

Improvement of Laccase Production in *Pleurotus pulmonarius*-LAU 09 by Mutation

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Abstract The strain improvement of *P. pulmonarius* LAU 09 for laccase production was carried out by UV- light exposure at 210nm (UV-sterilizer, Millipore xx63 70000) for 90minutes. The highest laccase activity of 2.5 Uml-1 was produced by LAU 90 (mutant) in comparison with obtained yield by wild type LAU 09 (1.75 Uml-1). The optimal physiological parameters for optimum laccase production by LAU 90 are 30OC of temperature, pH of 5.0 and incubation of 168hours. The mutant strain gave the highest total enzyme activity and enzyme specific activity of 71.7 x 10-4 mol/min and 89.63X 10-4 IU/mg of purified laccase enzyme respectively, with molecular weight of 97KDa. The activity of the purified enzyme was strongly inhibited by Sodium azide and EDTA at 1ml/0.1mM and 0.5ml/100mM respectively. The result obtained shown an improved performance of the mutant (LAU 90) strain of *P. pulmonarius* in laccase production over the wild (LAU 09) type.

Keywords Strain, Improvement, Mutation, Laccase, Enzyme

1. Introduction

Laccases are multinuclear copper-containing glycoproteins that belong to the family of enzymes known as oxidases, more specifically “blue” oxidases[25], and polyphenol oxidases[8]. Laccases are one of six enzyme classes capable of reducing dioxygen to water, five of which belong to the multicopper oxidase family (the only enzyme not in this class being cytochrome-c oxidase, a heme/copper containing enzyme). Laccase is a polyphenol oxidase, indicative of the fact that laccases can oxidize a phenolic substrate that in turn can initiate a polymerization reaction[8]. Laccases from various sources vary greatly with respect to their degree of glycosylation, molecular weight and kinetic properties[25].

Basidiomycetes fungi especially *Pleurotus* species are the most efficient lignin-degrading organisms that produce mainly laccases (EC 1.10.3.2), lignin peroxidase (EC 1.11.10.14) and manganese peroxidase (EC1.11.1.13). These enzymes present a non-specific biocatalyst mechanism and have been used for bioremediation process due to their ability to degrade azo, heterocyclic, reactive and polymeric dyes[1, 6]. Prospection for fungi is the ability to secrete high levels of lignin-degrading enzymes and novel enzyme variants, with desirable properties for biotechnological applications. On the other hand, alternative low cost substrates like agricultural residues for enzyme production using solid

state fermentation (SSF) offer economic and environmental advantages. The main technological applications of laccases are in the textile, dye or printing industries, in processes related to decolouration of dyes[4], in the pulp and paper industries for the delignification of woody fibres, particularly during the bleaching process[3,13]. In most of these applications, laccases are used together with a redox mediator. At the end of 2005 three industrial processes were using laccases: dye bleaching, lignin bleaching and bleaching of cork for bottled wine[19].

Laccases exhibit a broad natural substrate range, which is a major reason for the attractiveness of laccases to biotechnological applications[16]. Even more interesting however is the application of laccase activity to a broader substrate range through the secondary activity of the free (cation) radical formed by oxidation of its substrates. The substrates that result in this type of activity towards other compounds are termed mediators. The industrial applicability of laccase may therefore be extended by the use of a laccase-mediator system. In view of the importance of laccase mentioned above, the improve production of the enzyme is inevitable.

Ultra violent radiation (UV- light) has been reported as one of the best physical method of strains improvement for better yield performance[10]. This method has been employed in improving enzyme production in *Aspergillus niger*[10], *Rhizopus oryzae*[22], mycelia cell and sporophore production in *P. florida* and *P. sajor-caju*[20]. However, no report is available on the improvement of laccase production by *P. pulmonarius* using UV- light radiation exposure.

The present investigation was undertaken to improve the laccase production by *P. pulmonarius* - LAU 09 strain

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through exposure to UV-light radiation.

2. Material and Methods

Mutation Induction

The actively growing culture (7 days old) of *P. pulmonarius* LAU 09 on PDA plates (90mm) were exposed to UV-light (210 nm, Millipore xx63 70000) for 90mins. The mutants were subcultured on the PDA with 5% of yeast extract agar (YEA), incubated at 25°C for 7days.

Enzyme Assays

The strains, both LAU 09 (wild) and LAU 90 (mutant) were grown in PDB with 5% of yeast extract (YE). The experiments were carried out in 250ml Erlenmeyer flask with 50ml of substrate, inoculated with a plug (6mm), incubated at 25°C for 5days at 150rpm. The culture were sieved (Whatman paper II), centrifuged and filtrate were used as crude enzymes. Laccase activity was determined via the oxidation of 2, 2-azino-bis (3-ethylbenzthiazoline)- 6-sulfonate (ABTS) (Sigma, America). The reaction mixture containing 0.1ml of 0.3mM ABTS in 100mM of Sodium acetate (pH 3.5) and 0.1ml of crude enzyme solution was incubated at 40°C for 1min. The ABTS oxidation was monitored by the increased in absorbance at 420nm ($\epsilon = 36000\text{M}^{-1}\text{cm}^{-1}$). One unit was defined as 1 μmol of ABTS oxidized per minute and activity was expressed in Uper ML per min[15].

Effect of temperature on enzymes production

The optima temperature for laccase enzymes for LAU 09 and LAU 90 strains were evaluated by incubating the enzymes at different temperature ranges (25°C, 30°C, 35°C, 40°C, 45°C and 50°C) with appropriate substrates and buffers as described above.

Effect of pH on enzymes production

The optima pH for laccase was determined by incubating the enzyme with their appropriate substrates at their optimum temperature using McIlvaine's method[17].

Effect of incubation period on enzymes production

The optimum incubation period for laccase production was determined by incubating the enzyme with their appropriate substrate at their optimum temperature for 9days[17].

Acetone precipitation

The crude enzymes (20% w/v), 10,000g supernatant was subjected to 80% acetone precipitation at -4°C. The contents were then placed in ice for an hour and then centrifuged at 10,000g for 30 min at -4°C. The pellet was re-dissolved in 0.1 M glycine-NaOH, pH 10.0.

Ion exchange column chromatography

Aliquots (3 mL) of the acetone precipitated proteins were applied to a column of Sephadex A-50 ion exchange resin (30 X 0.8 cm) equilibrated with 0.1 M citrate buffer, pH 5. The protein sample volume was adjusted to 10% of the column bed volume. The ion exchange

Chromatography of the proteins was carried out at room temperature (35°C). Following sample application, the column was washed with the equilibrating buffer, until the absorbance of the wash at A280 was <0.05. The adsorbed

proteins were then eluted using a linear gradient of NaCl (0–1.0 M) in 0.1 M glycine-NaOH buffer, pH 10.0 at a flow rate of 0.9 mL/min. Fractions of 2mL were collected manually. Protein content of each fraction was evaluated at A280 employing a UV-vis double beam spectrophotometer (Spectra scan UV 2700: Thermo Scientific). Laccase activity in the fractions was determined using the enzyme assay as described above.

Sephadex G-100 gel permeation column chromatography

Sephadex G-100 was allowed to swell in 0.1 M glycine-NaOH buffer, pH 10.0 for 72 h at room temperature and then packed on to a glass column (30 X 0.8 cm) with a flow rate of 0.9 mL/min. Active fractions of ion exchange chromatography were loaded onto the column. Fractions of 2mL were collected and the absorbance in each fraction was measured at 280 and 469 nm for the enzyme activity. The molecular weight was determined using 10% sodium dodecyl sulfate – polyacryl-amide gel electrophoresis (SDS-PAGE).

Determination of protein concentration

The concentration of protein was determined by Lowry's Method[14].

Laccase enzyme inhibitor Studies

The effects of Sodium azide and EDTA on purified enzyme activity were tested using ABTS as substrate after pre-incubating the enzyme for 10mins at 25°C with inhibitors before addition of substrate[21].

SDS-PAGE

Molecular weight of the purified enzyme was determined under the reducing condition of the SDS-PAGE according to Laemmli method[12].

DNA extraction and ITS amplification

Mycelia were grown on potato dextrose agar, harvested using a scalpel, transferred into Eppendorf tubes, small amount of autoclaved refined sand (Sigma) was added and ground to fine paste with pestle-like stick (High Media), 400 μl of DNA Extraction buffer pH 8 (1M Tris-Cl pH 8.0; 1M NaCl; 200mM EDTA pH 8.0; 10%SDS; 0.1% β - Mercaptoethanol) was added and centrifuged at 4°C (12000g) for 10mins. To the collected supernatant 300 μl Phenol and 300 μl Chloroform: Isoamylalcohol (24:1) were added and mixed gently. This was centrifuged (12000g, 4°C for 10mins), and aqueous phase was collected and 500 μl chilled Isopropanol was added and incubated at -20°C overnight. After the incubation, it was centrifuged (12000g, 4°C for 10mins), and the pellet was washed with chilled 70% ethanol centrifuged for 5mins. The dried pellet was resuspended in 50 μl of Tris EDTA (10Mm Tris and 1mM EDTA, pH 8.0) buffer.

Amplification of the ITS region of the rRNA gene was carried out with a modified method of Gerdes and Bruns[7], using primers ITS1-F and ITS4-B. The final concentration of 25 μl PCR reaction volume were; 200 μM each of dATP, dCTP, dGTP and dTTP, 2.5mM MgCl₂, 10X Taq.DNA Polymerase and 20Pico mole of each of the two primers (Banglore Genei). The PCR profiles were initial denaturation step of 94°C for 85s followed by 25 amplification cycles of

denaturation, annealing and extension. The temperature and times for these steps were 95°C for 35s, 55°C and 55s and 72°C for 2mins with further incubation at 72°C for 10mins. The amplified PCR products were resolved on a 1.2% agarose gel, and stained with Ethidium bromide. A 1kb ladder DNA marker (GeneRuler™) was used as a size standard.

Sequencing and Phylogenetic Analysis

The PCR products were purified using Exonuclease I and Shrimp Alkaline phosphatase in buffer (EXOSAP Kits). Both strands of the amplified region were sequenced using fluorescent dye terminator chemistry and were run on ABI 3130 (4 capillary) or 3730XI (96 capillary) Automated Sequencer (Perkin Elmer Applied Biosystems, Foster City, CA), following the manufacturer's protocols. Sequencing primers were ITS1-F, 5.8S, 5.8SR and ITS4-B. Oligonucleotide sequences for primers 5.8S and 5.8SR were given in Vilgaly and Hester[23]. Sequence contigs were assembled and edited using Sequencer 3.0 software (Gene codes Corporation, Ann Arbor, MI).

Phylogenetic trees were constructed by using all cloned sequences together with all non redundant large subunit (nLSU) sequences of named *Pleurotus* species obtained from GenBank. The multiple alignments of all the sequences were performed using CLUSTAL W (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>), followed by manual adjustments. The phylogenetic analyses was carried out using sequence data of ITS 5.8s and 28s ribosomal RNA gene from LAU 09 (wild) of *P. pulmonarius* and corresponding GenBank data of related species. The identical sequences were merged into one input sequence when running the computer programs to generate the phylogenetic trees constructed by UPGMA, Neighbor-joining (NJ) and parsimony methods. The bootstrap test for estimating the reliability of phylogenetic tree topology was performed using 100 replications by the SEQBOOT program[5]. The consensus tree was obtained by running the consense program[5].

3. Results and Discussion

The laccase enzyme activity of the wild and mutant strains of *P. pulmonarius* was shown (Figure 1), with highest yield (2.5 Uml⁻¹) obtained by LAU 90 (mutant) comparing with (1.75 Uml⁻¹) obtained by LAU 09 (wild). The results obtained by the LAU 90 strain have shown an improved enzymes production over wild type (LAU 09).

The highest production of laccase, enzyme could be as result of efficient lignin-degrading ability of *P. pulmonarius*. Basidiomycetes fungi are the most efficient lignin-degrading organisms that produce mainly laccases, manganese peroxidase, and lignin peroxidase[9]. These enzymes present a non-specific biocatalyst mechanism and have been used for bioremediation process due to their ability to degrade azo, heterocyclic, reactive and polymeric dyes[1,6]. The increased yield in laccase production by LAU 90 is an evidence of true strain improvement. This correlate with work of Kang et al[10], who reported an improving enzyme pro-

duction in *Aspergillus niger* through UV-light mutation, *Rhizopus oryzae*[22], mycelia cell and sporophore production in *P. florida* and *P. sajor-caju*[20].

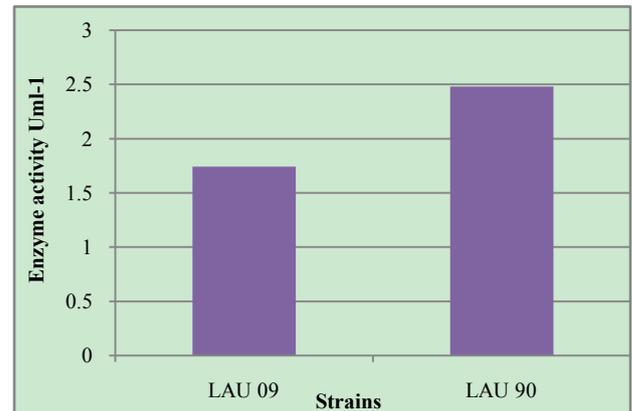


Figure 1. Laccase activity of wild and mutant strains of *Pleurotus pulmonarius*

The production of laccase enzyme was significantly increased in mutant (LAU 90) and of *P. pulmonarius* in comparison to their wild type (LAU 09) (Figure 2). All the evaluated temperature ranges (25°C- 50°C) supported the laccase production satisfactorily, with highest yield at 30°C for both mutant and hybrid strains. The optimum temperature obtained for laccase production by mutant and hybrid strains of *P. pulmonarius* is 30°C whereas Okamoto et al[17] reported 50°C as active optimum temperature for laccase production from *P. ostreatus*. Gomes et al[9] reported the optimum temperature for laccase production by the following organisms; *Lentinus strigellus* (55-60°C), *Picnoporus sanguineus* (60-70°C), *Phenllinus rimosus* (60°C).

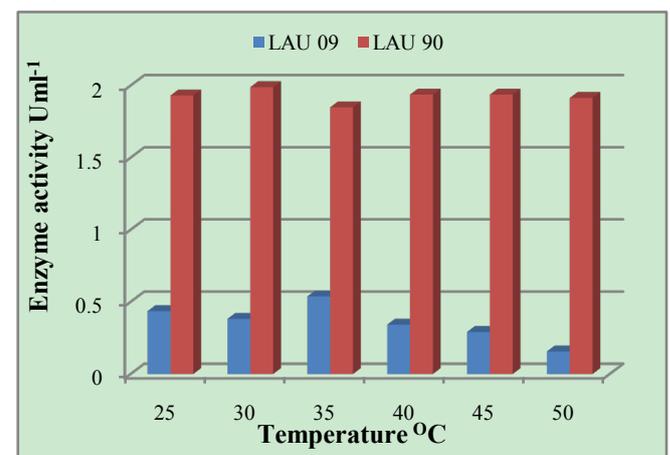


Figure 2. Effect of temperature on laccase activity of wild and mutant strains of *P. pulmonarius*

The optimum pH for laccase enzyme for mutant strain of *P. pulmonarius* is pH 5, due to highest yield of laccase enzyme obtained at pH 5 for mutant strain (Figure 3). The sharp decreases in the yield of laccase were observed in both wild and mutant strains above pH 5. In the present study pH5 was obtained as optimum pH for laccase production while Okamoto et al[17] reported pH6 as optimum pH value for laccase production by *P. ostreatus*.

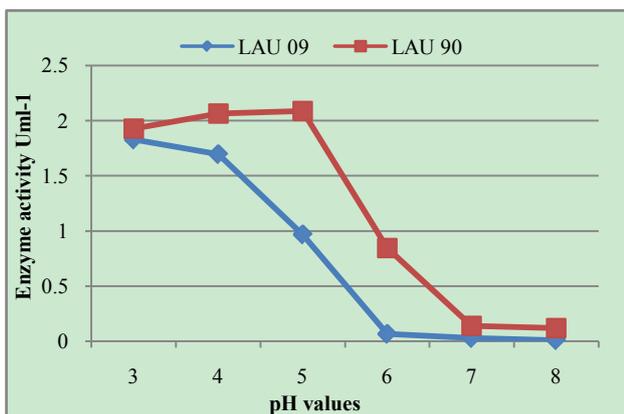


Figure 3. Effect of pH on laccase activity of wild and mutant strains of *P. pulmonarius*

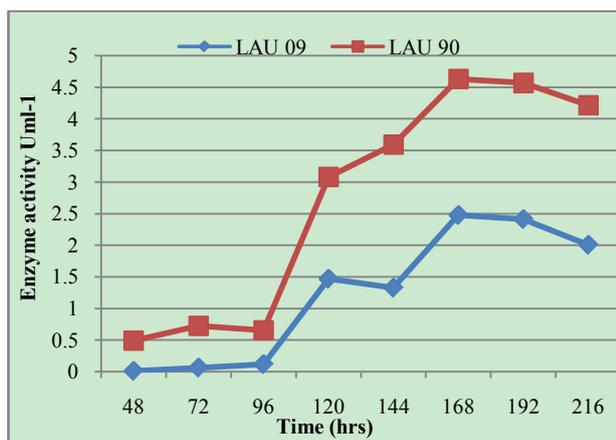


Figure 4. Effect of incubation period on laccase activity of wild and mutant strains of *P. pulmonarius*

The mutant (LAU 90) strain of *P. pulmonarius* has shown an improved yield of laccase enzyme production over the wild types (LAU 09) (Figure 4).

The optimum incubation period for laccase by LAU 90 and LAU 09 were obtained at 144hrs and 168hrs respective, while the laccase yield reduced below or after these incubation periods. The optimal condition for laccase production might be as a result of exponential growth of the organisms which has previously determined[26].

The purification fold, total enzyme activity, protein concentration and specific enzyme activity of purified laccase were shown Table 1. The acetone precipitation at single fold of enzyme purification, with highest total enzyme activity (1640×10^{-4} IU/mg) obtained by LAU 90, while the wild strain (LAU 09) produced 360×10^{-4} IU/mg. The result obtained in this study is higher than that of Kumar and Sri-kumar[11], which reported 2.8×10^{-4} IU/ml and 5.6×10^{-4} IU/ml for laccase enzyme activity and specific activity respectively from *Opuntia vulgaris*. The highest enzyme activity and specific activity obtained in this study may be as result of acquired potential by mutant (LAU 90) strain of *P. pulmonarius* for increased enzyme production. As the enzyme becomes more purified at step two and three purification methods (Ion exchange Sephadex A-50 and Gel filtration Sephadex G-100 chromatograph), the total enzyme

activity decreased, while specific enzyme activity increased accordingly (Table 1). The specific enzyme activity obtained in LAU 90 (mutant) is higher than that of LAU 09 (wild type), which implied that the purified laccase of mutant strain might be more active than that of wild type strain. Kumar and Sri-kumar[11] reported a purified laccase at purification folds of 11.6 and 11.9 with yields of 18.6 and 15.5% respectively, using sephadex G-100 gel permeation chromatograph.

Table 1. Purification fold of laccase enzyme

Steps	Total enzyme activity	protein conc (mg)	Specific activity (IU/ mg) $\times 10^{-4}$	purification fold
	(mol/min) $\times 10^{-4}$			

Acetone

Precipitation				
LAU 09	360.3	116.20	3.10	1
LAU 90	1640.0	230.0	7.13	1
Ion exchange Chromatograph (Sephadex A-50)				
LAU 09	209.3	5.6	37.38	17
LAU 90	352.0	8.4	41.9	5.0
Gel filtration Chromatograph (Sephadex G-100)				
LAU 09 (97KDa)	50.6	0.6	84.33	7.5
LAU 90 (97KDa)	71.7	0.8	89.63	8.0

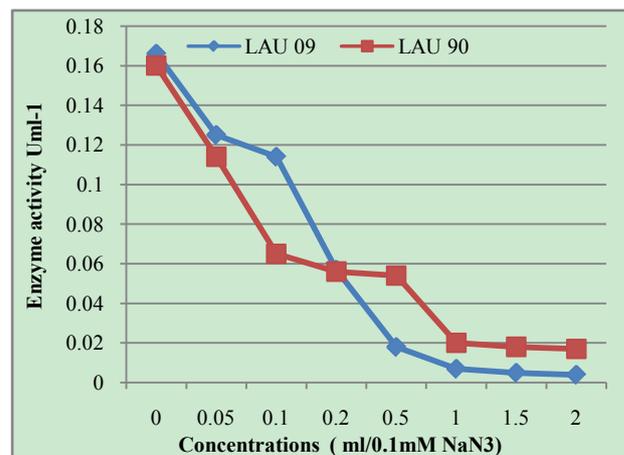


Figure 5. The laccase enzyme inhibition test by Sodium azide

The activity of purified laccase from wild type (LAU 09) was highly inhibited by sodium azide, while mutant was lesser inhibited (Figure 5). The sharp inhibition was observed at 0.1ml/0.1mM Na_3N_3 in wild type, while mutant was inhibited sharply at 0.5ml/ 0.1mM Na_3N_3 . The activity of purified laccase was inhibited by 100mM EDTA, using ABTS as substrate, the rate of enzyme inhibition increased as the concentration of EDTA is increased (Figure 6) in all the strains (both wild and mutant) laccase inhibition was observed at low concentration of 0.1ml/100mM EDTA, and sharp increased in enzyme inhibition was obtained at 0.5ml/100mM EDTA. The result obtained in present study agrees with Soden et al[21] and Pang et al[18] with reported EDTA and sodium azide as strong inhibitory agents for laccase enzyme.

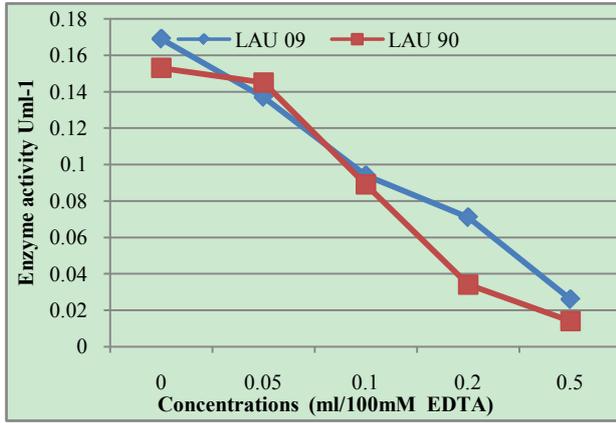
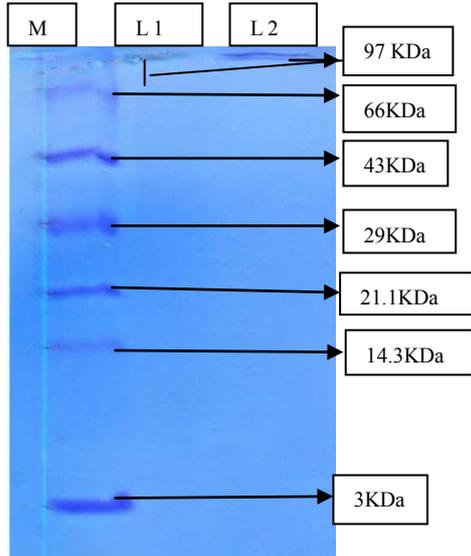


Figure 6. The laccase enzyme inhibition test by EDTA



LM = protein molecular weight, L 1= LAU 09, L 2 = LAU 90

Figure 7. Protein (laccase) Molecular Weight as visible on 10 % SDS-PAGE after staining with Coomassie Brilliant Blue

The molecular weight of the purified laccase was deter-

mined in comparison with the molecular weight of the standard proteins such as Phosphorylase b (97.4KDa), Bovine serum albumin (66KDa), Ovalbumin (43KDa), Carbonic Anhydrase (29KDa), soybean trypsin inhibitor (21.1KDa), lysozyme (14.3KDa) and aprotinin (6.5KDa). The molecular weight of the proteins was calculated to be approximately 97KDa for LAU 09 and LAU 90 (Figure 7). Kumar and Srikumar[11] reported different bands (43KDa, 90KDa and 137KDa) for laccase species under non-reducing SDS – PAGE, whereas a molecular weight of 72KDa, 46KDa and 44KDa were previously reported by Pang et al[18] for different species of laccase enzymes. The same molecular weight of 97KDa obtained in both LAU 09 (wild) and LAU90 (mutant) suggested that the enzyme produced by both wild and mutant strains may not be differed.

The phylogenetic analyses using sequence data of ITS 5.8s and 28s ribosomal RNA gene from *P. pulmonarius* (LAU 09) and corresponding GenBank data of related species was shown in Figure 8. The outgroup arising from the root of the phylogenetic tree for the *P. pulmonarius* (accession number: JF736658) for phylogenetic evaluation is *P. tuberregium* (AY450344) from GenBank database. *P. pulmonarius* LAU 09 formed a phylogenetic cluster with *P. pulmonarius* complex at top of phylogenetic tree with bootstrap value of 77%, which confirmed the close evolutionary lineage of the strain with *P. pulmonarius* complex from GenBank database. The cladistic position of the *P. pulmonarius* (LAU 09) strain in the phylogenetic trees suggested that there was common ancestor, sub ancestors diverged majorly at early stages of evolution[24]. The phylogenetic analyses based on the PCR-RFLP data of the partial 26srDNA has also reported[2] which revealed that 9 of the biological species, the *P. cornucopiae* complex, *P. cystidiosus* complex, *P. salmoneos-tramineus*, complex, *P. dryinus*, *P. nebrodensis*, *P. smithii*, and *P. ulmarius* were congruent with independent phylogenetic lineages.

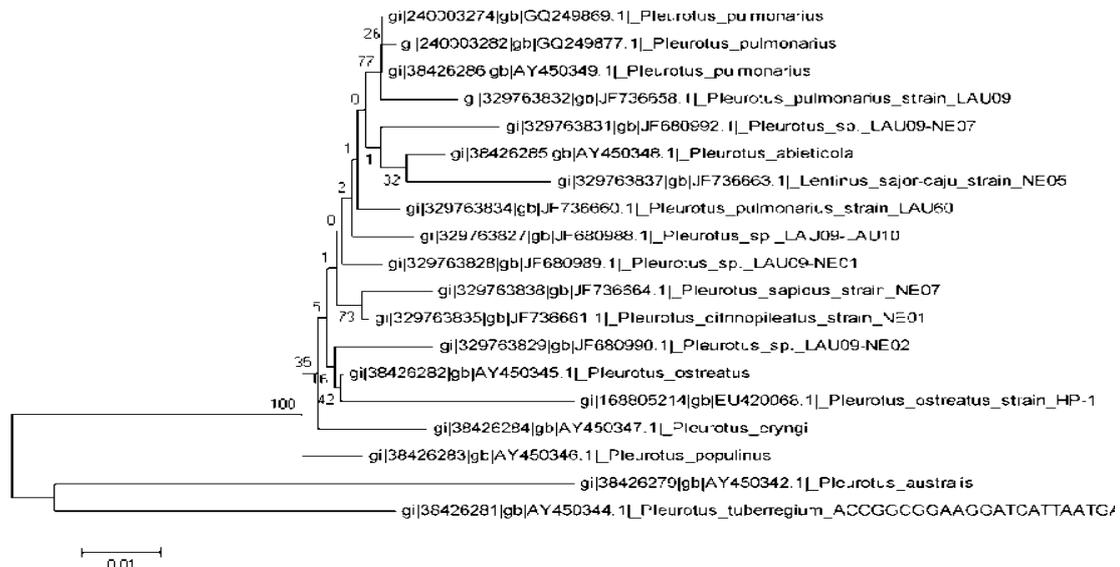


Figure 8. The Phylogenetic trees of LAU 09 (wild) constructed by UPGMA, with bootstrap values from 100 replicates showing maximum similarity with LAU 09 (wild) and the homogeneous strains of *Pleurotus* species

Table 2. The total length (1150) of nucleotides sequence of LAU 09 (wild)

```
GCGGAAGGATCATTAATGAATTCACATGAGGTTGTTGCTG
GCCTTAGGGGCATGTGCACGCTTACTAGTCTTCAACCAC
CTGTGAACCTTTGATAGATCTGTGAAGTCGTCCTTCAAGTCG
TCAGACTTGGTTTGTGGGATTTAAACGCTCTCGGTGTGACAA
CGCAGTCTATTACTTAACACACCCCAAATGTATGTCTACGA
ATGTCATTTAATGGCCTTGTGCCTATAAACATAATACAAC
TTTCAACAACGGATCTCTGGCTCTCGCATCGATGAAGAAC
GCAGCGAAATGCGATAAGTAAATGTGAATTGCAGAATTCAGT
GAATCATCGAATCTTGAACGCACCTTGCGCCCTTGGTATT
CCGAGGGGCATGCCTGTTGAGTGTCAATTAATTTCAAAC
CACATTTATTTGTGATGTTGGATTGTTGGGGGTTGTCTGGCT
GTAAACAAGTCGGCTCCTCTTAAATGCATTAGCAGGACTTCTC
ATTGCTCTGCGGATGATGTGATAATTATCACTCATCAATAG
CACGCATGAATAGAGTCCAGCTCTTAATCGTCCGCCAGGC
GAATTTGACAATTTGACCTCAAATCAGGTAGGACGTCCGG
GTGAACCTAAGCATATCAATAAGGGGAGGAAAAGAAAATA
ACAAGGATCCCCTAGTAACTGCGAGTGAAGCGGGAAAAG
CTCAAATTTAAAATCTGGTGGTCTTTGGCCATCCGAGTTGTA
ATCTAGAGAAGTGTATCCGCGCTGGACCGTGTACAAGTCC
CCTGGAATGGGGCGTCATAGAGGGTGAGAATCTCGTCTTTG
ACACCGACTACCAGGGCTTTGTGATGCACTCTCAAAGAGTC
GAGTTGTTGGGAATGCAGCTCAAATGGGTGGGAAATTC
ATCTAAAGCTAAATATTGGCGAGAGACCGATAGCGAACAA
GCGCAGTGAGGGAAAAGATGAAAAGAACTTTGAAAAGAGAG
TTAAACAGTACGTGAAATTTGTTGAAAGGAAACGCTTGAAG
TCAGTCGCGTCTGCCAGGGATCAACCTACTTTTGTGGGGCG
TACTTCTGGTTGATGGGTGAGCATCAGTTTTGTGCTCGTGA
TAAAGGTCAGAGAAATGTGGCACCTTCGGGTGTG
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The edited nucleotide sequence of LAU 90 (mutant) show no similarity with any available data at GenBank database after blasting at national centre for Biotechnology information (NCBI) website. The nucleotide sequence of LAU 09 (wild) and LAU 90 (mutant) were shown in Table 2 and 3 with total number of sequence nucleotides very high (1150) in LAU 09 (wild) compare to 750 obtained in LAU 90 (mutant). Beside the shorter length of nucleotides sequence in the mutant strain, the arrangement of the nucleotides sequence in the mutant strain has widely altered.

Table 3. The total length (750) of nucleotides sequence of LAU 90 (mutant)

```
AGCCAACCCGAGGGAGTAAAAGGATTTTTTTCTAAGTTGTT
GCTGGCCCTCCCGTAGAGACGCGGGTACTAGTTTCTC
ATATCCGCTATGCACTGAAGTGGTGTGATTACAACAGGT
CTTTATAGACTTGGGAAATCTGTCTATGCTTTTATGGGAC
TTCACCTTTTATAATGTCTTCCATACACGCATTTATTTCA
ACTTTCATCTACGGATCTGTTGGCTCCGCATCAAAAACAA
CTCCCCACTTGCAACAGAAGTGAATTGGCTAATTCAAG
GAACCAGCCGCTATGAAAAGAGATATCGCCCTTTGGAA
TTCAGTAAGGCATGCCAATTTGACAGCCAGCCCCCTCGGA
TAGGCATAAGCAGTGGGTGTGTGAGTTGTTGAAATTTGCTC
ATTCTCAAATTTATTTGATATGTTAATTGCATCCCCTTCC
TCGATGTGACCAAGAGCACCGAATTTAAATTTAGGTTTTT
CCGCTTCCCCTAAGTAAGGAGGGGATCCCTGGTTAGTTTC
TTCCCCCCTCATTAAATAGGCTTAAATTCACGGGGTATC
CCTACCTGATTGAGGGCAGAATGGTCAAATTTGGCCGGCG
GAGGATTAACCCAGCTGGAATTTGTTTACCGCGCTACTAA
CGAGTGAAAATTTATCACAATGCGCAGTGGCAATGATAA
CTCTGGCAATTTGCTTTAAGAAGAAGTGGAGACGTTAC-
CATCACCTGCCACCACCTCCCTAC
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The result obtained in this study has shown the improved performance in laccase production by the mutant (LAU 90) strain over the wild (LAU 09) strain. So also, the molecular variation with evolutionary divergence of the mutant strain from the wild type is true evidence of a novel strain of *P. pulmonarius*.

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