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Journal of Advanced Scientific Research

Available online through http://www.sciensage.info

ISSN
0976-9595
Research Article

COMPARATIVE EXPRESSION ANALYSIS OF LACCASE GENES IN *ASPERGILLUS NIDULANS* MTCC-344

Baviskar Jayashree Waman[1], Khandelwal Sharad Ratan*[1], Aniwale Akshay Gopalroa[2], Akolkar Dadasaheb Bhausaheb[2]

[1]*H.P.T Arts and R.Y.K Science College, Nasik, Maharashtra, India*

[2]*Datar Cancer Genetics Pvt. Ltd. Ambad F-8, D Road, Nasik, Maharashtra, India*

**Corresponding author: baviskarjayashree@gmail.com*

ABSTRACT

Laccases belong to the family of multicopper oxidoreductases. They act on large variety of substrates particularly phenols and are biotechnologically more applicable than other enzymes. Laccases produce only carbon dioxide and water as by product after biodegradation. Among white rot fungi *Aspergillus nidulans* produces three extracellular laccases. It is an ideal organism to study genes for industrial purpose. Scanty research is available on isoenzymes of laccase belonging to *A. nidulans.* In this work laccases were induced using Guaiacol as inducer. *A. nidulans* MTCC-344 showed highest laccase enzyme activity of 2.09 U/ml using Guaiacol as a substrate on the 5th day after induction. This study aimed at selection and detection of three inducible genes *viz: lcc*B, *lcc*C, &*lcc*D for expression studies. Universal primers were designed for detection of laccases in *Aspergillus* family. Total mRNA was isolated and converted to cDNA using RT-PCR. Gene expression analysis was carried out usingsemi-quantitative PCR based approach for conserved regions of *lcc*B. *lcc*C, *lcc*D sequences. Results revealed comparatively high rate of expression in *lcc*B. Full length *lcc*B. *lcc*C, *lcc*D genes were also amplifiedand sequenced. The genes were found to be of size between 1.7 to 2 kb.BLAST analysis reviled identity to *Aspergillus* family laccases. The gene sequences were submitted to Genebankwith accession number for *lcc* B: MG783369,*lcc* C: MG783370, *lcc* D: MG783371. Thus the isolated genes can be a benchmark of gene expression analysis and protein expressions studies.

Keywords: *Aspergillus,* Laccase, Expression RT-PCR. Guaiacol.

1. INTRODUCTION

Laccase enzymes are widely distributed in plants, fungi and bacteria [1, 2]. White rot fungi are the potent producers of laccases [3]. Laccases have high stability in solutions thus have high biotechnological potential [4, 5]Although there are reports on applications of laccases, Genomic research is yet to be explored in terms of expression studies. Laccases are involved in bioremediation of recalcitrants and are used in pharmaceutical, food, textile and drug industries [6, 7]. Laccases have been also reported in the field of nanotechnology and designing of biosensors [8, 9].Thus laccases are receiving much attention from industrialist as well as researchers around the globe because of its multifunctional activity. *A. nidulans* is extensively studied organisms in fungi and is considered as an experimental model in filamentous fungal genome research [10]. *A. nidulans* genome was sequenced and compared with *A. fumigatus* and *A. orazae*and it was found that they share 70% identity [11]. *A. nidulans*produces six putative laccases of which extracellular secretion signal peptides are found in yA laccase produced in conidia, TilA i.e. fungal tip laccase and *lcc B, lcc C* and *lcc D*except for *lcc* A [12]. The study was focused on *lcc B, lcc C* and *lcc D*genes for expression analysis. Thus the laccase isotypes can be explored for substrates specificity and their applications. Understanding the concept of sequence, structure and function will help in heterologous expression of fungal laccases. On the basis of present findings *Aspergillus nidulans* strain can be exploited for Biotechnological applications.

2. MATERIAL AND METHODS

2.1. Material

Reagent grade chemicals like potato dextrose agar, Sabourauds broth, Guaiacol, sodium acetate, K_2HPO_4 KH_2PO_4, $MnSO_4$, $ZnSO_4$, $MgSO_4$ and $CaCl_2$, were procured from Himedia, Mumbai. RNA latter solution was procured from Sigma-Aldrich, TRIZOL® Reagent from Life Technologies Invitrogen, USA), SYBER safe

DNA gel stain from InvitrogenOne Taq RT- PCR Kit, DNA purification kit was procured from New England Biolabs and were used according to manufacturer's instruction

2.2. Laccase Induction and production

Standard culture of *A. nidulans* was procured from Microbial Type Collection Center, Chandigarh (MTCC-344). The culture was revived and maintained on potato dextrose agar. The fungal culture was screened for Laccase production, 5 mycelial Discs from one week old mycelia was inoculated in 50 ml of Potato Dextrose Broth in 250mL Erlenmeyer flasks,incubated at 27°C on rotary shaker. After 48 hours 50ul/100ml of Guaiacol was added as an inducer for production of Laccases.

2.3. Extracellular Laccase enzyme assay

Fungal spores of 10^6spores/ml were inoculated in 100ml of Sabourauds broth and were incubated at 27°C. The culture supernatant was treated as the enzyme source. The reaction mixture contained 3ml 100mM acetate buffer (pH-5.0), 1ml 10mM Guaiacol and 1ml Enzyme sample. The change in absorbance of the reaction mixture containing Guaiacol was monitored at 470nm for 60 minutes of incubation using UV Spectrophotometer (Shimadzu). Enzyme activity was measured in U/ml.

$$\text{Volume Activity U/ml} = A \times V / t \times \varepsilon \times v$$

V- final volume of reaction mixture(5ml)

v= Sample volume (1ml)

ε= extinction coefficient of Guaiacol (0.6740 μM/cm)

t= incubation time

2.4. Primer designing and BLAST analysis

Nucleotide sequence for *lcc B, lcc C and lcc D* were retrieved from *Aspergillus* genome database with accession number AN9170, AN5397 and AN0878. Primers were designed using primer-BLAST, International database of National center for biotechnology information (NCBI)(https://blast.ncbi.nlm.nih.govBlast.cgi). The first set of primers was designed to identify the presence of three laccases and second set of primers were used for full length amplification of genes. Primers were also designed for control gene i.e. GAPDH and its sequence was retrieved from NCBI gene bank with accession number FJ410422.1. Primers were synthesized form Eurofins Scientific Ltd, Bangalore.

2.5. Total RNA Isolation

Mycelia was harvested before 48 hours and after induction of laccases at 72 hours. It was Ultra centrifuged (Thermo Scientific FRISCO 21) at 16000xg at room temperature. To remove traces of media,mycelia was washed with Phosphate Buffer saline prior to RNA extraction,and was stored in RNA latter solutionfor downstream processing. Total RNA was isolated from 0.25g of fungal pellet using 1ml of TRIZOL® Reagent according to manufacturer's instructions. Concentrations were determined using NANO DROP spectrophotometer (Thermo Scientific 2000c V.I, USA)

2.6. cDNA synthesis using RT-PCR and amplification

The total RNA was reverse transcribed into the first strand of cDNA using oligonucleotide primers on Gene Amp* PCR Systems 9700 (Applied Biosystems) with One Taq RT- PCR Kit. The cDNA product was stored at -20°C for downstream PCR amplification.PCR amplification for conserved sequences was accomplished with annealing of 55°C, and 45°C for full length gene amplification respectively. The PCR products were stored at -20°C for downstream processing.

2.7. Agarose Gel Electrophoresis and Gene expression analysis

The integrity of amplified fragments was estimated using 2% agarose gel for short fragments and 0.8% for full length amplicons. Gel bands were analyzed using IMAGE J software, where the grey scale gel image was selected using a rectangle band,thus the profile plots with Peaks were obtained. For extraction, gel was observed under E-Gel TM IMAGER system with UV light base (Life Technologies). The expected bands were removed and gel purified using DNA purification kit. Nucleic acid concentration was determined using NANO DROP spectrophotometer.

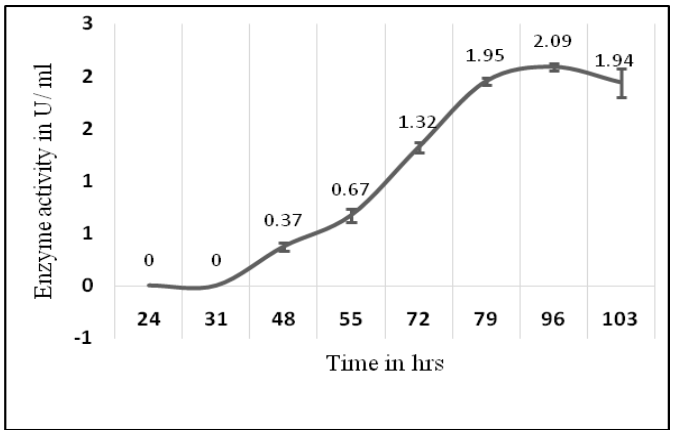
2.8. Sequencing of full length genes and characterization

DNA sequence was determined by sanger sequencing method at the Chromous Biotech (Bangalore) and were BLAST against *lcc B, lcc C and lcc D* of *A. nidulans* FGSC A4. Sequences were aligned using CLUSTAL V algorithm. The partial coding nucleotide sequence of all three genes of *A.nidulans*MTCC-344 was deposited in the EMBL/GenBank Database.

3. RESULTS

3.1. Laccase Induction and Enzyme assay

After 48 hours of incubation, laccase enzyme production which produced brown coloration due to oxidationof Guaiacol. This indicates presence of enzymes extracellularly. Enzyme activity gradually increased from third day and was found to be highest i.e. 2.09 U/ml on 5th day of incubation as shown in Graph. 1. The enzyme activity decreased on 6th day may be due to depletion of micro and macronutrients.



Graph 1: Enzyme activity of *A. nidulans* in batch culture with induction at 48hrs

Data represents the mean of triplicate results of enzyme activity. The incubation temperature was maintained at 30 ˚C. This was the highest activity reported at this temperature.

Fig. 1: Conserved laccase and GAPDH sequences for primer designing

3.2. Primer designing and BLAST analysis

The oligonucleotide primers were highly specific for the laccase sequences viz. *lcc B, lcc C, lcc D* and GAPDH gene as per Fig. 1. BLAST analysis of primers revealed no identity within the internal sequence of gene as well as with whole *aspergillus* genome sequence. Two set of primers, were designed to amplify conserved short stretches to identify laccases in *A. nidulans* and full length gene sequences as listed in Table 1 & 2. Primers for conserved sequences can be used as universal primers for isolation of laccase genes from *Aspergillus* family.

Table 1: Primers for amplification of conserved stretches

Sr.No	Gene	Orientation	Sequence	Amplicon length	Tm in ˚C
1	Lac B	Fw	CTGAACCGTGATAACCCGCC	159 bp	64
2		Rw	ACGCTCGACAAACTGGAGGG		64
3	Lac C	Fw	GCAGGGAATGGTGCGACTA	166bp	60
4		Rw	ATCGTTGGACCAGGGATCGT		62
5	Lac D	Fw	CTGACGTGGGAGGATCGAAA	159bp	62
6		Rw	ACCATGGAAATGTACCGACG		60
7	GAPDH	Fw	TGGGTGTCAACAACGAGACC	169bp	62
8		Rw	GACGACCTTCTGGGTAGCAG		64

**Fw= Forward primer, *Rw=Reverse primer*

Table 2: Primers for amplification of Full length amplicons

Sr.No	Gene	Orientation	Sequence	Amplicon length	Tm in ˚C
1	Lac B	Fw	ATGGCGCAACTTTGGGGATGG	1713bp	63
2		Rw	TTAGATACCAGAATCATCCTGATGG		62
3	Lac C	Fw	ATGCTGCGTTCTTCCTTTCTTC	1995bp	60
4		Rw	CTAGACACCCGAATCATACTG		59
5	Lac D	Fw	ATGAAAGGTTTACAACACGGATTG	1791bp	60
6		Rw	CTAGAGTGCCGCTGCCGCT		63

**Fw= Forward primer, *Rw=Reverse primer*

3.3. Isolation and Expression analysis using IMAGE J software

Agarose gel electrophoretic images showed distinct bands for conserved sequences at expected position 150bp to 200bp for *lcc* B, *lcc* C, *lcc* D and *GAPDH* control gene (Fig. 2a). Bands were observed between 1650kb to 2000kbfor full length gene amplification of *lcc* B, *lcc* C, *lcc* D and GAPDH (Fig. 2b).

The reproducible gel bands were considered for analysis using IMAGE J software reveled relative intensity in terms of peaks (Graph. 2). The area obtained were 2254.89, 8247.38, 3011.22, and 4869.88 for GAPDH,*lcc* B, *lcc* C and, *lcc* D gene respectively. Results indicated high expression of *lcc* B than other laccases using semi quantitative PCR gene expression.

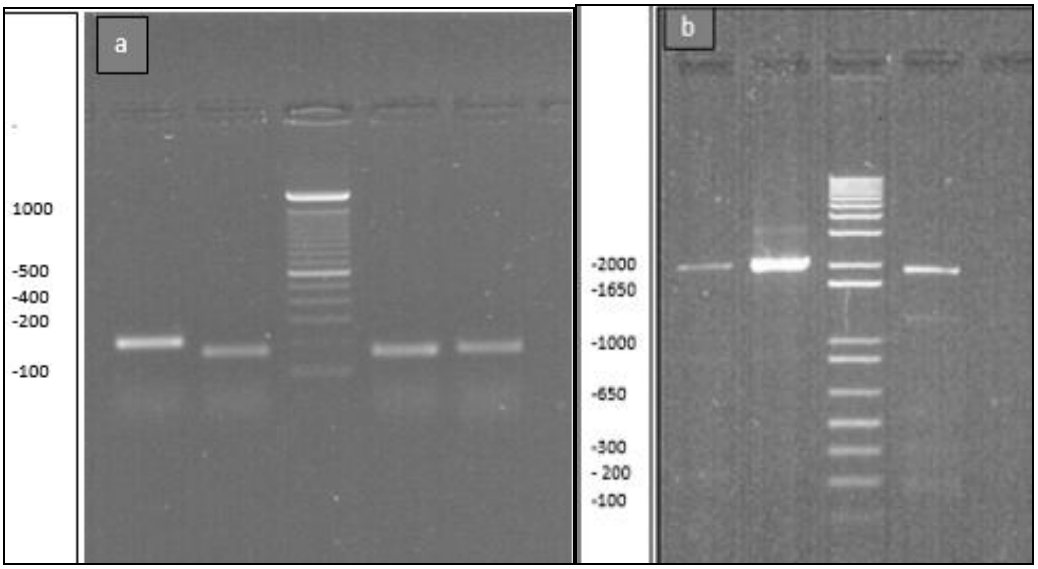
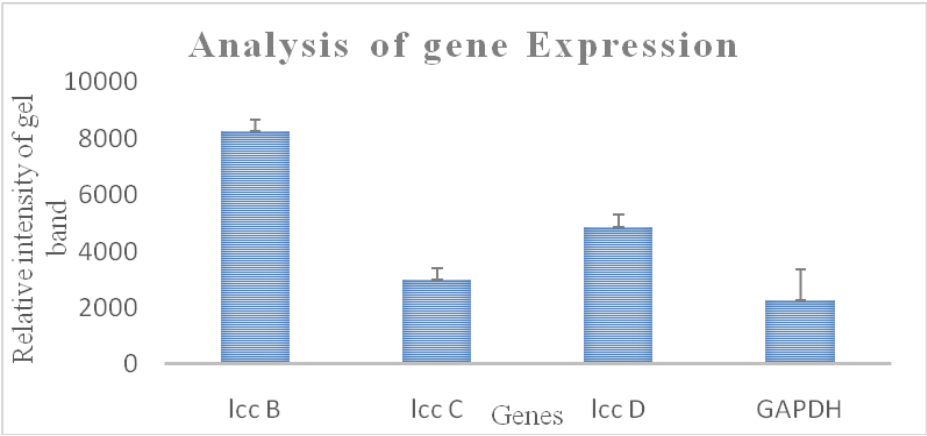


Fig. 2: Analysis of PCR-amplified products by agarose gel electrophoresis

(a) 1st lane lcc B, 2nd lane lcc C, 3rd lane 100kb ladder, 4th lane lcc D and 5th lane GAPDH for short conserved fragments (b). 1st lane lcc B, 2nd lane lcc C 3rd lane 1000kb Plus ladder and 4th lane lcc D for full length gene amplicons



Graph 2: Relative intensities of the GAPDH and laccases genes.

Data represents the results taken in triplicates

3.4. Sequencing of laccase genes

Laccase genes were characterized using sanger Sequencing. The sequenced data is submitted to GenBank with accession number for *lcc* B:MG783369,*lcc* C: MG783370, *lcc* D: MG783371. Sequencing reviled *lcc* B with 1720bp length, *lcc* C 1961bp and *lcc* D with 1820b.

The sequenced data of gene *lcc* B when studied using BLAST showed 94.8% identity to *A. nidulans* FGSC A4 at chromosome VI. A region. Similarly, *lcc* C showed 95.1% identity to chromosome V. A region and *lcc* D with 96.5% identity to chromosome VIII. A.

4. DISCUSSION

More than 60 fungal Isolates belonging to *Ascomycetes*, *Deuteromycetes* and *Basidiomycetes* group were screened and found to produce laccase. Production media generally include minimal media as well as starch and yeast extract, wheat and rice bran, Olga liquid medium [13]. In this work sabarouds broth was used for expression of laccasemRNA. For laccases induction many inducers were tried, commonly reported substrates include Bromophenol blue dye and ABTS (2,20-azinobis 3-ethyl-benzothiazoline-6-sulfonate) [14] 4,4'- [azinobis (methanylylidene)] bis (2,6-dimethoxyphenol) i.e. Syringaldazine is also reported as best substrate for laccase assay [15]. In this work Guaiacol was exploited as inducer for laccase genes induction where it was reduced to brown color after the gene products were synthesized. In the present study *A. nidulans* was effective in production of laccase.

Organisms have been reported to produce many isoforms of laccases. They contain four copper and are divided into three clusters type I, II and III [16]. *A. nidulans* produces six isoforms of laccases. We selected three laccase genes from *A. nidulans* whose products are extracellular enzymes.

Genes were analyzed for synthesis of degenerate primers on gel electrophoresis for many organisms like *Ganoderma lucidum*, *Grifola frondosa*, *Lentinula edodes*, and *Lentinus tigrinus*. However, bands were not observed for *Pleurotus ostreatus* [17]. The issue was focused by emphasizing on synthesis of universal primers. Thus we could report primers for conserved sequences of genes for their isolation and identify sequence of up to 200bp in length. Primers were also constructed for full length gene amplification. Compared to previous published research the expected length of gene was observed on gel electrophoresis was upto 2kb due to potential to produce laccase from many fungi. Research has made extensive use of RT- PCR, Multiplex Reverse Transcription-PCR and Quantitative real-time polymerase chain reaction (qPCR) were used to identify and isolate laccase genes [18]. In this work we tried to isolate three genes i.e. *lcc* B, *lcc* C *and lcc* D with the help of RT-PCR, and were quantified using semi-quantitative technique using image J software. Gel quantification software was used to analyze the bands within a very less time and gives automated counting of pixels' percent [19]. For accurate interpretation of gene amplification, we selected GAPDH as reference gene to study expression of *lcc B, lcc C and lcc D.*Out of all laccases only *Lcc* C is being exploited by cloning and expression and then immobilization with hydrophobins on hard polystyrene surfaces, and was found to be with less redox potential [20]. In this study *lcc* B was seen to be highly expressed so it can be used for heterologous expression studies**.** Laccase production is studied extensively in *Cerrena. species* with its application in Pharmaceutical industry. The laccase engineering database (LccED. http://www.lccd.uni-stuttgart.de) has designed tools for sequence based classification of laccases [21].

5. CONCLUSION

Scanty research is available on isoenzymes of laccase belonging to *A. nidulans.* In this work we isolated three laccase genes ie. *lcc*B. *lcc*C, &*lcc*D from *A. nidulans.* Their products beingextracellular produce brown coloration when reacted with Guaiacol as substrate. Semi quantitative PCR analysis showed high expression of *lcc* B. Sequenced genes can be exploited for heterologous expression for high yield of enzymes for various purposes like bioremediation, medicine and biotechnology. More research is needed to experimentally validated use of different control genes at different stages of fungal cell cycle.

6. ACKNOWLEDGEMENTS

We gratefully acknowledge the support of Datar cancer Genetics Ltd. Nasik. We are grateful to Dr. Datar for providing access to their laboratory to carry out molecular level work. We also thank Dr, Pooja Fulali, Suraj Chauhan, and Datar Genetics team for their timely help. We are thankful to H.O.D and staff of Department of Microbiology for their kind support.

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