

# PHYLOGENETIC ANALYSIS AND HOMOLOGY MODELLING OF LACCASE GENE OF FUSARIUM OXYSPORUM Lcc2.

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## ABSTRACT

Morphological identification of fungi is the first and the most difficult step in the identification process. This is especially true for *Fusarium* species. *Fusarium* has a cosmopolitan distribution. *Fusarium* sp was isolated from Anamalai Hills region and Subjected to Morphological identification using standard microbiological methods. Genotypic identification was carried out by PCR amplification and partial sequencing of the 16S rRNA for the confirmation of morphological identity. The rDNA sequence of 16S ribosomal RNA gene was amplified by PCR and the PCR product .The fungus, was bi directionally sequenced using forward and reverse primers. Using BLAST Tool the sequence was searched for the similarity matches .*F.oxysporum* shows maximum similarity. The Gene of the species was obtained from Uniprot. Since the unavailability of the 3D structure of Fusarium Laccase gene this attempt was carried out and Homology modeling was performed using Modeller Tool.

**Key Words:** Homology Modelling, Laccase, Lcc2 gene, *F.oxysporum*.

## 1.INTRODUCTION

Morphological identification of fungi is the first and the most difficult step in the identification process. This is especially true for *Fusarium* species. *Fusarium* has a cosmopolitan distribution. Although morphological observations may not suffice for complete identification, a great deal of information is usually obtained on the culture at this stage. Morphological and biochemical distinctiveness of fungi are universally used for their identification, but differentiation of closely related cultures require extensive molecular techniques (Shahriarnour *et al.*, 2011). Many researchers turn to molecular systematics as an alternative or a complementary tool to classical taxonomy.

Central to this is the decision over which molecular marker to use, that will give the closest interpretation of the conventional morphological data. The internal transcribed spacer (ITS) regions of the ribosomal DNA (rDNA) has become a more popular marker for systematics and phylogenetic studies of closely related species of animals, plants and fungi (Von der Schulenburg *et al.*, 2001).

PCR amplification with universal primers targeted to conserved regions within the rRNA complex and subsequent DNA sequencing of the internal transcribed spacer (ITS) regions, shows promise to identify a broad range of fungi to the species level (Chen *et al.*, 2001). The PCR primer sets routinely used for amplification of ITS regions and rDNA are known to be ITS1 and ITS4 (White *et al.*, 2001)

One of the most widely used methods for detecting homologous sequences is Blast. The Blast suite of programs is used to find local sequence similarities, which might lead to evolutionary clues about the structure and/or function of the query sequence. The detected sequences can then be used e.g. to build a multiple alignment of complete sequences (MACS), which represents an ideal workbench to study all the information related to a set of homologous sequences. (Anne Friedrich *et al.*, 2007)

To experimentally discover functionality of any protein, the information of its 3D structure remains an indispensable fact, which is achieved using techniques like X-Ray Crystallography or NMR spectroscopy. Experimental techniques are very tedious and prolonged and not always succeed in determining structure for all proteins especially membrane proteins (Johnson *et al.* 1994). Moreover, the rate at which protein sequence data is accumulating is far more than the structural information available, thus creating a gap between available sequences and experimentally solved structures. Computational methods like homology modeling can help reduce this gap. It is known that existing proteins are result of continuous evolution of previously existing ones, thus proteins can be grouped into families Homology modeling methods use the fact that evolutionary related proteins share a similar structure. Therefore, models of a protein with unknown structure (target) can be built based on an alignment of a protein of known structure (template). This typically involves four steps (Sánchez

and Sali 1997; Marti-Renom *et al.* 2000): (1) identification of homologs that can be used as template(s) for modeling; (2) alignment of the target sequence to the template(s); (3) building a model for the target based on the information from the alignment(s); and (4) evaluation of the model. Finally, all four steps can be repeated until a satisfactory model is obtained.

## II. MATERIALS AND METHODS

### 2.1 Genotypic Identification of Fungus

The fungus, which was observed to produce positive result in the screening test for laccase was subjected to genotypic identification. Genotypic identification was carried out by PCR amplification and partial sequencing of the 16S rRNA for the confirmation of morphological identity. The rDNA sequence of 16S ribosomal RNA gene was amplified by PCR and the PCR product. The fungus, was bidirectionally sequenced using forward (ITS1-5'-TCC GTA GGT GAA CCT GCG G-3') and reverse (ITS4-5'-TCC TCC GCT TAT TGA TAT GC-3') primers (Bakri *et al.*, 2010). Since, fungal ITS sequences generally provide greater taxonomic resolution than sequences generated from coding regions (Anderson *et al.*, 2003). Primers were synthesized and PCR amplifications with subsequent sequencing were carried out by Synergy Scientific Services, Chennai, India.

### 2.2 Softwares and Tools Used

#### BLAST

Sequences of unknown isolates were compared with sequences in the NCBI GenBank database using BLAST algorithm at the website <http://www.ncbi.nlm.nih.gov>, but due to the lack of quality control on the correct identification of sequences in the GenBank database (Geiser *et al.*, 2004), CBS Fungal Biodiversity Center (<http://www.cbs.knaw.nl/fusarium>) was also used along with NCBI.

#### Multialin

This software will allow to align simultaneously several biological sequences. It is the arrangement of several protein or nucleic acid sequences with postulated gaps so that similar residues are juxtaposed. A positive score is attached to identities, conservative or non-conservative substitutions (the score amplitude measuring the similarity) and a penalty to gaps; an ideal program would maximize the total score, taking account of all possible alignments and allowing for any length gap at any position. It is undeniably useful to have an automatic system available for multiple sequence alignment to provide a starting point for a more human analysis. Available at <http://multalin.toulouse.inra.fr/multalin/>.

#### MEGA

MEGA is an integrated tool for conducting automatic and manual sequence alignment, interfering phylogenetic trees, mining web based database, estimating rates of molecular evolution, interfering ancestral sequences, and testing evolutionary hypotheses. MEGA is a multithreaded windows application. It runs on all releases of Microsoft Windows operating system. Available at <http://www.megasoftware.net>.

#### SEQUIN 12.21

Sequin is a stand-alone software tool developed by the NCBI for submitting and updating entries to the GenBank sequence database. It is capable of handling simple submissions that contain a single short mRNA sequence, and complex submissions containing long sequences, multiple annotations, gapped sequences, or phylogenetic and population studies. A single Sequin file should contain less than 10,000 sequences for maximum performance. Available at <http://www.ncbi.nlm.nih.gov/Sequin/>

#### PROTEIN DATA BANK (PDB) and UniProt.

The Protein Data Bank (PDB; <http://www.pdb.org/>) continues to be actively involved in various aspects of the informatics of structural genomics projects developing and maintaining the Target Registration Database (TargetDB), organizing data dictionaries that will define the specification for the exchange and deposition of data with the structural genomics centers and creating software tools to capture data from standard structure determination applications. Three dimensional structural data has grown from 7 files in 1971 to a database containing over 83768 structures as of August 2012. The Universal Protein Resource (UniProt) is a comprehensive resource for protein sequence and annotation data. The UniProt databases are the UniProt Knowledgebase (UniProtKB), the UniProt Reference Clusters (UniRef), and the UniProt Archive (UniParc). The UniProt Metagenomic and Environmental Sequences (UniMES) database is a repository specifically developed for metagenomic and environmental data.

#### ClustalX

Multiple sequence alignment program. It provides an integrated environment for performing multiple sequence and profile alignments and analysing the results. <http://www.clustal.org/>

### Modeller

MODELLER is a computer program for comparative protein structure modeling. In the simplest case, the input is an alignment of a sequence to be modeled with the template structures, the atomic coordinates of the templates, and a simple script file. MODELLER then automatically calculates a model containing all non-hydrogen atoms.

Apart from model building, MODELLER can perform additional auxiliary tasks, including fold assignment, alignment of two protein sequences or their profiles, multiple alignment of protein sequences and/or structures, calculation of phylogenetic trees, and de novo modeling of loops in protein structures. <http://salilab.org/modeller/>

### Procheck

The *PROCHECK* suite of programs provides a detailed check on the stereochemistry of a protein structure. Its outputs comprise a number of plots in PostScript format and a comprehensive residue-by-residue listing. These give an assessment of the overall quality of the structure as compared with well refined structures of the same resolution and also highlight regions that may need further investigation. The *PROCHECK* programs are useful for assessing the quality not only of protein structures in the process of being solved but also of existing structures and of those being modelled on known structures. <http://www.ebi.ac.uk/thornton-srv/software/PROCHECK/>

## III. RESULT AND DISCUSSION

Microscopic observation revealed the taxonomic identity of the fungus, which was observed to produce laccase. The fungus strain has shown the production of conidia. In general the aerial mycelium first appears white and then changes to purple colour.

Their conidiophores, the means through which *F. oxysporum* asexually reproduce, are short, single, lateral monophialides (flask-shaped projections) in the aerial mycelium, later arranged to densely branched clusters. Their macroconidia are fusiform, slightly curved, pointed at the tip, mostly three septate, basal cells pedicellate, 23-54 x 3-4.5 µm. Microconidia are abundant, never in chains, mostly non-septate, ellipsoidal or cylindrical, straight or curved, 5-12 x 2.3-3.5 µm. Chlamydospores are terminal or intercalary, hyaline, smooth or rough-walled, 5-13 µm.

The above mentioned characteristics designated the fungus to be *Fusarium sp.* in taxonomy. The morphological identification of *Fusarium sp.* was further substantiated with molecular study. PCR amplification of ITS region yielded a fragment of 609 bp length from the isolate of *Fusarium sp.* The sequence of the isolate studied was compared with that of NCBI databases using BLAST network. The isolate was analyzed based on the similarity with the best-aligned sequence of the BLAST search (Singh *et al.*, 2006). Based on BLAST search of partial 18S rRNA gene sequence, partial 5.8S rRNA gene sequence and complete sequence of ITS region, the fungus was found to be closest homolog (83% identity) to *Fusarium oxysporum* and it was submitted to GenBank using Sequin and the accession number is JQ950134.

Similar sequences which is obtained from tBlast are copied along with the query and saved as "hits.txt". Multiple sequence alignment is carried out in multalin, output file is downloaded in Fasta format (alin results.txt), align results.txt is converted to mega format as "sequences.meg". The Phylogenetic tree is constructed using MEGA tool (Fig 1b)

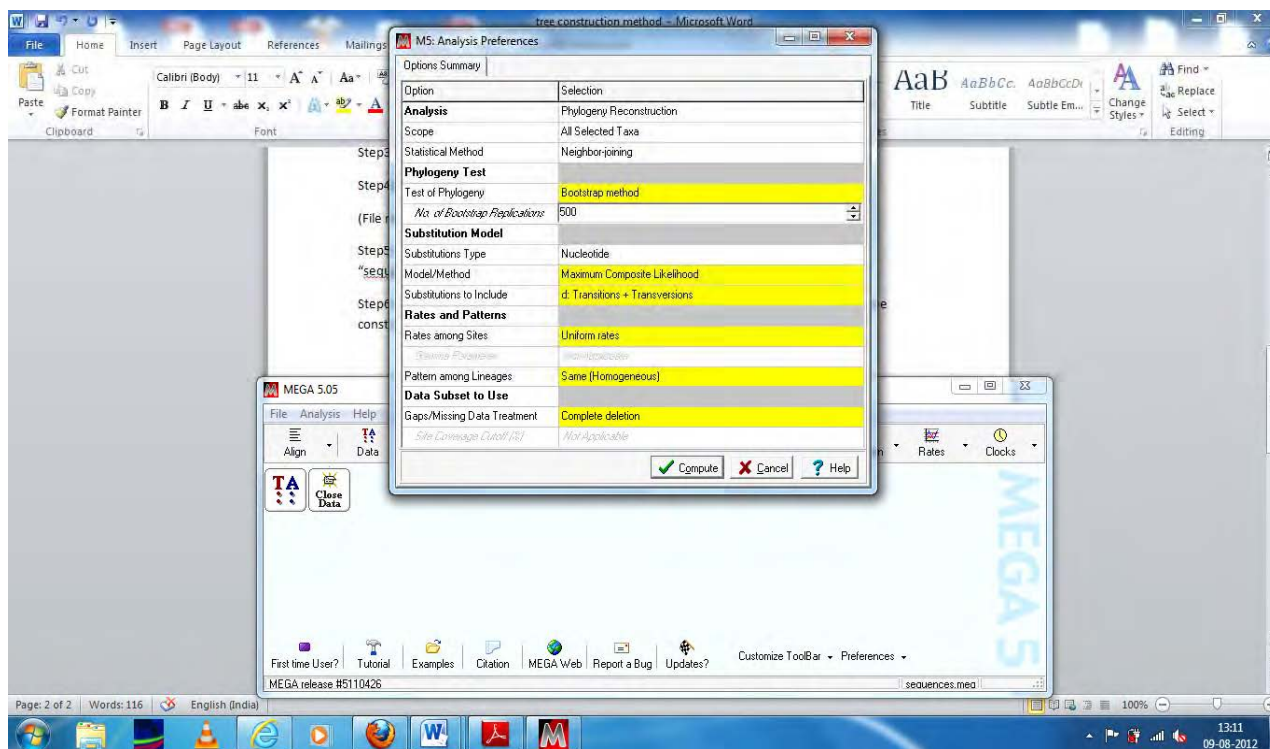


Figure: 1a. The parameters used for the tree construction in MEGA

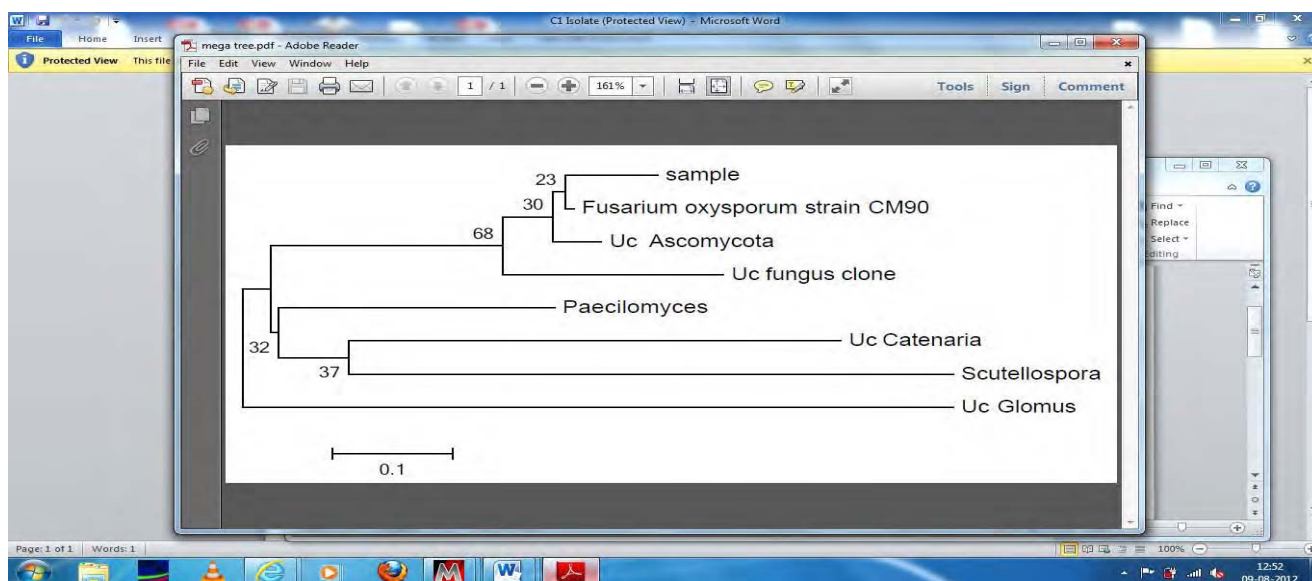


Figure: 1b. Phylogenetic tree of Sample

### 3.1MODELLING

The amino acid sequence of *fusarium oxysporum* laccase was obtained from Swissprot database. The Laccase sequence is blasted against PDB using BlastP. The Top 6 similar sequences are taken and aligned with Laccase sequence using ClustalX. A phylogenetic tree is generated using Phylip to know the nearest homologue. The results shows that 3SQR is the nearest homologue to laccase of *Fusarium oxysporum*. Crystal structure of laccase from *Botrytis aclada* at 1.67 Å resolution is taken from the protein data bank (PDB ID: 3SQR) and used as the template for building the homology model. Having Query coverage of 97% and Identities of 40% are also reasons for considering 3SQR as template.

Source : Uniprot.org

Protein Sequence ID : tr|A7LBK5



Sequence length : 640 Residues

### Query

MWITDACKAVVLSFVAVFHYPVSDEGPHQDVMFTFHGHSTPKIDYPYFDSPPSGPDQDGKVFTCNYPD  
LKGWKPCTTADDRSCWLKGPYQNFNTINDYENFWPIGITREYHLNVDQSDINGDGVNPFQKVFNGK  
YGPWVIQACWGDLIKVTVHNHLKYNGTTIHWGLRQLETFEMDGVNGVTQCPIAPGDSYTYTFRAVQ  
YGTSWYHSHYSLQYADGLAGPITIYGSSAPFDEGRNPILITDWSHRSFAFQSWQRELVPNPTPMMNGV  
LINGVGNFAGSFPFRERFNMVTVKGRKYVLRVINTSVDTTWIFSIDNHNFTVMSTDFVPIHPYSVSHIVLG  
IGQRYHIVLDANPTNTTKMPANPDGNYWIRTVGATRCKGFEPGNEPDERQGILRYNASSKLVPTTFRED  
YSLECRDEAYDRLSPVYEWVNDPVKINYTKSQFDIGLAKFAHRPELMDNFTWWAFGENPLWLNFSNP  
TILNLKNTTWNPDYAVDLVVPDSKKDKWVYIAITAPPAPIQTKPNRAFAPVAHPLHLHGHDFFALLAQG  
TNFSDLDTGKVNLFNPPRRDVALLPAGGYLVVAFKADNPGSWLFFHCHIAWHASSGLAIQILEQQTV  
CDNWNKWFKNNTNLWNAHIFQDDSGI.

For docking purpose the availability of the 3D structure is essential. There is no 3D structure available for laccase of *Fusarium oxysporum*. Hence we have taken the above mentioned sequence from uniprot database and proceeded for Homology modeling

BLASTP was carried out in order to get the similar structure for the query against the PDB Database. (Fig 2 & 3)

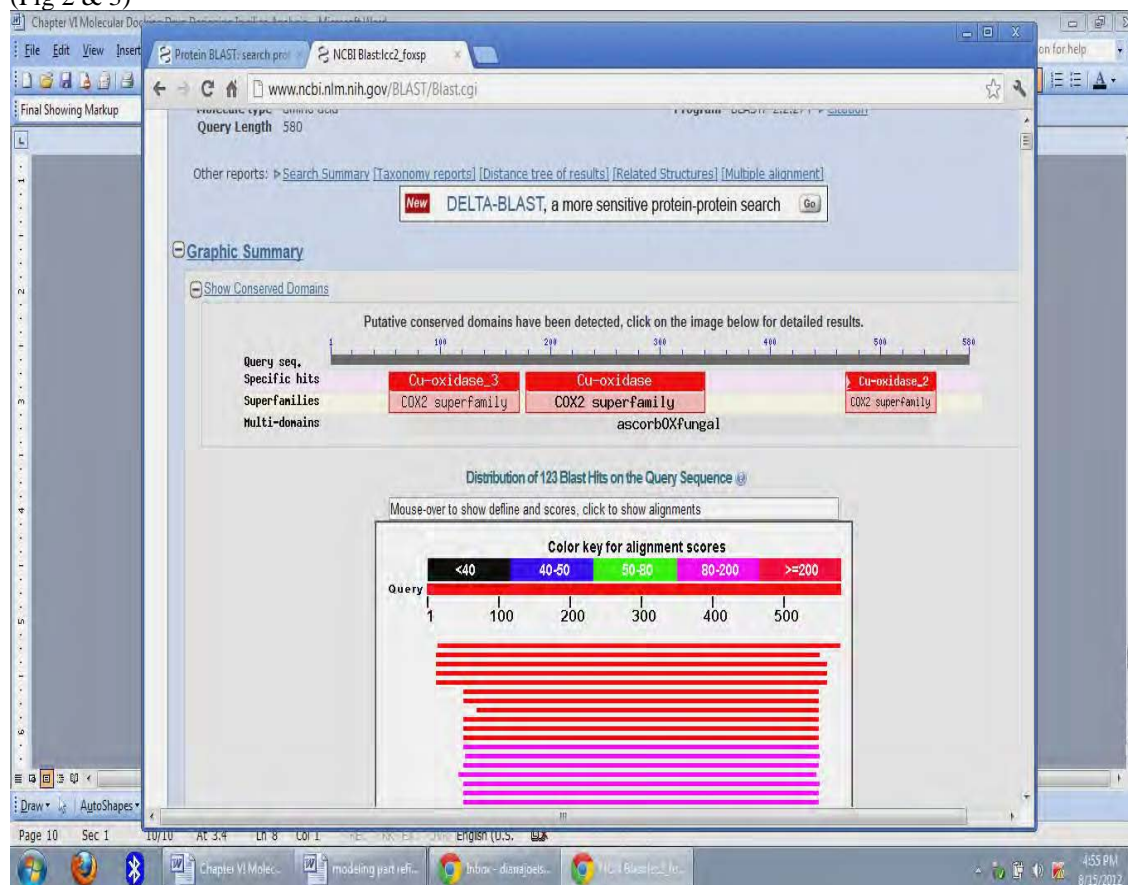


Figure 2 : similar structure for the query against the PDB Database

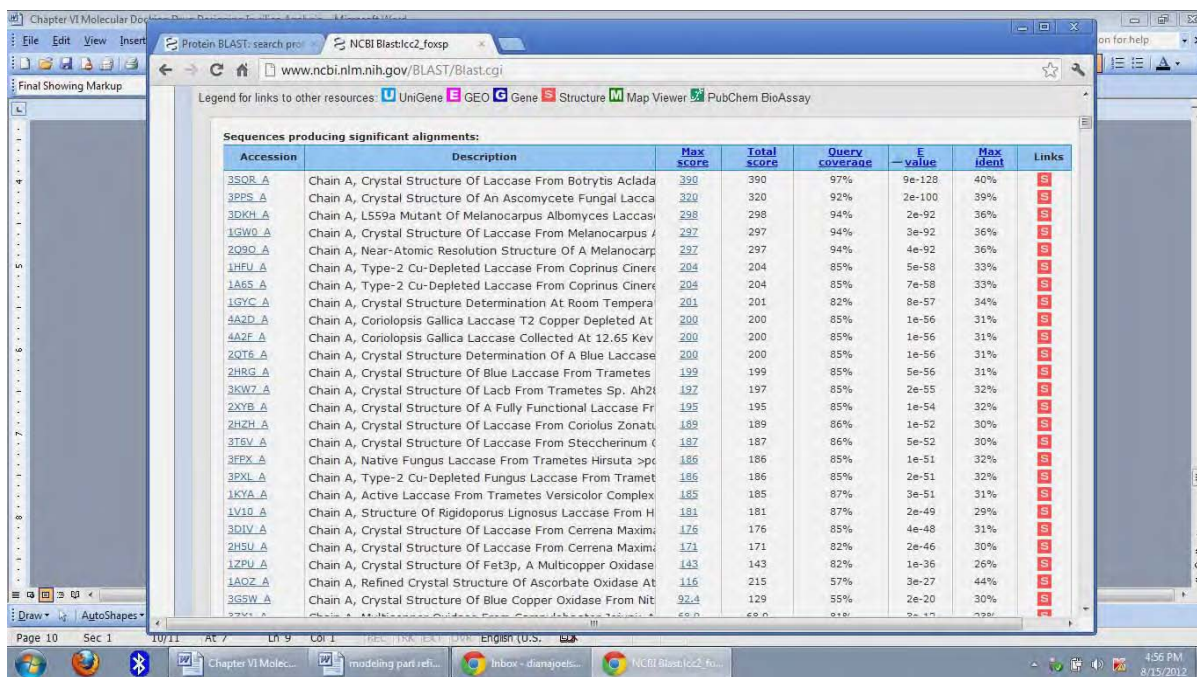


Figure : 3 Most similar sequences against the Query was selected from the Blast result.

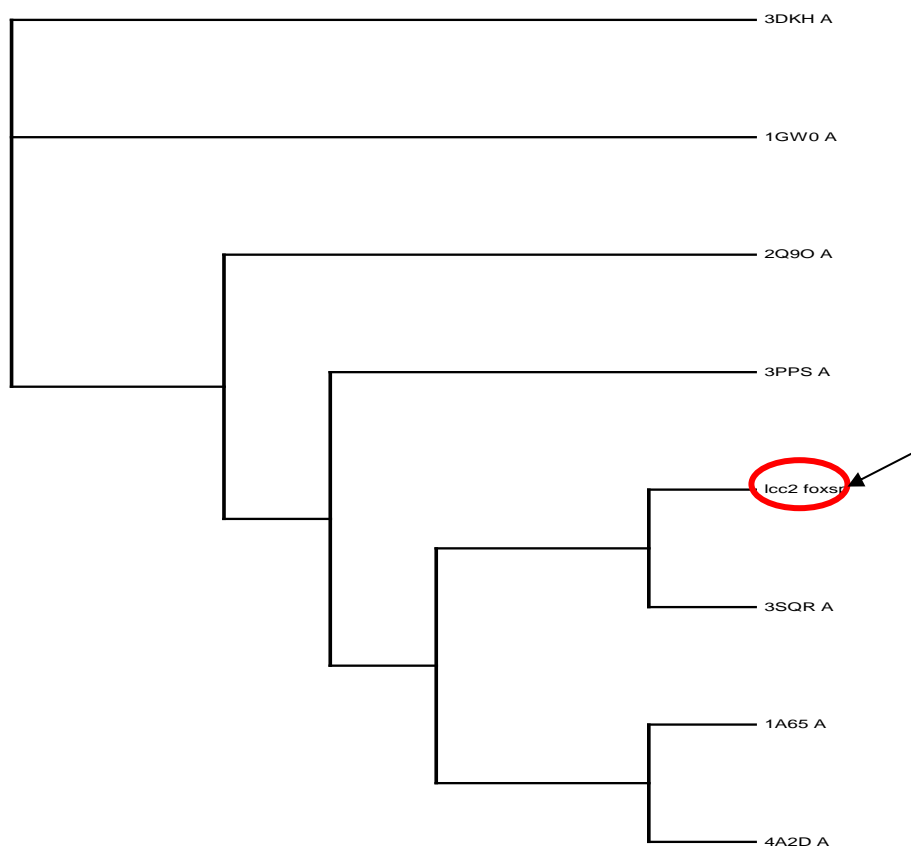


Figure : 4 Phylogenetic tree of Laccase genes of Selected Templates of BLAST search Result.

Sequence alignment is carried out by using Clustal X and all the alignment files are attached in the folder. Using the multiple sequence alignment and Phylip tools a phylogenetic tree was constructed (Figure : 4). and based in that the nearest homologue 3SQR model's A chain abd 3PPS A Chain has been identified as nearest homologue (Figure : 5). So 3SQR model is used in Homology modelling using Modeller.

The Initial model is generated with the help of modeller. Modeller generates the 3D models by optimization of molecular probability density functions. The optimization process consists of applying the variable target function as well as conjugated gradients and molecular dynamics with simulated annealing

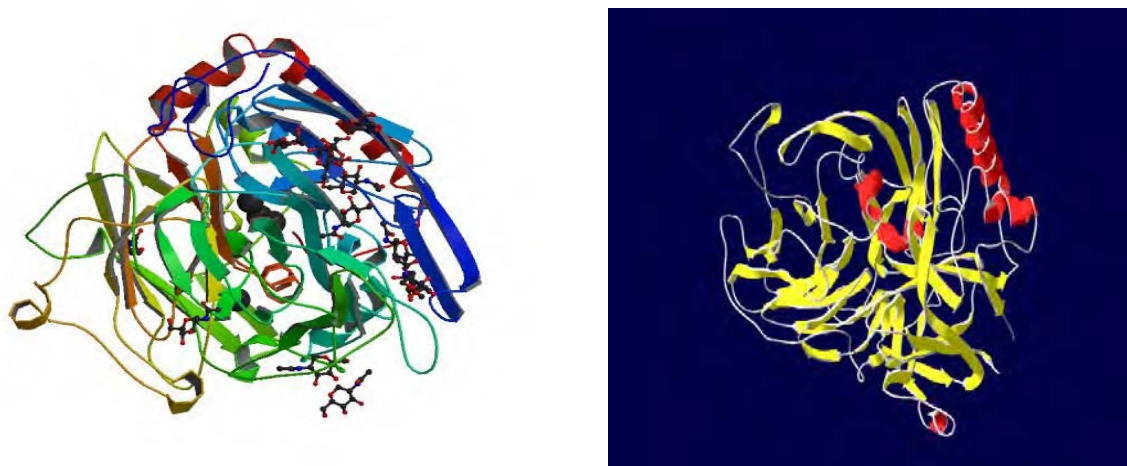


Figure: 5 Template Structure of 3PPS\_A Chain and 3SQR\_A from PDB.

- Using modeller9.7 version 15 structures were generated using 3SQR\_A and 3PPS\_A.

```

model-multiple.py - C:\Users\VATRAGADDAN\Desktop\lac default\lac\model-multiple.py
File Edit Format Run Options Windows Help
# Homology modeling with multiple templates
from modeller import * # Load standard Modeller classes
from modeller.automodel import * # Load the automodel class

log.verbose() # request verbose output
env = environ() # create a new MODELLER environment to build this model in

# directories for input atom files
env.io.atom_files_directory = ['.', './atom_files']

a = automodel(env,
  alnfile = 'align-multiple.ali', # alignment filename
  knowns = ('3SQR', '3PPS'), # codes of the templates
  sequence = 'lfdx', assess_methods=(assess.DOPE, assess.GA341) # code of the target

a.starting_model= 1 # index of the first model
a.ending_model = 15 # index of the last model
# (determines how many models to calculate)
a.make() # do the actual homology modeling
  
```

Figure: 6 Program in Modeller 9v7

DOPE scores were as follows

```

Python Shell
File Edit Shell Debug Options Windows Help

<< end of ENERGY.
DOPE score          : -64958.511719
>> Model assessment by GA341 potential

Surface library      : C:\Program Files (x86)\Modeller9.10/modlib/surf5.de
Pair library         : C:\Program Files (x86)\Modeller9.10/modlib/pair9.de
Chain identifier     :
% sequence identity  : 42.115002
Sequence length      : 640
Compactness          : 0.012994
Native energy (pair) : -5.239815
Native energy (surface) : -4.295139
Native energy (combined) : -0.643697
Z score (pair)       : -8.014526
Z score (surface)    : -6.370114
Z score (combined)   : -9.298693
GA341 score          : 1.000000

>> Summary of successfully produced models:
Filename            molpdf      DOPE score      GA341 score
-----
1fdx.B99990001.pdb  22161.45117   -66546.60156    1.00000
1fdx.B99990002.pdb  22229.91602   -66234.20312    1.00000
1fdx.B99990003.pdb  22472.96094   -65454.98047    1.00000
1fdx.B99990004.pdb  23151.92383   -64459.22656    1.00000
1fdx.B99990005.pdb  22505.34375   -64683.97656    1.00000
1fdx.B99990006.pdb  23199.66797   -64882.77734    1.00000
1fdx.B99990007.pdb  22437.26758   -66516.86719    1.00000
1fdx.B99990008.pdb  22101.24414   -66356.82812    1.00000
1fdx.B99990009.pdb  22672.34961   -65459.14062    1.00000
1fdx.B99990010.pdb  22557.32812   -66505.10938    1.00000
1fdx.B99990011.pdb  22786.82422   -66798.64844    1.00000
1fdx.B99990012.pdb  22853.96094   -66199.07031    1.00000
1fdx.B99990013.pdb  22162.79688   -66165.51562    1.00000
1fdx.B99990014.pdb  22046.57812   -66698.12500    1.00000
1fdx.B99990015.pdb  22921.30664   -64958.51172    1.00000

>>>

```

- Due to lack of similar sequence the amino acids from 1 to 68 are deleted manually for all 15 structures using pymol.
- All the 15 structures were submitted to SAVS and rampage servers for “Verify\_3D”, “Errat” and “Ramachandran plot” validation.(Figure 7)



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S
1	model.no	molepdf	dope	Verify_3D	Errat	R.all	R.g.all	R.dis.all											
2	1	22161.45117	-66546.60156	76.79%	49.823	524(91.9)	29(5.1)	17(3.0)											
3	2	22229.91602	-66234.20312	76.27	48.227	527(92.5)	31(5.4)	12(2.1)											
4	3	22472.96094	-65454.98047	78.53	42.021	528(92.6)	29(5.1)	13(2.3)											
5	4	23151.92383	-64459.22656	77.84	44.128	527(92.5)	23(4.0)	20(3.5)											
6	5	22505.34375	-64683.97656	78.88	40.391	524(91.9)	34(6.0)	12(2.1)											
7	6	23199.66797	-64882.77734	82.2	41.844	523(91.8)	27(4.7)	20(3.5)											
8	7	22437.26758	-66516.86719	85.69	50.98	517(90.7)	38(6.7)	15(2.6)											
9	8	22101.24414	-66356.82812	81.33	43.085	531(93.2)	29(5.1)	10(1.8)											
10	9	22672.34961	-65459.14062	82.02	45.39	527(92.5)	29(5.1)	14(2.5)											
11	10	22557.32812	-66505.10938	77.14	44.742	529(92.8)	23(4.0)	18(3.2)											
12	11	22786.82422	-66798.64844	82.02	53.226	524(91.9)	33(5.8)	13(2.3)											
13	12	22853.96094	-66199.07031	79.58	46.809	521(91.4)	33(5.8)	16(2.8)											
14	13	22162.79688	-66165.51562	83.94	48.582	519(91.1)	33(5.8)	18(3.2)											
15	14	22046.57812	-66698.125	81.85	48.49	525(92.1)	33(5.8)	12(2.1)											
16	15	22921.30664	-64958.51172	74.69	44.326	533(93.5)	24(4.2)	13(2.3)											
17																			
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Figure: 7 Values of Generated Structures

- Based on “Dope Score”, Verify\_3D”, ”Errat” and “Ramachandranplot” values model 11(1fdx.B99990011) was the best model among the 15 models.
- Model 11 was submitted to “modloop” for building loops (amino acid residues are as follows)  
212::216::  
355::359::  
381::385::  
413::417::
- modloop job ID is loop\_661460.



Figure 8: Best Model for Lcc2 Gene

### 3.2 Model Evaluation

Models so produced were ranked on QMEAN server. The QMEAN score ranges between 0 and 1 with higher value to reflect a better quality of the model. The QMEAN score for the current computational model is 0.67. Models were evaluated on basis of geometrical and stereochemical constraints using ProCheck and factors like unfavorable atomic contacts, side chain planarity problems, connections to aromatic rings out of plane etc were assessed using WhatCheck (WhatIf) utilities available at SIB server. The final structure obtained was checked by Ramachandran's map using PROCHECK (Programs to check the Stereochemical Quality of Protein Structures) and environment profile using ERRAT graph (Structure Evaluation server).

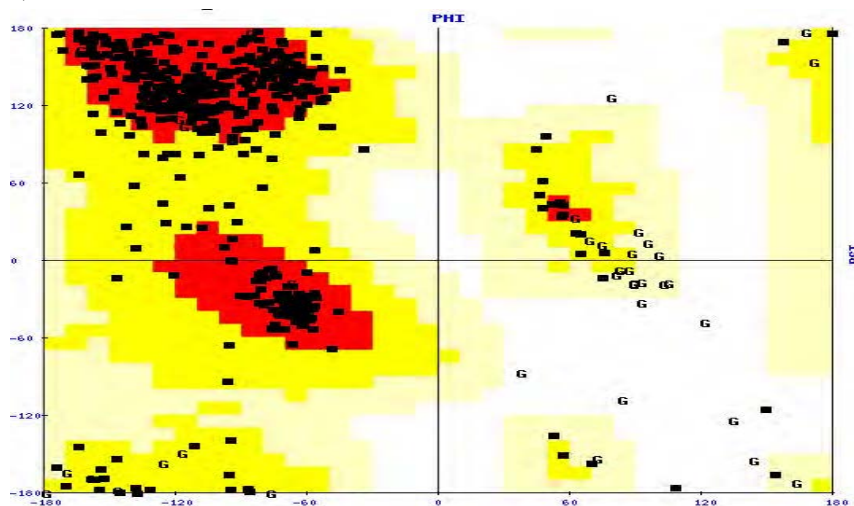


Figure:9 Ramachandranplot results for best model for Lcc2 gene created employing Modeller

The stereochemical quality and accuracy of the predicted model was evaluated after the refinement process using Ramachandran Map calculation with the PROCHECK program (Laskowski 1993). The structure of the protein revealed many segment of helix and the Ramachandran plot showed a tight grouping or clustering of  $\phi \sim -50$  and  $\psi \sim -50$ . In the Ramachandran plot analysis, the residues were classified according to its regions in the quadrangle. The red regions in the graph indicate the most allowed regions whereas the yellow regions represent allowed regions. Glycine is represented by G and other residues are represented by

squares. PROCHECK analysis reveals in Ramachandran plot concluding phi and psi angles to contribute in conformation of amino acids excluding glycine and proline with 84.8% (413 amino acid) residue in most favoured region, 13.3% (65 amino acid) in additional allowed region, 1.7% (7 amino acid) generously allowed region and 0.4% (2 amino acid) residue in disallowed region (Fig. 9).

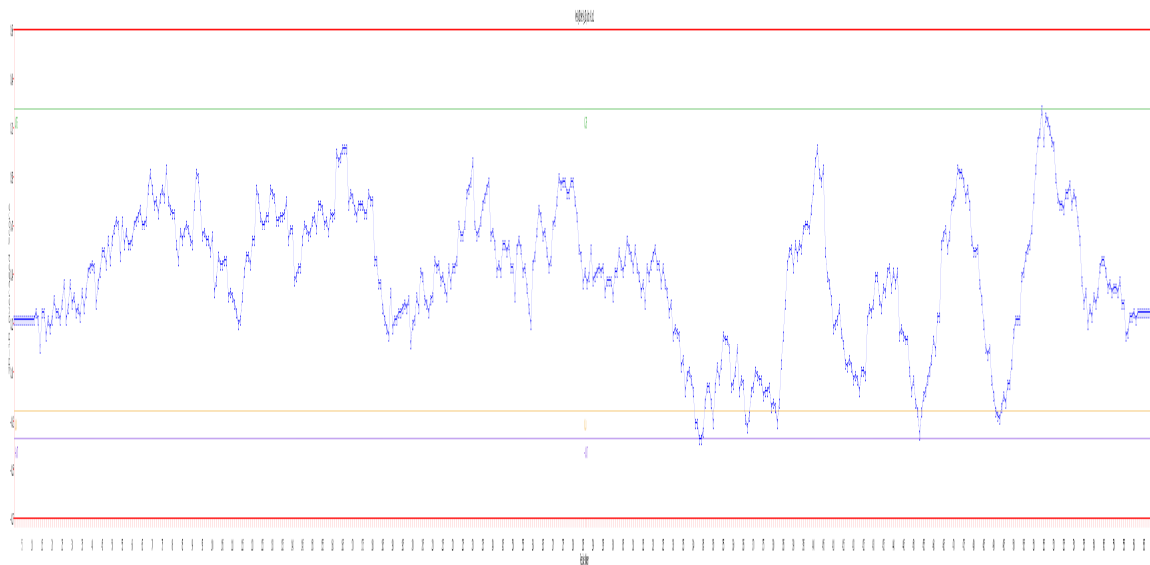


Figure. 10: VERIFY 3D plot analysis of computed lcc2 protein model

### 3.3 Homology models validation

The overall PROCHECK G-factor of lcc2 was  $-0.37$  and verify3D environment profile was good.

The quality of predicted structure of lcc2 was further assessed and confirmed by VERIFY 3D. The residues falling in the area where the orange line crosses 0.0 have low prediction accuracy and less stable conformation whereas, most of the residues fall above 0.22-0.6 so we can say that the model is of good quality (Fig.10) The Ramachandran plot of the structure shows satisfactory results..

## IV. CONCLUSION.

The overall results provided the evidences that the lcc2 protein of *F.oxysporum* is a stable protein having scidic nature. Their physiochemical characterization had shown the deep view of the protein. The predicted secondary structure of fur protein characterize the dominant coil region. Validation and evaluation result of 3-D structure of lcc2 protein

shows that predicted model is the best structural model and of good quality because it shows maximum residues (84.8%) in most favoured region.

The model was constructed for the target protein using MODELLER 9v2 and was deposited to the Protein Model Data Base (PMD) (<http://www.caspar.it/PMD/>) to obtain PMD identifier PM0078408

Sequence alignment, based on the matching of residue identity, is a widely used tool in similarity studies. This is due to many reasons, the most important of which are the wealth of methodology developed over many years, a negligibly small number of solved protein structures ( $\sim 44\ 000$  PDB entries) on comparison with the number of known protein sequences (some 4 300 000 unique sequences in UniProt database (Leinonen et al., 2003), and computational efficiency. While the last reason becomes less important in view of computer progress and modern efficient tools for structural alignment, the others will remain for many years to come.

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