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Phylogenetic Analysis of The Internal Transcribed Spacer (ITS) Region in Laccase Producing *Pseudomonas aeruginosa* ADN04 by Predicted rRNA Secondary Structure

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Secondary structure and phylogenetic efficacy of the internal transcribed spacer-1 (ITS1) in laccase producing organism were studied among 35 species through molecular phylogenetic analyses. The sequence of laccase gene were taken from the NCBI databases and compared with the identified sequence that was coded for laccase. Similar sequences of laccase synthesizing organisms were downloaded from the database and multiple sequence alignment was performed. The most closely related sequences were taken from each organism and RNA structure was predicted. This structure was used for comparative approach of rRNA sequences to be similar for the aligned sequence. Then the phylogenetic analysis was carried for the above sequences and the results were compared with the RNA structure for comparative analysis. During the evolution, the laccase genes from the prokaryotes were differentiated to the higher organisms was reported.

Key words: *Pseudomonas aeruginosa* ADN04, Secondary structure, rRNA, Phylogenetic analysis.

Laccase

Laccase (EC 1.10.3.2, benzenediol: oxygen oxidoreductase) is a multi-copper oxidase enzyme which reduces phenolic compounds from one electron oxidases and reduced to water molecule. (Arora and Sharma, 2010). The catalytic motif in multicopper oxidases includes a type I (T1) copper and the trinuclear center (TNC) comprised of a type II (T2) copper and a pair of type III (T3) coppers (Sakurai,T., Kataoka,K 2000; 2007; 2011). Based on the reaction type of laccase enzymes they are oxidoreductases that transfer electrons from a substrate to an acceptor. Even though laccases catalyze a wide range of enzymatic reactions, they have huge biotechnological significance (Giardina *et al*., 2010). Many researchers reported that laccases are involved in the degradation of complex organic substances (Sathiskumar *et al*., 2013). Till date laccase are reports in both prokaryotes and also in eukaryotes. The nuclear internal transcribed spacer (ITS) regions, which are interspersed among the rRNA genes, have been sequenced widely because of their relatively high variability and

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facility of amplification. Phylogenetic analysis of the enzymes of the members of the multi-copper protein family that have developed from small-sized prokaryotic azurins to eukaryotic plasma proteins (Harald Claus, 2003). The internal transcribed spacer (ITS) regions, which are scattered among the mature rRNA genes are because they enable their own excision from the RNA transcript, have been sequenced widely because of their relatively high variability and facility of amplification. (Joseph *et al*., 1999). Hence based on the gene level studies using the comparative analysis of both prokaryotic and eukaryotic genes and the RNA secondary structure prediction was done along with the phylogenetic analysis.

Phylogenetic analysis

Phylogenetic methods can be used for explore morphological and different types of molecular data. In phylogenetic analysis the concept is to understanding the results of the analysis mean, and to avoid errors and to share a number of primary conventions. The maximum Parsimony method for the analysis was used because it implies that simpler hypotheses that prefer to more complicated ones. Maximum parsimony is a character-based method that infers a phylogenetic tree by minimizing the total number of evolutionary steps required to explain a given set of data, or in other words by minimizing the total tree length. (Felsenstein and Joseph, 2004, Hillis *et al*., 1996, Hennig, 1966)

RNA secondary structure prediction

RNA secondary structure prediction is used for the complex process of 3- dimensional modeling and to compare sequence analysis (Naimi *et al*., 1997; Major *et al*., 1991). So based on the phylogenetic analysis and the RNA secondary structure prediction comparative sequence analysis can be done.

MATERIALS AND METHOD

Isolation

The bacterial culture was isolated from the soil sample (Harur forest, Tamil Nadu) was screened for the activity of laccase using ABTS assay and preserved as pure culture in the Department of Biotechnology, Sathyabama University, Chennai.

Identification

The identification of microorganism was done by both biochemical and also 16S rRNA sequencing. The molecular characterization was done by using Universal primer for amplification and further it was purified and sequenced. The neighboring known relatives of the isolate were determined by performing a sequence database search. The sequences of closely associated strains were reclaimed from GENBANK and the Ribosomal Database Project (RDP) libraries. After the confirmation the Sequence is submitted in NCBI (Arunkumar *et al*., 2014).

Sequence alignment

The sequence alignment was performed manually by BioEdit version 7.0.0, Ibis Therapeutics, a division of Isis Pharmaceuticals, Inc. which is a biological sequence editor and is intended to provide basic functions for protein and nucleic sequence editing, alignment, manipulation and analysis.

Phylogenetic Tree:

The genotypes were transformed according to PHYLIP package version 3.57c. These data were collected and used in the MIX program also from PHYLIP with which the evolutionary relationships were estimated based in the Wagner parsimony method. The significance of the branching in the Wagner network was done by bootstrapping. SEQBOOT of the PHYLIP package was used to perform 1,000 bootstraps.

Secondary Structure perdition using Mfold

MFE (Minimum Free Energy) RNA structure prediction algorithm. The mfold is to provide easy access to RNA and DNA folding and hybridization software to the scientific community. By making use of universally web Graphical User Interfaces, the server avoid the problem of manageability of the software. Detailed output, in the form of structure plots with or without reliability information, single strand frequency plots and 'energy dot plots', are available for the folding of single sequences (Zuker, 2003).

RESULT AND DISCUSSION

Identification of the organism using 16s rRNA sequencing

The samples collected from different parts of Dharmapuri district, Tamilnadu were

Table 1. The Organism used in this study, and the Genbank ID along with the Authors

S.No	Given name	Organism name	Genbank ID	Author
Isolated Bacteria				
1	Pseudo	Ps.aeruginosa ADN04	KM491169	Arunkumar,T., Alex Anand,D. and Narendrakumar,G.
BACTERIA				
1	B1	Azospirillum lipoferum	M59061.1	Woese,C.R.
2	B2	Bacillus subtilis	KJ722789.1	Fan,L., Wang,Y. and Zhao,M.
3	B3	Caulobacter crescentus	gi470469325	Iniesta,A.A., McGrath,P.T., Reisenauer,A.,McAdams,H.H. and Shapiro,L.
4	B5	Marinomonas mediterranea	AF184209.1	Solano,F. and Sanchez-Amat,A.
5	B8	Streptomyces cyaneus	HQ857207.1	Moya,R., Hernandez,M. and Arias,M.E.
6	B10	Stenotrophomonas maltophilia	FJ514243.1	Lucas-Elio,P., Galai,S. and Sanchez-Amat,A.
7	B12	Streptomyce lavandulae	AB092576.1	Suzuki,T. and Ito,M.L
8	B13	Thelephora terrestris	gi399572840	Tourtellot,S.G., Horton,T.R., Powell,W.A. and Maynard,C.A.
FUNGI				
1	F3	Aspergillus niger	XM_001389488.2	*****
2	F6	Coprinus cinereus	EF175934.1	Hoegger,P.J., Kilaru,S., Navarro-Gonzalez,M. and Kues,U.
3	F7	Coriolopsis byrsina	FN666089.1	Meskys,R.
4	F8	Coriolus hirsutus	AY081775.2	Koroleva,O.V., Rebrikov,D.V., Stephanova,E.V., Pegasova,T.V., Landesman,E.O. and Gavrilova,V.P.
5	F12	Ganoderma lucidum	FJ473385.2	You,L.F., Liu,Z.M., Lin,J.F., Guo, L.Q., Huang,X.L. and Yang,H.X.
6	F13	Heterobasidion annosus	Y16951.1	Asiegbu,F.O., Wattad,C., Boyd,C., Keen,N.T.K. and Johansson,M.
7	F14	Lentinus tigrinus	AY914796.1	Schmatchenko,V.V., Leontievsky, A.A. and Golovleva,L.A.
8	F17	Phanerochaete chrysossporium	AY532142.1	Larrondo,L.F., Canessa,P., Vicuna,R., Stewart,P., Vanden,Wymelenberg,A. and Cullen,D.
9	F18	Phlebia radiata	X52134.2	Saloheimo,M., Niku-Paavola,M.L. and Knowles,J.K.
10	F19	Pleurotus ostreatus	AY450404.1	Zhang,Y.B., Jiang,M.L., Hu,X.J., Zhang,G.M. and Ma,L.X.
11	F20	Pycnoporus cinnabarinus	AF170093.1	Otterbein,L., Record,E., Longhi,S., Asther,M. and Moukha,S.
12	F21	Pycnoporus sangunieus	HM106997.1	Kurt Gur,G., Yazgan Karatas,A., Sannia,G. and Tamerler,C.
13	F23	Thelephora terrestris	gi399572840	Tourtellot,S.G., Horton,T.R., Powell,W.A. and Maynard,C.A.
14	F24	Trametes hirsutus	EU492907.1	Cherkashin,E.A., Stepanova,E.V., Landesman,E.O., Koroleva,O.V. and Tishkov,V.I.
15	F25	Trametes ochracea	gi58081983	Vasilenko,O.V., Koroleva,O.V. and Psurtseva,N.
16	F26	Trametes trogii	AJ294820.1	Colao,M.Ch., Garzillo,A.M.,

				Buonocore,V., Schiesser,A. and Ruzzi,M.
17	F27	Trametes versicolor	JQ828930.1	Li,Q., Zhao,L. and Chai,C.
INSECTS				
1	I1	Bombyx mori	NM_001109925.1	Yatsu,J. and Asano,T.
2	I2	Dorsophila melanogaster	NM_001273780.1	Hoskins,R.A., Carlson,J.W., Kennedy,C., Acevedo,D., Evans-Holm,M.,Frise,E., Wan,K.H., Park,S., Mendez-Lago,M., Rossi,F., Villasante,A., Dimitri,P., Karpen, G.H. and Celniker,S.E.
3	I3	Manduca sexta	AY135185.1	Suderman,R.J., Jiang,H. and Kanost,M.R.
4	I5	Musca domestica	XM_005184817.1	*****
5	I6	Nysius plebeius	AB586069.1	Futahashi,R., Tanaka,K., Matsuura,Y., Tanahashi,M., Kikuchi,Y. And Fukatsu,T.
6	I7	, Papilio palytes,	AB531135.1	Shirataki,H., Futahashi,R. and Fujiwara,H.
7	I8	Riptortus pedestris	AB586067.1	Futahashi,R., Tanaka,K., Matsuura,Y., Tanahashi,M., Kikuchi,Y. And Fukatsu,T.
PLANTS				
1	P1	Pseudoplatanus	U12757.1	LaFayette,P.R.,Eriksson,K.E. and Dean,J.F.
2	P3	Lentinula edodes	AB055157.1	Sakamoto,Y., Nakade,K., Yano,A., Nakagawa,Y., Hirano,T., Irie,T., Watanabe,H., Nagai,M. and Sato,T.
3	P9	Schinus molle	gi7595535	Soltis,P.S., Soltis,D.E. and Chase,M.W.

Sequence alignment

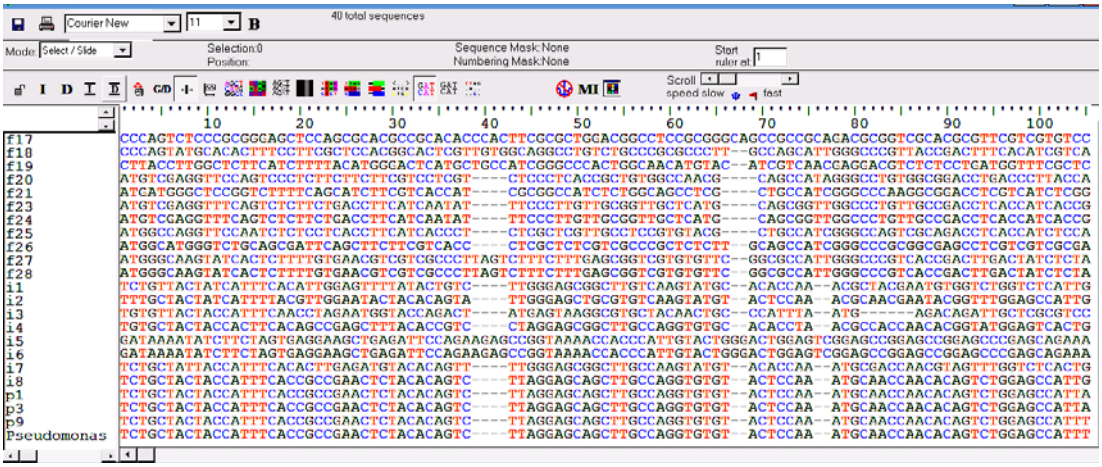

Fig.1. Part of the alignment of the 16S ITS region of the 35 laccase producing stains isolated in this study compared to *Pseudomonas aeruginosa* AND04

subjected for serial dilution and grown on Plate Count Agar (PCA), after incubation these organisms were purified and screened for the ability of producing laccase. The isolated organism *Pseudomonas aeruginosa* was proved to have maximum ability of producing laccase was confirmed by conventional method and further by 16SrDNA sequencing and the sequence was submitted in GENBANK and the Accession number was provided as KM491169.

The sequence identified was

atggctcaga ttgaacgctg gcggcaggcc taacacatgc aagtcgagcg gatgaaggga gcttgctcct ggattcagcg gcggacgggt gagtaatgcc taggaatctg cctggtagtg

Phylogenetic tree

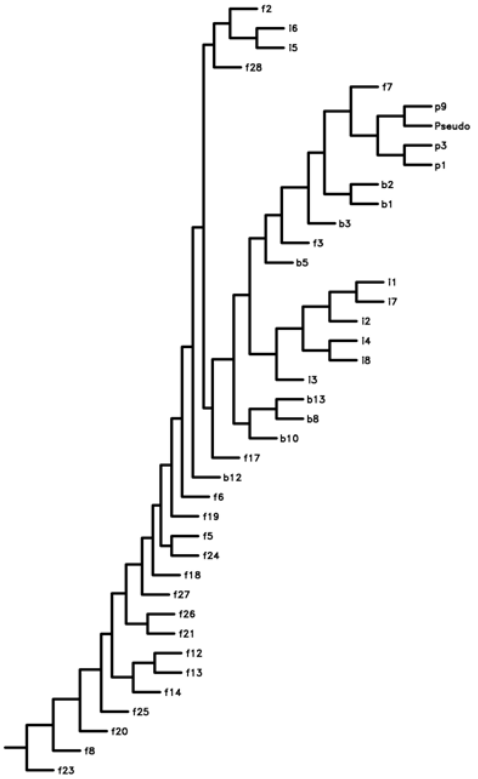

Fig. 2. Phylogenetic tree derived from Maximum parsimony analysis of ITS nucleotide sequences

rRNA secondary structure

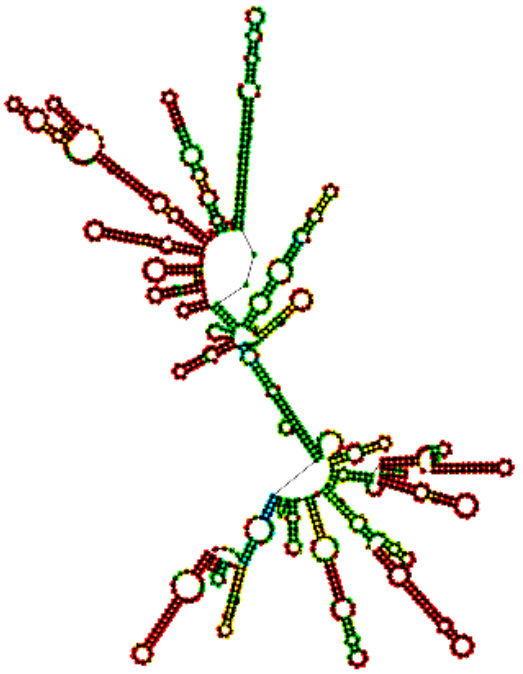

Fig. 3. Predicted secondary structure for the Pseudomonas aeruginosa AND04 ITS regions Illustration produced using the program mFold.

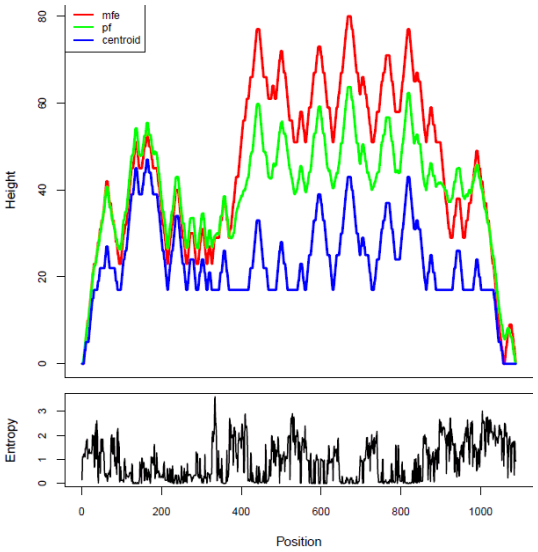

Fig. 4. Mountain plot representation of the MFE structure

ggggataacg tccggaaacg ggcgctaata ccgcatacgt cctgagggag aaagtggggg atcttcggac ctcacgctat cagatgagcc taggtcggat tagctagttg gtggggtaaa ggcctaccaa ggcgacgatc cgtaactggt ctgagaggat gatcagtcac actggaactg agacacggtc cagactccta cgggaggcag cagtggggaa tattggacaa tgggcgaaag cctgatccag ccatgccgcg tgtgtgaaga aggtcttcgg attgtaaagc actttaagtt gggaggaagg gcagtaagtt aataccttgc tgttttgacg ttaccaacag aataagcacc ggctaacttc gtgccagcag ccgcggtaat acgaagggtg caagcgttaa tcggaattac tgggcgtaaa gcgcgcgtag gtggttcagc aagttggatg tgaaatcccc gggctcaacc tgggaactgc atccaaaact actgagctag agtacggtag agggtggtgg aatttcctgt gtagcggtga aatgcgtaga tataggaagg aacaccagtg gcgaaggcga ccacctggac tgatactgac actgaggtgc gaaagcgtgg ggagcaaaca ggattagata ccctggtagt ccacgccgta aacgatgtcg actagccgtt gggatccttg agatcttagt ggcgcagcta acgcgataag tcgaccgcct ggggagtacg gccgcaaggt taaaactcaa atgaattgac gggggcccgc acaagcggtg

gagcatgtgg tttaattcga agcaacgcga agaaccttac ctggccttga catgctgaga actttccaga gatggattgg tgccttcggg aactcagaca caggtgctgc atggctgtcg tcagctcgtg tcgtgagatg ttgggttaag tcccgtaacg agcgcaac

Taxon sampling

Complete ITS nucleotide sequences were obtained from prior phylogenetic work in Laccase producing organisms. .Thirty five taxa were sampled out, including the Eight monotypic genera from bacteria, Seventeen species of fungi, and seven species of Insects and Three species of plants that was compared with *Pseudomonas aeruginosa* ADN04.

Structural modeling of *Pseudomonas aeruginosa* sequences was executed using the algorithm of Zuker *et al.* (1999) and RNA secondary structures was predicted.(Nicholas and Donald, 2008)

Mountain plot representation of the MFE structure

Figure 4 represents mountain plot of the MFE structure, the thermodynamic ensemble of RNA structures, and the centroid structure and additionally the positional entropy for each position was determined.

The phylogenetic analysis of the given sequences of different organisms was represented as phylogenetic tree by clade 1 and clade 2. Total 17 OTUs which include 16 fungus OTUs and 1 bacteria OTUs were grouped under clade 1. Total 21 OTUSs that includes 2 fungus (F3 and F7), 7 bacteria (B10, B13, B8, B5, B3, B1, B2), 8 insects (I1, I2, I3, I4, I5, I6, I7, I8), 3 plants (P1, P9, P3) and *Pseudomonas aeruginosa* ADN04 come under clade 2. So the gene of Pseudomonas aeruginosa ADN04 which is included in clade 2 was found associated with plant ribosomal RNA genes and with fungus the overall variation exhibited in the alignment which differentiated this two clade. This differences are due to the sequence alignment of the basepair from 252-256,343-346,430-437,470-478,560-572,600-608,640-655 and 691-700.and finally it will had a prominent base 20per deletion at 106 to 108 in the Pseudomonas aeruginosa ADN04 main features.

16s rRNA sequence alignment and the secondary structure prediction using mfold algorithm with minimum energy reveals that the *Pseudomonas* species was related with 3 plant species namely P1, P3 and P9 and fungal species namely F7. This taxonomic clustering was due to the base pair deletion of 134th base pair in the 3rd stem with 2 deletions, 261st base pair in the 6th stem with 2 deletions, 313th base pair in the 6th stem with 2 deletions, 319th base pair in the 6th stem with 4 deletions.

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