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Full Length Article

Biochemical and Molecular Analysis of Laccase Enzyme in Saprobic Fungus; *Sordaria fimicola*

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Abstract

Saprobic fungi play an important role in decomposition and thus contributing to the global carbon cycle. *Sordaria fimicola* strains collected from diverse environment were evaluated for their laccase enzyme activity, while *Aspergillus niger* was used as control fungus. In laccase assay, *S. fimicola* strain N6 collected from the station 6 located at the Northern Facing Slope (NFS) of the Evolution Canyon 1 (EC 1), showed the maximum laccase enzyme activity (1.1 ΔA/min). In the next step, complete laccase gene (1.917 Kb) was amplified and sequenced from the biochemically efficient isolates of *S. fimicola* viz. S1, S2, SF13, N6 and N7 and on comparison with reported sequence of *Neurospora crassa* (Accession no. M18334.1) 245 point mutations, 65 amino acid and post-translational modifications (PTMs) changes were detected in the *in silico* translation proteins. In phylogenetic analysis, S1, S2, SF13, N6 and N7 strains of *S. fimicola* were found in clade-I alone, while reference sequence of *N. crassa* (Accession no. M18334.1) was present in clade-II. The laccase gene sequences were submitted to NCBI data base under accession numbers KM282173, KM282174, KM282172, KM282175 and KM282176 for *S. fimicola* strains S1, S2, SF13, N6 and N7, respectively. The biochemical as well as molecular data of the study depicted that environmental stresses affected the specific genes by bringing in mutations, which may result in genomic diversity among the organisms and their frontier molecules such as proteins. The observed laccase enzyme activity of *S. fimicola*-a non-pathogenic fungus, was found even better and comparable to *A. niger,* a pathogenic fungus. Therefore, being a saprophytic with short life cycle *S. fimicola* can become a fungus of choice to produce laccase enzyme at large scale.

Keywords: *Sordaria fimicola*; Laccase assay; Laccase gene; Phylogenetic analysis

Introduction

Laccases (EC 1.0.3.2) are used as a biocatalyst in many industrial processes including biopulping, bioleaching and treatment of industrial waste water (Bourdais e*t al*., 2012). Laccase has ability to oxidize compounds like polyphenols, cyclic diamines, methoxy substituted phenols and other compounds (Baldrian, 2006). Fungi secrete enzymes in the growth media and purification of these secreted enzymes is considered relatively easier than from bacterial or plant sources, in which enzymes mostly are retained inside the cell as described by different workers (Archer *et al*., 2008; Upadhyay *et al*., 2016). Due to higher oxidation reduction potential (+800 mV) as compared to bacteria and plant, the fungal laccases have more application in biotechnology and food industry (Minussi *et al*., 2002). Saprophytic fungi are unable to produce their own food and depend upon their enzymatic system, which breaks the complex biopolymers into simple nutritional components and subsequently absorbed from their surroundings (Alexopolous *et al*., 1996). Therefore, saprophytic fungus has potential to produce different extracellular enzymes, including laccases (Tellez-Jurado *et al*., 2005). Laccase from *Neurospora crassa* is an inducible secretory enzyme and other fungi that have laccase activity include *Trichoderma* and *Botryosphaeria* (Pointing *et al*., 2005; Ishfaq *et al*., 2016a).

Genes encoding laccase enzymes have been studied in different filamentous fungi like *Aspergillus niger*, *A. oryzae* and *Trichoderma reesei* (Hoffmeister and Keller, 2007; Gomaa and Momtaz, 2015). Fungal species belong to Ascomycota have different laccase encoding genes which are involved in the oxidization of syringaldazine dye (Dedeyan *et al*., 2000). In the current research *S. fimicola* fungus was evaluated for its laccase enzyme potential, further laccase gene analysis was carried out in order to exploit them to produce laccase enzyme at large scale.

Materials and Methods

Procurement of Fungal Strains and Culturing

Strains of *S. fimicola* (Roberge ex Desm.) Ces. & De Not.,

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used in the study was isolated from the EC 1, located at the Mount Carmel, Israel. Whereas, the EC I have two opposite slopes, the Southern Facing Slope (SFS) with dry and harsh environment, which is quite different in flora and fauna from the NFS with moist and lush green environment (Nevo, 1995). On the SFS station 1 and 2 are located, while station 6 and 7 are present on the NFS and from each station 15 sample of *S. fimicola* were included, while one *S. fimicola* strain SF13 has been isolated from the premises of the University of Illinois at Urbana-Champaign (UIUC), USA. Potato Dextrose Agar (PDA) medium and PD broth were used for the culture reviving and sub-culturing of *S. fimicola*.

Laccase Assay

Fungal filtrate was separated from fungal mass by using Whatman filter paper No. 1. Bradford assay (Bradford, 1976) was used to estimate total secretory proteins from fungal filtrate. Laccase enzyme activity of *S. fimicola* was evaluated by using syringaldazine dye and absorbance was taken at 525 nm. The reaction mixture components were 0.1 mL syringaldazine (0.1 mM), 0.85 mL sodium citrate (0.1 M, pH-5.0) and 0.05 mL of the fungal growth medium filtrate containing 50 µg of total proteins. Absorbance value was read at 525 nm by using UV spectrophotometer (UV 1800 SHIMADZU), while 0.85 mL of 0.1 M sodium citrate (pH 5.0) and 0.1 mL of 0.1 mM syringaldazine was used as blank. Fifteen continuous readings were taken for each fungal strain filtrate sample with 1 min time interval. The growth medium filtrate if showed high absorbance at 525 nm in different time intervals then it indicated the high enzyme activity in that fungal strain (Kahraman and Gurdal, 2002).

DNA Extraction and Ribotyping

The manual DNA isolation was carried out by 1% CTAB method with some modifications (Saghai-Maroof *et al*., 1984). The quality of extracted DNA was checked by 1% agarose gel electrophoresis and quantification was carried out by taking absorbance at 260 nm using UV spectrophotometer (UV 1800 SHIMADZU). *S. fimicola* strains found efficient in laccase enzyme production were subjected to ribotyping by amplification of 431 bases long hypervariable (V4) region of 18S rRNA gene as described earlier (Machouart-Dubach *et al*., 2001).

Laccase Gene Amplification and Sequencing

S. fimicola strains found efficient in laccase assay were further subjected to laccase gene targeting. Different primers were designed by using the *N. crassa* laccase gene (Accession no. M18334.1) as reference (Table 1). Amplification conditions were optimized by using different melting temperatures and $MgCl_2$ concentration (1.5–3.0 mM) during PCR, while all other parameters of reaction mixture were kept constant including Go *Taq* flexi® Buffer (1x), dNTPs mixture (0.2 mM), Go *Taq*® DNA Polymerase (2.5 U), 25 pmol of each upstream as well as downstream primers and template DNA (100 ng) was used in 50 µL reaction mixture. Temperature cycling condition used were; initial denaturation at 94°C for 4 min, followed by 40 cycles of denaturation temperature at 94°C for 1 min, annealing temperature at 50–60°C for 1 min, primer extension at 72°C for 1 min and final extension temperature was 72°C for 7 min, followed by hold at 4.0°C, while lid temperature was adjusted to 105°C to stop the evaporation. After laccase gene amplification, the PCR products were subjected to 1% agarose gel electrophoresis. The amplified DNA bands of laccase gene were purified from the gel and quantified by using *NanoDrop* ND-1000 (SPECTRAmax Plus) and were got sequenced in both directions from the Core Sequencing Facility available at UIUC, USA.

Bioinformatics Tools

The sequencing results of laccase gene from different strains of *S. fimicola* were submitted to NCBI database after peaks analysis by Chromas software. The laccase gene sequences were aligned by using ClastalW software in order to determine sequence variations. For finding protein sequence variations, gene sequences were translated by using protein translation and ExPASy Protparam tools. The post-translational modifications (PTMs) potential was predicted for laccase protein in *S. fimicola* by using different servers like *LysAcet* and *PredMod* for Acetylation, *BPS* for Methylation, *DISPHOS* and *YinOYang* for Phosphorylation and for Glycosylation *NetNGlyc 1.0* and *YinOYang* were used. Molecular Evolutionary Genetics Analysis (MEGA 6.0.5) software was used for phylogenetic analysis (Tamura *et al*., 2013).

Results

Among the sixty one *S. fimicola* strains evaluated for their laccase enzyme activity, the isolate N6 collected from the station 6 of the EC 1 showed the maximum laccase enzyme activity (1.1 ΔA/min) (Fig. 1). The isolates N7 (1.0 ΔA/min), S1 (0.95 ΔA/min), IQ36.5 (0.9 ΔA/min), S2 (0.85 ΔA/min) and SF13 (0.82 ΔA/min) were found to be second, third, fourth, fifth and sixth most efficient isolates for laccase production (Fig. 1).

Five *S. fimicola* strains with maximum laccase enzyme activity from different environment viz. S1, S2, N6, N7 and SF13, were shortlisted for laccase gene amplification and sequencing. As regards DNA isolation, a genomic DNA band of ~15 Kb was observed from *S. fimicola* strains by using 1% gel electrophoresis. After DNA extraction *S. fimicola* strains were confirmed by ribotyping for their purity of cultures. Ribotyping results showed that sequences of V4 domain of 18S rRNA gene were 100% similar with

Table 1: Primers used to amplify laccase gene in *S. fimicola*

Sr. No.	Name	Sequence (5'-3')	Expected PCR Product (bp)
1	LACU-F	TCC AGA CTC GGA GGT GAA	798
2	LACU-R	GAA ATG CGA GTG GTA CCA C	
3	LACM-F	GGC ATG CAC CAG CGC AAC	799
4	LACM-R	TGA TGG GGA TCG TGT TGC	
5	LACL-F	GAA CAC CAA CAG CAT CGC	753
6	LACL-R	ACC AAG ACC AAC ACC AGC	

each other as well as with previously reported sequence of *S. fimicola* (Accession No AY545724.1). The sequences of V4 domain of *S. fimicola* strains S1, S2, N6, N7 and SF13 were submitted to NCBI data base under accession numbers KF487278, KF487279, KF487281, KF487282 and LM654514, respectively.

After amplification, complete laccase gene including exons and introns were got sequenced from all five shortlisted strain of *S. fimicola* and compared with the reference gene sequence of *N. crassa* (Accession no. M18334.1) by using ClastalW. The deduced laccase proteins from all five strains of *S. fimicola* as well as in reference gene of *N. crassa* (Accession no. M18334.1) were consisted of 619 amino acids (Table 2; Fig. 2). The PTMs of the laccase proteins in *S. fimicola* strains from the opposite slopes of the EC 1 were compared with each other and these were found to be common among all the five strains of *S. fimicola* except for the acetylation by server *predMod* (Table 3). The phylogenetic analysis of Laccase gene using 12 nucleotides sequences available at NCBI, placed the *S. fimicola* strains in clade-I and *N. crassa* in clad-II (Fig. 3).

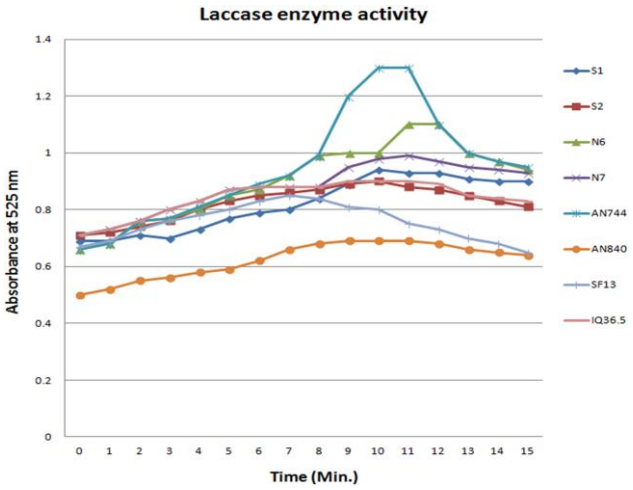

Fig. 1: Comparison of *S. fimicola* and *A. niger* laccase enzyme activity

Key: S1: *S. fimicola* from the station 1. S2: *S. fimicola* from the station 2. N6: *S. fimicola* from the station 6. N7: *S. fimicola* from the station 7. AN744 and AN840: Wild isolates of *A. niger*. SF13: *S. fimicola* from UIUC. IQ36.5: *S. fimicola* strains from the station 2

Discussion

Out of total sixty one strains of *S. fimicola*, the strain N6 collected from the station 6 of the mild NFS showed the maximum laccase enzyme activity, while *S. fimicola* strains IQ36.6, S2, N7 and S1 were found second, third, fourth and fifth most efficient strains for laccase enzyme activity. The average laccase enzyme activity of *S. fimicola* was found comparable to the *A. niger* as described earlier (Ishfaq *et al*., 2014). Laccase enzyme production has been reported from soil Ascomycota species (Levasseur *et al*., 2010). Laccases are distributed in *Ascomycetes*, *Deuteromycetes* and *Basidiomycetes*, being particularly abundant in many white rot fungi, which are involved in lignin metabolism (Gochev and Krastanov, 2007).

Ribotyping results of S1, S2, N6, N7 and SF13 revealed that sequences of hypervariable V4 domain of 18S rRNA gene were 100% similar with each other (Ishfaq *et al*., 2016b) as well as with previously reported sequence of *S. fimicola* (Accession No AY545724.1), which confirmed that *S. fimicola* strains were original and pure without any contamination. This was in line with the finding of other coworkers, who used 18S rRNA gene sequencing for species identification in different studies (Caetano-Anolles, 2002; Meyer *et al*., 2010).

Genomic sequence of *Sordaria macrospora* was found most closely related to *N. crassa* (Nowrousian *et al*., 2010) than other sequenced filamentous fungi; therefore, in the current research, gene sequences of *N. crassa* were used to design the primers for the amplification of *S. fimicola* genes. The complete laccase gene including exons and introns were amplified from all five strains of *S. fimicola* and sequencing results were aligned with the reported laccase gene of *N. crassa* (Accession # M18334.1). In all five strains of *S. fimicola,* a total of 245 base substitutions were observed in the exonic region of laccase gene. As a result of base substitutions, a total of 65 amino acids changes were observed in all five strains of *S. fimicola* (Fig. 2). These amino acids changes were common in all strains of *S. fimicola* with 100% prevalence except M(109)R amino acid shift which was present only in strain N6 and N7 with 40% prevalence (Table 2; Fig. 3) and this amino acid change may have impact on laccase enzyme activity of strains N6 and N7 as evident in biochemical assay performed for laccase enzyme. The sequence variations in laccase genes and their proteins were reported earlier in Ascomycetes fungi (Lyons *et al*., 2003). The laccase genes amplified and sequenced from *Ganoderma*

Table 2: Analysis of laccase proteins derived from different strains of *S. fimicola* by ExPASy Protparam tool

Strains	No. of Amino acid.	Mol. Wt. of Protein (Da)	PI. of Protein	Gene length (Exons) (bp)	Dominated Amino acid (%)
RefE	619	68120.7	7.67	1857	Gly (G) 9.5
LACS1	619	67956.9	8.31	1857	Gly (G) 9.7
LACS2	619	67956.9	8.31	1857	Gly (G) 9.7
LACN6	619	67981.9	8.50	1857	Gly (G) 9.7
LACN7	619	67981.9	8.50	1857	Gly (G) 9.7
LACSF13	619	67956.9	8.31	1857	Gly (G) 9.7

Key: Laccase from Strain S1: LACS1; Laccase from Strain S2: LACS2; Laccase from Strain N6: LACN6; Laccase from Strain N7: LACN7 and Laccase from Strain SF13: LACSF13. RefE: Laccase complete reference gene protein of *N. crassa* (Accession no. M18334.1); PI: Isoelectric point of protein

Table 3: Predictions of PTMs in laccase proteins by using different servers

Servers used to predict PTMs	Serial no.	Laccase			
		S1	N7	S2	N6
		Acetylation			
	1	K38	K38	K38	K38
LysAcet	2	K104	K104	K104	K104
	1	K2	K2	K2	
	2	Nil	K104	Nil	
	3	Nil	K114	Nil	
	4	K174	K174	K174	
PredMod	5	K256	K256	K256	
	6	K272	K272	K272	Nil
	7	K362	K362	K362	
	8	K461	K461	K461	
	9	K601	K601	K601	
	10	K616	K616	K616	
		Methylation			
	1	K274	K274	K274	K274
BPS	2	K611	K611	K611	K611
		Phosphorylation			
	1	T84	T84	T84	T84
DISPHOS	2	T88	T88	T88	T88
	1	S186	S186	S186	S186
YinOYang	2	S499	S499	S499	S499
		Glycosylation			
	1	N139	N139	N139	N139
NetNGlyc 1.0	2	N282	N282	N282	N282
	1	T81	T81	T81	T81
YinOYang	2	S186	S186	S186	S186

Note: Amino acids abbreviations, K-Lysine (Lys); T-Threonine (Thr); S-Serine (Ser); N-Asparagine (Asn); Key: The grey highlighted shows the PTMs changes in N7 and S2 strains of *S. fimicola*

lucidium, *Phlebia brevispora* and *Trametes* exhibited 65–74% nucleotide sequence homology (Galhaup *et al*., 2002). Laccase enzyme gene and protein isolation has been reported in different fungi, including *A. niger* and *A. oryzae* (Couto and Toca-Herrera, 2006, 2007).

The deduced laccase proteins consisted of 619 amino acids in all strains of *S. fimicola* as well in reference gene (Accession no. M18334.1) of *N. crassa* (Table 2); but *N. crassa* protein has different molecular weight (68120.7 Da) as compared to laccase proteins derived from strains of *S. fimicola* (Table 2). This difference in molecular weight is attributed to the amino acids substitutions at 65 different positions in all five samples of *S. fimicola* as compared to reference control protein (Fig. 2). This is also evidenced from the laccase protein molecular weight, where molecular weight of the S1, S2 and SF13 were same (67956.9 Dalton); while the molecular weight of N6 and N7 were same (67981.9 Dalton) (Table 2). The laccase protein from *Cryphonectria parasitica* was 591 amino acids long and has 57% similarity with *N. crassa* as reported earlier by Choi *et al*. (1992). Several reports can be referred, on laccase genes in Ascomycota such as *Gaeumannomyces graminis* (Edens *et al*., 1999), *Magnaporthe grisea* (Iyer and Chattoo, 2003) and *Mauginella* (Palonen *et al*., 2003). Laccase is monomers having a molecular mass in the range of 40000–130000 Da with a covalently linked carbohydrate content of 10–25% in fungi and 20–45% in plants (Claus, 2003). As regard isoelectric point (pI) value of laccase proteins, N6 and N7 strains showed 8.50, while strains S1, S2 and SF13 showed 8.31; while the (pI) value of reference control laccase protein was 7.67 (Table 2). However, this finding was found in contrary to an earlier

CLUSTAL 2.1 multiple sequence alignment

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LACN6           MKFFGIAALVAGLLAPSLVLGAPALGNDRVNLLTPVDKRQDFVAPGFGGGFGGNCNSPTN 60
LACN7           MKFFGIAALVAGLLAPSLVLGAPALGNDRVNLLTPVDKRQDFVAPGFGGGFGGNCNSPTN 60
LACSF13         MKFFGIAALVAGLLAPSLVLGAPALGNDRVNLLTPVDKRQDFVAPGFGGGFGGNCNSPTN 60
LACS2           MKFFGIAALVAGLLAPSLVLGAPALGNDRVNLLTPVDKRQDFVAPGFGGGFGGNCNSPTN 60
LCAS1           MKFFGIAALVAGLLAPSLVLGAPALGNDRVNLLTPVDKRQDFVAPGFGGGFGGNCNSPTN 60
RefE            MKFLGIAALVAGLLAPSLVLGAPAPGTEGVNLLTPVDKRQDSQAERYGGGGGGGCNSPTN 60
                ***:******************** *.: ************  *  :*** **.******

LACN6           RQCWSLGFNINTDFELGTPTTGNTRRYTLTLTETDNWIGPDGVKKDKVRMVNNKIIGPTL 120
LACN7           RQCWSLGFNINTDFELGTPTTGNTRRYTLTLTETDNWIGPDGVKKDKVRMVNNKIIGPTL 120
LACSF13         RQCWSLGFNINTDFELGTPTTGNTRRYTLTLTETDNWIGPDGVKKDKVMMVNNKIIGPTL 120
LACS2           RQCWSLGFNINTDFELGTPTTGNTRRYTLTLTETDNWIGPDGVKKDKVMMVNNKIIGPTL 120
LCAS1           RQCWSLGFNINTDFELGTPTTGNTRRYTLTLTETDNWIGPDGVKKDKVMMVNNKIIGPTL 120
RefE            RQCWSPGFNINTDYELGTPNTGKTRRYKLTLTETDNWLGPDGVIKDKVMMVNDNIIGPTI 120
                ***** *******:*****.**:****.*********:***** **** ***::*****:

LACN6           QANWGDYLEITVINRLKANGTSIHWHGMHQRNTNLHDGVNGVTECPIPPNGGSKVYRFRA 180
LACN7           QANWGDYLEITVINRLKANGTSIHWHGMHQRNTNLHDGVNGVTECPIPPNGGSKVYRFRA 180
LACSF13         QANWGDYLEITVINRLKANGTSIHWHGMHQRNTNLHDGVNGVTECPIPPNGGSKVYRFRA 180
LACS2           QANWGDYLEITVINRLKANGTSIHWHGMHQRNTNLHDGVNGVTECPIPPNGGSKVYRFRA 180
LCAS1           QANWGDYLEITVINRLKANGTSIHWHGMHQRNTNLHDGVNGVTECPIPPNGGSKVYRFRA 180
RefE            QADWGDYIEITVINKLKSNGTSIHWHGMHQRNSNIQDGVNGVTECPIPPRGGSKVYRWRA 180
                **:****:******:**:***************:*::**************.*******:**

LACN6           TQYGTSWYHSHFSAQYGNGIVGSIVVNGPASANYDVDLGPFPLMDYYYETADRLVLLTQR 240
LACN7           TQYGTSWYHSHFSAQYGNGIVGSIVVNGPASANYDVDLGPFPLMDYYYETADRLVLLTQR 240
LACSF13         TQYGTSWYHSHFSAQYGNGIVGSIVVNGPASANYDVDLGPFPLMDYYYETADRLVLLTQR 240
LACS2           TQYGTSWYHSHFSAQYGNGIVGSIVVNGPASANYDVDLGPFPLMDYYYETADRLVLLTQR 240
LCAS1           TQYGTSWYHSHFSAQYGNGIVGSIVVNGPASANYDVDLGPFPLMDYYYETADRLVLLTQR 240
RefE            TQYGTSWYHSHFSAQYGNGIVGPIVINGPASANYDVDLGPFPLTDYYYDTADRLVLLTQH 240
                **********************.**:***************** ****:**********:

LACN6           AGPPPSDNVLFNGFAKHPTTGAGQYATVSLTKGKKHRLRLINTSVENHFQLSLVNHSMTI 300
LACN7           AGPPPSDNVLFNGFAKHPTTGAGQYATVSLTKGKKHRLRLINTSVENHFQLSLVNHSMTI 300
LACSF13         AGPPPSDNVLFNGFAKHPTTGAGQYATVSLTKGKKHRLRLINTSVENHFQLSLVNHSMTI 300
LACS2           AGPPPSDNVLFNGFAKHPTTGAGQYATVSLTKGKKHRLRLINTSVENHFQLSLVNHSMTI 300
LCAS1           AGPPPSDNVLFNGFAKHPTTGAGQYATVSLTKGKKHRLRLINTSVENHFQLSLVNHSMTI 300
RefE            AGPPPSNNVLFNGFAKHPTTGAGQYATVSLTKGKKHRLRLINTSVENHFQLSLVNHSMTI 300
                ******:************************************************

LACN6           IAADLVPVQPYKVDSLLLGVGQRYDVIIDANQAIGNYWFNVTFGGSGLCGLSNNPAPAAI 360
LACN7           IAADLVPVQPYKVDSLLLGVGQRYDVIIDANQAIGNYWFNVTFGGSGLCGLSNNPAPAAI 360
LACSF13         IAADLVPVQPYKVDSLLLGVGQRYDVIIDANQAIGNYWFNVTFGGSGLCGLSNNPAPAAI 360
LACS2           IAADLVPVQPYKVDSLLLGVGQRYDVIIDANQAIGNYWFNVTFGGSGLCGLSNNPAPAAI 360
LCAS1           IAADLVPVQPYKVDSLLLGVGQRYDVIIDANQAIGNYWFNVTFGGSGLCGLSNNPAPAAI 360
RefE            ISADLVPVQPYKVDSLLLGIGQRYDVIIDANQAVGNYWFNVTFGGNDLCGTSDNKYPAAI 360
                *:*****************:*************:***********..*** *:*  ****

LACN6           FKYQGAPNGLPTNKGVAPPDHQCLDLVDLKPVLQRSLNTNSIALNTGNTIPITLDGFVWR 420
LACN7           FKYQGAPNGLPTNKGVAPPDHQCLDLVDLKPVLQRSLNTNSIALNTGNTIPITLDGFVWR 420
LACSF13         FKYQGAPNGLPTNKGVAPPDHQCLDLVDLKPVLQRSLNTNSIALNTGNTIPITLDGFVWR 420
LACS2           FKYQGAPNGLPTNKGVAPPDHQCLDLVDLKPVLQRSLNTNSIALNTGNTIPITLDGFVWR 420
LCAS1           FKYQGAPNGLPTNKGVAPPDHQCLDLVDLKPVLQRSLNTNSIALNTGNTIPITLDGFVWR 420
RefE            FRYQGAPKALPTNKGVAPPDHQCLDLNDLKPVLQRSLNTNSIALNTGNTIPITLDGFVWR 420
                *:*****:.***************** ********************************

LACN6           VNGTAIDVNWNKPVLEYVMTGLTNYSESDNIVKVDGVNQWKYWLIENDPDGPFSLPHPIH 480
LACN7           VNGTAIDVNWNKPVLEYVMTGLTNYSESDNIVKVDGVNQWKYWLIENDPDGPFSLPHPIH 480
LACSF13         VNGTAIDVNWNKPVLEYVMTGLTNYSESDNIVKVDGVNQWKYWLIENDPDGPFSLPHPIH 480
LACS2           VNGTAIDVNWNKPVLEYVMTGLTNYSESDNIVKVDGVNQWKYWLIENDPDGPFSLPHPIH 480
LCAS1           VNGTAIDVNWNKPVLEYVMTGLTNYSESDNIVKVDGVNQWKYWLIENDPDGPFSLPHPIH 480
RefE            VNGTAININWNKPVLEYVMTGNTNYSQSDNIVQVEGVNQWKYWLIENDPDGAFSLPHPIH 480
                ******::************** ****:*****:*:****************.********

LACN6           LHGHDFLILGRSPDVPAISQTRYVFNPAVDMPRLKGNNPTRRDVAMLPAKGWLLIAFRTD 540
LACN7           LHGHDFLILGRSPDVPAISQTRYVFNPAVDMPRLKGNNPTRRDVAMLPAKGWLLIAFRTD 540
LACSF13         LHGHDFLILGRSPDVPAISQTRYVFNPAVDMPRLKGNNPTRRDVAMLPAKGWLLIAFRTD 540
LACS2           LHGHDFLILGRSPDVPAISQTRYVFNPAVDMPRLKGNNPTRRDVAMLPAKGWLLIAFRTD 540
LCAS1           LHGHDFLILGRSPDVPAISQTRYVFNPAVDMPRLKGNNPTRRDVAMLPAKGWLLIAFRTD 540
RefE            LHGHDFLILGRSPDVTAISQTRYVFDPAVDMARLNGNNPTRRDTAMLPAKGWLLIAFRTD 540
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LACN6           NPGAWLMHCHIAWHVSGGLSNQFLERAQDLKNGISNADKQAFNNNCNAWRAYFPDNAPFP 600
LACN7           NPGAWLMHCHIAWHVSGGLSNQFLERAQDLKNGISNADKQAFNNNCNAWRAYFPDNAPFP 600
LACSF13         NPGAWLMHCHIAWHVSGGLSNQFLERAQDLKNGISNADKQAFNNNCNAWRAYFPDNAPFP 600
LACS2           NPGAWLMHCHIAWHVSGGLSNQFLERAQDLKNGISNADKQAFNNNCNAWRAYFPDNAPFP 600
LCAS1           NPGAWLMHCHIAWHVSGGLSNQFLERAQDLKNGISNADKQAFNNNCNAWRAYFPDNAPFP 600
RefE            NPGSWLMHCHIAWHVSGGLSNQFLERAQDLRNSISPADKKAFNDNCDAWRAYFPDNAPFP 600
                ***:**************************:*.** ***:***:**:**************

LACN6           KDDSGLRSGVKAREVKMEW- 619
LACN7           KDDSGLRSGVKAREVKMEW- 619
LACSF13         KDDSGLRSGVKAREVKMEW- 619
LACS2           KDDSGLRSGVKAREVKMEW- 619
LCAS1           KDDSGLRSGVKAREVKMEW- 619
RefE            KDDSGLRSGVKAREVKMKW- 619
                *****************:*
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Fig. 2: Multiple sequences alignment of laccase proteins isolated from different strains of *S. fimicola* along with reference protein derived from nucleotide sequence of *N. crassa* laccase complete gene (Accession no. M18334.1) with exons only

Key: RefE: Laccase protein derived from gene of *N. crassa* (Accession no. M18334.1); Laccase from strain S1: LACS1; Laccase from strain S2: LACS2; Laccase from strain N6: LACN6; Laccase from strain N7: LACN7 and Laccase from strain SF13: LACSF13

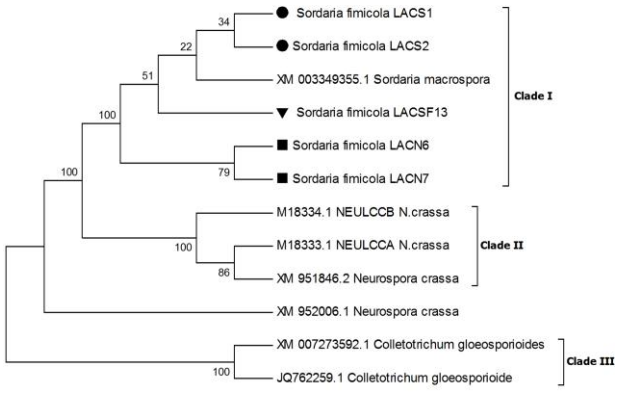

Fig. 3: Phylogenetic relationship of *S. fimicola* based on the laccase gene by using Maximum likelihood method

report of Terraza *et al*. (2007), according to which (pI) of fungal laccase was in range of 4–5.

As regards PTMs, these were common among all strains of *S. fimicola* except for the acetylation, which was predicted in N7 strain at K104 and K114 (Table 3). *Ceriporiopsis subvermispora* has a single laccase gene and its multiple isoforms are formed by the process of PTMs possibly by glycosylation and phosphorylation as reported by different coworkers (Larrondo *et al*. 2003; Feng *et al*., 2015).

The Phylogenetic analysis placed the S1, S2, SF13, N6, and N7 in the clade-I along with *S. macrospora* (XM003349355.1) depicting no or negligible intraspecific variation between *Sordaria* spp., while reference sequence of *N. crassa* (Accession no. M18334.1) was found in clade-II (Fig. 3). The fifty specific laccase gene sequences from different organisms including fungi were compared by multiple sequence alignment and results of the phylogenetic analysis reported diversity of the derived proteins (Satpathy *et al*., 2013). The protein coding genes have been used to study the phylogenetic analysis among Ascomycetes and Zygomycetes fungi as reported earlier by several co-workers (Diezmann *et al*., 2004; Tanabe *et al*., 2004). The biochemical and molecular analysis revealed the laccase activity of *S. fimicola* strains better and comparable to *A. niger*. Therefore, being a non-pathogenic with a short life cycle *S. fimicola* can be used as an alternative fungus for *A. niger* to produce laccase on a larger scale.

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