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Use of Gene Specific Universal Primers for Isolation of DNA Sequences Encoding Laccase Enzyme from a Wild Isolate of *Schizophyllum commune*

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Authors' contributions

This work was carried out in collaboration between all authors. Authors VPK and MS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors APK and AD assisted in conduct of PCR and also managed the analyses of the study. Authors VPK and CN managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Laccase enzymes plays a vital role in innumerable biotechnological applications and hence their large scale production has stimulated considerable research. In the present study, degenerate universal primer pairs were employed to isolate laccase gene from a wild isolate of laccase producing white rot fungi *Schizophyllum commune*. Primer pairs for this fungal isolate were also designed using laccase specific consensus sequences for fungi. The PCR product of 1000 bp

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amplicon was visualized on agarose gel using the degenerate primer pair Cu1F/Cu3R. Matching of the sequenced gel purified DNA sequence resembled most of the putative phosphatases involved in cell cycle with 100% identity to *S. commune.* Here we report the false negative results obtained upon use of laccase specific degenerate primers as well as other primers specific for the genus. These failed to contribute towards isolation of laccase gene even after optimization of PCR conditions in terms of reaction volume, annealing temperature, number of cycles, touchdown PCR and gradient PCR. These findings constitute a practical guide for researchers addressing amplification of transcripts of this biotechnologically important enzyme from the not so well characterized genus of *Schizophyllum.*

Keywords: Schizophyllum commune; laccase gene; lignin degradation; cloning; sequence analysis.

1. INTRODUCTION

Lignin biodegradation has attracted the attention of many researchers in recent years, on account of its significance in efficient bioconversion of lignocellulosic residues into useful chemicals as well as the ability to enhance the digestibility of crop residues as feed for ruminants. Filamentous microorganisms are of particular importance in biotechnological processes as they are responsible for the production of a vast number of primary and secondary metabolites such as antibiotics, enzymes and vitamins. Basidiomycetous fungi, especially those belonging to the white rot group are known to produce extracellular ligninolytic enzymes viz., laccases, lignin peroxidases and manganese peroxidases with the ability to selectively mineralize all components of lignin to carbon dioxide and water. Most experiments on bio degradation using white rot fungi have been carried out using *Trametes versicolor and Pleurotus ostreatus* which are well known for their ability in improving the nutritive value of the fodder [1,2].

Laccases, or the blue copper oxidases, and one among the ligninolytic enzymes (benzenediol/oxygen oxidoreductases, EC. 1.10.3.2) are a group of multicopper oxidases which are potentially useful in a number of industrial as well as environmental applications. Laccase like Multicopper Oxidases (LMCOs) catalyzes the oxidation of various phenolic compounds and also some non phenolic substrates with the concomitant reduction of molecular oxygen to water [3]. The biological role of laccase enzymes though not fully well elucidated, appears to vary depending on the type of the organism (Thurston, 1994). Laccases belong to the large blue copper protein group, which include the plant ascorbate oxidases and the mammalian plasma protein ceruloplasmin. The one cysteine and ten histidine residues, involved in binding of four copper atoms in a majority of laccase molecules, are highly conserved [4,5].

Though laccases are present in almost all domains of life [6], they have been most extensively studied in the ligninolytic fungi [7]. Although laccases are commercially available for their innumerable applications, there is immense scope for production of better isoforms in terms of activity, pH and heat stability, shelf life etc. In the frame of the development of a bioprocess using the competence of laccase producing white rot fungi, an attempt was made in the current study to isolate and clone laccase gene from a wild new strain of *Schizophyllum commune* [8]. PCR reactions utilizing already reported universal primer sets [9-14] as well as those specifically designed for the present study by targeting the copper binding domains of laccase from *Schizophyllum commune* [15] were employed with aim to efficiently degrade lignin in crop residues and enhancing their digestibility for livestock feed.

2. MATERIALS AND METHODS

2.1 Organism, Culture Conditions and Laccase Activity Measurements

The wild strain of *Schizophyllum commune,* NI-07 (Gen Bank accession number KF911323, MTCC accession number 118930) was isolated from Dubare forests, Karnataka and cultured in basal medium [16]. The flasks inoculated with fungal mycelia were incubated at 30℃ (120 rpm) for 7 days. After four days of incubation 0.1 mM $CuSO_4$ and 2.5 mM p-anisidine (Sigma-Aldrich, USA) were added to the flasks, to induce laccase production. The cell free culture supernatant was used to measure laccase activity and the cells were collected on the day when maximum laccase activity was obtained in the culture supernatant, frozen in liquid nitrogen and stored

at -80℃ until further use. Laccase activity was monitored by the oxidation of ABTS [17]. One unit of laccase activity was expressed as change in absorbance of 0.001 min^{-1}.

2.2 RNA Isolation and cDNA Construction

Total RNA was extracted from the mycelium of NI-07 ,the wild *S. commune* strain, collected after six days of cultivation under submerged culture conditions (30℃, 120 rpm) in the production medium [18] using Nucleopore RNA Sure Plant Kit (Genetix) as per the manufacturer's instructions. 100 mg of tissue sample was homogenised in a DEPC treated, pre sterilized, pre cooled mortar and ground well. 350 µl of RLB1 buffer and 3.5 µl of β-mercapto ethanol were added to the lysate, transferred to a clean tube and vortexed. The lysate was then passed through a RNA Sure Shredder placed in a 2 ml clean tube and centrifuged (Thermo Fisher Scientific Sorvall ST 16R) to obtain a homogenous mixture. To the filtrate, 350 µl of ethanol (70%) was added and the whole revortexed. The mixture was then loaded onto a RNA Sure Plant Column, placed in a clean tube and centrifuged. All the centrifugation steps were carried out at 11,000×g for a duration of 1 min. The flow through was discarded, and 350 µl of desalting buffer (RDB) was added before centrifugation. The second flow through was again discarded and 95 µl of DNase (10 U/µl),in DNase reaction buffer was added carefully into the centre of the column and incubated at room temperature for 15 min. Guanidium thiocyanate buffer (200 µl) was added, centrifuged and the filtrate was discarded. Buffer (600 µl) reconstituted with ethanol (100%) was added to this and then re centrifuged for 3 min. The filtrate was discarded and the aforementioned step was repeated once again. The column was placed in a nuclease free 1.5 ml collection tube and RNA was eluted into 60 µl RNase free water. The concentration of RNA was recorded using a nanodrop spectrophotometer. All the isolation steps were carried out at 4℃. qPCR RT master mix with genomic DNA (g DNA) remover kit (Toyobo, Japan) was used for the construction of cDNA. To avoid the amplification of genomic DNA if present along with cDNA, genomic DNA degradation step using gDNA remover was performed. Total RNA was used directly as the template for cDNA preparation. The RNA solution was first incubated at 65^0 C for 5 min and then immediately placed in ice. The reaction mixture (8 µl) was prepared by adding 2 µl of 4XDN Master Mix (buffer solution containing RNase inhibitor), RNA template (2 µl, 20 µg) and nuclease free water (4 µl). This was incubated at 37^0 C for 5 min. To the reaction mixture 2 µl of 5 Master Mix (Reverse Transcriptase enzyme, RNase inhibitor, Oligo dT primers, Random primer and dNTPs) were added bringing up the final volume to 10 µl. The reaction mixture devoid of reverse transcriptase served as control. The mixture was then incubated under the following conditions: 37℃ for 15 min, 50℃ for 5 min and 98℃ for 5 min. The obtained cDNA preparation was stored at -20℃.

2.3 Isolation of Laccase Gene

All the PCR reactions were performed using Peqlab 96 Universal Gradient PEQ starthermocycler. The primers used in the present study were got synthesized from Eurofins (India). To amplify laccase gene from cDNA, different laccase specific degenerate primers Cu1F (CAY TGG CAY GGN TTY TTY CA), Cu2R (G RCT GTG GTA CCA GAA NGT NCC) and Cu3R (TG ICC RTG IAR RTG IAN IGG RTG) [9-14] as well as primers specifically designed for *S. commune* laccase gene [15] in the present study (SET1 F/R, SET2 F/R, SET3 F/R) (Table 1) were used. The primer pairs were used in combination and PCR conditions were optimized to amplify the produced complementary DNA. PCR master mix (Thermo Scientific) was used in all the PCR reactions. The reaction buffer (1X) contained Taq DNA Polymerase (Thermo Prime, 0.625 U), Tris-HCl (75 mM, pH 8.8 at 25℃), $(NH_4)_2SO_4$ (20 mM), $MgCl_2$, (1.5 mM, 0.01 % v/v), Tween 20 with 0.2 mM each of dATP, dCTP, dGTP and dTTP. The amplified products were loaded and visualized (Biorad Mini sub cell GT) on 1% agarose gels stained with ethidium bromide. Sample was loaded into each well by mixing 1 µl of loading buffer with every 5 µl of sample. After electrophoresis the gel images were captured using a Gel Doc (Biorad Gel Doc XJ equipped with Image Lab software).

2.4 Cloning PCR Products, Preparation and Transformation of Competent *E. coli* DH5α Cells

pGEM-T Easy vector system (Promega) was used for cloning the gel purified (Appendix-I) PCR product. The pGEM-T Easy vector and control insert DNA were briefly centrifuged to collect the contents sedimenting at the bottom of the tube. The size of the vector was 3015 bp. The ligation mixture was prepared by adding 5 µl

Table 1. List of laccase specific degenerate primer pairs and primers designed for isolation of laccase gene laccase gene from basidiomycete *Schizophyllum commune* coding sequence

Primer name	Primer sequence 5'→ 3'	References
Laccase specific degenerate primers		
Cu1F	CAY TGG CAY GGN TTY TTY CA	7,8,9
Cu1AF	ACM WCB GTY CAY TGG CAY GG	10,11,12
Cu2R	G RCT GTG GTA CCA GAA NGT NCC	7,8,9,10,11,12
Cu3R	TG ICC RTG IAR RTG IAN IGG RTG	10
Primers designed in the present study		
SET 1 F	5'GCG AAT TCA TGG CAG CGC TGC TGG3'	
SET 1 R	5'AGC GGC CGC TCA CAA ATA CTG GTC A3'	
SET 2 F	5'GCG CAT CCA TGG TCT CCC CAT TT3'	
SET 2 R	5'AGC GGC CGC TCA CAA GAA CGT GTT3'	
SET 3 F	5'GCG AAT TCA TGC GTG TCT CAT TG3'	
SET 3 R	5'AGC GGC CGC CTA CAT CAT CAG AC3'	
CDS* F	5' GCG GAATTC ATG CGC GTC TCA TTG ATC ATA G 3'	
CDS R	5' GCG GCGGCCGC CTA GTC AAC GAG TCC ACC CAA 3'	
Oligo dT	TTTTTTTTTTTTTTTTTTTTGACC	

CDS: Coding sequence; F-Forward/Sense primer; R-Reverse/Antisense primer

of 2X Rapid ligation buffer, 1µl of pGEM-T vector (50 ng), 2 µl PCR product, 1µl T_4 DNA ligase and deionised water to a final volume of 10 µl. Ligation mixture without the PCR product served as a background control. The reaction mixture was mixed thoroughly by pipetting and was incubated overnight at 4℃.

A single bacterial (*E. coli* DH5α) colony (2-3 mm in diameter) was picked up from the LB agar plate that had been incubated for 16-20 hours at 37℃. It was then transferred into 25 ml of LB broth in a 250 ml flask. The culture was incubated for 6-8 hours at 37℃ under vigorous shaking (250-300 rpm). This starter culture was used to inoculate three 1 litre flasks, each containing 250 ml of medium (Appendix-I). To the first flask 10 ml, to the second flask 4 ml and to the third flask 2 ml of starter culture was added and the flasks incubated overnight at 18-22℃ under moderate shaking (150-200 rpm). The culture flask attaining an OD of 0.55 (600 nm), was transferred onto an ice bath. The cells were harvested by centrifugation (2500×g, 10 min, 4° C) and gently resuspended by swirling in 80 ml of ice-cold Inoue transformation buffer (prepared by dissolving $MnCl2.4H_20$ (10.88 g, 55 mM); $CaCl_2.2H_20$ (2.20g, 15 mM); KCl (18.65 g, 250 mM) and PIPES (0.5 M, pH 6.78, 20 ml, 10 mM in 1000 ml of pure water). The cells were re - harvested by centrifugation (2500×g, 10 min, 4° C). The supernatant was discarded completely and cells were resuspended in 20 ml of ice-cold Inoue transformation buffer and 1.5 ml of DMSO (dimethyl sulfoxide) was added. The bacterial suspension was mixed by swirling and stored on ice. Immediately 50 µl aliquots of the suspension were dispensed into chilled, sterile micro centrifuge tubes and stored at -80℃.

A tube containing competent *E. coli* DH5α cells was removed from storage in a -70℃ freezer and thawed on ice. Five µl of transforming DNA was added to the tube using a chilled sterile pipette tip. The mixture was kept on ice for 1 h. The tube was then transferred to a pre heated block (Dry Bath, AccuBlock Digital Dry, Lab net) maintained consistently at 42℃. Heat shock treatment was given exactly for 45 sec after which the tube was immediately transferred to an ice bath and cooled for 10 min. 450 µl of SOC medium (Appendix- I) was then added to the tube and incubated for 1 h at 37℃ (200 rpm). After incubation the tube was centrifuged for 5 min (8000 rpm) (Spinwin, Tarsons, MC-00/MC-02) and 400 µl of the media supernatant was carefully aspirated. The remaining 100 µl of transformed cells were gently mixed and each of the 50 µl aliquots was spread onto LB/Ampicillin/IPTG/X-Gal plates using a sterile L-shaped bent rod and incubated overnight at 37℃.

Single colonies from transformed plates were picked by touching sterile tooth picks on to the colony and scraping it at the bottom of a 1.5 ml sterile tube. This tube was used to make glycerol stocks of the clones. The same tooth pick was then dipped into 20 µl PCR reaction mix containing Dream taq master mix (10 µl), forward and reverse primers (1 µl each) and nuclease free water (8 µl) and PCR performed. The

touchdown PCR conditions used for amplifying the PCR product cloned into *E. coli* DH5α cells were: 94℃ for 10 min (to disrupt the cells and release DNA) followed by10 cycles of denaturation (94℃, 45 sec), annealing (58-49℃, 1 min/decrease in 1℃/cycle) and elongation (72 ^{0}C, 2 min) and 30 cycles of denaturation (94℃, 45 sec), annealing (54℃, 1 min) and elongation (72℃, 2 min) and a final extension for 10 min at 72℃.

2.5 Plasmid Isolation

Transformed *E. coli* DH5α cell plasmid was isolated using Gene JET plasmid miniprep kit (Thermo Scientific, USA). A single colony was picked from the transformed *E. coli* cells on LB/Ampicillin/IPTG/X-Gal plate and incubated for 6-8 h in LB/Ampicillin broth at 37℃ (160 rpm). Cells were harvested by centrifugation at 8000 rpm for 2 min and the supernatant was discarded. All the isolation steps were carried out at room temperature. The pellet was resuspended in 250 µl of resuspension solution containing RNase A and the cell lumps were dissolved completely by vortexing. To this mixture 250 µl of lysis buffer was added, mixed thoroughly by inverting the tube 4-6 times until the solution became viscous and slightly clear. Immediately 350 µl of neutralization solution was added into the same tube and mixed thoroughly and gently. The neutralized bacterial lysate became translucent. It was then centrifuged for 5 min, to pellet cell debris and chromosomal DNA. All the centrifugation steps were performed at 13,000×g. The supernatant was carefully transferred to the Gene JET Spin column and centrifuged. The flow through was discarded and the column was placed on to the same collection tube. To the spin column, 500 µl of wash solution was added, centrifuged (1 min) and the flow through was discarded. The wash procedure was repeated twice to remove the residual wash solution. The Gene JET spin column was transferred to a fresh 1.5 ml micro centrifuge tube and plasmid DNA was eluted by adding 50 µl of elution buffer into the centre of the column membrane and centrifuged. The plasmid DNA was stored at -20℃.

3. RESULTS AND DISCUSSION

3.1 Organism, Culture Conditions and Laccase Activity Measurements

S. commune a dikaryon exhibits vegetative growth. Isolated NI-07 strain was a sole producer of laccase, secreting only this enzyme into the culture medium while no other ligninolytic enzymes were detected. The basal medium after fermentation showed a bluish tinge on the mycelial inoculum disc and a change in colour of the media to yellowish green was observed with progress in culture duration (Fig. 1a and b). Highest laccase activity was obtained on the seventh day in the cell free culture supernatant as indicated by ABTS assay. The maximum activity of 737.78±42.1 Uml^{-1} in submerged fermentation was obtained on day 7 followed by 723.32±13.6 Uml^{-1} on day 6.

Fig. 1. *Schizophyllum commune* NI-07 strain in submerged cultivation (a) 5 days (b) 7 days

3.2 RNA Isolation and cDNA Construction

Laccases are blue copper oxidase enzymes which catalyse the oxidation of both phenolic and non phenolic lignin related compounds with the concomitant reduction of molecular oxygen to water. They are comprised of four regions which bind the copper atoms of the active centre. These copper binding regions are known to be strongly conserved in all laccases. As induction of laccase synthesis by copper is known to act on the gene transcription levels [19], copper sulphate was added to the production medium during fermentation.

RNA was isolated from *Schizophyllum commune* NI-07 strain after six days of cultivation and the quality of RNA obtained was good with 28S and 18S subunits being quite prominent after electrophoresis on agarose gel (Fig. 2). The A_{250} values of RNA extracted from the cells were measured using a nanodrop. Total RNA was directly used as a template for cDNA construction. RNA solution was first incubated at 65℃ for 5 min and then kept on ice, a step known to increase the efficiency of reverse transcriptase of RNA templates that form secondary structures.

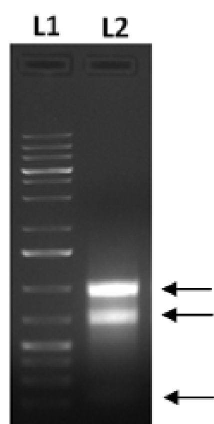

Fig. 2. RNA sample electrophoresed on 1% agarose gel (RNA isolated from *S. commune* NI-07 strain). Lane 1: Molecular weight marker (Gene Ruler, 1Kb plus DNA ladder) (0.5 µg/lane). Lane 2: RNA sample. Arrows read from top to bottom indicate 28S, 18S and 5.8S subunits respectively

3.3 Laccase Gene Isolation

Laccase specific gene sequences from mRNA transcripts of *Schizophyllum commune* NI-07 strain were isolated using cDNA as a template. The degenerate primer pair Cu1F/Cu2R is known to be specific for basidiomycetes and amplifies fragments of around 200 bp and 142 bp for laccase fragments between the copper binding regions cbr1 and cbrII [10]. In addition primer pairs (SET 1, 2 and 3 F/R) were specifically designed in the present study for amplifying laccase gene sequences from the wild *S. commune* isolate [16]. A complete coding sequence for laccase from *S. commune* available in the databases was used as a reference standard to design the primers using Primer 3 software.

cDNA corresponding to mRNA transcripts used as a template in the PCR reactions was amplified using the degenerate primer pair Cu1F/Cu2R [10]. The PCR primer set Cu1F/Cu2R targets the two conserved copper binding domains CuI and CuII. In all the reactions, 40 ng of cDNA was used as it is known that 20 ng of cDNA is sufficient for a 25 µl reaction. Different concentrations of cDNA (40, 100, 250, 500, 1000 and 1500 ng) however, were also used to check whether the concentration of cDNA influenced amplification efficiency. For this 50 µl reaction was prepared (Master mix-25 µl, FP-1 µl, RP-1 µl, Template DNA- 0.4 µl (40 ng) and volume was made up with nuclease free water. As expected the amplicon size between the copper binding domains Cu1F and Cu2R could not be detected (data not shown).

Performance of PCR reaction is affected by several factors and primer pair combinations. Magnesium as a cofactor for polymerase enzyme is known to affect the primer annealing in PCR. Hence $MgCl_2$ concentrations were varied in the reaction mix. PCR reactions contained 1 µl of cDNA, 10XTaq buffer (5 µl), dNTPs (4 µl), forward and reverse primers (1 µl each), Taq DNA polymerase (0.2 µl) and $MgCl_2$ in a reaction mix. $MgCl_2$ (25 mM) was prepared and added so as to obtain final concentration s of 2, 2.5, 3, 3.5 and 4 mM. Increase in Magnesium concentration also did not influence amplification efficiency (data not shown).

Enhancers are known to improve the efficiency of amplification. Hence in the present study three different enhancers DMSO (5%), glycerol (20%) and PEG (5%) were used in the PCR reaction in place of nuclease free water. Though a smear was observed when PEG was used, there was no clear amplification. To check if PEG contributed to clear band intensity, different concentrations of PEG (40%) were used in the PCR reaction so that the final PEG concentration was 5, 10 and 15%. The addition of enhancers into the reaction also failed to elicit any amplification with little or no decrease in background noise (data not shown). The smear in the gel was observed even after different concentrations were tested. Moreover, no linear increase in amplification intensity was observed with increase in the concentration of enhancers.

Independent PCR reactions were set using annealing temperature as 40°, 52°, 54℃ etc. As no amplification was observed and the primer set was a standard degenerate one, gradient PCR was performed with the following program: 95℃ for 2 min with 35 cycles of denaturation, annealing and elongation (95℃ for 30 sec, gradient 55.0℃±5.0℃, 72℃ for 1 min) with a final extension for 5 min at 72℃. However gel electrophoresis of the PCR product failed to show any amplification. The T_m of Cu1F was 48.1℃ and Cu2R was 56.9℃. Since there was a variation in the Tm by >3℃, touchdown PCR was adopted. The Touchdown PCR was performed using the following program: 95℃ for 2 min followed by 16 cycles of denaturation, annealing and elongation (95℃ for 30 sec, 61℃

for 30 sec with a decrease of 0.5℃/cycle, 72℃ for 60 sec) and again 19 cycles of denaturation, annealing and elongation (95℃ for 30 sec, 53℃ for 30 sec and 72℃ for 60 sec) with a final extension of 5 min at 72℃.

However, as expected the changes made in the PCR program using these primer sets, did not yield any amplification of cDNA. Different enhancers and metal ions used to fine tune the amplification process also failed to yield positive results as expected. No PCR product was detected with RNA extracts when Cu1F/Cu2R degenerate primer pair was used. The same trend was exhibited when *S. commune* laccase specific primers designed in the present study were used. Additional PCR reactions were performed using variations in the T_m values as well as the number of cycles in touch down PCR under various conditions. However there was no proper amplification using these set of primers indicating a loss of specificity in the PCR. All these permutations and combinations failed to amplify the laccase gene.

Consequently, primer pair Cu1F/Cu3R which is specific for the conserved copper binding regions of CuI and CuIII was used to amplify laccase gene using cDNA as the template by touchdown PCR. A 20 µl PCR reaction was prepared using PCR master mix (10 µl), forward and reverse primer (1 µl each), template DNA (3 µl) and nuclease free water (5 µl). Cu3R is more degenerate as compared to Cu2R. A faint band was observed when Cu1F/Cu3R degenerate primer pair along with the same touchdown PCR protocol was used giving a positive signal. The annealing temperature was optimized for each experiment with the respective set of primers so that bands appeared clearly without much noise. A fragment of 1000 bp was visualized (Fig. 3.) when PCR conditions were modified and the annealing temperatures were varied from 61 to 48℃. These PCR conditions used were: 94℃ for 3 min followed by10 cycles of denaturation (94℃, 45 sec), annealing (61-53/58-54/56-48/58-49℃, 1 min/decrease in 1℃/cycle) and elongation (72℃, 2 min) and 30 cycles of denaturation (94℃, 45 sec), annealing (54℃, 1 min) and elongation (72℃, 2 min) and a final extension for 10 min at 72℃. The band so obtained was in agreement with the expected fragment size between Cu1F and Cu3R primer pair when electrophoresed on 1% agarose gel.

The laccase encoding gene was isolated from the aquatic fungus *Myrioconium* sp. UHH 1-13-18-4 using Cu1F/ Cu3R primer pair [20]. In our study degenerate primers Cu1F and Cu3R, designed based on amino acid and nucleotide sequences [12] showed amplification of a 1000 bp product along with a few other nonspecific bands when touchdown PCR was used.

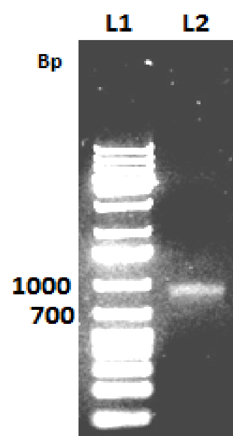

Fig. 3. Amplified PCR band using the primers Cu1F and Cu3R on 1% agarose gel. L1- Gene ruler 1kb plus DNA Ladder, L2- PCR product (2 µl) showing amplification of a 1000 bp insert in *E. coli* DH5α cells

3.4 Preparation and Transformation of Competent *E.coli* DH5α Cells

The 1000 bp gel purified PCR product was cloned into pGEM-T Easy vector for sequencing. Appearance of clones (Fig. 4.) confirmed the efficiency of transformation when PCR product with the pGEM-T vector was ligated and transformed into *E. coli* DH5α chemically competent cells. From the transformed plates, ten white colonies were picked and colony PCR was performed. Colony PCR confirmed the presence of insert in all the selected clones (data not shown).

3.5 Plasmid Isolation and Sequencing

Two clones containing the inserted fragments were isolated and verified by DNA sequencing (ABI 3100 sequencer, Applied Biosystems, USA). The sequencing results revealed that both the clones contained the same sequence of putative phosphatases somehow involved in cell cycle with very high identity match with *Schizophyllum commune.* The nucleotide sequence has been submitted in GenBank (GenBank accession number MG214559).

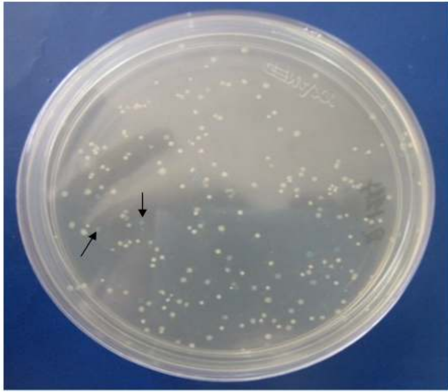

Fig. 4. Plate showing Blue/white colonies on LB agar/Ampicillin/X-Gal/IPTG plates. arrows indicate blue colonies

It is a well known fact that amplification using high degeneracy primers leads to the formation of many nonspecific PCR products. Till date, laccase gene employing these degenerate primers has not been isolated from *S.commune,* and our finding is in agreement with earlier reports which stated that laccase specific universal primers amplified laccase gene from almost all basidiomycetes isolated by them [10, 11] but failed to amplify laccase gene of *S. commune*. On account of the high degeneracy in laccase specific gene sequences, universal primers failed in isolating *S. commune* laccase gene as was also the case in the current study. Unlike the high activity obtained in the NI-07 strain , laccase activity measurements performed for the *E.coli* clone in LB broth recorded almost undetectable amount of laccase (12.22±1.5 Uml^{-1}), reinforcing the claim that the primers did not actually amplify laccase gene.

Primer pairs based on *S. commune* coding sequences were also designed for the present study. Even these primers failed to amplify the laccase gene. Though PCR product was formed when the Cu1F/Cu3R primer pair was used, this did not match with the laccase transcripts. Thus the primers designed for amplification of laccase specific gene sequences are not universal for all the white rot species isolated from different geographical regions. Moreover data available on laccase sequences from *Schizophyllum commune* is very limited. Use of different enhancers, metal ions also failed to contribute towards significant changes in the amplification of laccase gene . Further studies are needed to gain in depth knowledge regarding the precise set of degenerate primers which can amplify different laccase genes from *Schizophyllum commune* or for successful heterologous expression, a codon optimized synthetic variant of *Schizophyllum commune* laccase gene may have to be adopted. The results of the current study are certainly meant to serve as a practical guide for researchers and to aid them in addressing issues concerning amplification of transcripts for this interesting and biotechnologically important enzyme.

4. CONCLUSION

An attempt was made in the present study to isolate laccase gene from a wild new strain of *Schizophyllum commune* using laccase specific universal degenerate primers. Earlier many researchers had attempted to amplify this gene using available degenerate primer pairs which was true in case of most basidiomycetous laccases but not for *S. commune*. Primer pairs designed in the present study for amplifying laccase specific *S.commune* sequences also failed to contribute towards gene isolation. A putative 1000 bp transcript obtained using Cu1F/Cu3R primer pair revealed 100% identity to *S. commune* phosphatses involved in cell cycle. This clearly indicates the vast diversity of *S. commune* laccase genes in the environment which are virtually undescribed. The degenerate primers available have limitations for their use in isolation of laccase genes from all the genera. Different splicing signals of complementary DNA may also contribute to these undefined results. Thus it can safely be concluded that this genus is not well characterized and research on amplification of laccase transcripts from *Schizophyllum commune* is not well addressed. These findings highlight the complexities involved in the use of gene specific primers in isolation of laccase gene from *S. commune* and provide a handy practical guide for researchers concerned with addressing amplification of laccase gene transcripts from this not so well characterized genus.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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APPENDIX I

Gel Purification Protocol

Gel was extracted using Gene JET gel extraction kit (ThemoScientific). All purification steps were carried out at room temperature. The gel slice containing the expected DNA fragment was excised using a clean scalpel and then placed into a pre-weighed 1.5 ml tube. The weight of the gel slice was recorded. Equal volume (1:1 v/w) of binding buffer was added to the gel slice. The gel mixture was incubated at 60℃ for 10 min, so that the gel slice wa s completely dissolved. The gel mixture was then vortexed and loaded on to Gene JET purification column and centrifuged at 13,000×g for 1 min. The flow through was discarded and the column was placed into the same collection tube. To the column 700 µl of wash buffer (diluted with ethanol) was added, and centrifuged for 2 min to remove the residual wash buffer completely. The Gene JET purification column was then transferred to a clean 1.5 ml micro centrifuge tube. 50 µl of elution buffer was added to the column and centrifuged for 1 min to elute the DNA. The Gene JET purification column was discarded and the purified DNA was stored at -20℃.

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