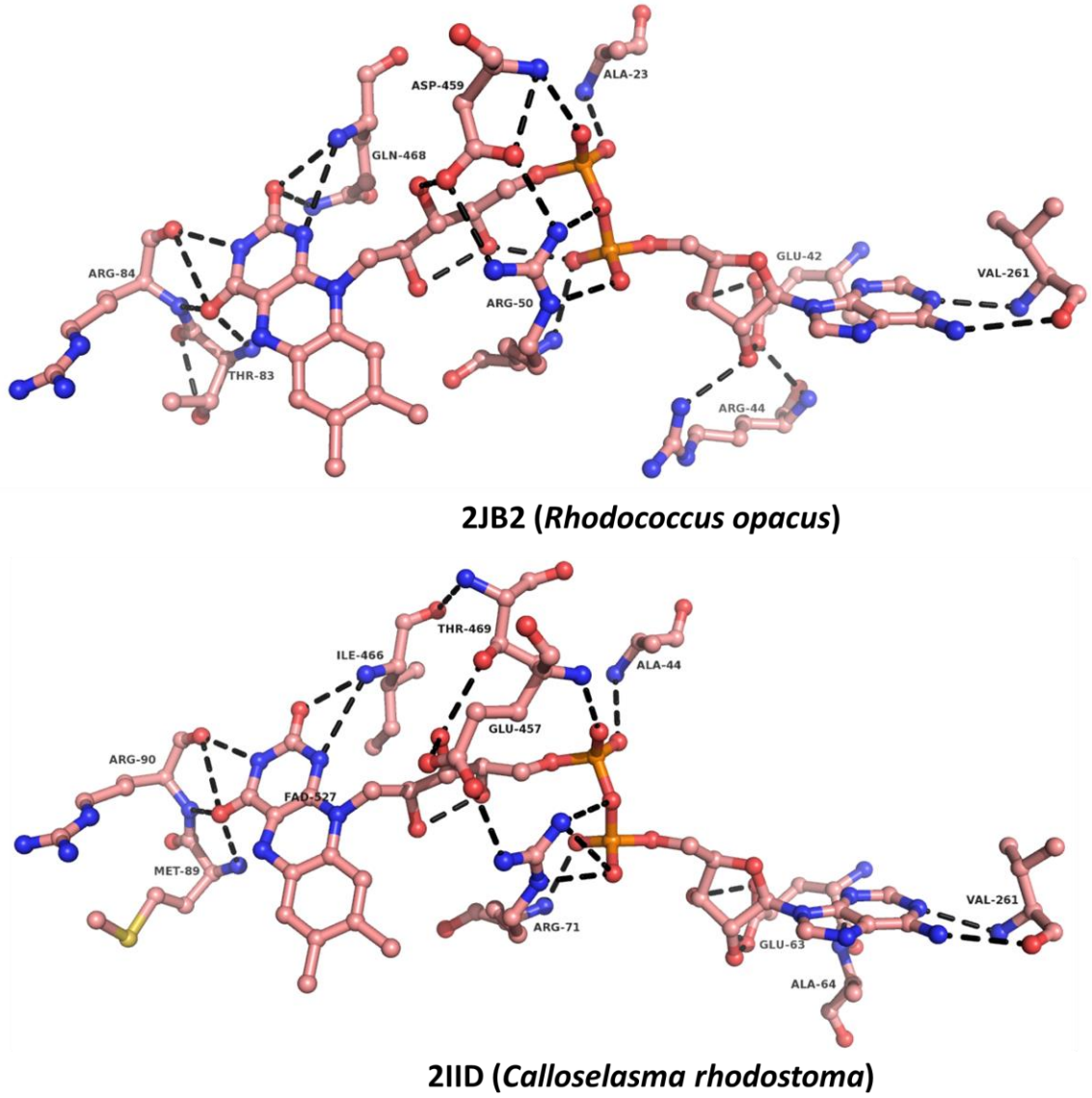


Figure 5.22. FAD binding motifs in various oxidases.

Based on the sequence alignment and available information from the literature the Li-rLAO structure was analysed for paths for various substrates and products. Based on the presence of solvent molecules, we identified three paths (Fig. 5.23).

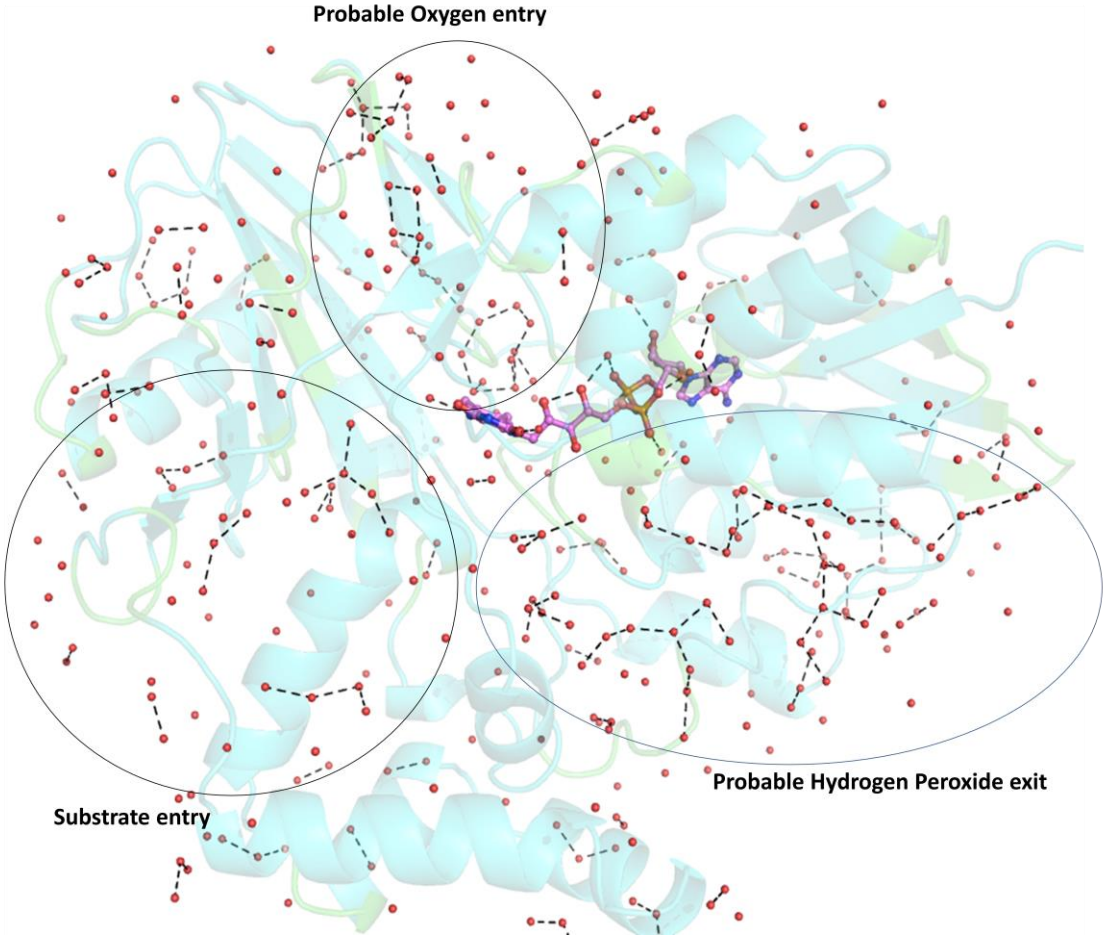


Figure 5.23. Overview of Solvent channels in Li-rLAO

In the peptides, spanning residues Thr212- Met218, Trp328-Lys333, Arg313-Ser317, and Lys301 and Phe303 form a narrow path filled with water molecules facing the N5 of FAD just below the plane of FAD with peptide stretch Gly88-Ala89-Glu90- Trp91 along the same side. The residue Ala89 methyl group forms a Vander - Waal contact distance of 3.4 Å along with Val433 with a distance of 3.9 Å such that no molecule can come and bind along this side of FAD; implying molecular oxygen can bind to the other side of FAD (Fig. 5.24A).

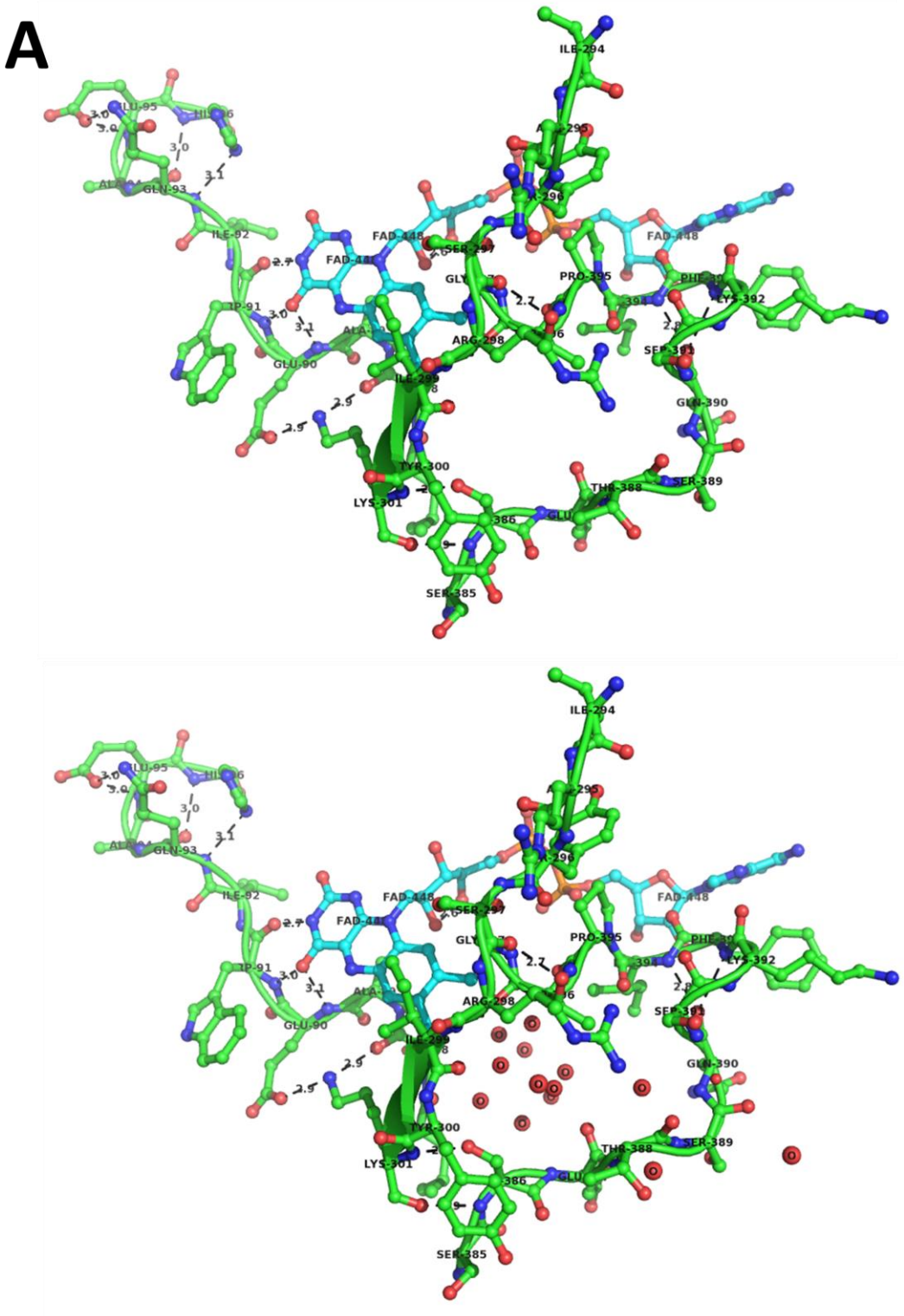


Figure 5.24. Solvent channels present in Li-rLAO.

Peptide stretch Ile294-Lys301, Ser385-Pro395, and Gly88-Glu95 form channel on the dimethyl side of isoalloxazine ring (Fig. 5.24A) that can act as potential molecular oxygen entry side.

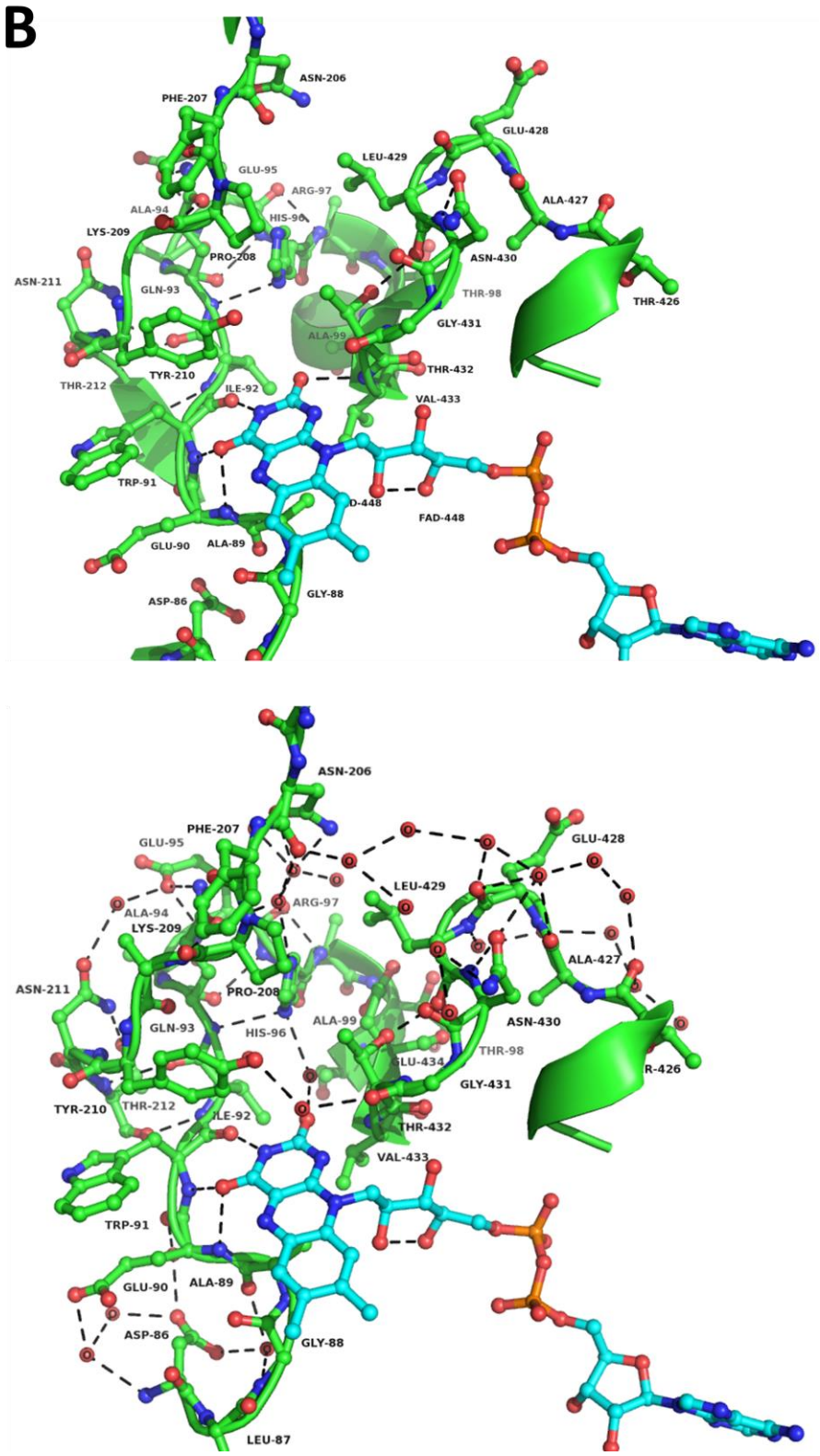


Figure 5.24. Solvent channels present in Li-rLAO.

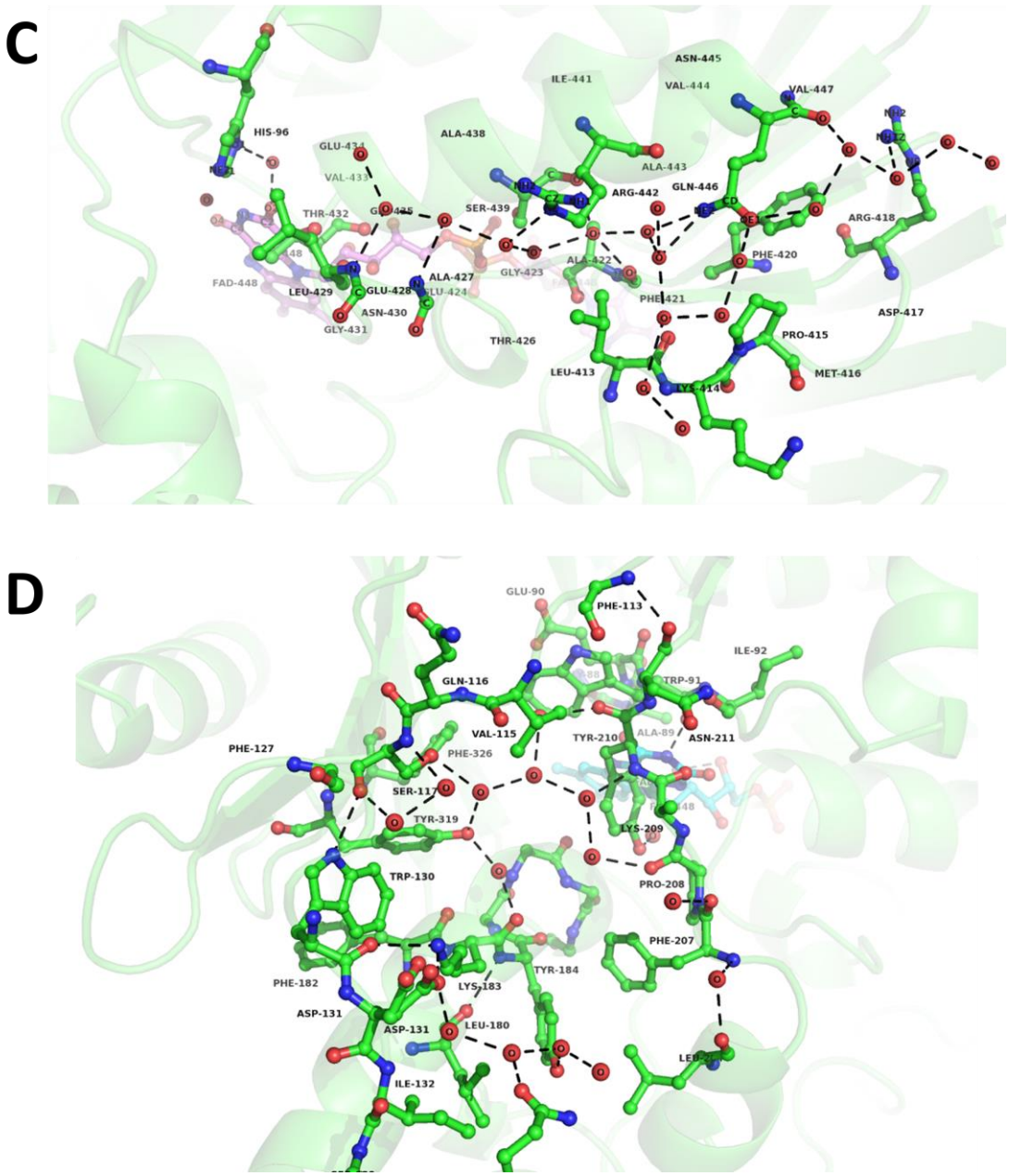


Figure 5.24. Solvent channels present in Li-rLAO.

Peptide stretch Asn206-Thr212, Thr426-Val433, and Gly88-Ala99 forms a channel on the C2 side of the isoalloxazine ring (Fig. 5.24B) that can act as a potential peroxide exit site. Peptide stretch Leu413-Val447 beneath the FAD on the C2 side of isoalloxazine ring with Leu429, Thr432 acting as a barrier, and residues Arg418, Arg442, and Gln446 can facilitate the exit of more polar hydrogen peroxide than the molecular oxygen (Fig. 5.24C).

Peptide stretch Leu204-Asn211, Leu180-Tyr188, Ser117-Val15, Gln137-Trp130, residues Tyr319, Phe127, Phe326 forms one more channel that may be a probable substrate path (5.24D). Though these paths can be identified whether they are NH_4^+ ion path or molecular oxygen path or hydrogen peroxide paths needs further investigation.

Further, during the reaction- initially, FAD undergoes reduction; during this process in total two electrons and two protons are transferred to N1 and N5 of FAD. The proton paths for this reduction process are predicted (Fig. 5.25) as a Hydrogen bond network (Ramasarma T. *et al.,* 2019). There are two paths predicted first for the proton transfer the first path involves

Lys301 (side chain) - Glu90 (side chain) - Arg213 (side chain) - Gly331 & Thr332 (backbone) - Asn83 (side chain) - Ser81 (side chain) - Asp79 (side chain) - H_2O -Gln258 (side chain) - H_2O - Arg75 (side chain) - H_2O - H_2O - Gly72 & Arg73(backbone) -O-P=O (from FAD) - H_2O - H_2O - Glu424 (side chain)- Gly431 & Asn430(backbone) - Tyr187(side chain) - H_2O - H_2O - Phe207 (backbone) - H_2O - His96(side chain) - H_2O.

alternatively Lys301 (side chain) - Glu90 (side chain) - Arg213 (side chain)- Glu215 (side chain) - - H_2O - H_2O - H_2O – Asp86 (side chain) – Gly88 & Leu87 (backbone) - H_2O - H_2O - H_2O - H_2O - Gly72 & Arg73(backbone) -O-P=O (from FAD) - H_2O - H_2O - Glu424 (side chain)- Gly431 & Asn430(backbone) - Tyr187(side chain) - H_2O - H_2O - Phe207 (backbone) - H_2O -His96(side chain) - H_2O.

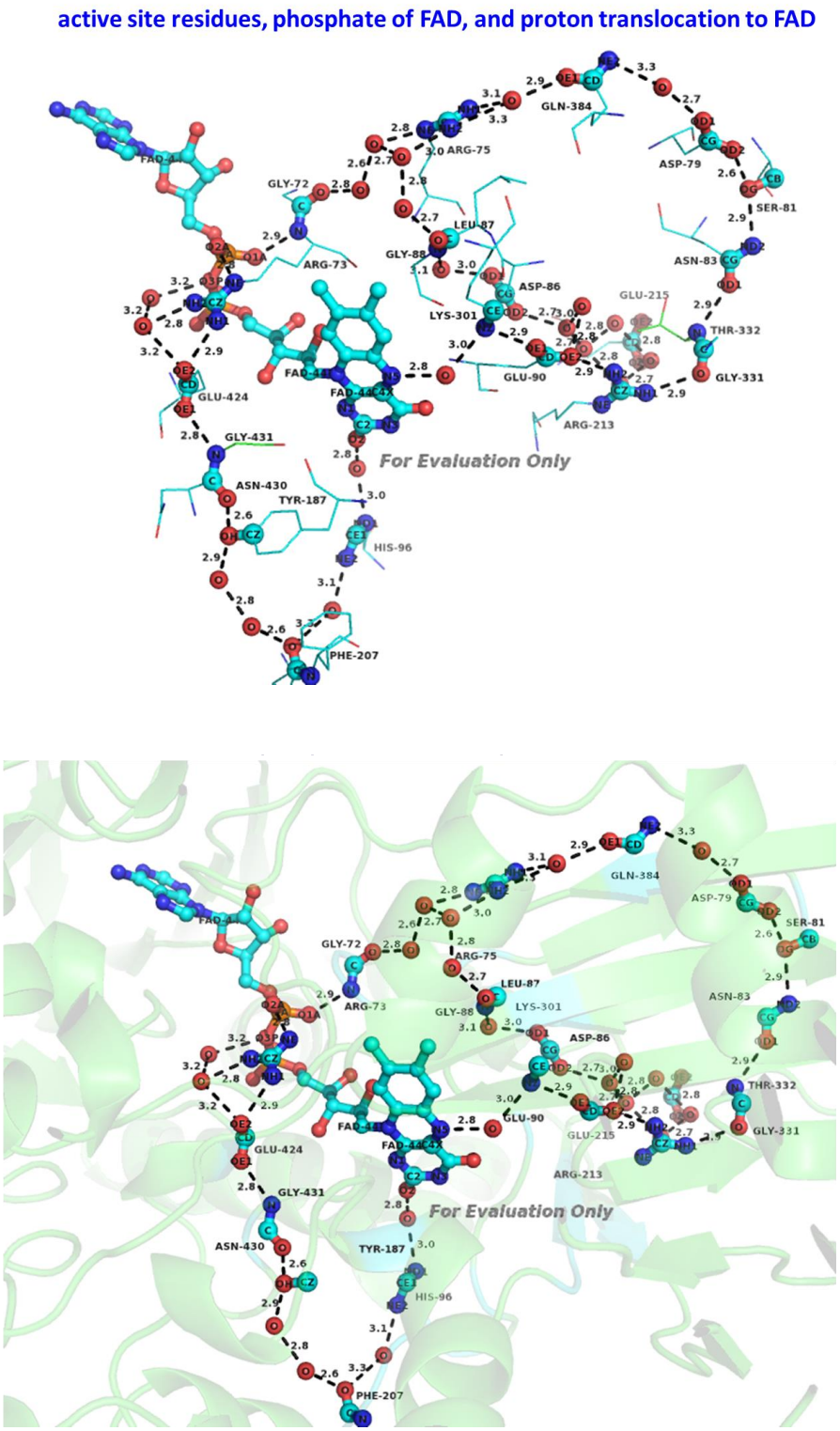


Figure 5.25. Predicted Hydrogen bond network in Li-rLAO.

5.4.12 L-amino acid oxidase and amine oxidase assay

Protein concentration was estimated by Bradford assay with bovine serum albumin as a standard (Fig 5.26).

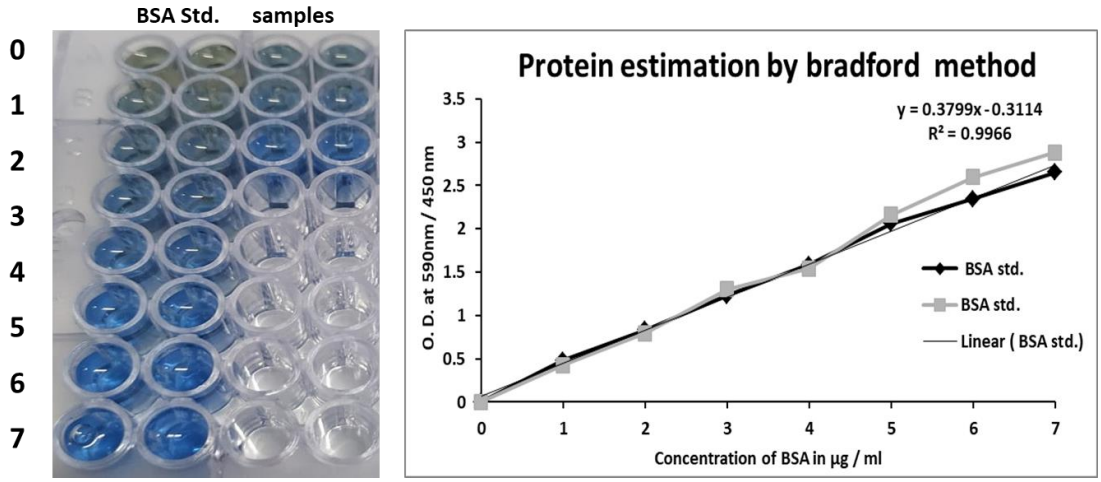


Figure.5.26. Protein estimation by Bradford method.

5mM of each of the amino acids and amines are used as the substrate to check the substrate specificity of LAO as given in the table by measuring hydrogen peroxide formed in the reaction by coupled enzyme assay with Horseradish peroxidase (Table 5.5 and Table 5.6).

Table 5.5. Amino acids tested as the substrates of Li-rLAO.

List of L-amino acids used as the substrate in the assay of Li-rLAO	
Glycine	Glutamate
Alanine	Aspartate
Leucine	Phenylalanine
Isoleucine	Tyrosine
Valine	Tryptophan
Proline	Asparagine
Serine	Arginine
Glutamine	Methionine
Threonine	
Histidine	

None of the substrates given in the table gave a reasonable activity. Since the absence of activity may be due to incorrect handling of assay components or maybe due to wrongly made reagents, we performed the standard calibration curve with the hydrogen peroxide as the standard for both amino acid oxidase assay reagents and amine oxidase assay regents.

Table 5.6. Amines tested as the substrates of Li-rLAO.

List of Amines used as the substrate in the assay of Li-rLAO	
Histamine	Putrescine
Spermine	Tryptamine
Spermidine	Dopamine

Standard curve for amino acid oxidase obtained using varying Hydrogen peroxide concentrations initially for the range of 0-7mM and later for the range of 0-3.5mM concentration (Fig. 5.27) using o-phenylene diamine and HRP.

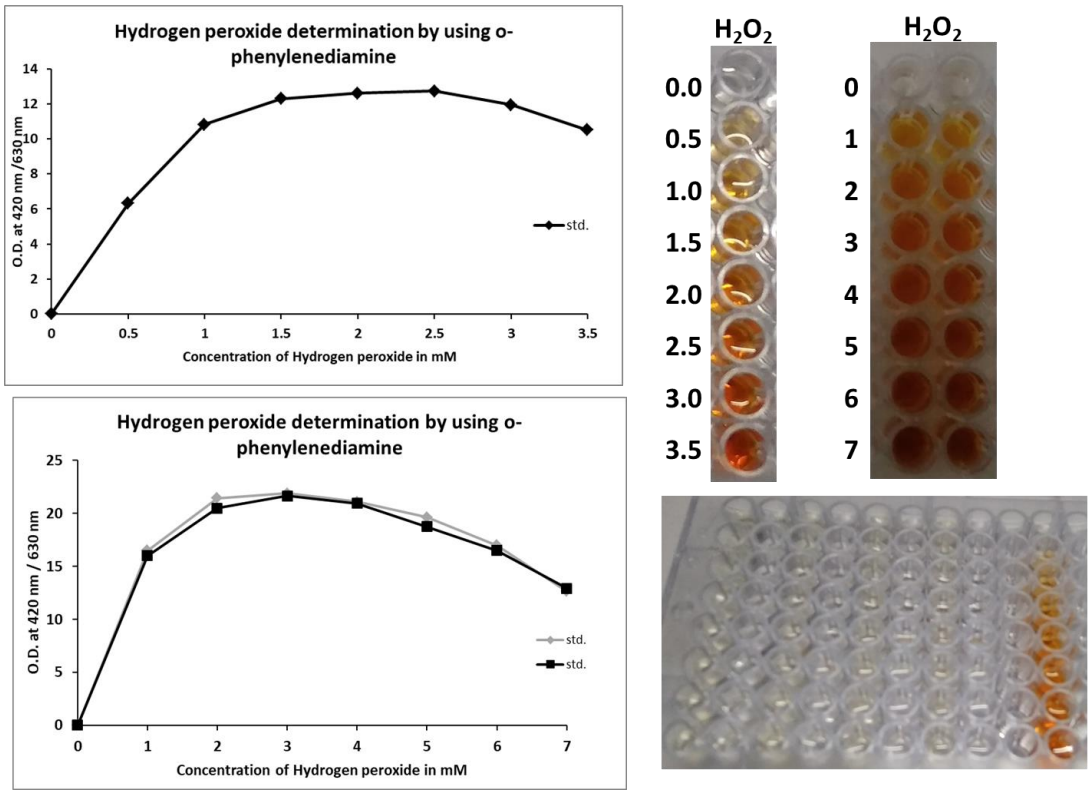
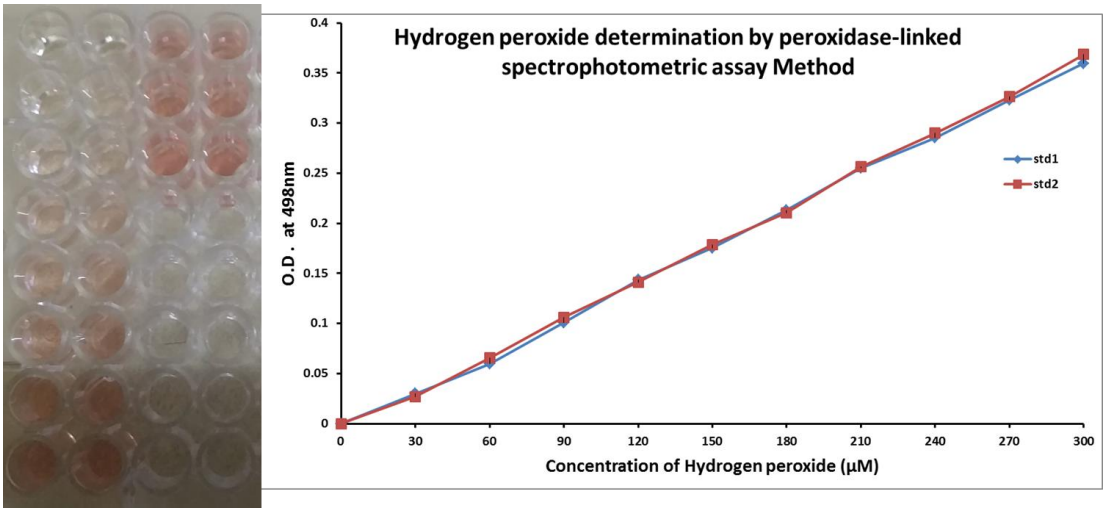


Figure 5.27. Standard curve for hydrogen peroxide determination by using Horseradish peroxidase.



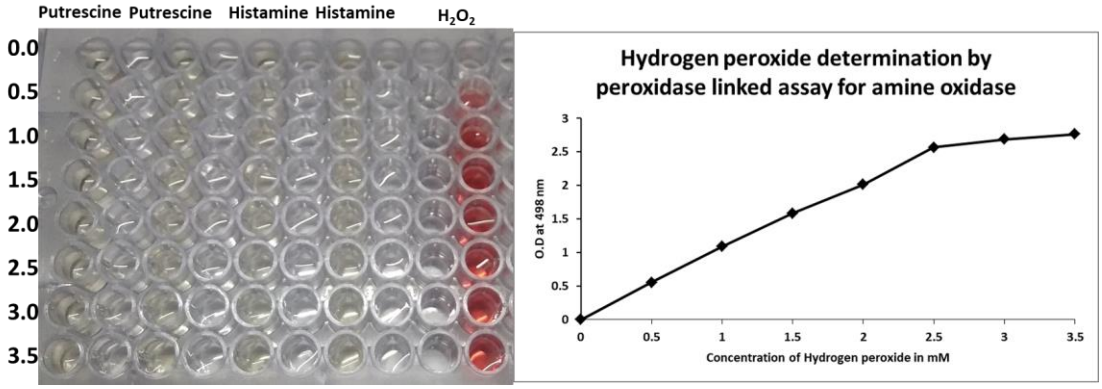


Figure 5.28. Standard curve for hydrogen peroxide determination by using Horseradish peroxidase.

The standard curve for Hydrogen peroxide concentrations ranging from 0-300µM and 0-3.5mM was obtained at 498nm which forms a blood-red color upon reaction with 4-aminoantipyrene and vanillic acid in presence of horseradish peroxidase (5.28).

5.5 Discussion

The absence of activity of Li-rLAO from Leptospira was surprising with most of the amino acids. In order to check the kind of amino acid that can fit into the active site the protein structure of *Calloselasma rhodostoma* L-amino acid oxidase (PDBID:2IID) and protein structure of *Rhodococcus opacus* L-amino acid oxidase (PDBID:2JB2) which are solved with amino acid ligand were analysed for substrate binding residues. The residues within 4Å distance that can bind and interact with the amino acid substrates are mapped (Fig. 5.29).

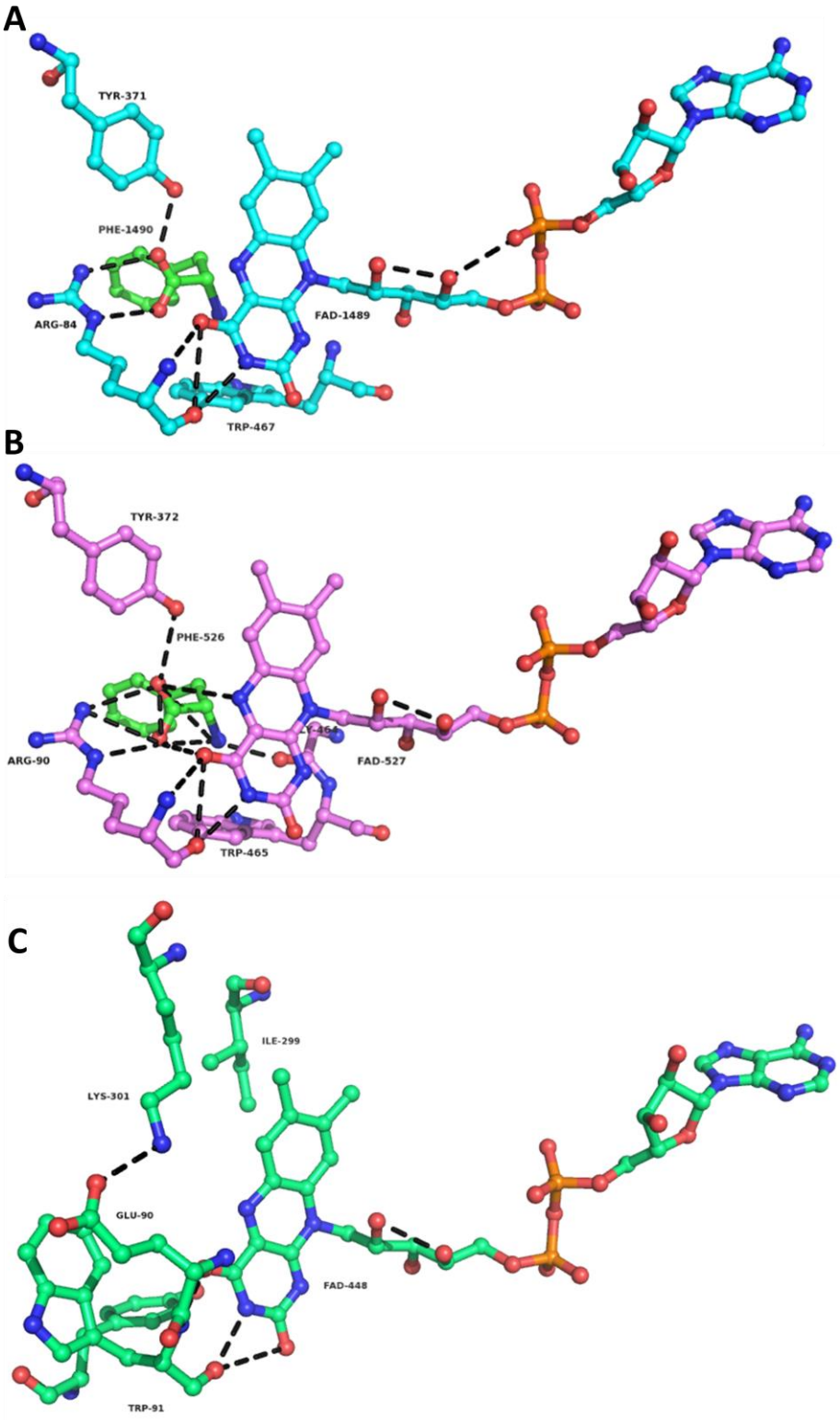


Figure 5.29. The active site residues binding the amino acid in *Calloselasma rhodostoma,* and *Rhodococcus opacus* L-amino acid oxidase.

After the mapping of the substrate-binding residues, the sequences of *Calloselasma rhodostoma* , *Rhodococcus opacus,* and Leptospiral L-amino acid

oxidase were compared, and equivalent substrate interacting residues are identified (Fig.5.30). Surprisingly, the substrate interacting residues are very well conserved in *Rhodococcus* and *Calloselasma* but not in *Leptospira*. However, in Li-rLAO, the FAD-binding motif GxGxxGx_{17}E was very much present.

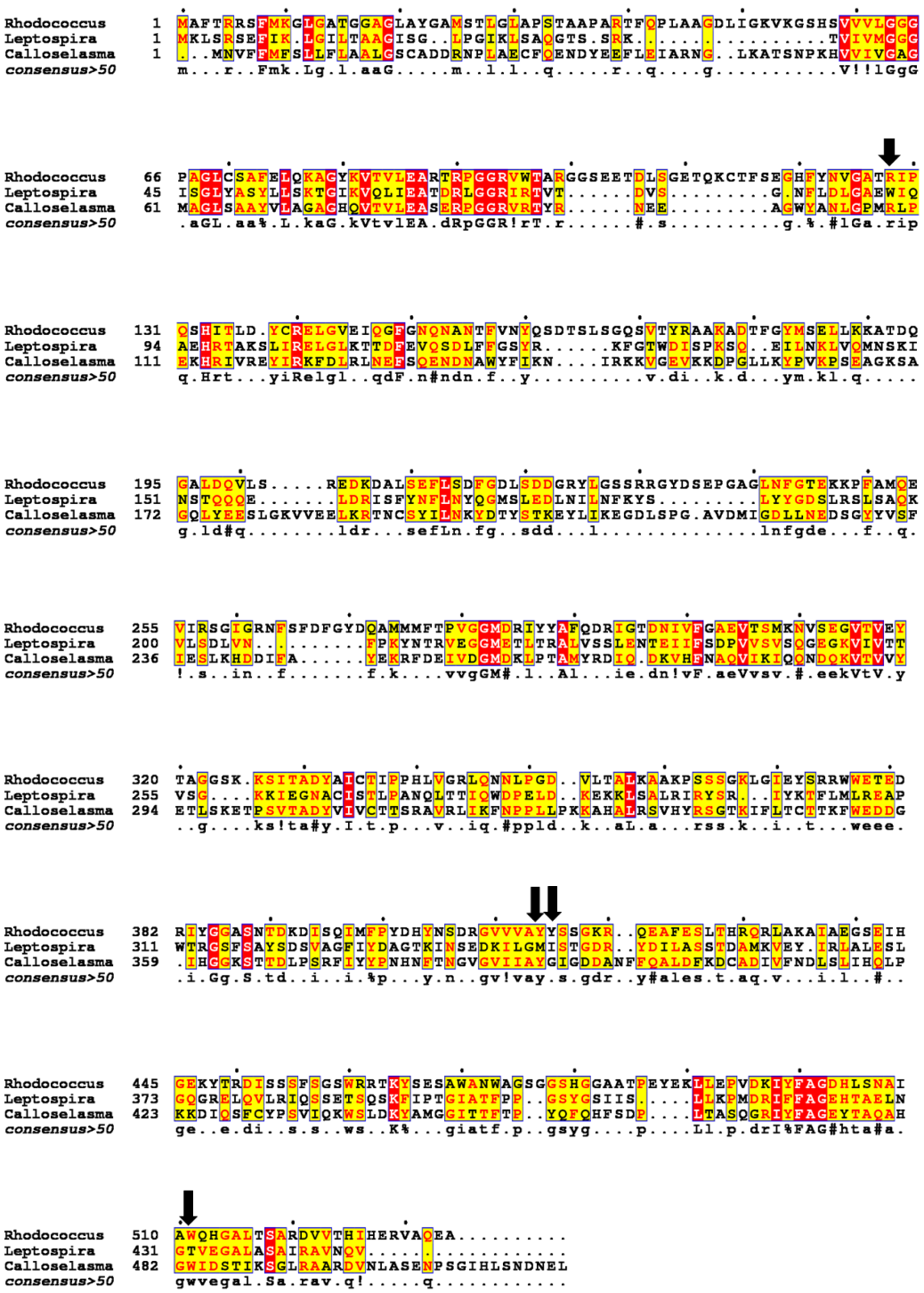


Figure 5.30. Sequence alignment of Rhodococcus and Calloselasma L-amino acid oxidase with Leptospiral L-amino acid oxidase sequence highlighting conserved active site residues.

In L-amino acid oxidase there is conserved arginine residue which is critical for holding the various amino acid substrates. The conserved arginine residue holds the amino acid substrates; by forming a hydrogen bond interaction with the carboxyl group of the amino acid substrates such that two hydrogen atoms can be removed (one from the amino group and the second one from the C-alpha atom) to form respective imino acid. The hydrogen atoms removed from the substrate reduces the FAD to $FADH_2$; which in turn reduces the oxygen to hydrogen peroxide. The imino acid subsequently takes up a molecule of water to form ammonium ion and respective keto acid.

One of the significances of the presence of L-amino acid oxidase in various organisms is to ensure the production of alpha-keto acid or ammonium ion that can be transported into the cell or hydrogen peroxide to the kill competing organism or to interfere with the host system to evade the immune system by degrading amines that are released by host immune system (Fig. 5.31).

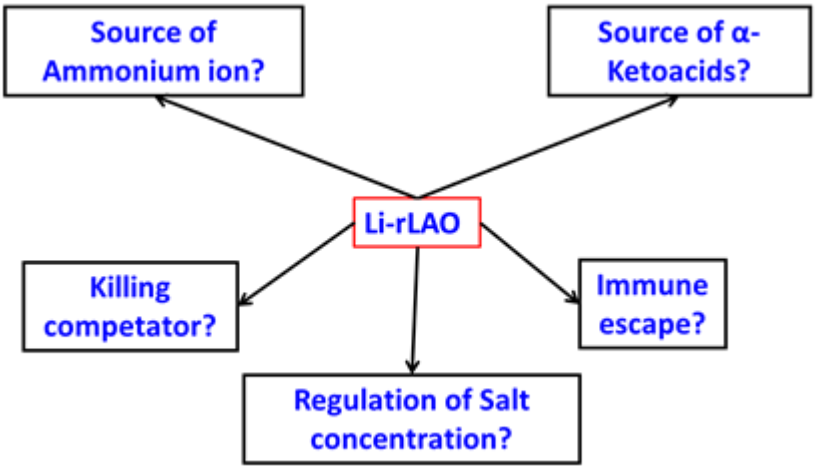


Figure 5.31. Predicted functional significance of Li-LAO.

In the case of the Li-rLAO, we further analyzed the genome of *Leptospira* for any amino acid transporter and ketoacids transporter. It was found that there were multiple amino acid transporters in the *Leptospira* (Table 5.7).

Table 5.7. Various amino acid transporters present in the leptospira.

Substrate uptake: Compounds transported into the cytosol	L. biflexa serovar Patoc strain Patoc 1 (Ames)	L. interrogans serovar Copenhageni str. 2006007831
a dipeptide	dipeptide/tripeptide permease	
a glutamate	amino acid permease proton glutamate symport protein	amino acid permease proton glutamate symport protein
a tripeptide	dipeptide/tripeptide permease	
ammonia	ammonia permease ammonia permease ammonia permease	Ammonia channel
an amino acid	amino acid transporter amino acid permease	amino acid permease
L-proline	sodium/proline symporter sodium/proline symporter	

Further, we also analyzed the Leptospira genome for the transaminase gene and found that at least 4 aspartate transaminases are encoded by the Leptospiral genome (Fig. 5.32); out of, three are exoproteins indicating that ketoacids can be easily formed from these transaminases.

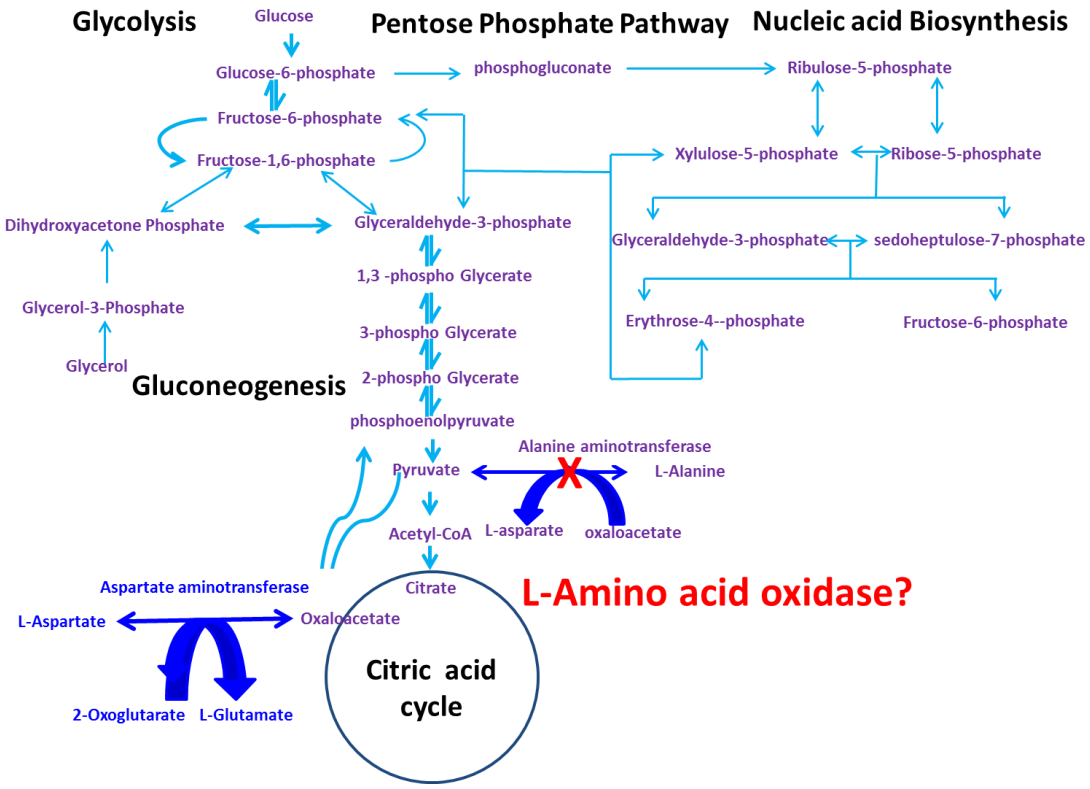


Figure. 5.32. Schematic diagram of central metabolic pathways with the emphasis on aminotransferases and L-amino acid oxidase present in Leptospira.

The significance of L-amino acid oxidase as a primary source for the production of alpha-ketoacids in presence of three exoprotein transaminase seems to become negligible.

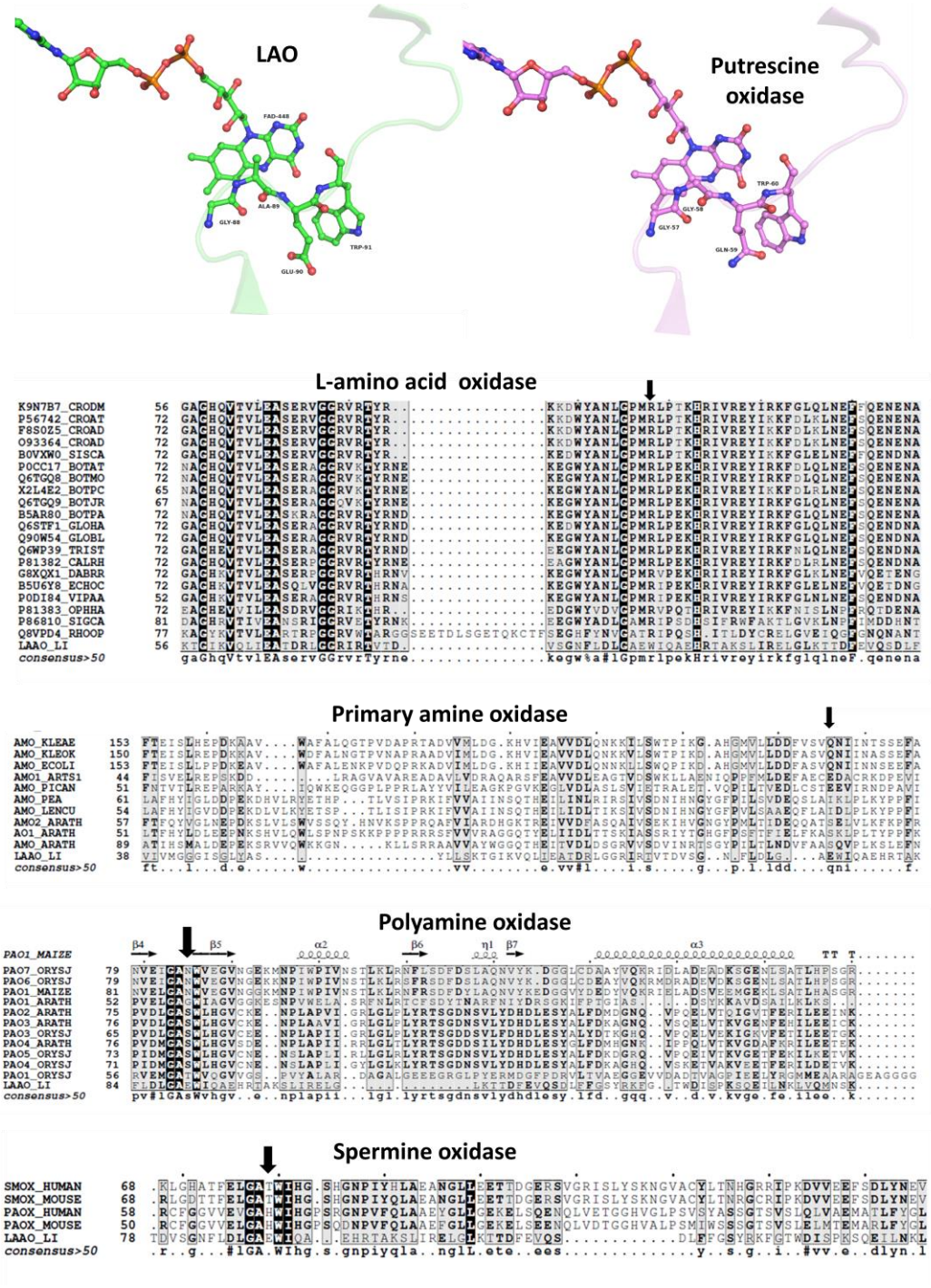


Figure 5.33. Comparison of residues (Active site) near FAD in various oxidases.

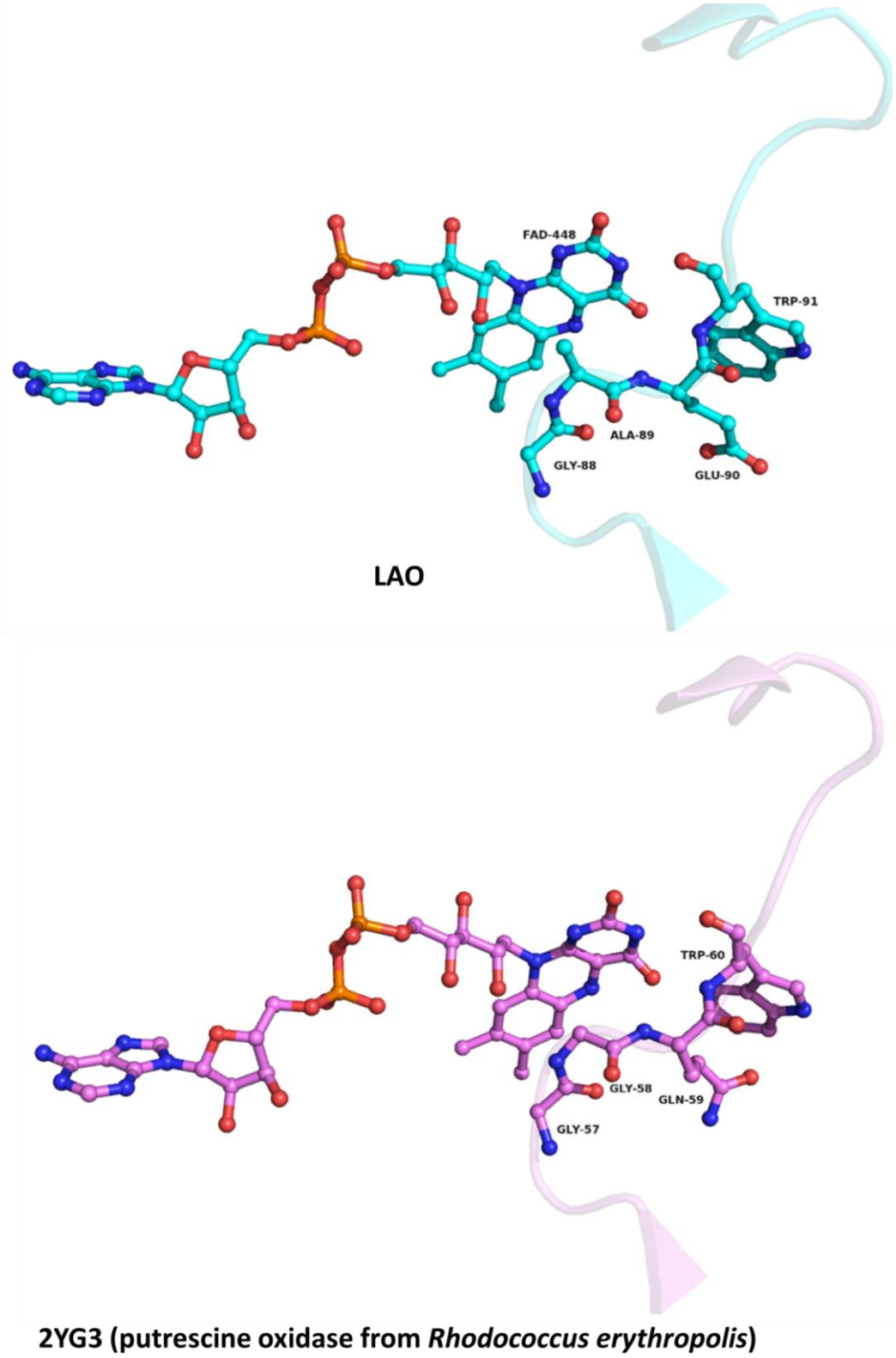
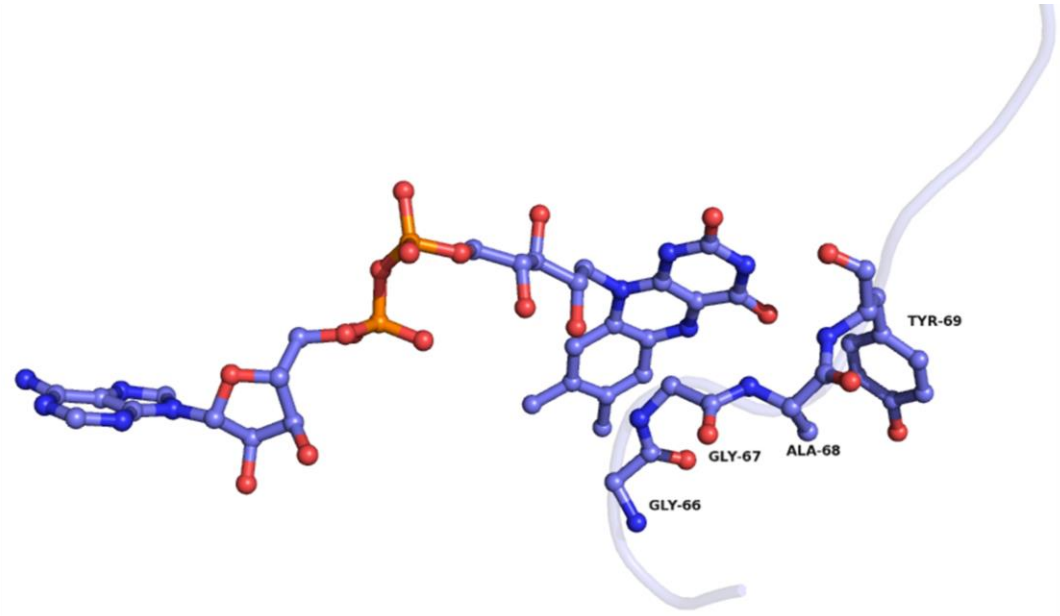
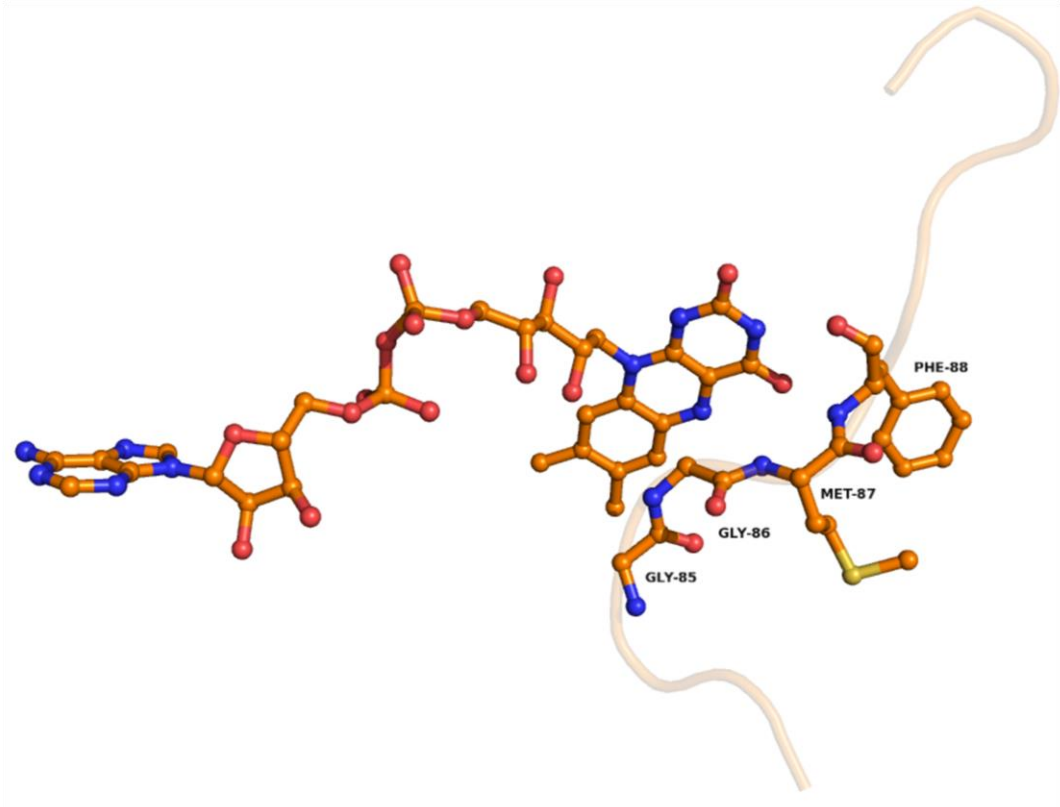


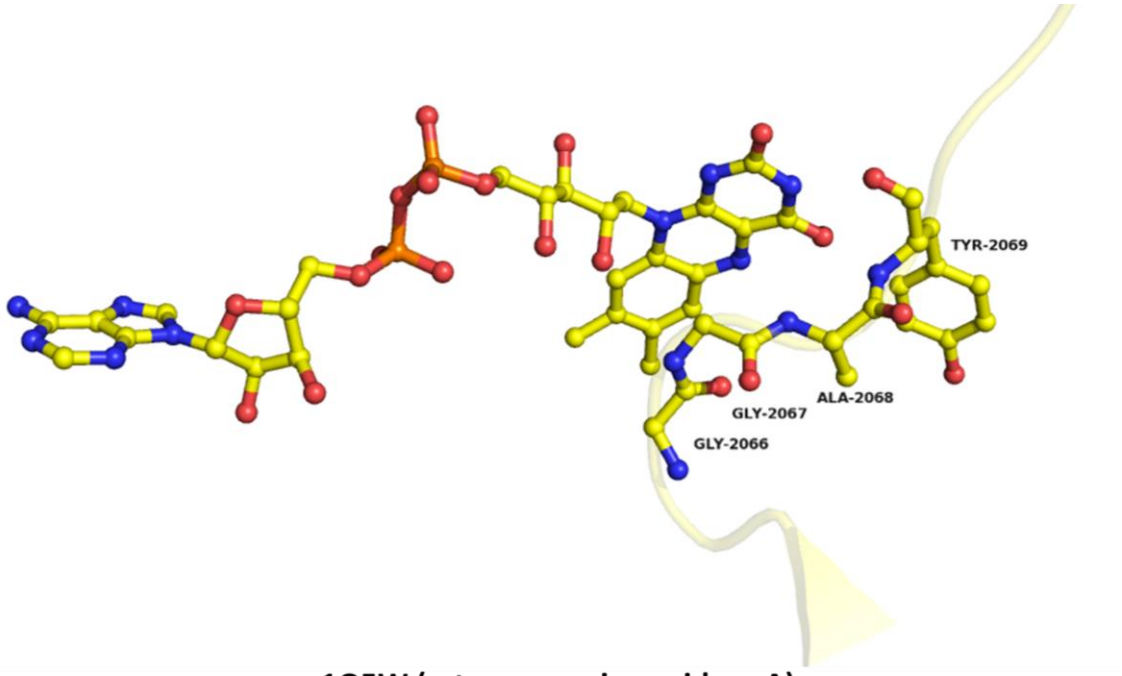
Figure 5.34. Comparison of residues (Active site) near FAD in various oxidases.



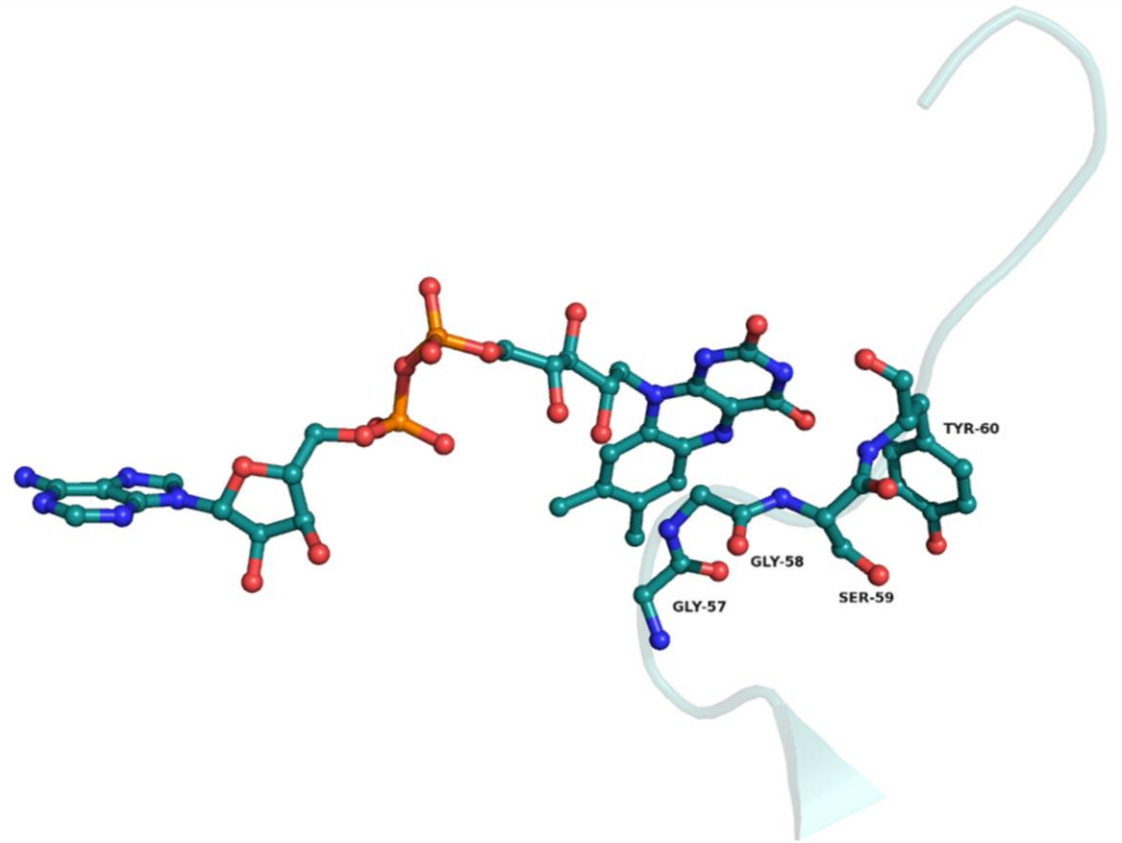
2Z5X (Human Monoamine Oxidase A)



4I58 (Cyclohexylamine Oxidase from *Brevibacterium oxydans*)



1O5W (rat monoamine oxidase A)



6FVZ (human monoamine oxidase B)

Figure 5.34. Comparison of residues (Active site) near FAD in various oxidases.

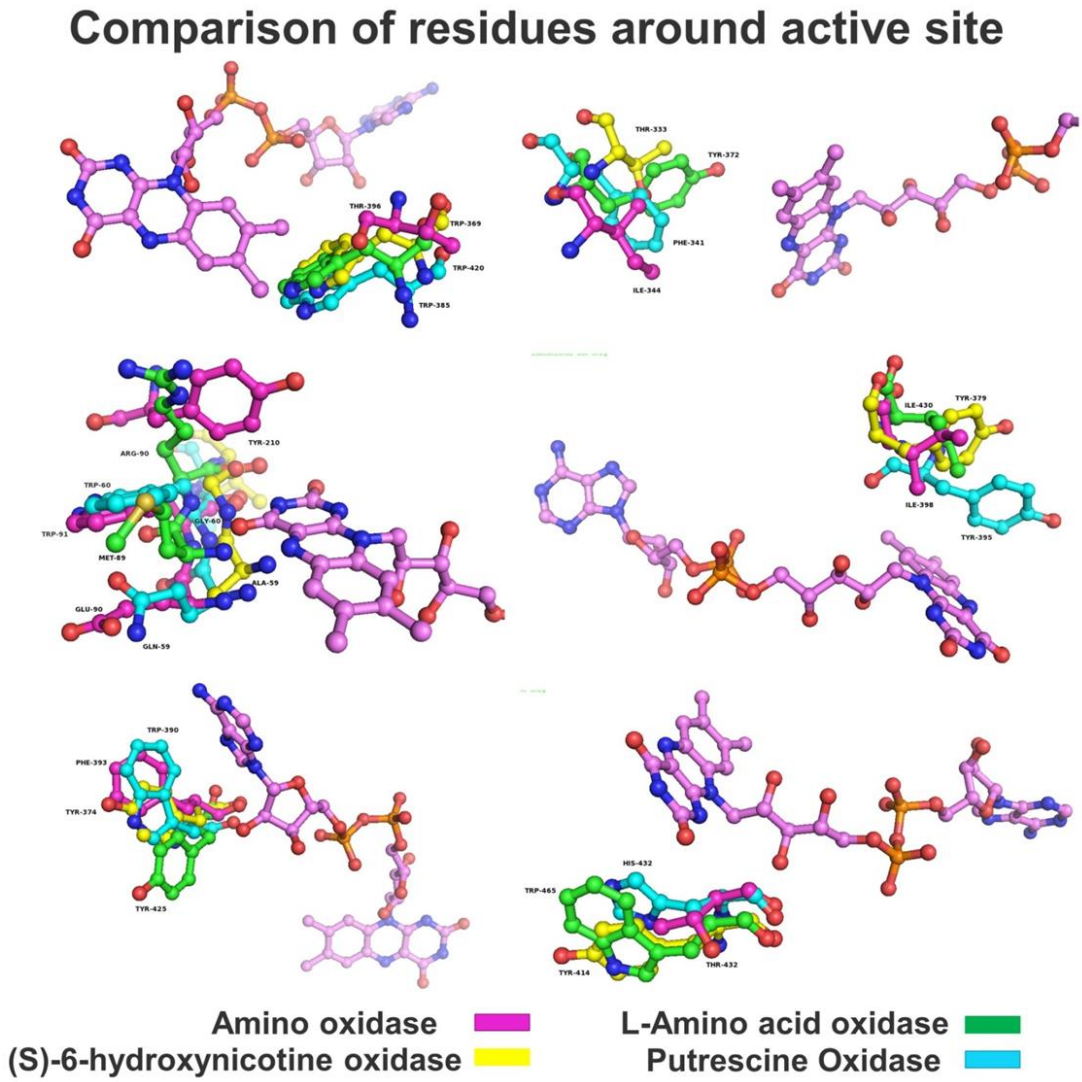


Figure 5.35. Comparison of residues (Active site) near FAD in various oxidases.

Further, the active site Li-rLAO was compared with various protein structures which showed the highest structural similarity. The structural analysis of Li-rLAO indicates that it resembles more of an amine oxidase rather than an amino acid oxidase (Fig. 5.33, Fig. 5.34, and Fig. 5.35). In Leptospira, the presence of three ammonia transporters indicates that Li-rLAO might be used as the source of ammonium ion needed for Leptospira. The habitat of Leptospira can be very useful in predicting the source of ammonia which can be used as a substrate by Li-rLAO. The complex habitat of Leptospira both inside and outside the host reservoir renders difficulty in identifying the proper substrate for Li-rLAO.

Considering the host habitat, the Li-LAO enzyme-substrate specificity was predicted assuming that it may be specifically acting few amino acids, instead of utilizing all amino acids (Fig. 5.36). Given such circumstances, the Li-LAO active site might be by holding the side-chain of amino acid substrates instead of the carboxyl group.

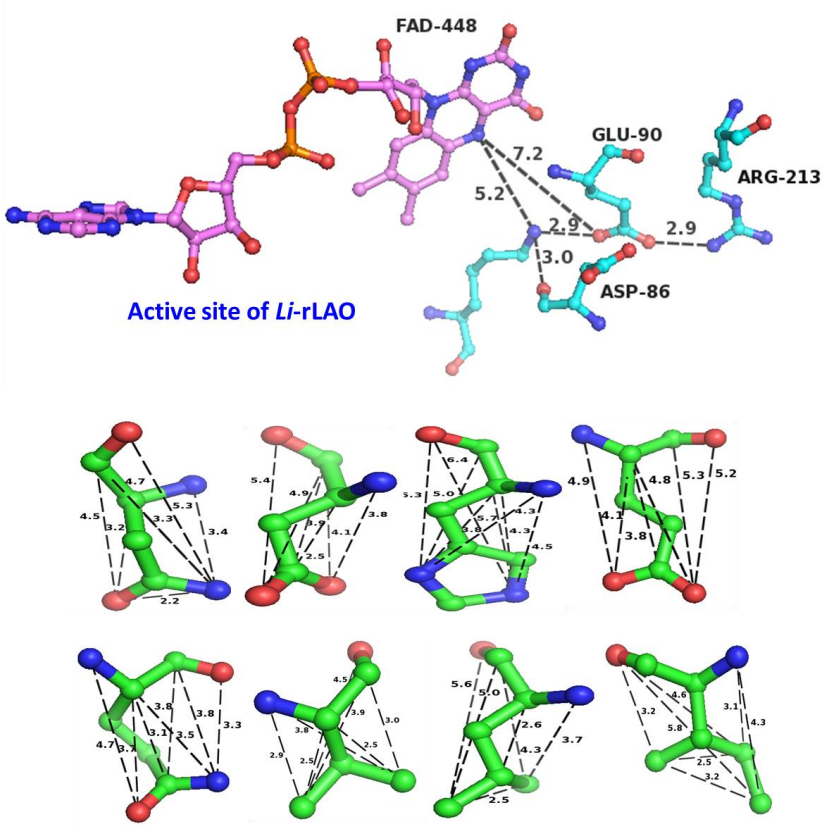


Figure 5.36. Prediction of substrate amino acid side chain groups that might be held by Li-rLAO in alkaline conditions.

Further assumptions were made that the organism while in the host reservoirs may not be in dearth of amino acids but outside the host, the organism might be facing the challenge for the Nitrogen source. The Li-rLAO was once again assayed with varying concentrations of amino acids, of the all amino acid tested, indeed, Li-rLAO showed very low non-specific activity with most of the amino acids (results were not reproducible for most of the amino acids tested) except for Arginine.

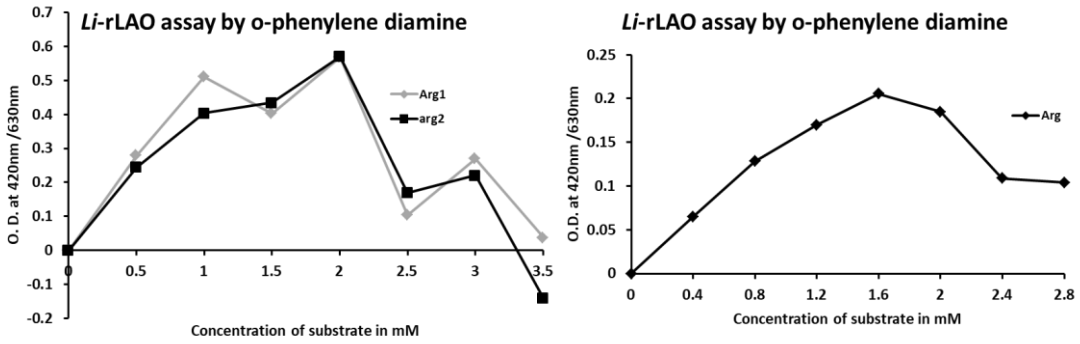


Figure 5.37. Assay of Li-rLAO with L-arginine.

The Li-rLAO showed low activity with the amino acid substrate L-Arginine, which was reproducible (Fig. 5.37). It is interesting to note that the Li-rLAO was active up to 2mM concentration of L-arginine, above this concentration the enzyme activity seems to be inhibited. In order to confirm that the substrate inhibition is not due to some other artifact; the assay was done with a different range of concentration. In the literature, it is well documented that substrate inhibition is very common in L-amino acid oxidase. Even though Li-rLAO seems to be acting specifically on L-arginine substrate, the overall activity cannot be appreciated, as in the enzymatic reaction; there was no visible color change which was expected; that might be attributed to the failure to identify the appropriate reaction condition in which the enzyme might be showing optimal activity.

5.6 Summary and Conclusion

Putative Li-rLAO was cloned expressed and purified to homogeneity. The recombinant protein was characterized by biophysical methods. The protein structure was determined by x-ray crystallography with 1.8Å resolution. The protein structure showed the highest similarity with putrescine oxidase with a Z score of 39.8 and RMSD of 2.7. Like other Flavin-dependent oxidases, the FAD-binding domain was well conserved. Comparative analysis of Li-rLAO structure with known L-amino acid oxidase structures revealed Li-rLAO lacks conserved substrate-binding residues, experimentally well supported by very low activity with L-amino acids. The enzyme also showed low or no activity with amines like putrescine, spermine, and spermidine (that were not reproducible). It showed limited activity with L-arginine which was reproducible. Further studies are needed to determine the appropriate conditions in the Li-rLAO that will be acting on its substrates.

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Contributions to the Scientific Literature

Publication

- Vaigundan Dhayabaran, Divya Chidambaram, Patnam Krishnaswamy*. Identification of compounds for improved growth of *Leptospira* in culture and isolation. Diagn Microbiol Infect Dis. 2020 Jan;96(1):114923. doi: 10.1016/j.diagmicrobio.2019.114923.

- Vaigundan Dhayabaran, Yuvaraj Iyappan, Sunita Prakash, Sekar K, MRN Murthy, Patnam R. Krishnaswamy*. Structural characterization of a putative recombinant L-amino acid oxidase from *Leptospira interrogans.* (Manuscript prepared).

- Vidhi Pareek, Vaigundan Dhayabaran, Hemalatha Balaram, Patnam R. Krishnaswamy. *Leptospira interrogans* triosephosphate isomerase: exploring the structural determinants of stability, high reaction rate and specificity doi: https://doi.org/10.1101/2020.12.04.412312.

Data Submitted to open data base.

Structural coordinates of the Protein Li-rLAO was submitted to Protein Data Bank.PDB ID is: 7EME

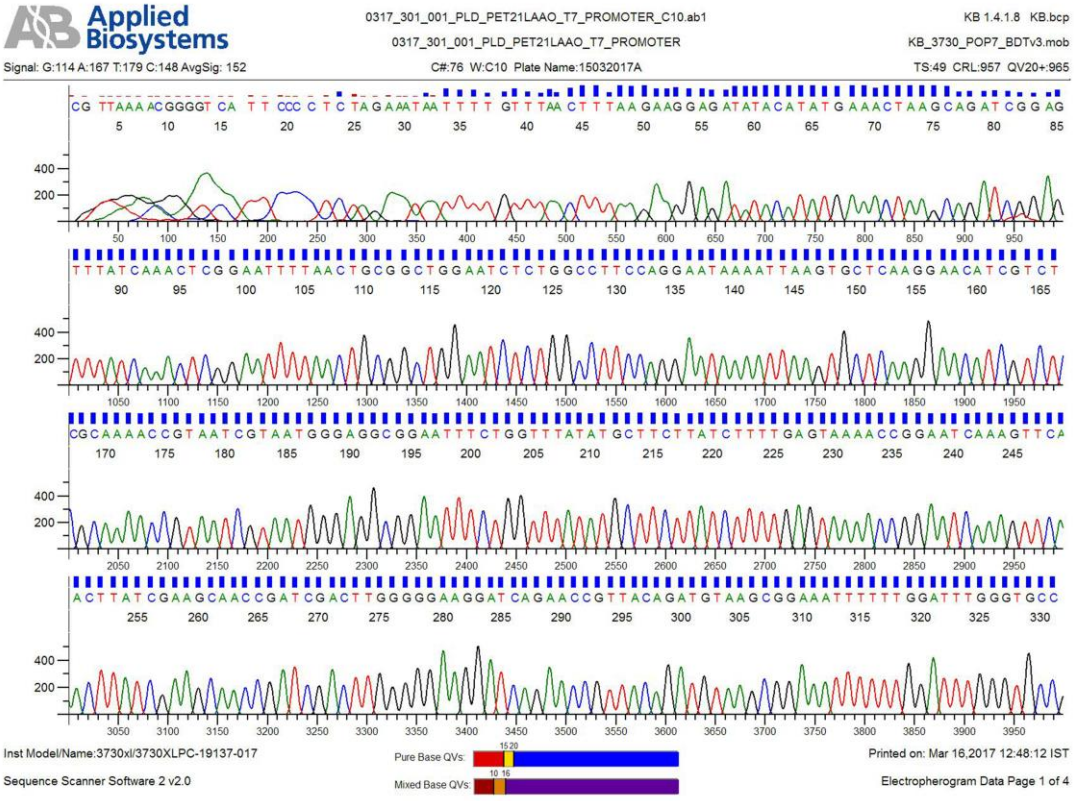
Conference / Webinar Participated

- ✓ Presented a poster titled "omic information driven, biochemical investigations of Leptospira" through the virtual communication platform "SLACK" in the international conference on "Recent Advances in Applied Sciences, Technology & Health" RASTH 2021, organised by SRM Institute of Science and Technology, Kattankulathur between March 03 and 05, 2021.

- ✓ Presented an e-poster titled "Visible culture growth possible or not? – a 'Omic' information driven investigation of Leptospira" in the international Webinar on "Alternative therapies to mitigate microbial resistance" organised by ICAR-Indian Veterinary Research Institute Izatnagar-243122 (Bareilly) UP India between 23-24 Feb. 2021.

Appendix-1

Sequencing results and analysis results of Leptospiral LAO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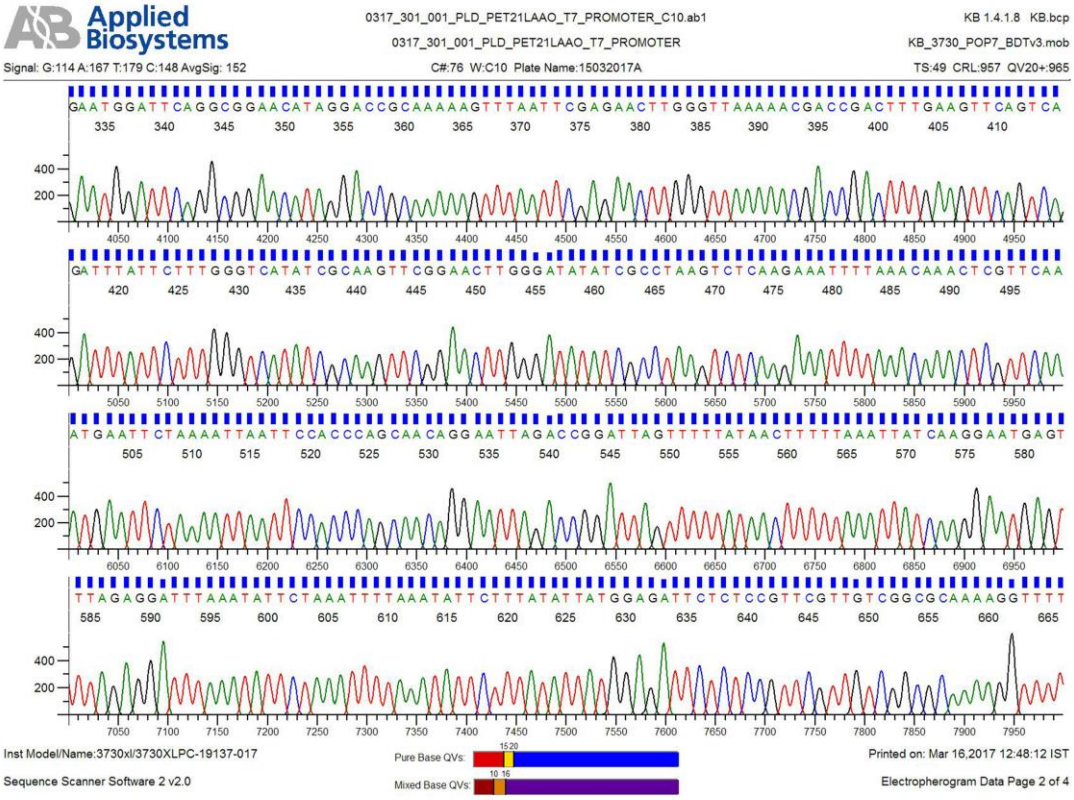
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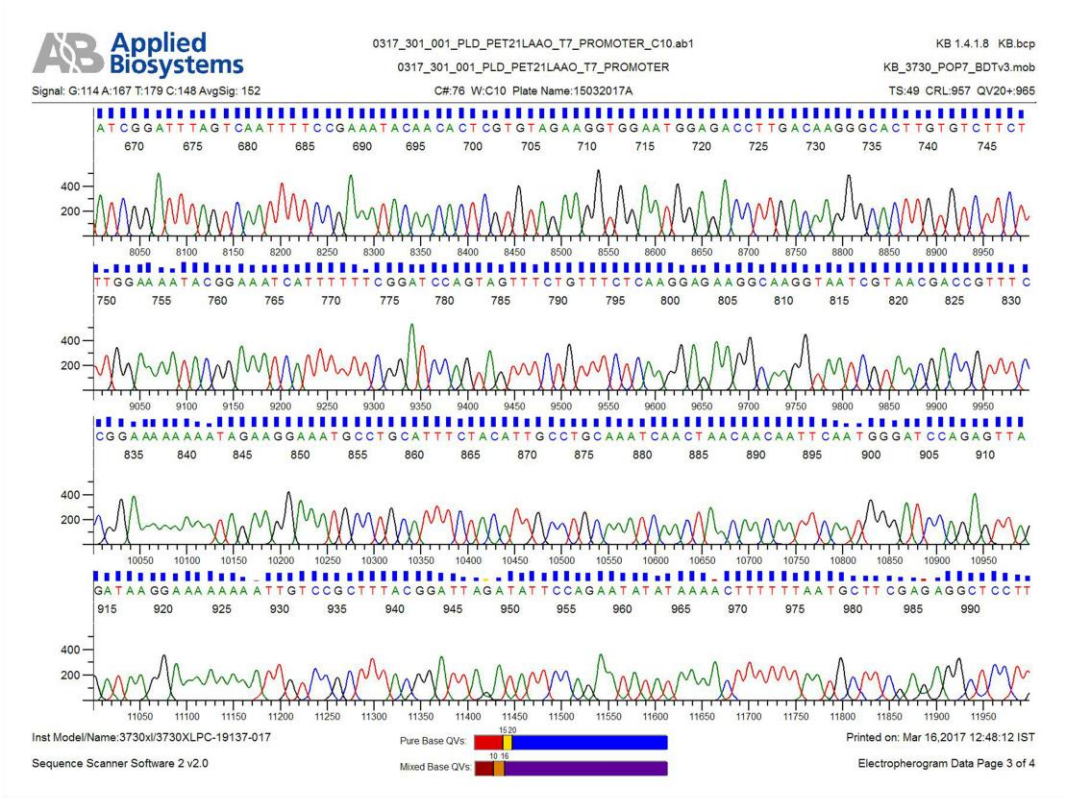
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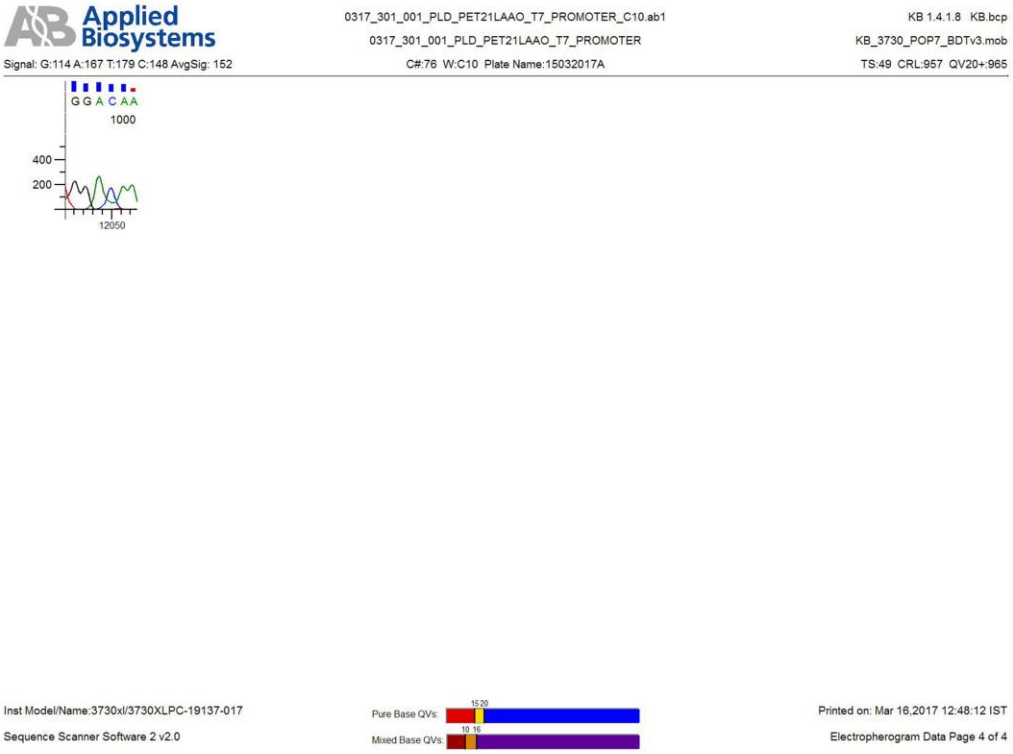
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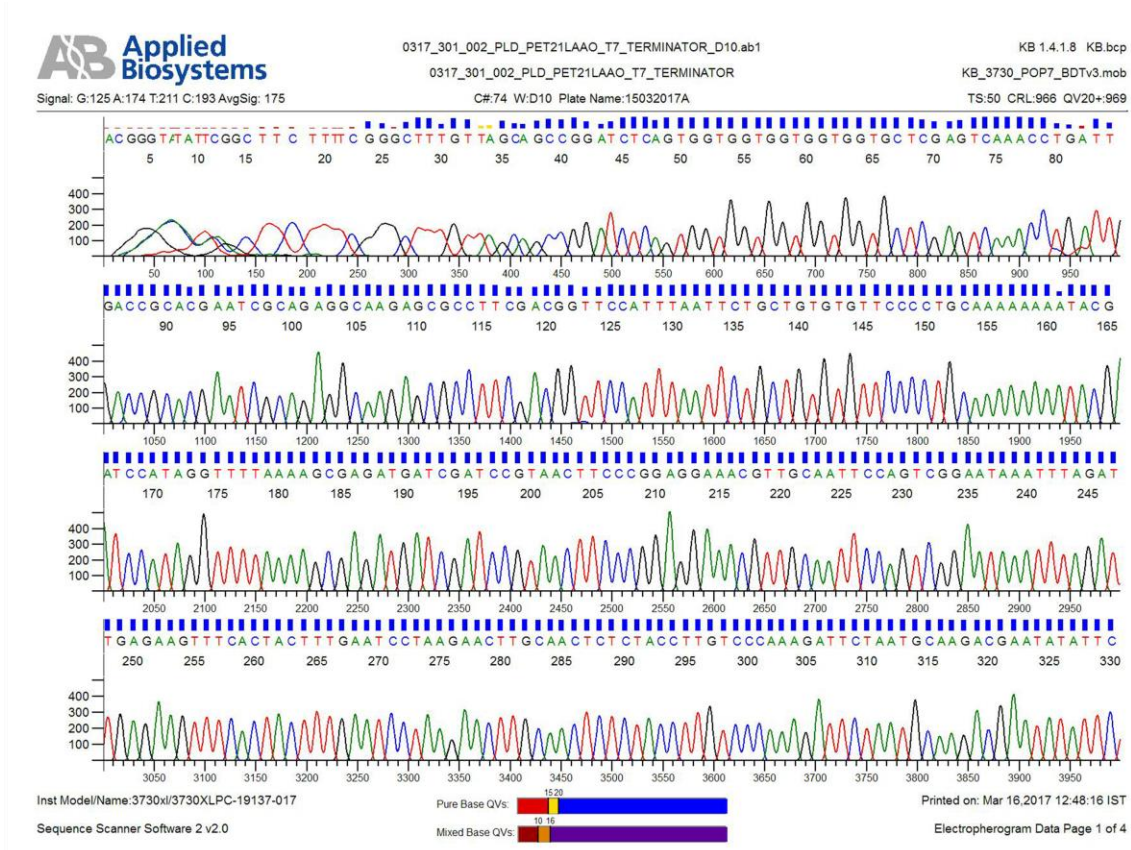
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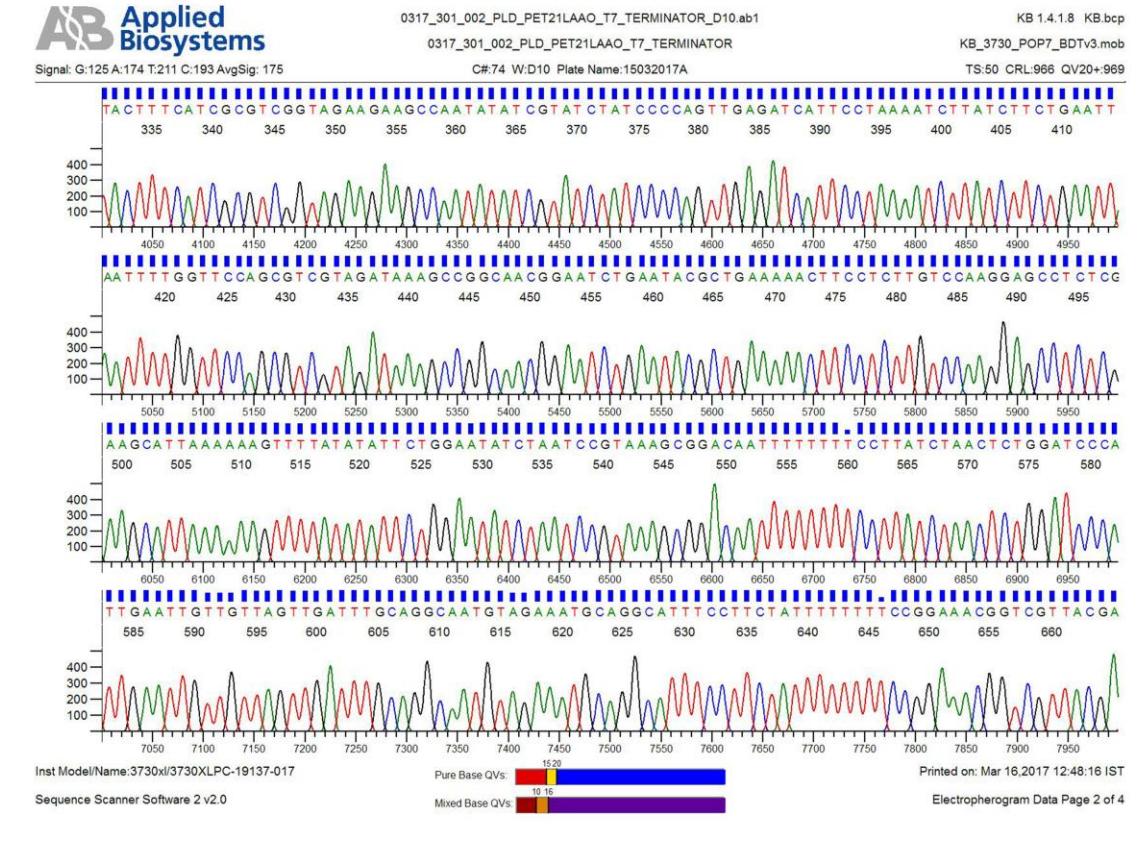
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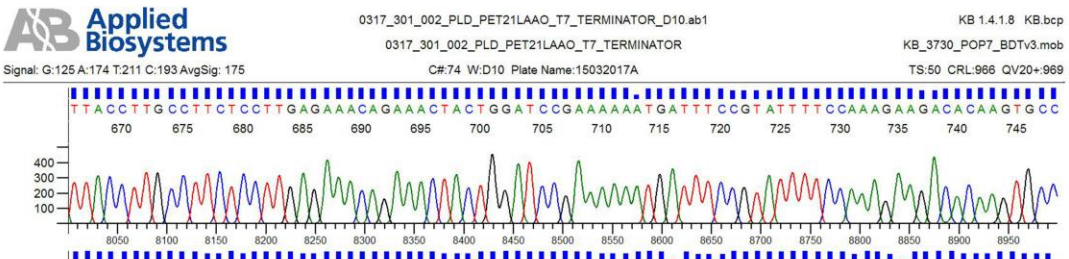
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Appendix 2

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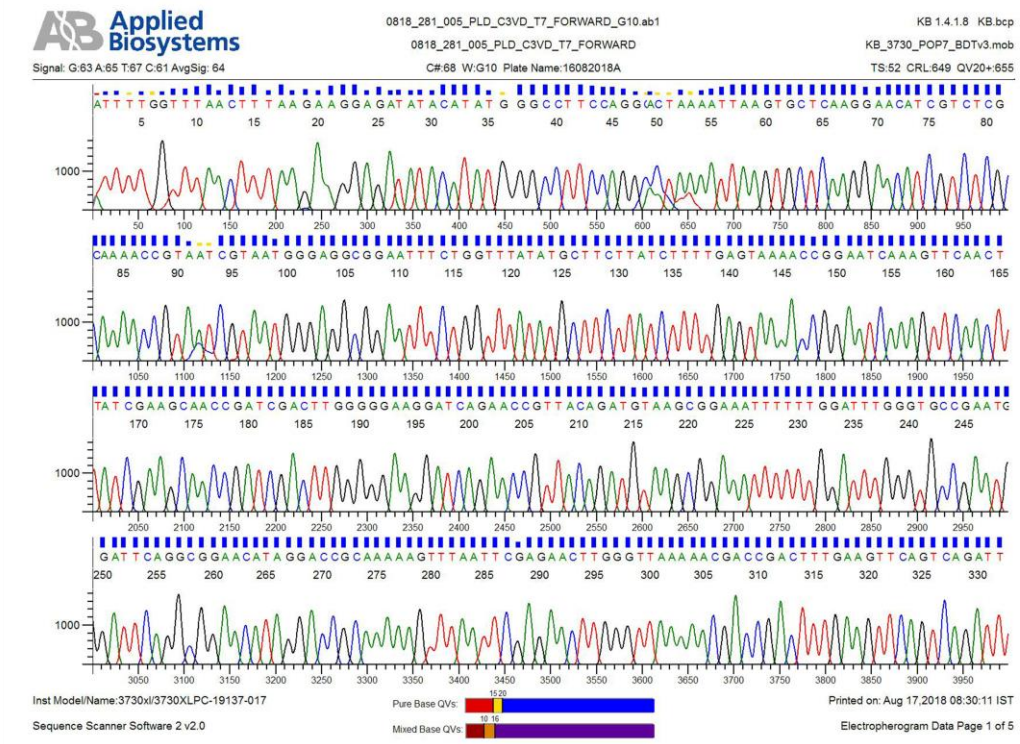
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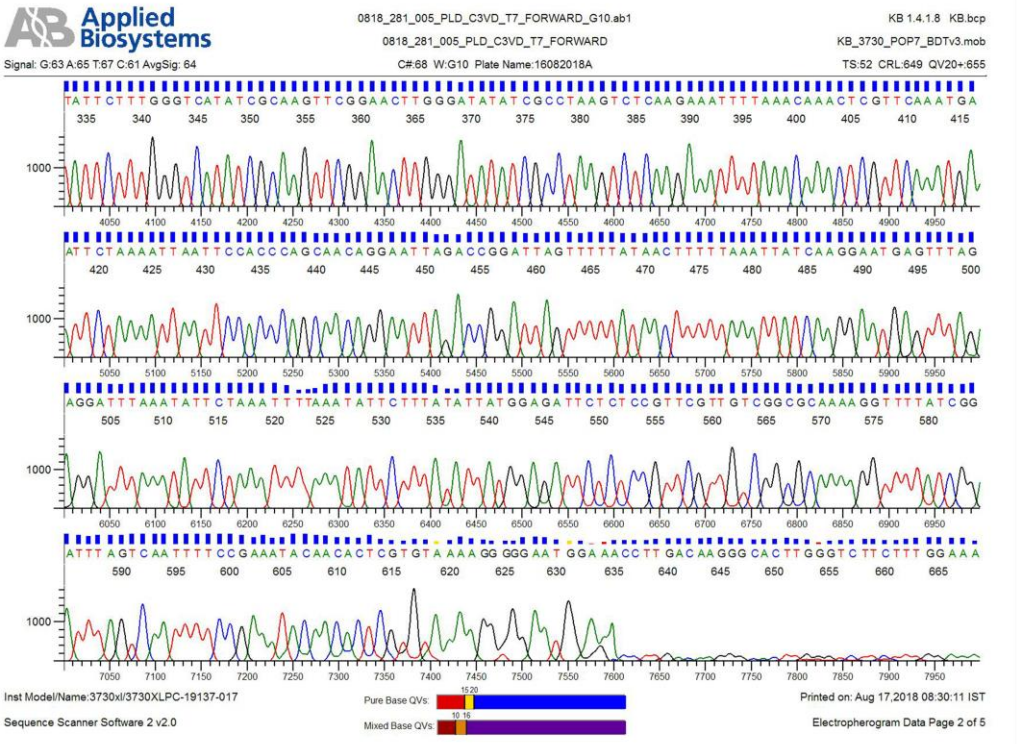
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gene          atgggaggcggaatttctggtttatatgcttcttatcttttgagtaaaaccggaatcaaa  180
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C3VD_T7F      TTGACAAGGGCACTTGGGTCTTCTTTGGAAAAA--------------------------  634
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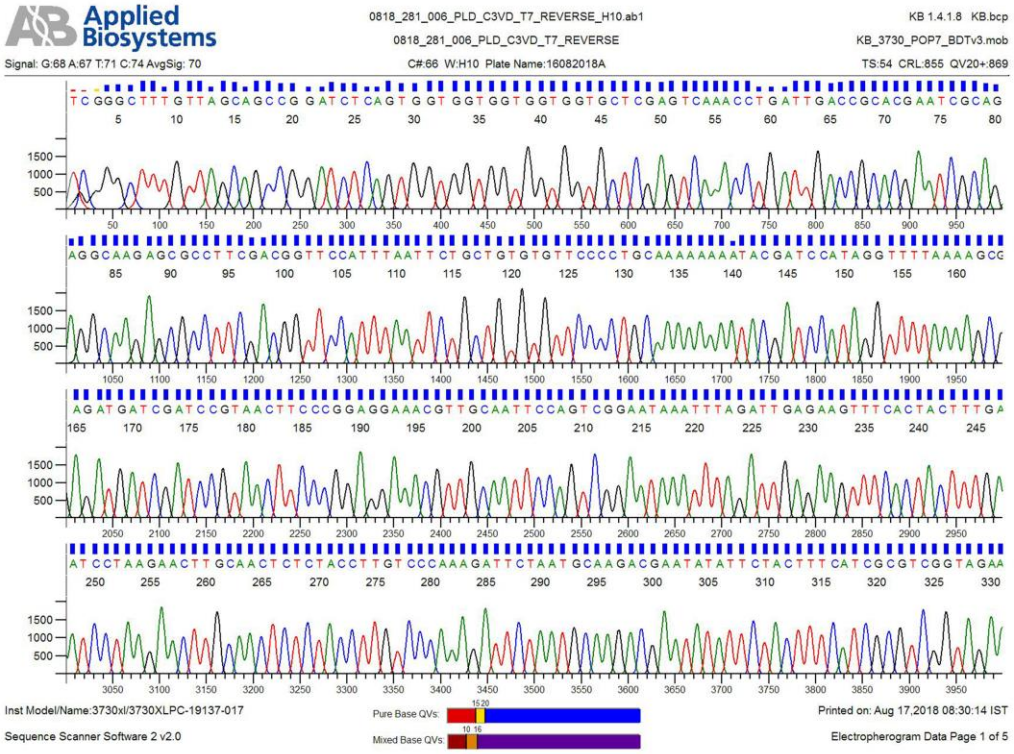
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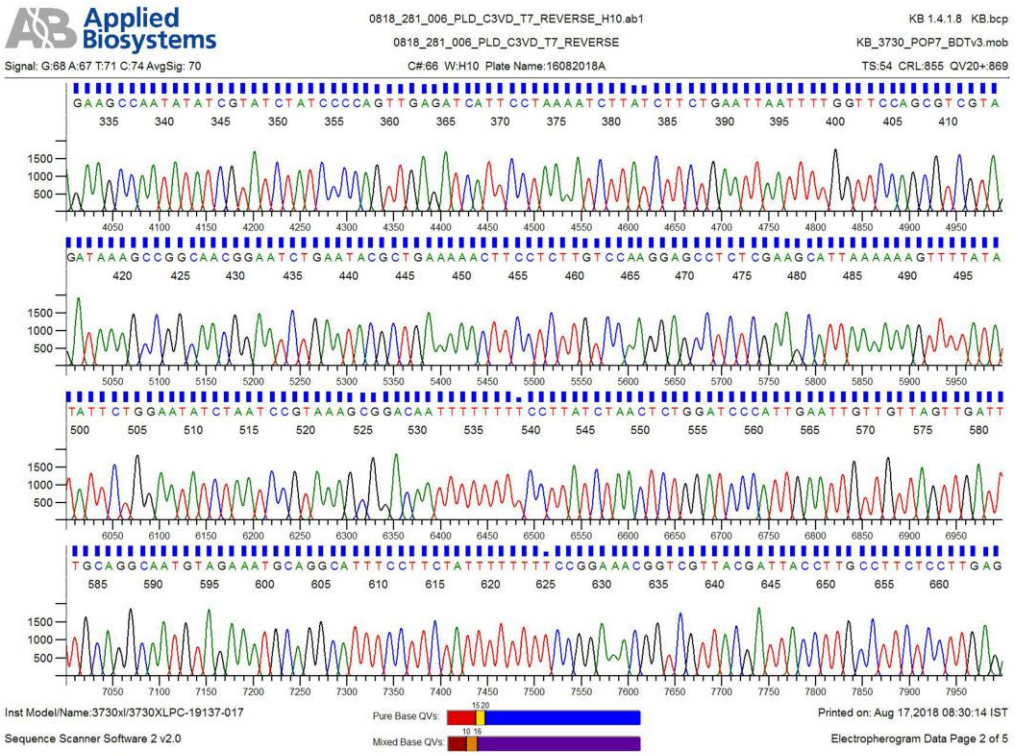
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fp        FGSYRKFGTWDISPKSQEILNKLVQMNSKINSTQQQELDRISFYNFLNYQGMSLEDLNIL      161
gene      FGSYRKFGTWDISPKSQEILNKLVQMNSKINSTQQQELDRISFYNFLNYQGMSLEDLNIL      180
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rp        -----------------------------------------------------SLEDLNIL      8
gene      FGSYRKFGTWDISPKSQEILNKLVQMNSKINSTQQQELDRISFYNFLNYQGMSLEDLNIL      180
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rp        NFKYSLYYGDSLRSLSAQKVLSDLVNFPKYNTRVEGGMETLTRALVSSLENTEIIFSDPV      68
gene      NFKYSLYYGDSLRSLSAQKVLSDLVNFPKYNTRVEGGMETLTRALVSSLENTEIIFSDPV      240
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rp        VSVSQGEGKVIVTTVSGKKIEGNACISTLPANQLTTIQWDPELDKEKKLSALRIRYSRIY      128
gene      VSVSQGEGKVIVTTVSGKKIEGNACISTLPANQLTTIQWDPELDKEKKLSALRIRYSRIY      300
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rp        KTFLMLREAPWTRGSFSAYSDSVAGFIYDAGTKINSEDKILGMISTGDRYDILASSTDAM      188
gene      KTFLMLREAPWTRGSFSAYSDSVAGFIYDAGTKINSEDKILGMISTGDRYDILASSTDAM      360
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rp        KVEYIRLALESLGQGRELQVLRIQSSETSQSKFIPTGIATFPPGSYGSIISLLKPMDRIF      248
gene      KVEYIRLALESLGQGRELQVLRIQSSETSQSKFIPTGIATFPPGSYGSIISLLKPMDRIF      420
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rp        FAGEHTAELNGTVEGALASAIRAVNQV 275
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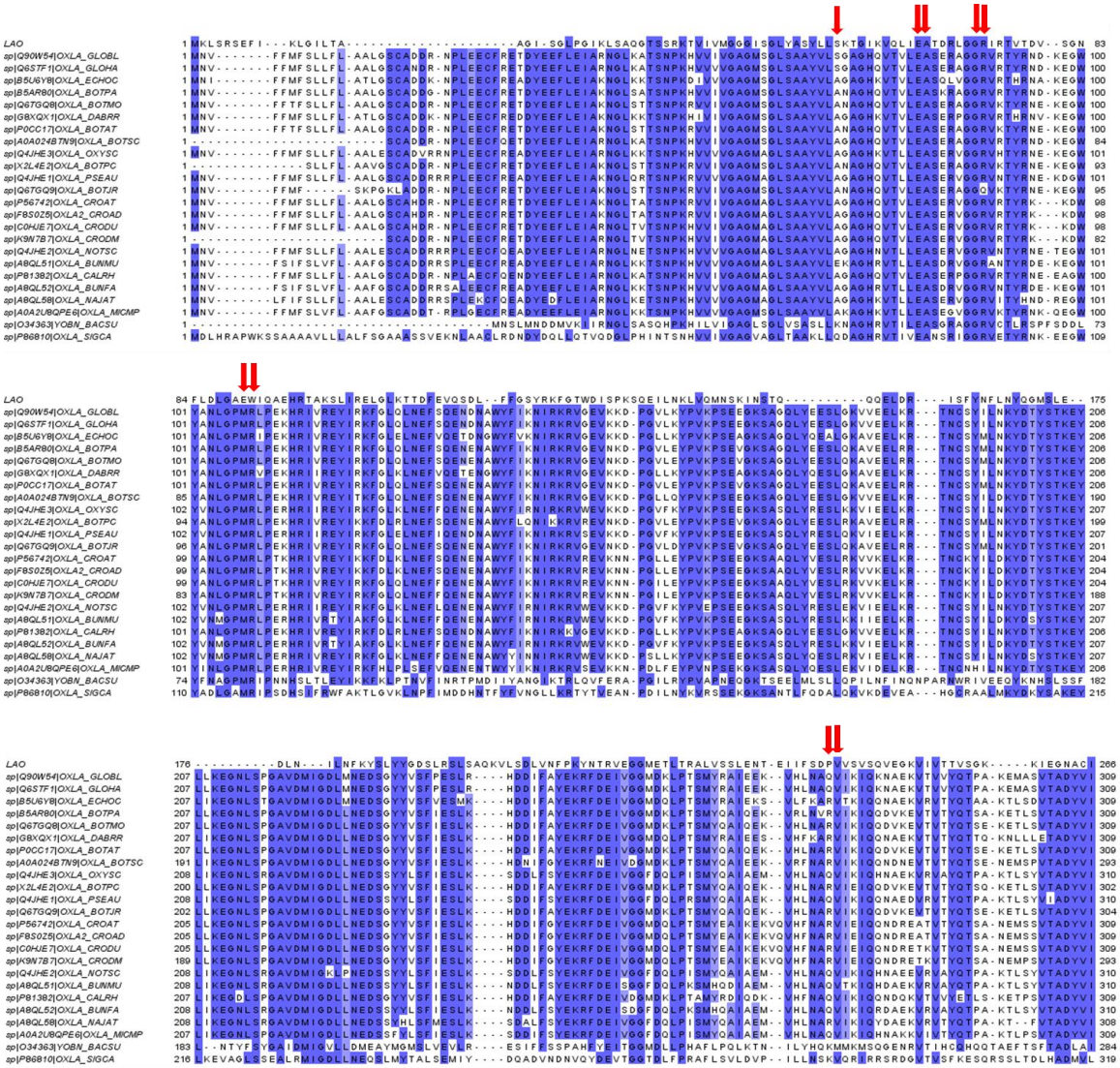
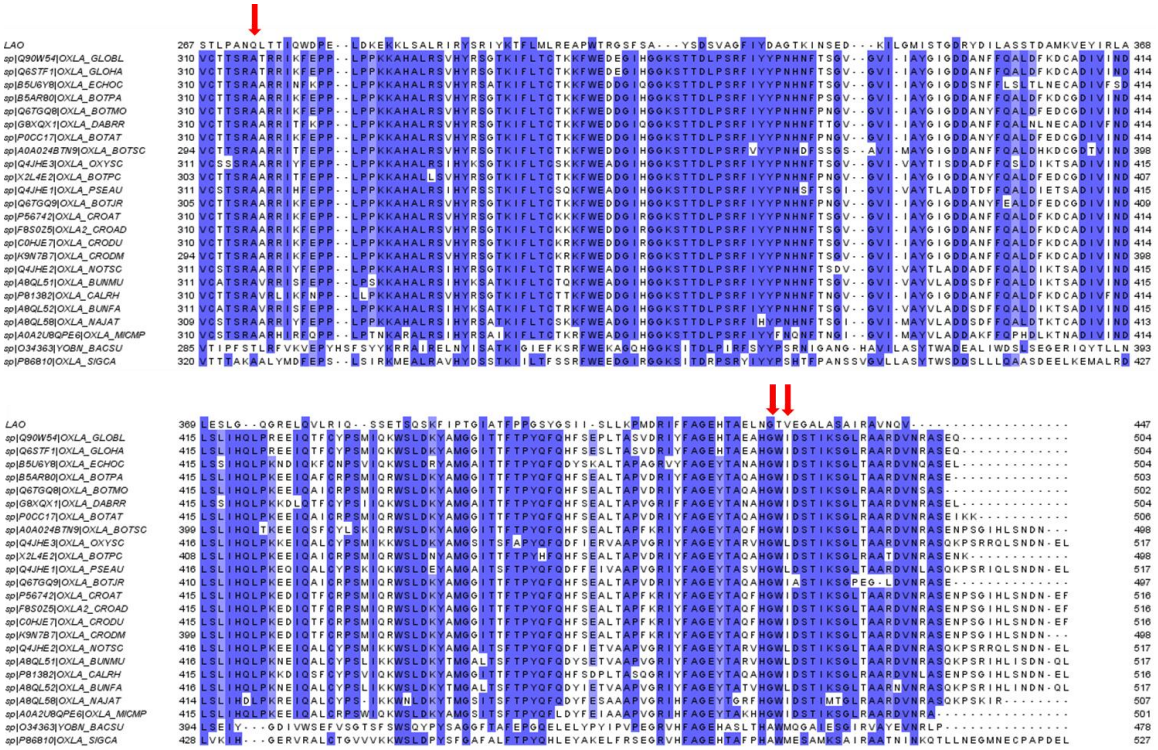
Appendix 3

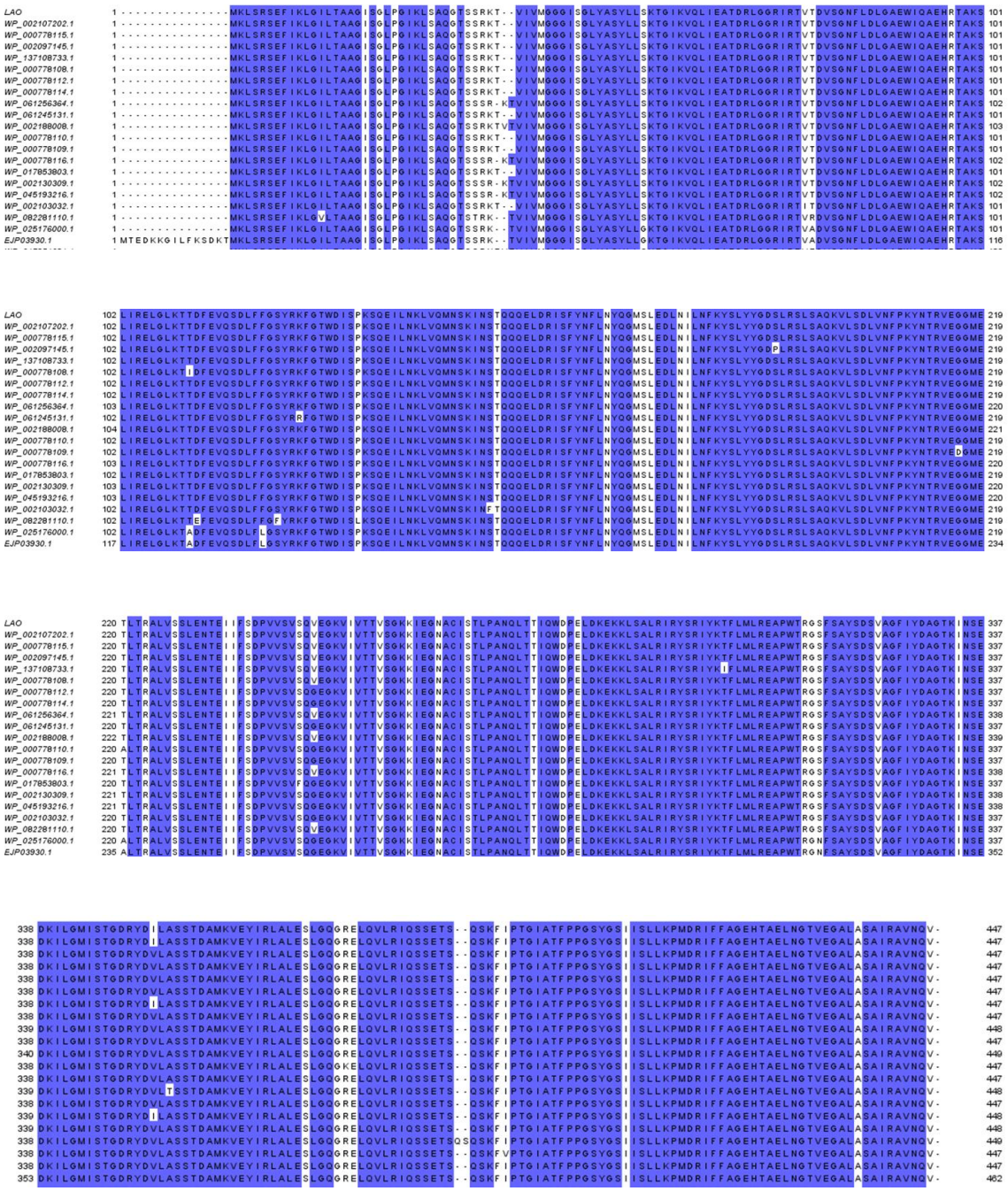
Alignment of leptospiral LAO (marked with FAD interacting residues) with L-amino acid oxidase





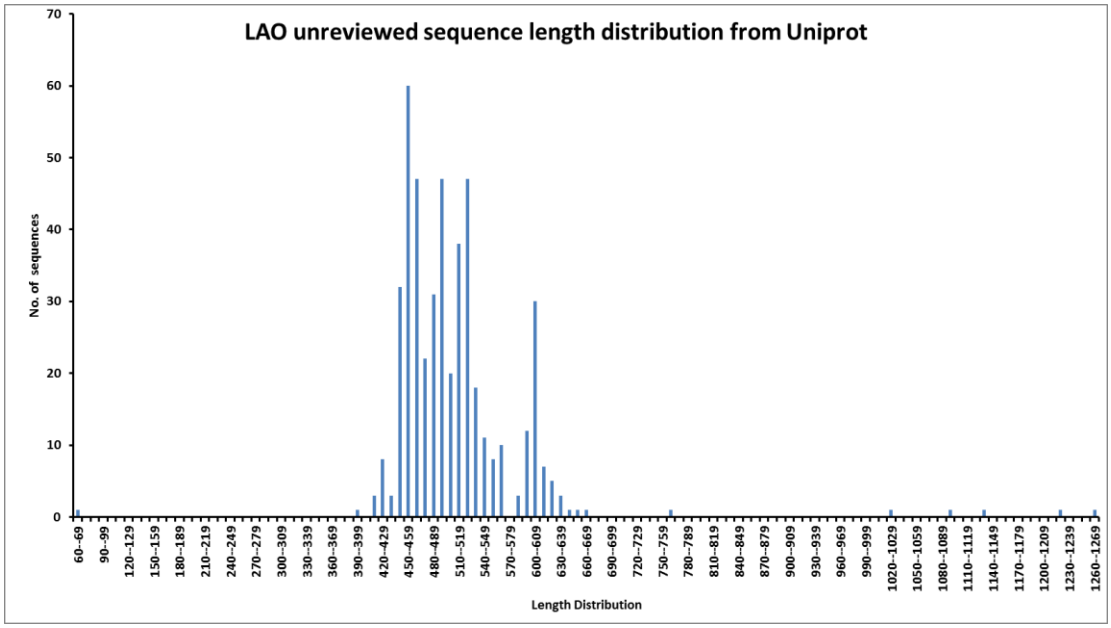
Appendix 4

Alignment of leptospiral LAO



Appendix 5

Number of Sequences available in the UniProt database with length distribution (Courtsey: Dr. Vijayasarathy)


LAO unreviewed sequence length distribution from Uniprot
No. of sequences
Length Distribution
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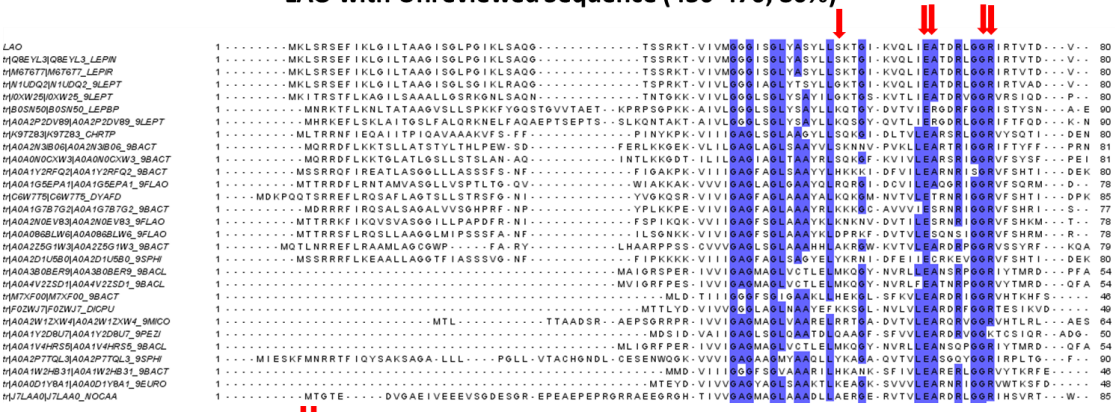
10
20
30
40
50
60
70
60–69
90–99
120–129
150–159
180–189
210–219
240–249
270–279
300–309
330–339
360–369
390–399
420–429
450–459
480–489
510–519
540–549
570–579
600–609
630–639
660–669
690–699
720–729
750–759
780–789
810–819
840–849
870–879
900–909
930–939
960–969
990–999
1020–1029
1050–1059
1080–1089
1110–1119
1140–1149
1170–1179
1200–1209
1230–1239
1260–1269

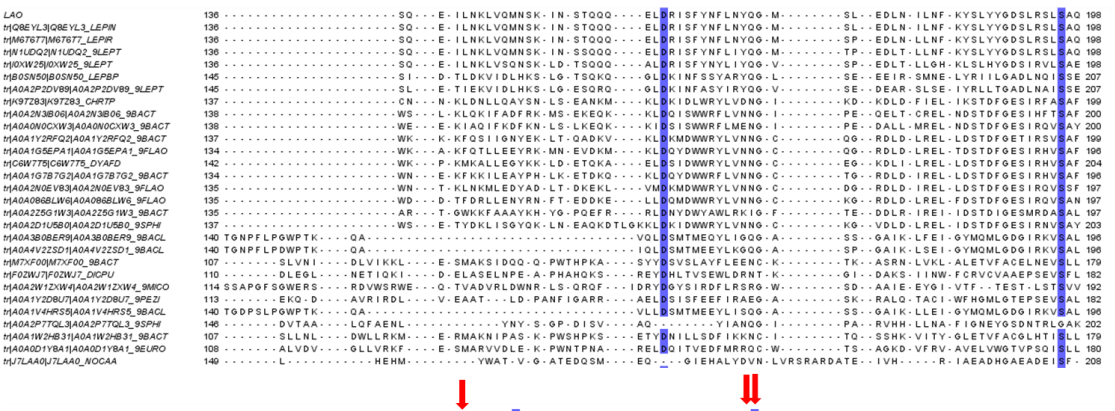
Appendix-6

Alignment of leptospiral LAO with unreviewed sequences from UniProt knowledge database
(Courtsey: Dr. Vijayasarathy)

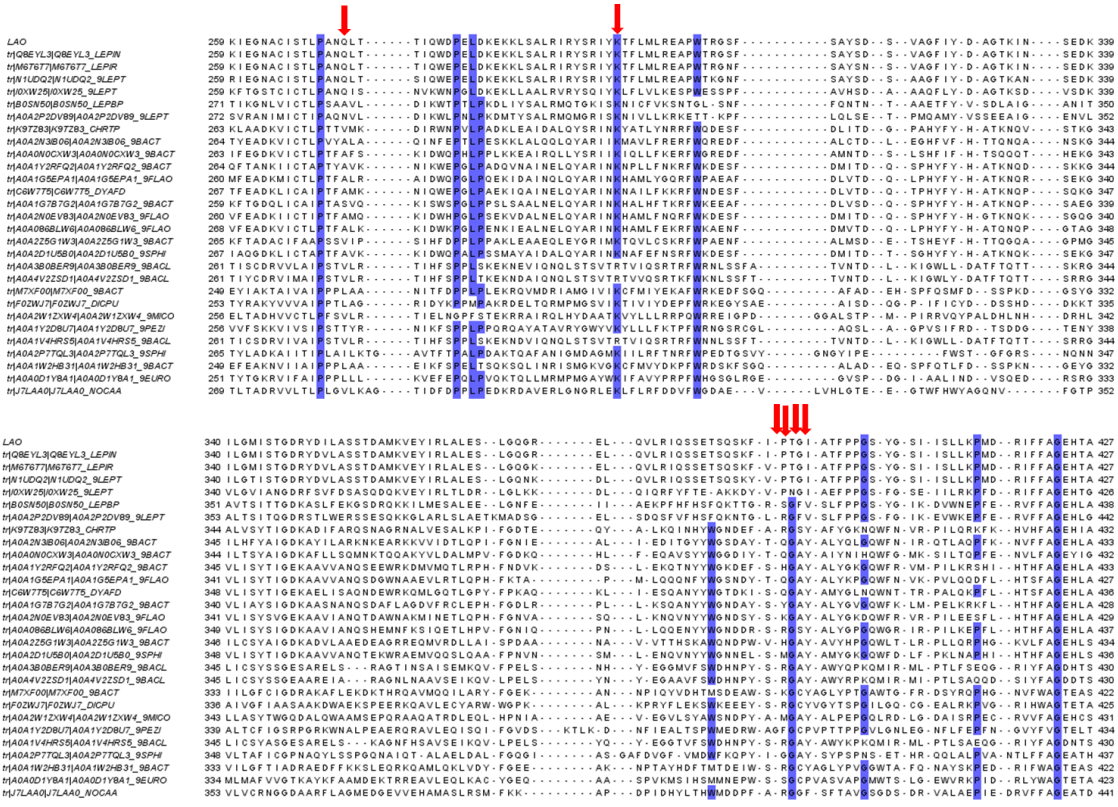
LAO with Unreviewed Sequence (430-470; 80%)



LAO with Unreviewed Sequence (430-470; 80%)



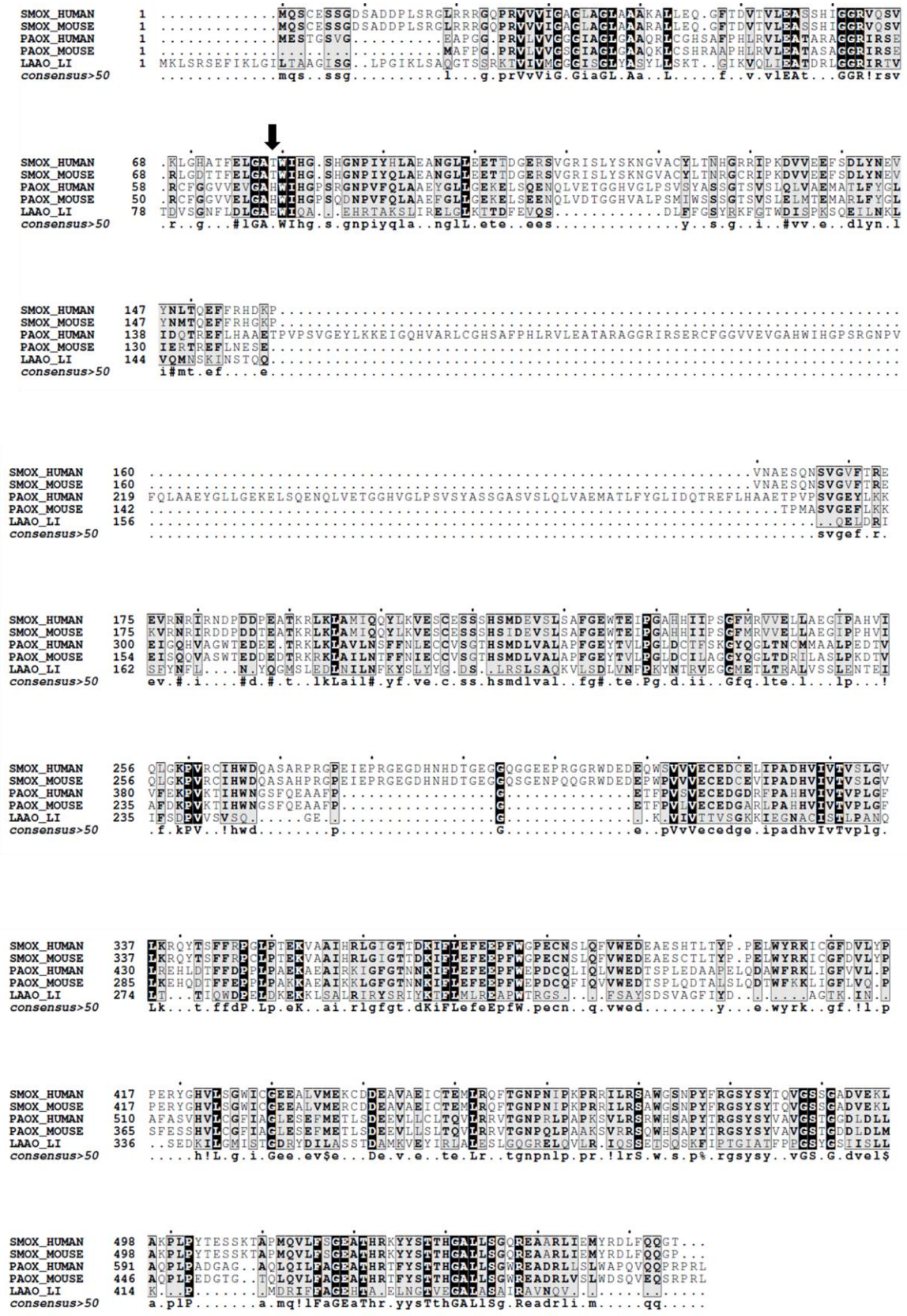
LAO with Unreviewed Sequence (430-470; 80%)



LAO with Unreviewed Sequence (430-470; 80%)

Appendix 7

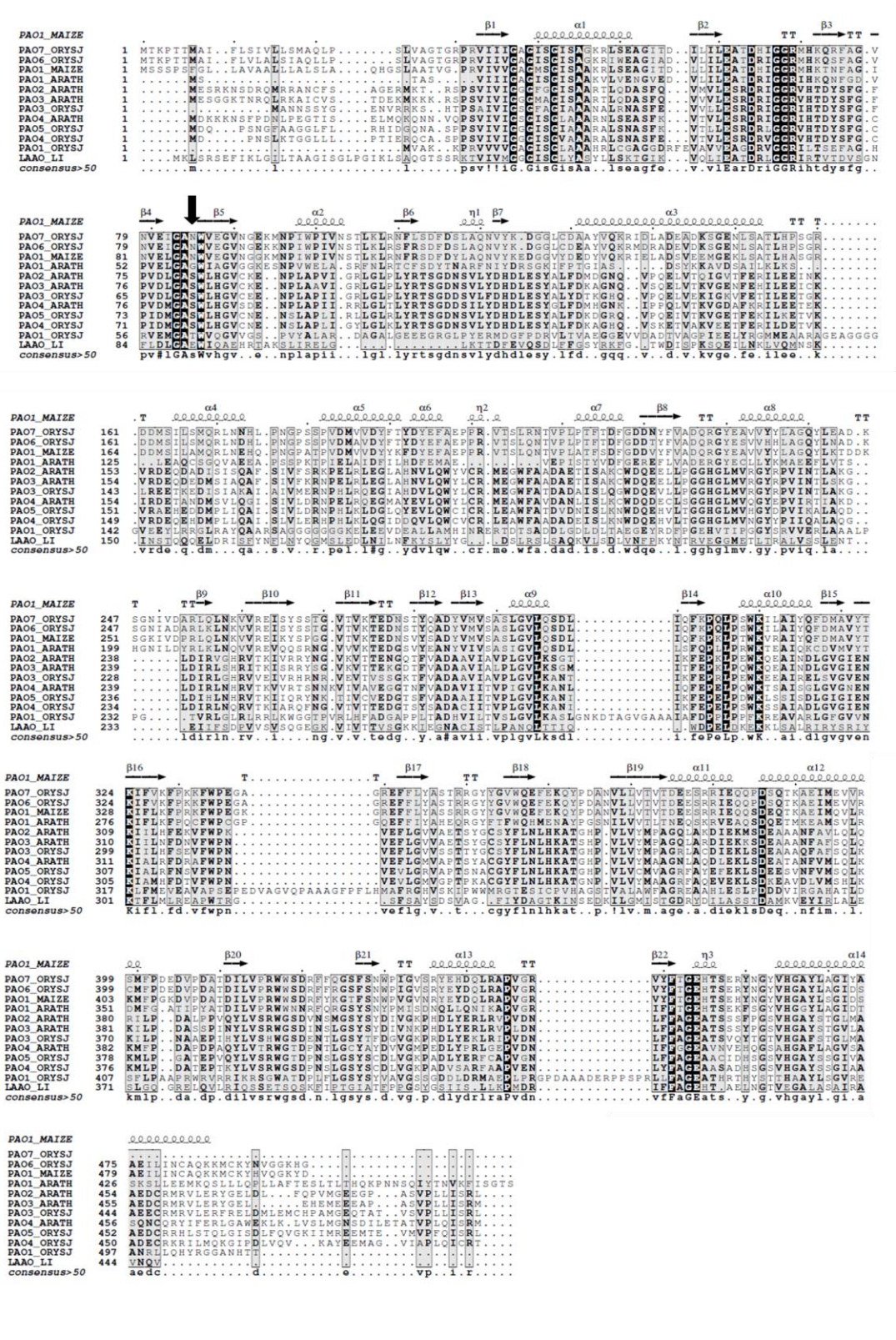
Alignment of leptospiral LAO with spermine oxidase



Alignment of leptospiral LAO with spermine oxidase.

Appendix-8

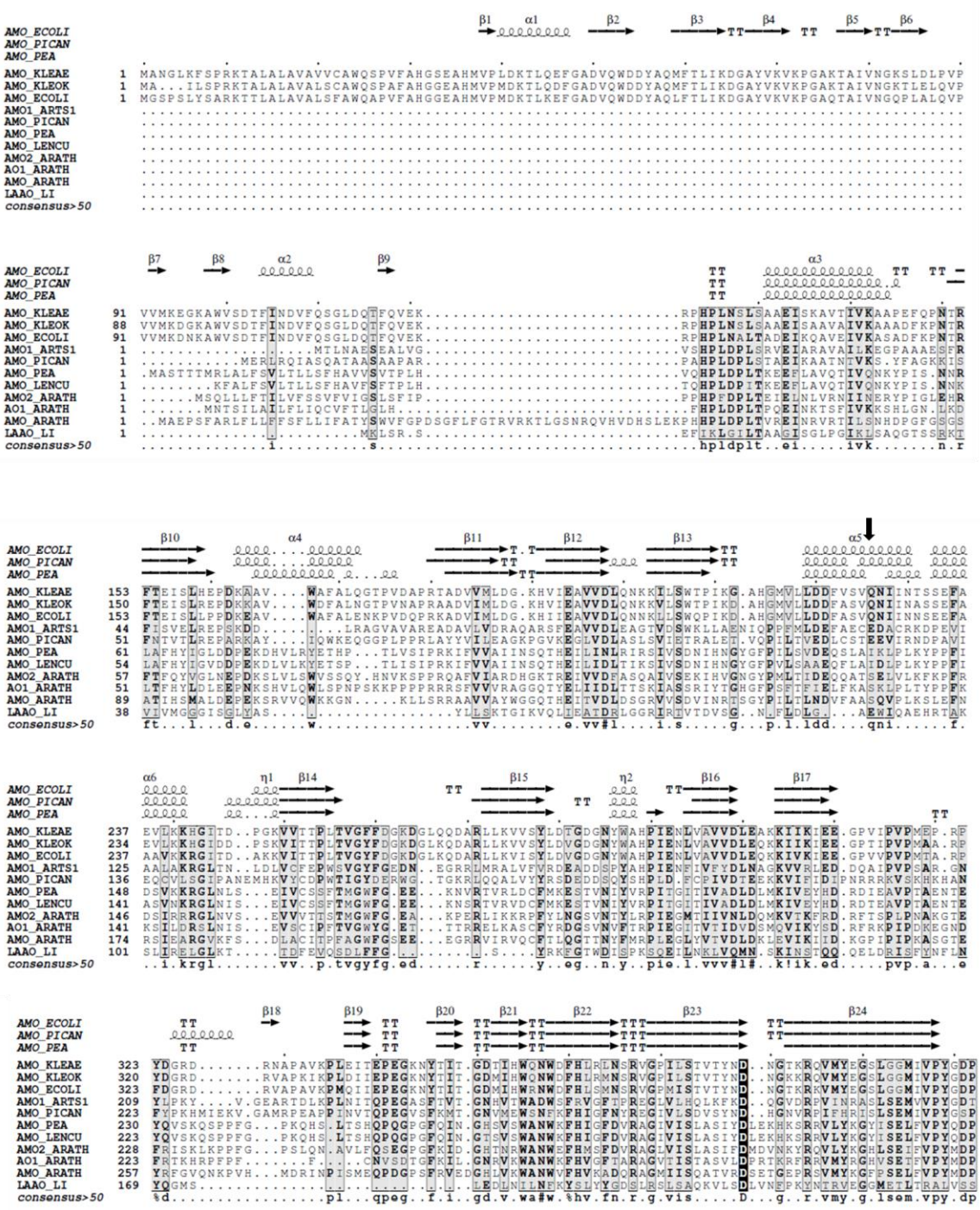
Alignment of leptospiral LAO with Polyamine oxidase



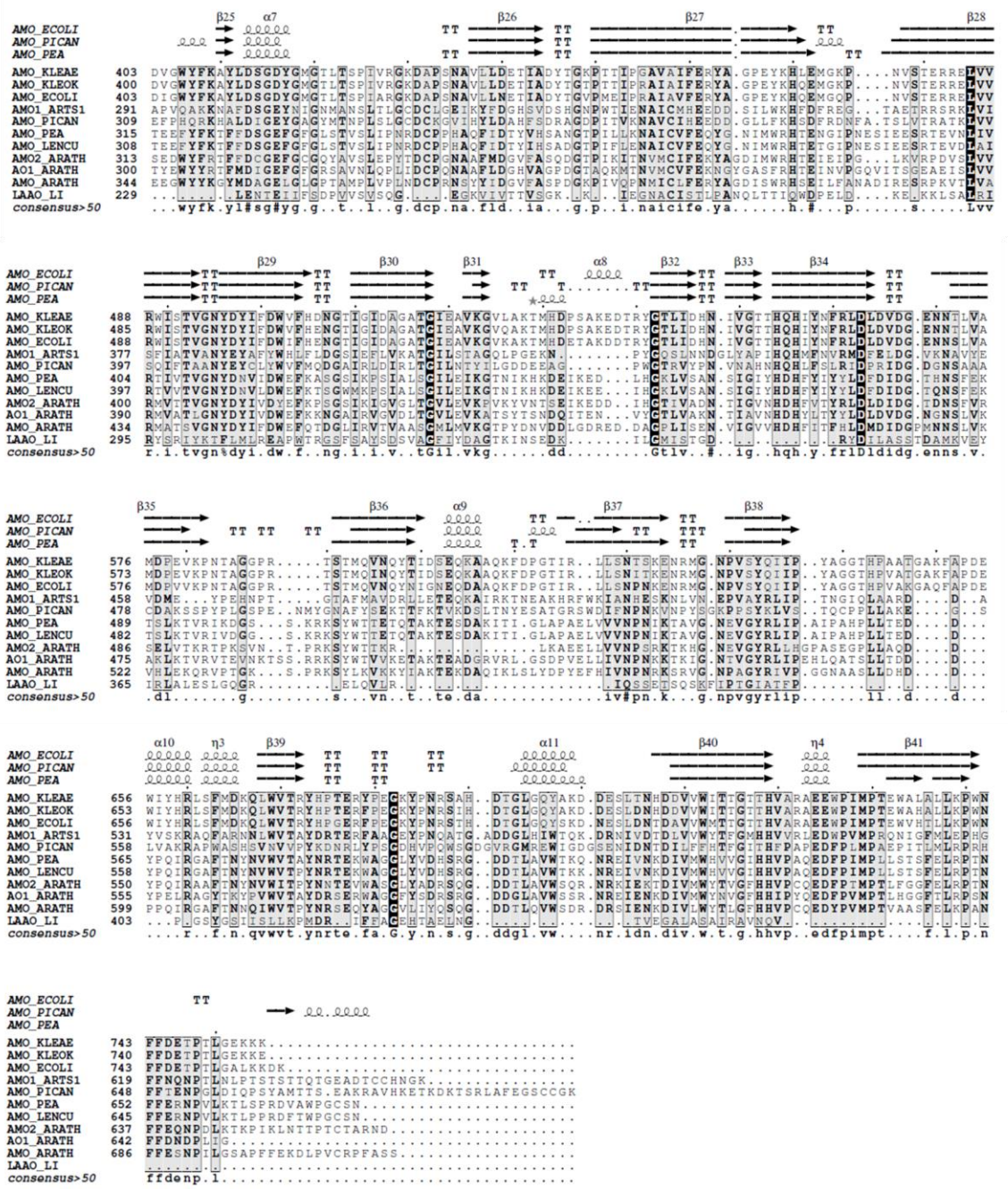
Alignment of leptospiral LAO with Polyamine oxidase.

Appendix 9

Alignment leptospiral LAO with Primary amine oxidase



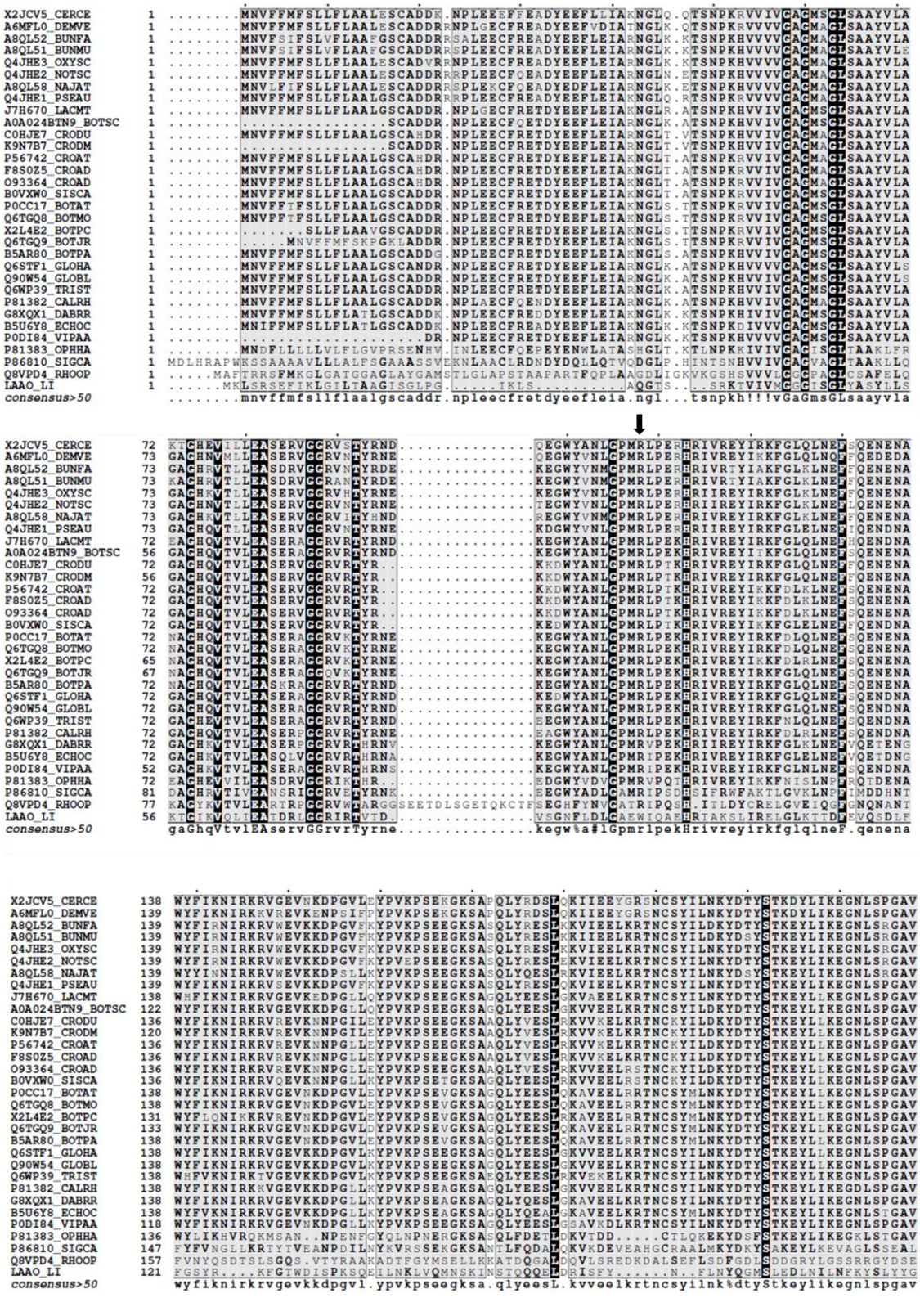
Alignment leptospiral LAO with Primary amine oxidase.



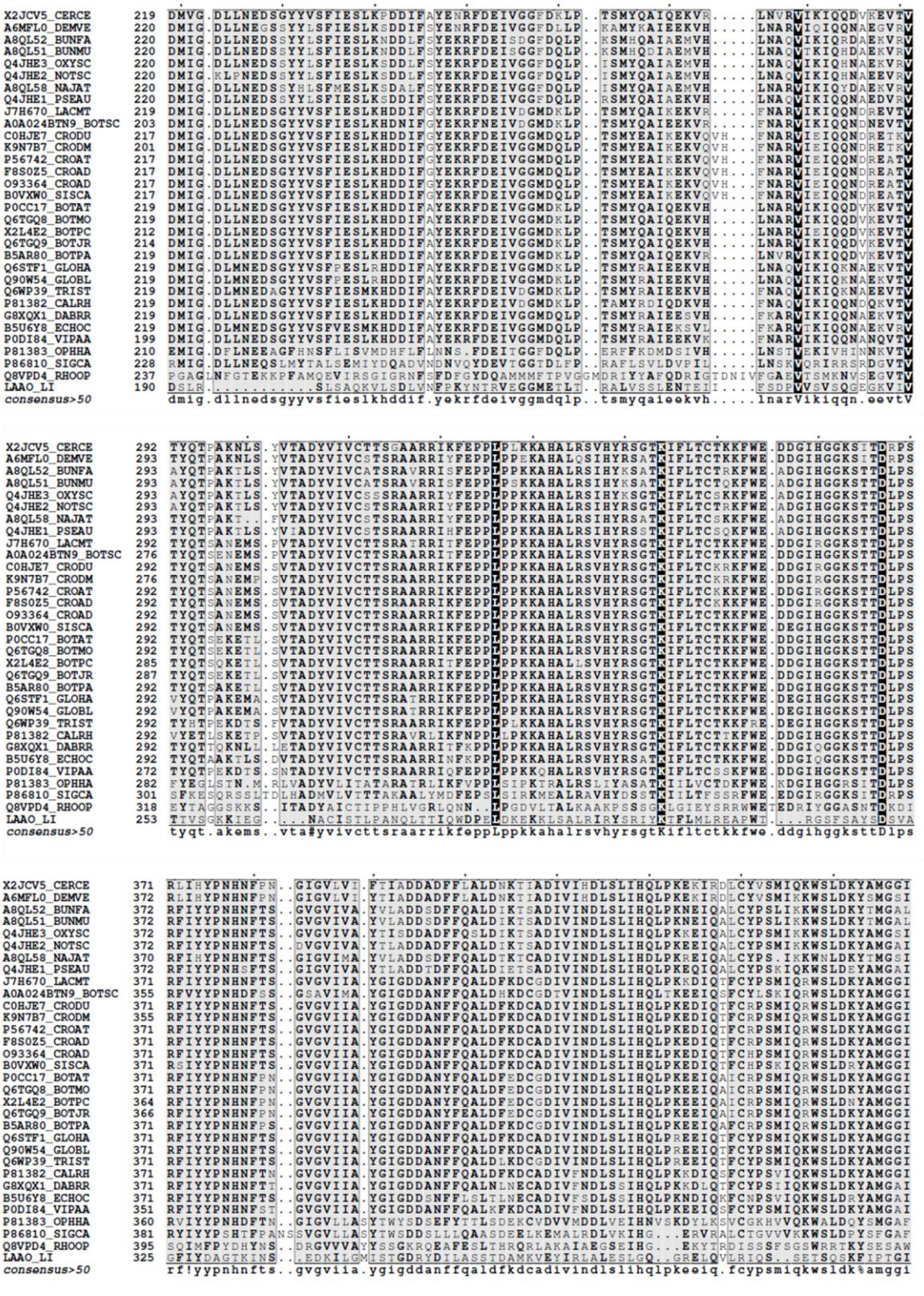
Alignment leptospiral LAO with Primary amine oxidase.

Appendix 10

Alignment leptospiral LAO with Amino acid oxidase



Alignment leptospiral LAO with Amino acid oxidase.



Alignment leptospiral LAO with Amino acid oxidase.

Ph.D. Thesis

On the Topic

"Studies on enzymes from *Leptospira interrogans* with special emphasis to Triosephosphate isomerase and putative L-amino acid oxidase"

Submitted to

Sri Devaraj Urs Academy of Higher Education and Research

for the award of the degree of

Doctor of Philosophy

in

Cell Biology and Molecular Genetics

Under the Faculty of Allied Health and Basic Sciences

by

VAIGUNDAN D.

13PhD2201

Under the Guidance of

Dr. Sharath B. and **Prof. P. R. Krishnaswamy**

Sri Devaraj Urs Academy of Higher Education and Research

Tamaka

Kolar-563101

Karnataka

Summary and conclusions drawn from the study

1. Nine serovars of Leptospira were tested for the visible growth in various combinations of media prepared using the wealth of information gained from; the genomic studies, proteomic studies, mode of infection, and the host environment that are available in the literature. The M12 medium reported in this study showed a visible growth (formation of the ring) of Leptospira at 40 hours of inoculation as might be helpful in the early diagnosis of Leptospirosis disease in the future.

2. Triosephosphate isomerase from *L. interrogans* was cloned, expressed, and purified. The purified enzyme LiTPI was kinetically characterized. The LiTPI is found to be an active dimer, with a k_{cat} of 1740 s−1 (D-GAP→DHAP and Km (D-GAP) of 0.21 mM, at 25 °C. The kinetic behaviour of LiTPI is similar to the TPIs from other organisms obtained from other studies.

3. Putative L- amino acid oxidase from *L. interrogans* was cloned, expressed, and purified. However the mass of the purified protein was lesser than the expected mass, due to abrupt cleavage of the N-terminal signal sequence (28 residues) in *E. coli*; further, the purified protein did not show any activity with any of the amino acid substrates. Recombinant putative Li-rLAO (without the 20 residues of N-terminal signal sequence) was cloned, expressed, and purified and the three-dimensional structure was determined by x-ray crystallography at a resolution of 1.8 Å. FAD is found to be the non-covalently bound cofactor. The activity of the purified recombinant enzyme with different amino acid substrates was not promising as other L-amino acid oxidases reported in the literature.

4. The kinetic behaviour of triosephosphate isomerase from *L. interrogans* was similar to that of isoforms of triosephosphate isomerase from humans the substrate specificities of L- amino acid oxidase from *L. interrogans* was different when compared to L- amino acid oxidase from the human. Phenylalanine is the preferred substrate of the human L-amino acid oxidase whereas as in the case of Leptospiral L-amino acid oxidase it seems to be L-arginine.

New Knowledge generated

1. A new medium has been developed for the growth of the bacterium Leptospira, which yields appreciable bacterial populations within 40 hours, for this hitherto "difficult to culture bacterium". The protocols have been developed based upon an analysis of available information in the literature, on the environmental conditions for bacterial growth *in vivo.*

2. Triosephosphate isomerase, a central enzyme in the glycolytic pathway has been cloned and characterized biochemically, from the organism *Leptospira interrogans*.

3. The gene for a novel, putative L-amino acid oxidase, identified from the available genomic sequence of *Leptospira interrogans*, has been cloned. Heterologous expression of the recombinant protein in *E.coli* has yielded pure recombinant protein, for further characterization. Protein mass spectrometry has been used to resolve the nature of the processing of the expressed precursor in *E.coli* and to identify the presence of the non-covalently bound cofactor, flavin adenine dinucleotide (FAD), establishing the likely oxidase activity of this new enzyme.

4. Diffraction quality crystals were obtained for this new oxidase, permitting three-dimensional structure determination by X-ray crystallography. The structure permits the definition of the FAD binding site and provides information on the active site of the enzyme, although the precise natural substrate remains unknown. *In vitro* experiments, using an oxidase assay system, suggest that of the several potential substrates examined, L-arginine appears to be a promising candidate. Bioinformatic analysis reveals a very low sequence homology between the new leptospiral oxidase and previously characterized bacterial and animal venom oxidases.

Strengths and Weaknesses of the study

1. The study has identified an M12 medium for a visible growth (formation of the ring) of Leptospira at 40 hours of inoculation. The sensitivity of the M12 medium has to be established with a different type of serovars at different concentrations and subjected to rigorous appropriate statistical analysis to use it for diagnostics purposes. Biomass quantification and morphological studies of the organism were not carried out.

2. The Leptospiral Triosephosphate isomerase was characterized by biochemical, biophysical methods, and kinetic parameters of the enzyme were determined. The study does not address the activity of TPI in presence of various other intermediates of glycolysis and the substrates of enzymes that uses DHAP or GAP as one of the substrates to address the allosteric regulation of TPI.

3. The crystal structure of Leptospiral L-amino acid oxidase was determined by x-ray crystallography and characterized Li-rLAO by biochemical and biophysical methods. Still, it is a challenging task to identify its primary substrate and the assay condition for the putative *L. interrogans* L-amino acid oxidase