Scientific and Technical Progress Report (STPR) [2017-2020]
(DBT R&D projects)

Section-A : Project Details

A1. Project Title: Biophysical Characterization of adenylosuccinate lyase- a potential drug target from Leishmania donovani – a computational and molecular approach

A2. DBT Sanction Order No. & Date: BT/PR15847/NER/95/21/2015 dated February 13, 2017

A3. Date of Project Initiation: 13/02/2017
A4. Date of project completion: 12/08/2020

A5. Name of Principal Investigator: Dr. Anupam Nath Jha
Name of Co-PI/Co-Investigator: Dr. Anju Pappachan

A6. Institute: Tezpur University
Central University of Gujarat

A7. Address with Contact Nos. (Landline & Mobile) & Email of Project Co-coordinator/ PIs and Co-PIs:

Dr Anupam Nath Jha
Assistant Professor
Molecular Biology and Biotechnology
Tezpur University
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Dr. Anju Pappachan
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School of Life Sciences,
Central University of Gujarat,
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A8. Total Approved Cost (including additional cost, if any):
41.80 Lakhs (Tezpur University)
27.85 Lakhs (Central University of Gujarat)

A9. Approved Duration: Three years

A10. Rational and background information of project (in brief Maximum 500 words): attached

A11. (i) Approved Objectives of the Project:
1. The sequence analysis from the available ADSL proteins and literature search
2. Identification of crucial residues involved in oligomerization and functional activity of LdADSL
3. Molecular Dynamics simulation of the mutants and control

(ii) Details of approved work Plan and milestone: attached

A12. Specific Recommendation(s)/ suggestion(s) made by the DBT Committee/ Task Force at the time of project sanction and/ or earlier progress review: NIL

Section-B : Scientific and Technical Progress

B1. **Progress made against the Approved Objectives, Targets & Timelines during the Reporting Period** (Please provide detailed report with full data set) **Attached**

B2. **Summary and Conclusions of the Progress made so far** (minimum 100 words, maximum 200 words): **Attached**

B3. **Details of New Leads Obtained, if any: NIL**

B4. **Details of Publications & Patents, if any (**Please compulsorily enclose the reprint of research publications (Also indicate Impact Factor)**/** details of patent & IPR generated from this project)**: attached**

Section-C: Details of Grant Utilization#

C1. **Equipment Acquired or Placed Order with Actual Cost:**
Tezpur University:
GPU based parallel computing system – Rs. 19.50 Lakhs
CUG:
Fluorescence spectrophotometer- Rs. 09.71683 Lakhs

C2. **Manpower Staffing and Expenditure Details:**
Tezpur University:
1. Nikita Bora – (JRF 13/02/18 to 31/01/20) – Rs. 5.89286 Lakhs
2. Titus B – (JRF, 10/02/2020 to 15/03/2020) – Rs. 0.14081 Lakhs

CUG:
1. Paula Rajiwala – (JRF 01/06/17 to 31/01/18) – Rs. 0.66 Lakhs
2. Krishna Kumar– (JRF, 21/09/2018 to 12/02/2020) – Rs. 4.56839 Lakhs

C3. **Details of Recurring Expenditure:**

Sl. No.	Recurring Head	Amount (Rs in Lakhs)	
		T.U	CUG
1	Consumables	2.93986	3.30872
2	Travel	1.11341	0.11355
3	Contingency	0.98485	0.09841
4	Overhead	3.00	1.080

[Signature(s) of the Investigator(s)]

Important Instructions:

(i) *STPR must be complete in all respect. All the information needs to be compulsorily provided, otherwise STPR will be treated as incomplete and will not be evaluated or considered further. In case of 'Nil' / 'Not Applicable' information, the same may please be indicated.*

(ii) *In case of multicentric network project, combined* Scientific and Technical Progress Report (*STPR) requires to be submitted incorporating the progress of all components. The Project Co-coordinator/ PI will be responsible for this.*

(iii) **Please indicate the reporting period [i.e. 1st year **(I)** / 2nd year **(II)** / 3rd year **(III)/ Completion Report etc.**]. In case of completion report, please indicate that it is the **'Project Completion Report'**.*

(iv) *Submission of STPR is linked with further continuation of the project, release of grant and final settlement of accounts.*

*(v) STPR need to be submitted by post and also by email in **pdf file**.*

A11. (ii) Details of approved objectives, work Plan and milestone:

Approved objectives, Targets and timelines

Tezpur University:

Period of study (in months)	**Achievable targets**
6	The sequence analysis from the available ADSL proteins and literature search
12	Identification of crucial residues involved in oligomerization and functional activity of LdADSL
18	Mutational studies to determine the-role of these residues
24	Molecular dynamics simulations of the native protein and mutants
30	Virtual screening of possible inhibitors
36	Prediction of possible drug ligands by different computational techniques (Docking, QSAR, ADMET)

Central University of Gujarat:

Period of study	**Achievable targets**
6 Months	Cloning of LdADSL gene and recombinant protein expression
12 Months	Generation of mutants (Mutations will be planned based on in-silico analysis)
18 Months	Biophysical characterization of the proteins and mutants using techniques like CD spectroscopy, Fluorescence spectroscopy, Gel permeation chromatography, etc
24 Months	Activity studies of native and mutant proteins using enzyme kinetic assays
30 Months	Testing selected inhibitor compounds on the activity of the protein using enzyme assays
36 Months	Communication of results

A10. Rational and background information of project

Background: Purine nucleotides are essential biological molecules needed for biosynthesis of nucleic acids and precursor coenzymes such as NAD, NADP, FAD, and CoA which play key roles in metabolism as well as signal transduction. Inhibitors of nucleotide biosynthesis are very toxic to cells and their toxicity has been manipulated in the treatment of cancer and certain diseases resulting from infections by viruses, bacteria, or protozoans. The parasitic protozoa, including *Leishmania* lack the enzymes to synthesize purine nucleotides de novo, therefore, they have to depend upon the purine salvage system to utilize purine bases from their mammalian hosts. Purine bases are translocated through the parasite cell surface by nucleoside transporters. This critical dependence of *Leishmania* for a purine salvage pathway offers an opportunity to target for chemotherapeutic intervention.

Rational of study: The purpose of the study is to identify the structural and functional details of the LdADSL enzyme. There is no work done on active site of enzymes from the current data, and no information available which residues involved in binding of substrate or inhibitor to the enzymes which cannot be analyzed in detail. However, we will apply both biochemical and multiple sequence alignment data, along with structural comparisons to other enzymes in the β-elimination superfamily, can give useful information about the residues involved in substrate binding and catalysis in ADSL. This approach gives to develop new drugs target and identify metabolic differences between the host and parasite and exploit those proteins which are crucial for the survival of the parasite at the same time not significant for the host as drug targets.

Objectives & methodology: Biophysical and functional characterization of these enzymes by cloning and expression of protein, mutational study biochemical and biophysical characterization & structural characterization x-ray crystallography.

Expected outcomes: The structure of LdADSL and their mutants have not been done so far. The sturdy will help us to understanding protein and their important residue for development of strong potential drug target.

1. Alvar, J., Yactayo, S., & Bern, C. (2006). Leishmaniasis and poverty. *Trends in parasitology*, *22*(12), 552-557.
2. Hammond, D. J., & Gutteridge, W. E. (1984). Purine and pyrimidine metabolism in the Trypanosomatidae. *Molecular and biochemical parasitology*, *13*(3), 243-261.
3. Fyfe, P. K., Dawson, A., Hutchison, M. T., Cameron, S., & Hunter, W. N. (2010). Structure of Staphylococcus aureus adenylosuccinate lyase (PurB) and assessment of its potential as a target for structure-based inhibitor discovery. *Acta Crystallographica Section D: Biological Crystallography*, *66*(8), 881-888.
4. Brosius, J. L., & Colman, R. F. (2002). Three subunits contribute amino acids to the active site of tetrameric adenylosuccinate lyase: Lys268 and Glu275 are required. *Biochemistry*, *41*(7), 2217-2226.
5. Boitz, J. M., Strasser, R., Yates, P. A., Jardim, A., & Ullman, B. (2013). Adenylosuccinate synthetase and adenylosuccinate lyase deficiencies trigger growth and infectivity deficits in Leishmania donovani. *Journal of Biological Chemistry*, *288*(13), 8977-8990.

Section-B: Scientific and Technical Progress (February 2017 –August 2020)

Biophysical Characterization of adenylosuccinate lyase- a potential drug target from Leishmania donovani – a computational and molecular approach

DBT Sanction Order No. & Date: BT/PR15847/NER/95/21/2015 dated Feb 13 2017

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B2. Summary and Conclusions of the Progress made so far

Considering the financial constraints happen because of the untimely release of the sanctions, we tried to achieve most of the objectives that we proposed. The major outcomes of the work done against the objectives are -

- The LdADSL protein from *Leishmania donovani* were cloned into pET28a vectors
- Protein expression and purification of LdADSL was done with standardize protocol.
- Enzyme kinetics assay of LdADSL is completed with different SAMP concentration.
- Generated five mutants in LdADSL genes (R40A, R40E, Y41A, E334A and N335Y) using SDM method
- Fluorescence spectroscopy was done with different concentration of LdADSL and mutant proteins.
- CD spectroscopy of LdADSL and mutant proteins were conducted.
- The selected double mutations i) N335Y and T367R and ii) S321C and E334A of LdADSL are reported to change its binding affinity with AMP molecule which infers a decreased substrate binding.
- A biochemical network of metabolic pathway "Purine salvage" has been constructed and analyzed.
- Pathway reactions showing higher sensitivity to the model have been sorted out with the proteins ADSL and IMPDH to be the most sensitive to the effect of concentration.

It would be interesting to see, whether the findings stay valid against in-vivo experiments!

B4. Details of Publications

1. Nikita Bora and Anupam Nath Jha. In silico metabolic pathway Analysis identifying target against Leishmaniasis – A Kinetic modeling approach. ***Frontiers in Genetics*** 2020; 11: 179.

2. Nikita Bora and Anupam Nath Jha. An integrative approach using systems biology, mutational analysis with Molecular Dynamics simulation to challenge the functionality of a target protein. ***Chemical Biology & Drug Design*** 2019; 93:1050-1060

I. Poster presented on "**Two protein from purine salvage pathway used as drug target in *Leishmania donovani*"** 9th Annual Edinburg Infection Disease Symposium -2020 (virtually) organized by The university of Edinburg, United Kingdom.

II. Poster presented on "**Adenylossucinate lyase and Adenylossucinate Synthetase, key enzymes in purine salvage pathway in *Leishmania donovani*"** Annual Molecular Parasitology & Vector Biology symposium-2021, organized by Center for tropical & Emerging Global Disease, University of Georgia, Athens, GA, USA.

<u>Section-B: Scientific and Technical Progress (2017 – 2020)</u>

B1. Progress made against the Approved Objectives, Targets & Timelines

Biophysical Characterization of adenylo succinate lyase- a potential drug target from *Leishmania donovani* – a computational and molecular approach

DBT Sanction Order No. & Date: BT/PR15847/NER/95/21/2015 dated February 13, 2017

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Approved objectives, Targets and timelines

Tezpur University

Period of study (in months)	**Achievable targets**
6	The sequence analysis from the available ADSL proteins and literature search
12	Identification of crucial residues involved in oligomerization and functional activity of LdADSL
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12	Generation of mutants(Mutations will be planned based on in-silico analysis)
18	Biophysical characterization of the proteins and mutants using techniques like CD spectroscopy, Fluorescence spectroscopy, Gel permeation chromatography, etc
24	Activity studies of native and mutant proteins using enzyme kinetic assays
36	Testing selected inhibitor compounds on the activity of the protein using enzyme assays.

Consolidated progress report

1. **Equipment purchasing & Manpower recruitment**

   Tezpur University

GPU-based parallel computing system (two NVIDIA Pascal P100 GPU cards in two Dell Poweredge R730) have been purchased and installed successfully.

1) One JRF (Ms. Nikita Bora) recruited (February 13th, 2017 to January 31st, 2019).
2) One JRF (Mr. Titus B) recruited (February 10th, 2020 to Marsh 15th, 2020)

Central University of Gujarat:

The remaining amount available in the grant was transferred from Indian institute of Advanced Research (IIAR), Gandhinagar to Central University of Gujarat (CUG), Gandhinagar on May 9th, 2018. So the project was initiated at CUG from May 10th 2018.

a. Purchase of equipment:

   Fluorescence spectrophotometer have been purchased and installed successfully.

b. One JRF (Mr. Paula Rajiwala) recruited (June 1st 2017 to January 31st 2018)

c. One JRF (Mr. Krishna Kumar) recruited (September 21st, 2018 to February 12th 2020)

2. **Attending conferences**

   Tezpur University:

i) Dr A N Jha (PI) has attended an International Conference on Intrinsically Disordered Proteins at IISER, Mohali, December 9-12, 2017.

ii) Ms. Nikita Bora (JRF) has attended two conferences:

a. Breaking Barriers Through Bioinformatics & Computational Biology, 31st July – 1st August, 2017, at ScFBio, IIT Delhi.

b. International Symposium on Systems, Synthetic & Chemical Biology, 5th – 7th December, 2017, at Bose Institute, Kolkata.

iii) Dr A N Jha (PI) has attended two conferences –

1) An International Conference on Bioinformatics (**InCoB-2018**) at JNU, Delhi, during 26-28 Sep, 2018.
2) Annual BSBE Winter Meeting on "Computational Biology in Disease Mechanisms" at IIT Kanpur during 07 – 09 Dec, 2018.

iv) Ms. Nikita Bora (JRF) has attended IFCAM Summer School on Mathematical and Computational Biology at IISc, Bangalore during 16-31 July, 2018

3. Research Work

Adenylosuccinate lyase (ADSL) is an enzyme catalyzing the formation of AMP in the purine nucleotide mechanism. The parasite *Leishmania donovani* is auxotrophic for purines and has to be host dependent for their nutritional requirements which is vital for the parasite's survival. The enzyme catalyzes the formation of AMP from adenylo succinate through a uni bi mechanism in which the β -H of the succinyl moiety of adenylo succinate is abstracted by a general base and the leaving group (AMP) is protonated at either the 1st / 6th position by a general acid (**Jennifer L. *et al*, 2002**). It proceeds by abstraction of a proton from Cβ atom of the succinyl group by a catalytic base leading to the formation of a carbanion intermediate that is stabilized by resonance. Protonation at N1 or N6 by a catalytic acid leads to Cα-N bond cleavage and release of AMP and fumaric acid as products **(Banerjee *et al*, 2014).** Affinity-labeling studies of *Bacillus subtilis* ASL have demonstrated that in all likelihood the two residues participating in this general acid–base mechanism are His68 and His141. These residues share identical numbering in *T. maritima* ASL. Subsequent mutagenesis and kinetic studies have shown that His141 functions as the general base and His68 as the general acid (**Toth and Yeates, 1999**). Mutagenesis studies have revealed additional amino acid residues, His89, Lys268, and Asn270 that assist in the catalytic reaction, most likely by contributing to substrate binding and orientation in the active site. Thr93, Ser94, Thr140, and Ser306 perform very important roles in the function of ASL through hydrogen bonding interactions directly with adenylo succinate, as well as indirectly through non bonded contacts to active site amino acid residues **(Segall et al, 2006). Banerjee *et al***, suggested His149 and either Lys285 or Ser279 of *Mycobacterium smegmatis* ADSL (Ms ADSL) are the residues most likely to function as the catalytic acid and base, respectively.

Worked carried out at Tezpur University

1. Sequence analysis from the available ADSL proteins

The sequence length of the adenylosuccinate lyase protein from *Leishmania donovani* is 479 amino acids with a molecular weight of 53.5 kDa. The computed PI for the protein is 5.79. To identify the functionally important residues of *L. donovani* ADSL sequence, a comparison of these reported acid and basic residues from *B. subtilis, M. smegmatis, T. maritima* to *L. donovani*

has been carried out as shown in **table 1.** It has been observed that they are conserved both at the sequence and the structure level and might be playing an important role in the catalysis process.

Table 1: Comparision of *Leishmania donovani* ADSL sequence to *B. Subtilis, Mycobacyterium smegmatis* and *Thermatoga maritime*

L.donovani	*B.subtilis*		*Mycobacyterium smegmatis*		*Thermatoga maritima*	
His 118	His 68	Acid			His 68	Acid
His 196	His 141	Base	His 149	Acid	His 141	Base
His144	His 89					
Lys327	Lys268		Lys285	Base		
Asn329	Asn270				Asn270	
Thr148	Thr93					
Ser149	Ser94					
Thr195	Thr140					
Ser366	Ser306					
Ser321			Ser279	Base		
Gln273					Gln212	

To check the similarity of the *L. donovani* ADSL sequence to adenylo succinate lyase sequences from other organisms, a phylogenetic analysis for the ADSL protein has been carried out with 19 ADSL protein sequences from known organisms. It has been observed that some of the sequences tend to be aligned more compared to the other sequences. Compared to the ADSL protein sequence of *Homo sapiens*, *Leishmania donovani* is showing an identity of 26 % which reveals that the both the proteins are less similar to each other. Several conserved regions are observed in the aligned residues. Literature studies reveal a signature region "GSSAMPHKVNPIDFEN" that is seemed to be conserved within the fumarase family which has been observed to be present in the ADSL too in region 320-335. Another motif observed in the multiple sequence alignment is "RTHGQPASPTN" lying in the region of 194-204. Both motif 1 and motif 2 lays within domain 1 region.

Fig1: Conserved regions: Motif 1 and Motif 2

The presence of the acid and the basic residues in *L. donovani* ADSL sequence has been analysed through a ligplot analysis of the active site from ChainA. It showed the presence of both acidic and basic amino acids from different chains at a closer proximity of the N1, or N6 atoms. The basic amino acids involved in Hydrogen bond formation are: His196, Arg361, Arg40, and Arg370, while the acidic amino acids involved are Gln273, Asp119. Targeting these residues might prove to be important in stopping the catalysis property of the enzyme.

2. Structural Analysis of ADSL

The crystal structure of the *L. donovani* ADSL protein (PDB id : 4MX2) showed that the protein is a homo-tetramer bearing four active sites. Pfam analysis revealed that there are two domains: domain 1 (39-348), domain 2 (357-473) in the ADSL protein belonging to the Lyase 1 and ASL C family. Part of both of these domains contributes towards the active site of the proteins. The active sites are formed through residues belonging to different chains. Active site on chain A is contributed from motif 1of chain B and His 196 belonging to the second motif from chain E. similarly active sites on chain B, E and F is contributed from motif 1 of chain A, F, E and His 196 from F, A, and B.

Residues involved in maintaining the tetramer structure (chain A, B, E, F) has been analysed through the PDBsum interaction server. Similar pattern of interaction has been observed for chain A:E and B:F, A:F and B:E , A:B and E:F. The pairs of chains that are facing each other A: E and B: F are seen to interact with each other through the formation of two salt bridges. Two residues involved in the salt bridge formation are Arg 215 and Asp 278 forming interaction with Asp 278 and Arg 215 respectively. Along with these some type of hydrogen bonding and other non-bonded contacts are also observed. Chains that are diagonally oriented to each other, A:F and B:E showed a similar pattern of interaction with the formation of 4

hydrogen bonds in both the cases. The interacting residues are similar in both the pairs. For chains lying side by side A:B and E:F, showed the same number of hydrogen bonding.

Table 2: The number of non-bonded contacts and hydrogen bonds

Chains	Salt Bridges	No of Hydrogen Bonds	No of Non-bonded contacts
A:E (facing)	2	54	518
B:F (facing)	2	56	543
A:F (diagonal)	-	4	91
B:E (diagonal)	-	4	95
A:B (side)	-	36	379
E:F (side)	-	36	395

3. Identification of important residues for our selected target protein ADSL

Sequence alignment of the 18 retrieved ADSL sequences from the listed organisms has shown conserved regions in the enzyme. The signature sequence "SSxxPxKxNxxxxE" (x representing any amino acid) of this protein in the range of 321 to 334 for *L. donovani*, belongs to a conserved structural region referred to as the C3 loop. This loop plays an active role in the catalysis process of the enzyme thereby closing the active site and bring ligand molecule near to catalytic residuesA set of residues based on literature search, sequence and structural analysis has been identified which play an important role in maintaining the structure as well as function of the protein.

Two sets of double mutant's have been selected to challenge the enzyme functionality. These selected residues have been used in pair for constructing a set of double mutants - (**N335Y** and **T367R**) and (**S321C** and **E334A**). The effects of these mutations on the stability of the protein structures were also checked through the mutational analysis servers DUET and Predict. The predications from these servers are destabilizing and deleterious for the selected mutations.

4Molecular Dynamics simulations

a) Structure preparation:

- The mutant structures for the selected residues were generated using Pymol.

b) Ligand Preparation:

- Coordinates for the molecule AMP were retrieved from the native crystal structure of L. *donovani* ADSL (PDB id: 4MX2).
- Known AMP topology ($C_{10}H_{14}N_5O_7P$) with -2 charges has been considered.
- Two AMP molecules were docked into two active site pockets of the receptor (native and mutants). The pose of the ligand similar to the native conformation has been chosen and the docked complex selected out for further analysis.
- Force-field parameters for the AMP molecule have been generated using the Automated Topology Builder Server (ATB) which supports the gromos54a7 force field. The partial charges of atoms were modified according to their chemical environment such that the overall charge of the molecules pertains to -2.

c) Molecular Dynamics (Wild Type + AMP, 2 mutants with and without AMP):

- All atom molecular dynamics simulation was carried out for 100ns using the gromos54a7 force field in GROMACS-2016.4 package.
- Simulation for the native ADSL protein was considered as control and used to compare with the simulations of mutated structures

d) Trajectory Analysis: RMSD, RMSF, PCA, MMPBSA, Catalytic residue Analysis, Domain Analysis.

Results:

A total of six molecular dynamics simulations (each of 100ns) of the native, mutant I and II structures both in the presence and absence of the ligand AMP has been carried out. The initial analysis of the MD trajectories (RMSD and RMSF) shows mutants behave differently from the native tetramer. The overall fluctuations in the proteins and protein-ligand complexes have been detected by performing a PCA analysis over the MD trajectories. It has been observed that the mutant structures (both in presence and absence of AMP) have greater amount of fluctuations compared to the native one. Moreover their trend of orientation differs from the control structure.

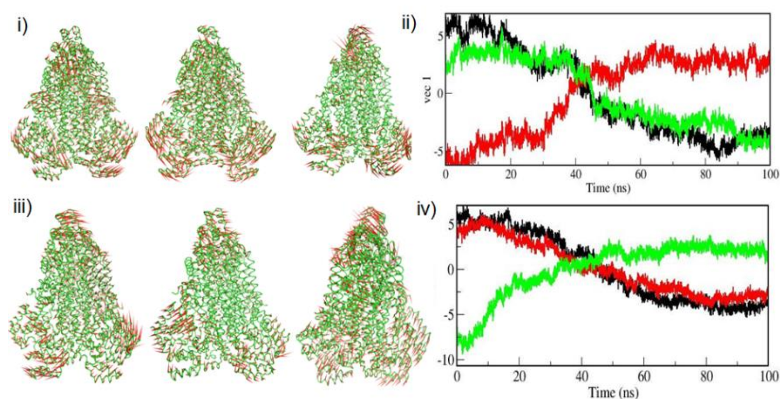

Fig 2:i) and iii) Porcupine plots for the native and the mutant structures in absence and presence of AMP. ii) and iv) represents the projection plot for the top eigen vector

MMPBSA calculations have been used to reveal the comparative binding of AMP with native and mutants. Energy components are able to justify the selection of these amino acids to affect the catalytic activity of the enzyme. Further the visualization of the binding cavity shows that the orientation of catalytic residues (His118 and His196) has changed during the course of simulation with respect to the ligand molecule. An important H-bond and π-π interaction formed by these residues with AMP are not able to retain during the simulation time. All these factors have brought down the interaction strength in the mutant structures leading towards decrement in the enzyme function comparative to native.

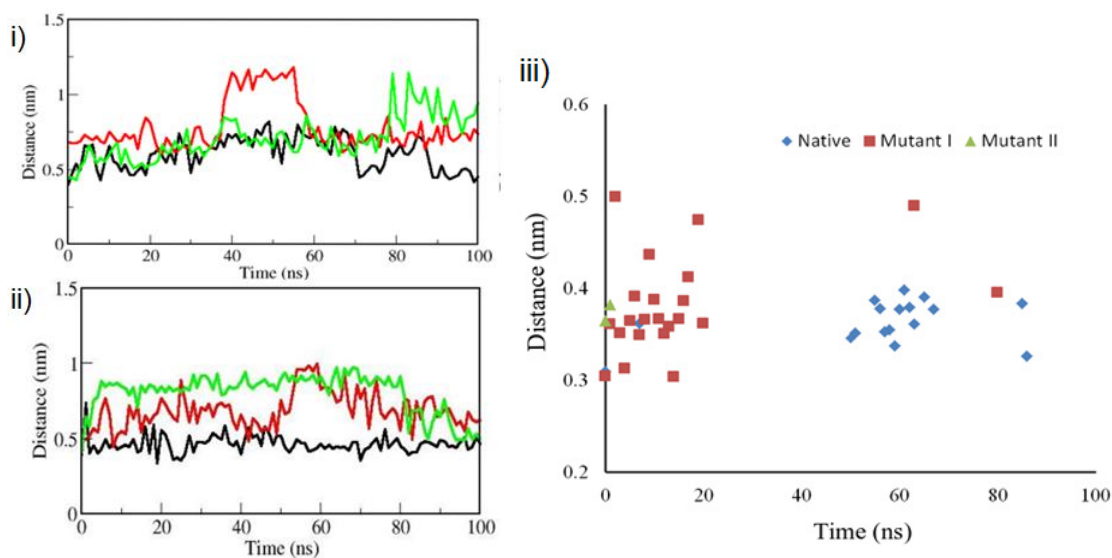

Fig 3: i) and ii): π-π interactionbetweenHis118 and AMP molecule for active site pocket 1 and 2, of the three simulations. **iii)** Presence of hydrogen bond between NE2 atom of the His196 and the N3 atom of the AMP in active site pocket for the three complexes

The conserved C3 loop is a part of active site whose closed conformation has been reported to be of catalytic importance. During the course of simulation, the movement in the loop structure (rmsf analysis and confirmed by DynDom server) domain is able to open the active site surface in the mutants and subsequently the ligand molecule fluctuates within the active site but not able to attain a stable conformation.

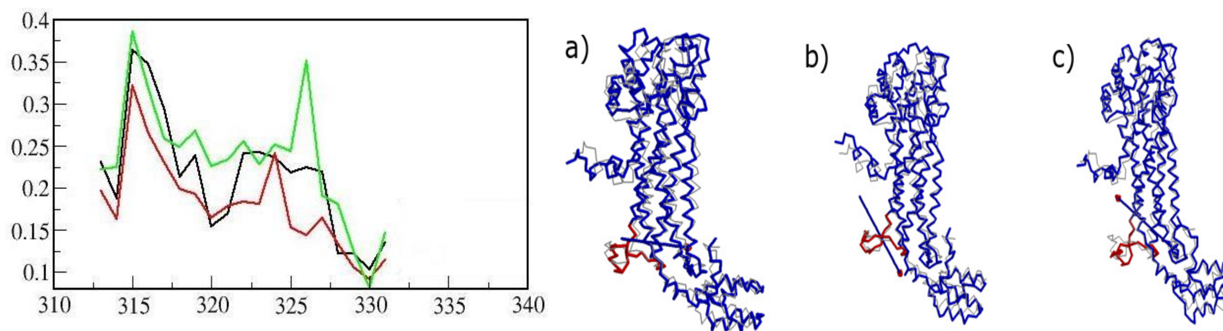

Fig 4: RMSF analysis and loop (shown in red) movement direction (arrow) for active site of a) native b) Mutant I and c) II of the C3 loop region for the three complexes

These comparative analyses are collectively displaying changes in the mutant structures induced through the *in-silico* mutations and among the two mutants the second one is showing slightly better performance towards removing the pathogenicity.

Work carried out at Central University of Gujarat, Gandhinagar–

Cloning of LdADSL gene and recombinant protein expression:

Leishmania donovani genomic DNA was used as the primary source for amplification of LdADSL gene. It was amplified by using Prime Star DNA polymerase and set of following specific primer at both 5' and 3' site. Forward primer: 5'-GCGGGATCATGTCCTGCCTTCGCAGAA -3' and Reverse Primer:5'-GCGAAGCTTTCAGGGATGCGCCGTGTAGCC-3'.The reaction condition for PCR amplification were as follows: 3 min initial denature at 95^{0}C followed by 35 cycles 1) denaturation at 95^{0}C for 1 min, 2) annealing at 60^{0}C for 30s 3) elongation at 72^{0}C for 1 min 30s and final elongation at 72^{0}C for 10 min (Fig 1). The LdADSL gene fragment (~1.4 kb) obtained by PCR was purified from 0.8% agarose gel, after that PCR product and pET28a plasmid digested with restriction enzymes *BamHI* & *HindIII*. The digested product was ligated into above

mentioned restriction site in pET28a expression vector. The resulting plasmid pET28a-ADSL, was transformed into DH5α *E. coli* strain for plasmid multiplication. After plasmid purification from bacterial cells, the positive clone of LdADSL gene was confirmed by plasmid shift assay, double digestion with same restriction enzymes and finally confirmed by DNA sequence analysis.

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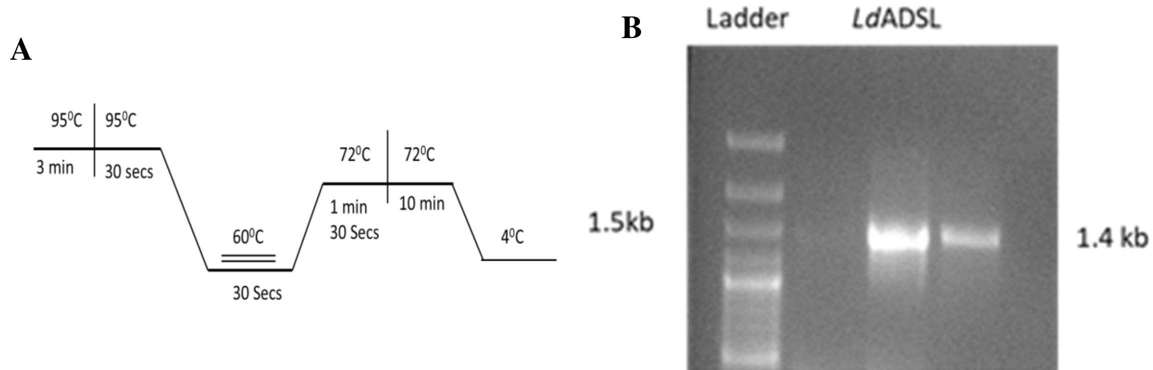


Fig 5: (A) PCR reaction step for amplification of gene. (B) PCR amplification LdADSL gene-lane 1containing 1kb DNA Ladder and Lane 2 containing amplified LdADSL gene

Confirmation of clone by Plasmid shift assay:

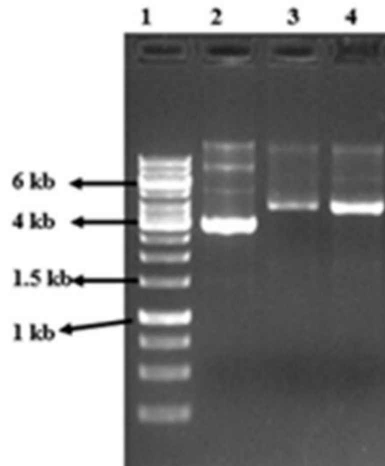


Fig 6: Plasmid shift assay. Lane 1-contaning 1kb DNA ladder, lane 2 containing Intact plasmid pET28a, Lane 3-4 pET28a-ADSL cloned plasmid, respectively.

Confirmation of clones by restriction digestion- The clones which showed shift in plasmid shift assay were further selected for confirmation by restriction digestion using enzymes *BamHI* and *HindIII.*

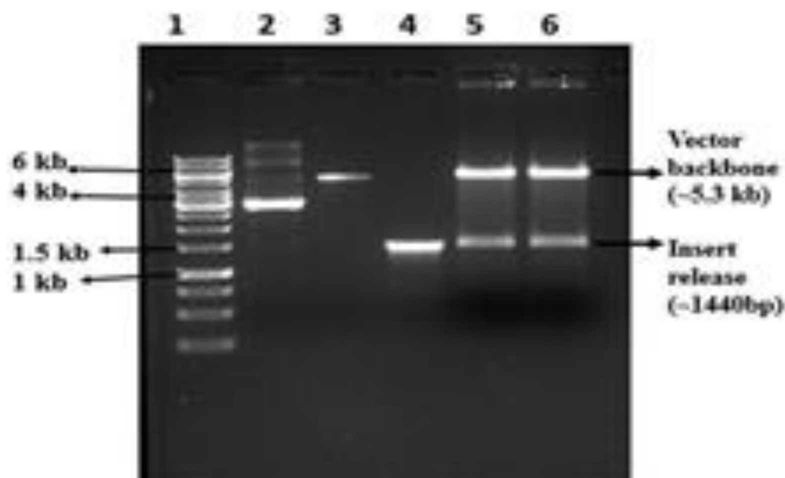


Fig 7: Clone conformed by restriction digestion: Lane 1-contaning 1kb DNA ladder, Lane 2 containing Intact plasmid pET28a, Lane 3 pET28a-ADSL cloned plasmid Lane 4 PCR amplified ADSL gene, Lane 5-6 double digested pET28a and ADSL gene, respectively

Recombinant LdADSL protein expression and purification:

Recombinant proteins were expressed in Rosetta strains of *E. coli* and expressed protein was purified by Ni-NTA affinity chromatography (Figures 1 & 2). LdADSL protein purification standardization is done. For the purification of LdADSL the cells after induction were lysed in buffer containing 50mM Tris HCL, pH 7.5, 300mM KCL, 5% glycerol, 1mM $MgCl_2$, 5mM BME and 1X Protein inhibitor. The soluble protein fraction was separated from cells debris by centrifugation and was loaded on a Ni-NTA column which had been previously equilibrate with buffer containing 50mM Tris HCL, pH 7.5, 300mM KCL and 10mM Imidazole then column was wash with 30mM Imidazole with same buffer and protein were eluted with 250mM Imidazole. After the elution proteins were dialyzed against 25mM Tris HCL pH 7.5, 150mM KCL, and then proteins were concentrated by using AMICON ultra-10. The entire purification process was carried out at 4^0C. The purity of protein was assessed using 12% SDS-PAGE.

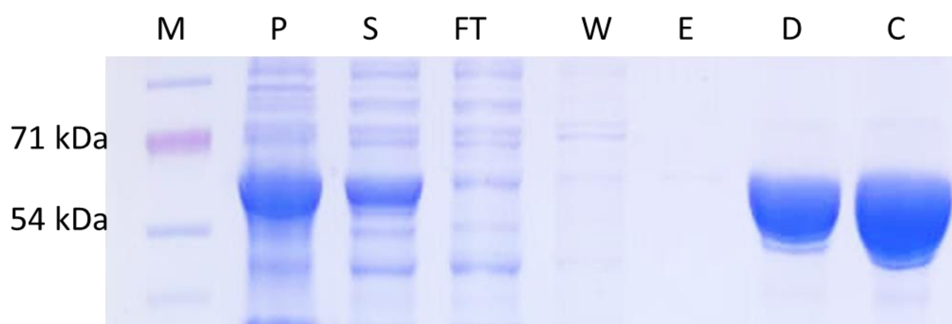


Fig 8: Recombinant protein expression and purification. Lane M protein marker, lane P pellet, lane S soluble fraction, lane FT flow through of Ni column, lane W Ni wash, lane E Ni-elution, lane after dialysis and lane C after concentrating.

Generation of mutants:

The following mutations were planned based on the structural analysis. The primers were designed such as to include the mutation in the gene.

Rational design of mutation in LdADSL & ADSS protein:

LdADSL: ADSL is a homotetramer from all species examined (Figure 1, A); In case of *Leishmania donovani* ADSL, each subunit contains 479 amino acids and has a molecular mass of approximately 54kDa. The enzyme has four active sites (Fig 5, B), and three subunits A, B and E contribute amino acids to each active site. We have designed 5 mutations (R40A, R40E, Y41A, E334A and N335Y) based on structural analysis which was done using CCP4 computer program. These amino acid residues are located at different region of enzymes. The R40A, R40E mutations were located in A/B subunit interface and also bind to AMP ligand. The R40 of subunit A make salt bridge with Glu54 & Glu113 of subunit B as well as make hydrogen bond with O1P of AMP ligand. The mutation of Y41A and N335Y are located in subunit A, has been strongly interacting with the ligand AMP through hydrogen bond and also involved in the inter-subunit non-bonded interactions withThr367 of subunit B. These interactions hold the tetramer structure and form the active site region necessary for the catalysis process. The E334A is located in subunit B which bind to His196 of subunit E, E334 being present in the active site and important for the catalysis of Adenylo succinate.

Mutation residue	Interacting residue
R40A, R40E (subunit A)	Glu54 & Glu113 (Subunit B) & AMP ligand
Y41A & N335Y (subunit A)	Thr367 (subunit B) & AMP
E33A (subunit B)	His196 (subunit E)

Table 2: Interactions of residues to be mutated

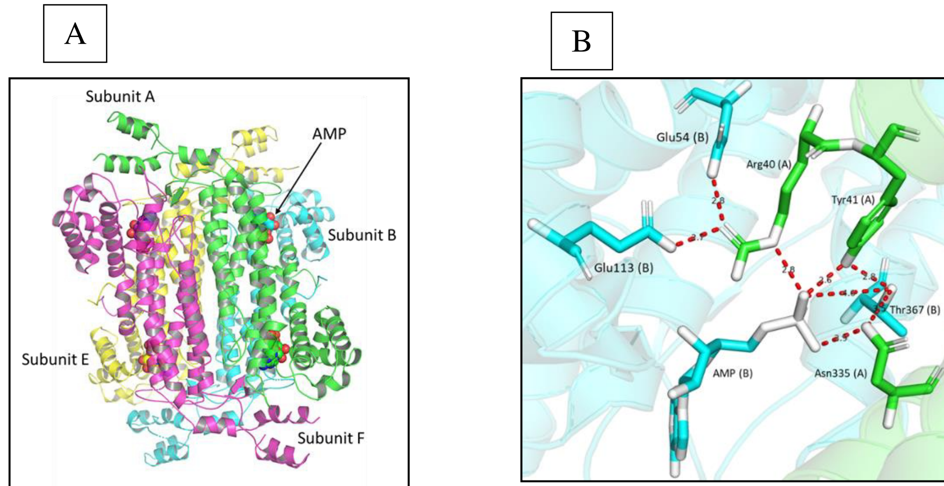


Fig 9: Crystal structure of LdADSL (4mx2): (A) tetramer of LdADSL (B) environment of AMP binding site and interacting residues (cyan colour subunit B) and (green colour subunit A).

Table3: Primers designed for LdADSL gene mutations

Mutation	Change code	Primer
R40A	CGC to GCC	FP: 5’CTCCCCTCGATGGTGCCTACAAGCGTGACACG 3'
		RP: 5' CGTGTCACGCTTGTGGCCACCATCGAGGGGAG 3'
R40E	CGC to GAG	FP: 5' CTCCCCTCGATGGTGAGTACAAGCGTGACACG 3'
		RP: 5' CGTGTCACGCTTGTACTCACCATCGAGGGGAG 3'
Y41A	TAC to GCC	FP: 5' CCCCTCGATGGTCGCGCCAAGCGTGACACGACC 3’
		RP: 5' GGTCGTGTCACGCTTGGCGCGACCATCGAGGGG 3'
E334A	GAG to GCA	FP: 5' GAACCCGATCGACTTCGCAAACGCTGAGGGCAACC 3’
		RP: 5' GGTTGCCCTCAGCGTTTGCGAAGTCGATCGGGTTC 3'
N335Y	AAC to TAC	FP: 5' CCGATCGACTTCGAGTACGCTGAGGGCAACCTG 3’
		RP: 5' CAGGTTGCCCTCAGCGTACTCGAAGTCGATCGG 3'

Site Directed mutagenesis:

Mutations were introduced in LdADSL& ADSS gene by PCR using the original LdADSL& ADSS clone as a template which was generated in pET-28a vector. The all primer purchased from Europhines Pvt. Ltd. All the primers for point mutants and the PCR protocol is listed in Table 2 & 3 respectively.

The PCR reaction mixture consisted of following protocol:

Reaction Mixture	Negative control (ul)	Experiment (ul)
Prime Star Max PCR MM	25	25
Mutant Forward primer (0.5µM)	-	1.25
Mutant Reverse primer (0.5µM)	-	1.25
Template (175ng/µl)	2.5	2.5
Water	22.5	20
Total Volume	50 µl	50 µl

Temperature	Time	Cycles
Initial denaturation 95^{0}C	3 min	1
Denaturation 95^{0}C	20 sec	35
Annealing 55^{0}C	20 sec	
Extension 72^{0}C	4 min	
Final extension 72^{0}C	10 min	1

Table 4: Reaction mix and protocol for PCR amplification

After the PCR run, the product was subjected to DpnI digestion to remove the parental plasmid, so as to select the mutation contain plasmid.

Table 5: Protocol for DpnI digestion:

Master Mix	Volume
Buffer (1X)	4 µl
PCR product	40 µl
DpnI (10U)	1 µl
Total volume	45 µl

This reaction mixture was incubated at 37^{0}C for 3-4 hours, followed by heat inactivation of DpnI at 80^{0}C for 20 min.

Transformation into competent DH5-α *E. Coli* strain:

2ul of DpnI digested product was mixed with 50ul of competent cells and placed into ice for 5min. This was followed by a heat shock at 42^{0}C for 45 sec and then kept in ice for 10min. 1ml of LB media was added to this and the cells were incubated at 37^{0}C, 200rpm for 2 hours for growth. After that, the cells were pelleted by spinning at 6000 rpm for 2min. 950ul of supernatant was discarded and 50ul of media was mixed with the pellet and dissolved. The transformed DH5α cells were grown on LB-agar plate containing kanamycin as a selection marker and plates were incubated for overnight at 37ºC. Positive growth was observed in plate contains colonies and in the control plate, growth was not observed.

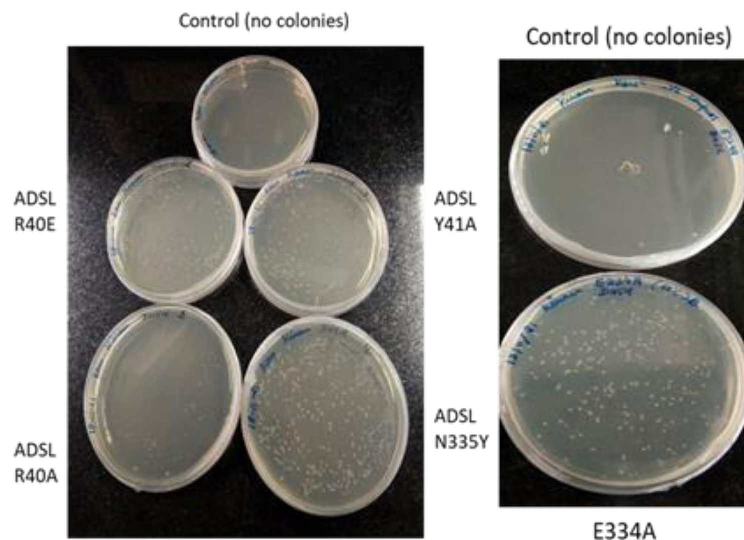


Fig 10: Transformation of *Ld*ADSL& ADSS mutant plasmid into competent DH5-α cells: plate containing no growth as control and plates containing colonies as positive plates for potential mutants.

Plasmid isolation by using Gene JET plasmid isolation kit (Thermo Fisher, K0502):

For plasmid isolation, 3 colonies were picked from respective mutant plates and grown in 10ml LB media containing kanamycin antibiotic and incubated at 37^{0}C for overnight. Next day, cells were harvested by spinning at 8000 rpm at 4^{0}C for 10min at 8000rpm. The supernatant was discarded, and the pellet was further processed for plasmid isolation as per manufacturer protocol. After that the isolated plasmid was visualized on 0.8% agarose gel.

Fig11:-

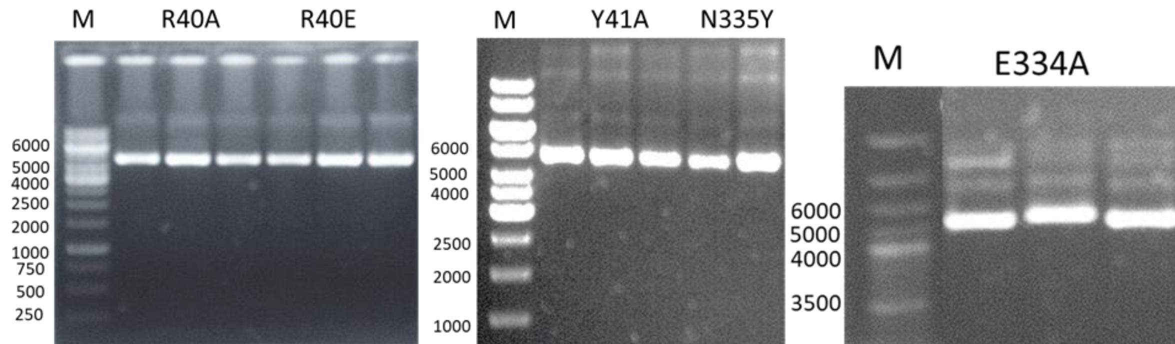


Fig 11: 0.8% agarose gel electrophoresis of LdADSL mutant plasmids: Lane M: 1kb ladder& positive plasmid containing mutation.

Sequencing results:

After the isolation and checking of putative mutant plasmids on agarose gel, 3 plasmids were sent for sequencing to confirm the introduction of point mutations in the gene sequence. The final sequencing result of suspected mutant was obtained and compared with original *Ld*ADSL& ADSS gene sequence by using Clustal omega sequence alignment online tools. The following are the sequencing results:

Fig 12: Sequencing result of LdADSL. The sequence obtained from the sequencing result (Seq.) was compared with the LdADSL gene sequence using the global alignment tool - Clustal omega

Biophysical characterization of the proteins and mutants using techniques like CD spectroscopy, Fluorescence spectroscopy, Gel permeation chromatography, etc

Fluorescence Spectroscopy of Wild type and mutant R40A & R40E LdADSL:

Fluorescence spectra were monitored at a protein concentration range from 1μM to 5μM in 25mM Tris–HCl, pH 7.5 using SHIMADZU RF-6000 spectrometer. The reaction mixture solutions were excited at 280 nm, which is specific for tyrosine residues, and the emission spectra were scanned and recorded in the range of 290-450 nm. Excitation and emission bandwidths were set to 1.0 nm and 1.5 nm, respectively. Baseline corrections were done by subtraction of the buffer spectrum.

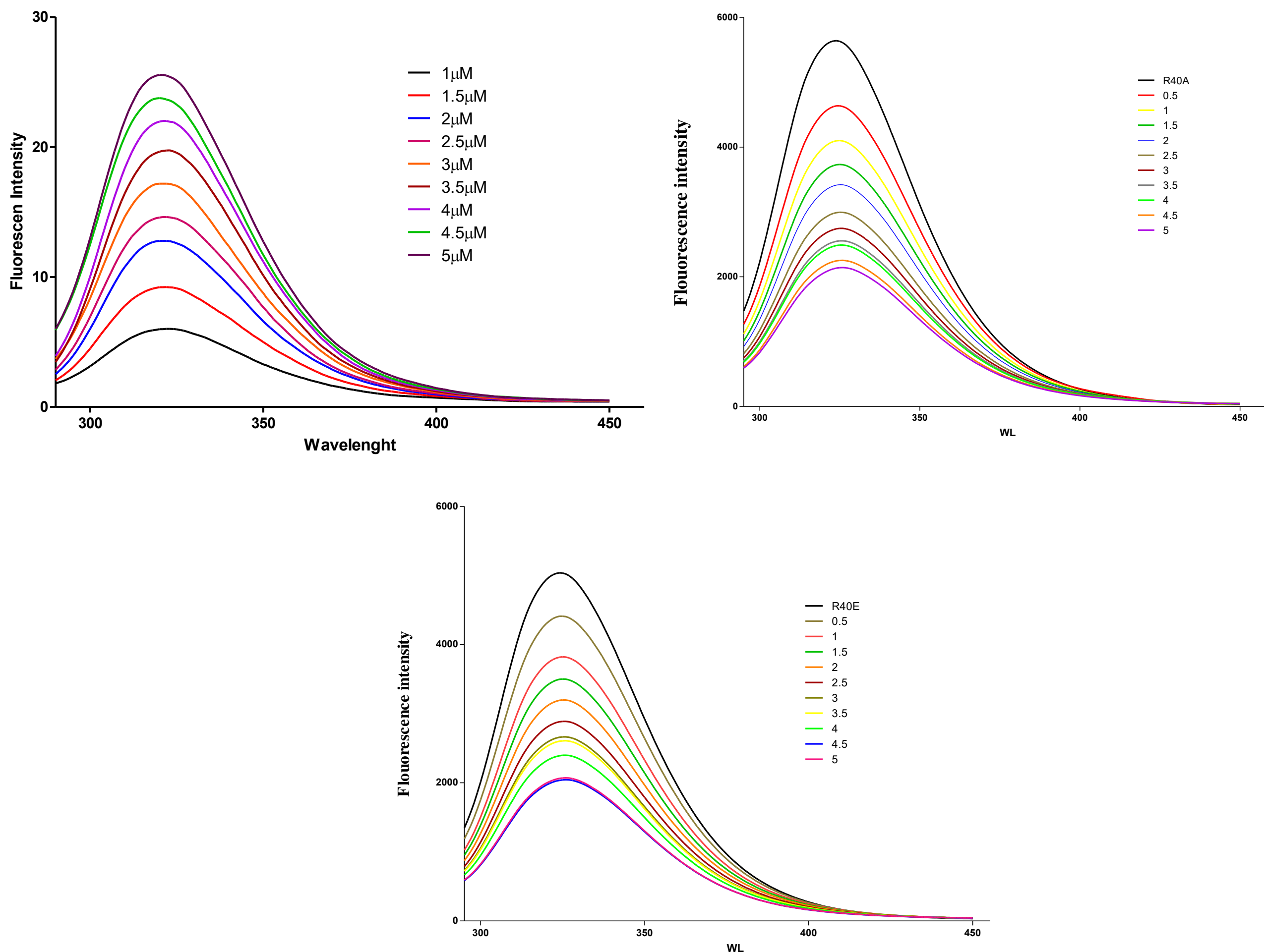
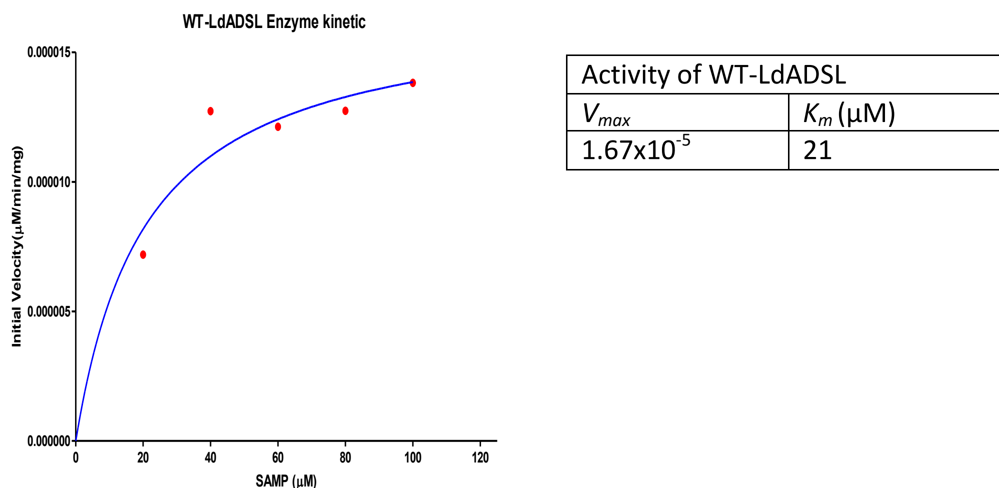


Fig 13: Fluorescent spectroscopy of LdADSL and mutants at different concentrations

Activity studies of native and mutant proteins using enzyme kinetic assays:

Enzyme assay of WT-LdADSL:

Protein concentration was measured by standard Bradford method, using BSA standard curve. For LdADSL, the enzymatic activity was performed in 25mM Tris-HCl buffer pH 7.5 at room temperature using Evolution 201/220 UV/Visible spectrophotometer (Thermo Fisher). The reaction mixture contains different concentration of SAMP that range from 20µM to 100µM in above mentioned buffer, OD reading was taken at 282nm after adding 100ug/ml LdADSL enzyme to mixture and incubating for 5min.The kinetic parameter as K_m, V_{max} value for SAMP was calculated by using extinction coefficient 10000 M^{-1} $cm^{-1}$for the amount of the substrate decreased. Activity expressed as One unit is defines as amount of micromole of SAMP decreased per min. The reaction volume was 750µl. All the kinetic data analyzed with computer program Graph Pad prism v5.0.



Activity of WT-LdADSL	
V_{max}	K_m (µM)
$1.67x10^{-5}$	21

Fig 14: Enzyme kinetic of WT-LdADSL: The initial velocity for SAMP converts AMP and fumarate. The substrate saturation curve follows Michalis-Menten hyperbolic graph.

CD spectroscopy of native and mutant LdADSL:

A Jasco J-815spectropolarimeter was used to carry out circular dichroism experiments. Each enzyme sample (1µM), in 25mMTris-HCL pH 7.5 was run in triplicate and averaged. Ellipticity as a function of wavelength was measured from 250 to 190 nm for each sample, in 0.1 nm increments. Individual samples were scanned 3 times and averaged, and the background buffer spectrum was subtracted out. The graph was plotted against mean molar ellipticity vs Wavelength using Graph Pad Prism v5.0.

Protein (LdADSL)	Estimated Secondary structure content (%)		
	Helix	Antiparallel	Parallel
WT	68.2	31.8	0.0
R40A	61.7	34.2	4.1
R40E	66.5	32.2	1.3

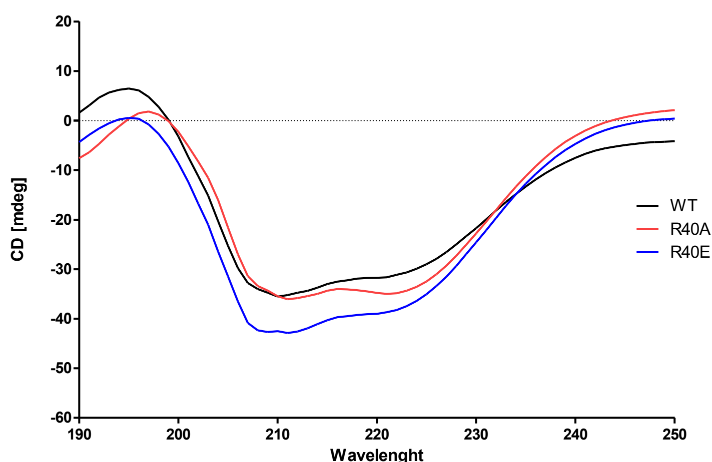


Fig 15: Circular dichroism spectra of the wild-type and R40A & R40E mutant enzymes. Each spectrum has been corrected for the background contributed by buffer and is normalized to the same enzyme concentration. Protein samples: wild-type black colour, R40A red colour and R40E blue colour.

References

1) Toth and Yeates, (2000). The structure of adenylo succinate lyase, an enzyme with dual activity in the de novo purine biosynthetic pathway. *Structure*, 8(2), 163-174
2) Jennifer L. *et al*. (2002). Three Subunits Contribute Amino Acids to the Active Site of Tetrameric Adenylo succinate Lyase: Lys268 and Glu275 Are Required. *Biochemistry*, 41, 2217-2226.
3) Banerjee *et al*, (2014). Structural and kinetic studies on adenylo succinate lyase from Mycobacterium smegmatis and Mycobacterium tuberculosis provide new insights on the catalytic residues of the enzyme. *FEBS Journal,* 281, 1642–1658.
4) Segall *et al*. (2007). Important roles of hydroxylic amino acid residues in the function of Bacillus subtilis adenylo succinate lyase. *Protein Science*, 16, 441–448.
5) Jennifer L. *et al*. (2000).A Key Role in Catalysis for His89 of Adenylo succinate Lyase of Bacillus subtilis. *Biochemistry*,39, 13336-13343.
6) Mehrotra and Balaram, (2007). Kinetic Characterization of Adenylo succinate Synthetase from the Thermophilic Archaea Methanocaldococcus jannaschii. *Biochemistry* 46, 12821-12832.
7) Jennifer L. *et al*. (2000). A Key Role in Catalysis for His89 of Adenylo succinate Lyase of Bacillus subtilis. *Biochemistry*,39, 13336-13343.
8) Sabrina A. *et al*. (2006). Proposal for a Hydrogen Bond Network in the Active Site of the Prototypic ç-Class Carbonic Anhydrase. *Biochemistry,* 45, 5149-5157.
9) Thompson *et al.* (1994). CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*, 22 (22): 4673-4680.
10) Boitz *et al*, (2012). Purine Salvage in Leishmania: Complex or simple by design. *Trends in Parasitology*, 28(8), 345-352.
11) Bulusu *et al*. (2009). Elucidation of the substrate specificity, kinetic and catalytic mechanism of adenylo succinate lyase from Plasmodium falciparum. *Biochimica et Biophysica Acta,* 1794, 642–654.
12)

FINALCONSOLIDATED STATEMENT OF EXPENDITURE
(FOR FINAL SETTLEMENT OF ACCOUNTS)

1. Title of the Project : **Biophysical Characterization of adenylosuccinate lyase- a potential drug target from Leishmania donovani – a computational and molecular approach**
2. Sanctioned Project Cost : 41.80 lakhs
3. Revised cost, if any : NA
4. Duration of the project : 3.5 years
5. Sanction Order No. & Date : BT/PR15847/NER/95/21/2015
6. Date of commencement of Project : 13 Feb 2017
7. Extension, if any : 06 months
8. Date of completion of project :12 Aug 2020

Details of grant, expenditure and balance

S. No.	Heads	Sanctioned Cost	Year-wise Releases made					Year-wise Expenditure incurred						
			1st yr.	2nd yr.	3rd yr.	4th yr.	Total	1st yr.	2nd yr.	3rd yr.	4th yr.	5th yr.	Total	Balance
			13 Feb 2017- 31 Mar 2017	01 Apr 2017- 31 Mar 2018	01 Apr 2018 - 31 Mar 2019	01 Apr 2019- 12 Aug 2020		13 Feb 2017- 31 Mar 2017	01 Apr 2017- 31 Mar 2018	01 Apr 2018 - 31 Mar 2019	01 Apr 2019- 31 Mar 2020	01 Apr 2020- 12 Aug 2020		
	A. Non-recurring page no.-													
	Equipment	19.50	19.50	0.00	0.00	0.00	19.50	0.00	19.50	0.00	0.00	0.00	19.50	0.00
	B. Recurring													
1.	Manpower	10.30	3.30	0.00	2.89	00.00	6.19	00.00	2.89286	3.00	0.08275	0.05806	6.03367	0.15633
2.	Consumables	5.00	1.50	0.00	1.45	00.00	2.95	00.00	1.44658	0.16705	1.32623	0.00	2.93986	0.01014
3.	Travel	1.50	0.50	0.00	0.50	00.00	1.00	00.00	0.61341	0.50	0.00	0.00	1.11341	-0.11341
4.	Contingency	1.50	0.50	0.00	0.50	00.00	1.00	00.00	0.50	0.13657	0.25428	0.094	0.98485	0.01515
5.	Overhead	4.00	2.00	0.00	0.99	00.00	2.99	00.00	2.00	0.62500	0.37500	00.00	3.00	0.01000
6.	Interest earned	0.00	0.01122	0.01075	0.06812	0.00901	0.09910	00.00	0.00	0.00	0.00	00.00	00.00	0.09910
	Total	22.30	7.81122	0.01075	6.39812	0.00901	14.22910	00.00	7.45285	4.42862	2.03826	0.15206	14.07179	0.15731
	Grand Total (A+B)	**41.80**	**27.31122**	**0.01075**	**6.39812**	**0.00901**	**33.72910**	00.00	**26.95285**	**4.42862**	**2.03826**	**0.15206**	**33.57179**	**0.15731**

(PROJECT INVESTIGATOR)
Dr. Anupam Nath Jha
Assistant Professor
Dept of Molecular Biology & Biotechnology
Tezpur University, Tezpur - 784028

(HEAD OF THE INSTITUTE)
Registrar
Tezpur University

(FINANCE OFFICER)
Finance Officer
Tezpur University

Appendix-A

Details of Assets acquired wholly or substantially out of Govt. grants Register to be maintained by Grantee Institution

1. Name of the Sanctioning Authority: Department of Biotechnology
2. Name of the Grantee Institution: Tezpur University
3. No. & Date of sanction order: BT/PR15847/NER/95/21/2015 dated February 13, 2017
4. Amount of the sanctioned grant: 27.30 Lakhs
5. Brief purpose of the grant: Biophysical Characterization of adenylosuccinate lyase- a potential drug target from Leishmania donovani – a computational and molecular approach
6. Whether any condition regarding the right of ownership of Govt. in the Property or other assets acquired out of the grant was incorporated in the grant-in-aid sanction order. Yes

*7. Particulars of assets actually credited or acquired.

Sl. No.	Asset	Status	Value
1	GPU based parallel computing system	Installed	19.50 Lakhs

8. Value of the assets as on 31/03/2020: Rs. 19.50 Lakhs
9. Purpose for which utilised at present: Installation has been done to perform long timescale MD Simulation
10. Encumbered or not: No
11. Reasons, if encumbered: NA
12. Disposed of or not: NA
13. Reasons and authority, if any, for Disposal: NA
14. Amount realised on disposal: NIL
15. Remarks: NO

(PROJECT INVESTIGATOR)
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2/12/2020
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Appendix-B

Utilisation Certificate

(Period from 1st April, 2020 to 30th Nov, 2020)

(Rs. in Lakhs)

1. Title of the Project/Scheme: **Biophysical Characterization of adenylosuccinate lyase- a potential drug target from Leishmania donovani – a computational and molecular approach**

2.	Name of the Organisation:	Tezpur University, Napam, Tezpur, Assam
3.	Principal Investigator:	Dr Anupam Nath Jha
4.	Deptt. of Biotechnology sanction order No. & date of sanctioning the project:	BT/PR15847/NER/95/21/2015 dated February 13, 2017
5.	Amount brought forward from the previous financial year quoting DBT letter No. & date in which the authority to carry forward the said amount was given:	0.30937 Lakhs
6.	Amount received from DBT during the financial year (*please give No. and dates of sanction orders showing the amounts paid)*:	Nil
7.	Other receipts/interest earned, if any, on the DBT grants:	Nil
8.	Total amount that was available for expenditure during the financial year (Sl. Nos. 5,6 and 7):	0.30937 Lakhs
9.	Actual expenditure (excluding commitments) incurred during the financial year (statement of expenditure is enclosed):	0.15206 Lakhs
10.	Unspent balance refunded, if any (Please give details of cheque No. etc.):	NA
11.	Balance amount available at the end of the financial year:	0.15731 Lakhs
12.	Amount allowed to be carried forward to the next financial year vide letter No. & date:	NA

1. Certified that the amount of Rs. **0.15206 Lakhs** mentioned against col. 9 has been utilised on the project/scheme for the purpose for which it was sanctioned and that the balance of Rs. **0.15731 Lakhs** will be adjusted towards the grants-in-aid payable during the next year.

2. Certified that I have satisfied myself that the conditions on which the grants-in-aid was sanctioned have been duly fulfilled/are being fulfilled and that I have exercised the following checks to see that the money was actually utilised for the purpose for which it was sanctioned.

Kinds of checks exercised:

1. Cash Book
2. Ledgers
3. Vouchers
4. Bank Statements
5. Any other

21/12/2020

(PROJECT INVESTIGATOR)

Dr.Anupam Nath Jha
Assistant Professor
Dept of Molecular Biology & Biotechnology
Tezpur University, Tezpur - 784028

(FINANCE OFFICER)

Finance Officer
Tezpur University

(HEAD OF THE INSTITUTE)

Registrar
Tezpur University

(To be countersigned by the DBT Officer-in-charge)

"Biophysical Characterization of adenylosuccinate lyase- a potential drug target from Leishmania donovani – a computational and molecular approach"

Annexure-II

Statement of Expenditure referred to in Para 9 of the Utilization Certificate

Showing grant received from the Department on Biotechnology and the expenditure incurred during the financial year 2020-2021

(Period from 1st April, 2020 to 30th Nov, 2020)

Rs. In Lakh

Items	Unspent balance carried forward from previous year	Grants received from the DBT during the year	Other receipts/interest earned if any, on the DBT grants	Total of col. (2+3+4)	Expenditure (Excluding commitments incurred during the year)	Balance (5-6)	Remarks
1	2	3	4	5	6	7	8
I. Non-Recurring (i) Equipments	0	NIL	NIL	0	0	0	
Sub Total (I)	0.00	NIL	NIL	0.00	0.00	0.00	
II. Recurring (i) Human Resource	0.21439	NIL	NIL	0.21439	0.05806	0.15633	
(ii) Consumables	0.01014	NIL	NIL	0.01014	0.00	0.01014	
(iii) Travel	-0.11341	NIL	NIL	-0.11341	0.00	-0.11341	
(iv) Contingency	0.10915	NIL	NIL	0.10915	0.094	0.01515	
(v) Overhead	0.00	NIL	NIL	0.00	0.00	0.00	
(vi) Interest Earned	0.08910	NIL	NIL	0.08910	0.00	0.08910	
Sub Total (II)	**0.30937**	**NIL**	**NIL**	**0.30937**	0.15206	**0.15731**	
Grand Total (I+II)	**0.30937**	**NIL**	**NIL**	**0.30937**	0.15206	0.15731	

Balance amount: Fifteen Thousand Seven Hundred and Thirty One only

PROJECT INVESTIGATOR
Dr.Anupam Nath Jha
Assistant Professor
of Molecular Biology & Biotechnology
Tezpur University, Tezpur - 784028

2/12/2020
FINANCE OFFICER
Finance Officer
Tezpur University

HEAD OF THE INSTITUTE
Registrar
Tezpur University

Annexure A

Manpower Staffing Details (In the financial year wise manner)

For the financial year 2020-2021

From 01-04-2020 to 30-11-2020

NAME OF THE PERSON	NAME OF THE POST	DATE OF JOINING	DATE OF LEAVING	TOTAL MONTHLY SALARY	TOTAL SALARY PAID DURING THE FINANCIAL YEAR	TOTAL SALARY PAID DURING PROJECT PERIOD
Titus B	JRF	10-02-2020	15-03-2020	Rs 12,000.00	Rs 5,806.00	Rs 5,806.00

(Signature of Principal Investigator)

Dr.Anupam Nath Jha
Assistant Professor
...nt of Molecular Biology & Biotechnolog...
Tezpur University, Tezpur - 784028

(Signature of Accounts Officer)

Finance Officer
Tezpur University

(SIGNATURE OF HEAD OF THE INSTITUTE)

Registrar
Tezpur University

Annexure B

Manpower Expenditure Details (In financial year wise manner)*:

For the financial year 2020-2021

(From 01-04-2020 to 30-11-2020)

SANCTIONED POSTS	NUMBER	SCALE OF PAY	ANNUAL OUTLAY	OUTLAY FOR THE ENTIRE PERIOD	REVISED SCALE, IF ANY	REVISED ANNUAL OUTLAY	REVISED PROJECT OUTLAY	ACTUAL RELASES BY DBT	ACTUAL EXPENDITURE	BALANCE
JRF	1	Rs 12,000	0.21439 Lakhs	0.21439 Lakhs	---	---	---	--	0.05806 Lakhs	0.15633 Lakhs

(Signature of Principal Investigator)

Dr.Anupam Nath Jha
Assistant Professor
Dept. of Molecular Biology & Biotechnology
Tezpur University, Tezpur - 784028

(Signature of Accounts Officer)

02/12/2020

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Tezpur University

(SIGNATURE OF HEAD OF THE INSTITUTE)

Registrar
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* Details of manpower salary/ fellowship revision alongwith due- drawn statement and arrears requested should be given separately, if applicable.

Due- Drawn Statement

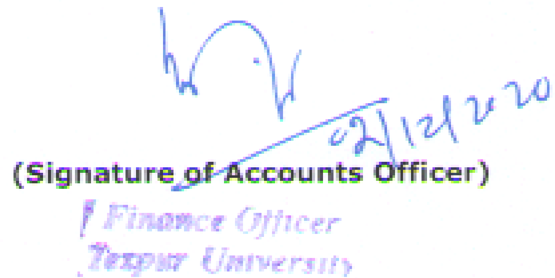
For the financial year 2020-2021

(From 01-04-2020 to 30-11-2020)

Name of the Project Staff	Month and Year	Due	Drawn	Difference
Titus B	NA	0.21439 lakhs	0.05806 Lakhs	0.15633 Lakhs

(Signature of Principal Investigator)

Dr.Anupam Nath Jha
Assistant Professor



(Signature of Accounts Officer)

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(SIGNATURE OF HEAD OF THE INSTITUTE)

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