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CLONING, EXPRESSION IN *E.coli* AND THE ENZYMATIC PROPERTIES OF LACCASE FROM *Hypsizygus ulmarius*

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ABSTRACT

In the present study a laccase from *Hypsizygus ulmarius* was cloned and expressed in *E.coli* **(**BL 21**).** The laccase gene (1.6 Kb) which was isolated from the mushroom *Hypsizygus ulmarius* was successfully cloned to pET-29a (+) and expressed in *E coli*. The sequencing results confirmed the expression of the gene with 98% homology to that of the original gene as retrieved from NCBI. The recombinant laccase was produced from the transformed colonies and it was purified upto 23 fold with 51% recovery. The optimum pH and temperature for the purified laccase was found to be 6.0 and 40^{o}C respectively. The molecular weight of the enzyme was found to be 63kDa. The enzyme activity was enhanced by the metal ions $Mn^{2+}$and Cu^{2+} whereas it was reduced by Fe^{2+}, Na^{2+} and Co^{2+}. These results suggest that the recombinant laccase purified from the mushroom *Hypsizygus ulmarius* can be used for various biotechnological applications.

KEYWORDS: Recombinant laccase, *Hypsizygus ulmarius, E.coli.*

INTRODUCTION

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) is a group of copper-containing polyphenol oxidases which can catalyze the four-electron reduction of O_2–H_2O with the concomitant oxidation of phenolic compounds. Laccase is one of the important ligninolytic enzymes responsible for the strong ability of lignin degradation of white-rot fungi [1, 2].

Laccase is also a very important and valuable enzyme for various biotechnological and industrial applications, such as biodegradation of lignin without polluting the environment, thorough degradation of different recalcitrant compounds, environmental protection and bioremediation, biological bleaching in paper industry, wine clarification and also in the textile dye decolorization [3].

Laccase production from native sources cannot meet the increasing market demand due to low yield, incompatibility of the standard industrial fermentation processes with the conditions required for the growth of many microorganisms. To make laccases available for industrial applications, cost effective methods like media optimization, novel fermentation methods and genetic modification for large scale production via eukaryotic recombinant strains are needed [4]. Much research has been done to identify effective methods for mass production of laccase using the above mentioned methods.

In recent years, more attention has been focused on recombinant laccase [5]. The yield of recombinant laccase could be increased significantly by construction of engineered strains using a highly productive strain as host [6], by setting stronger promoters to control the desired gene [7], by recombining more gene copies [8] and by selecting the gene sequence.

In order to obtain large amounts of enzyme for application, several laccase genes have been cloned from different fungal sources [9–13] and heterologously expressed in *Pichia pastoris* [14,15], *Pichia methanolica* [16], *Saccharomyces cerivisiae* [17–19], *Kluyveromyces lactis* [20], *Yarrowia lipolytica* [21], *Aspergillus nidulans* [22], *Aspergillus niger* [23], *Aspergillus oryze* [24] and *Trichoderma reesei* [25,26]. Thus, studying laccase cloning the laccase gene and studying its sequences is the best way to optimize recombinant laccase production when compared to other methods like media optimization and other fermentation methods. Keeping these points in view, the current study was initiated and the isolation of 1accase gene from *Hypsizygus ulmarius* with its properties has been reported.

MATERIALS AND METHODS

Organism

Hypsizygus ulmarius was obtained from the mushroom research centre, Tamil Nadu Agricultural University, Coimbatore, India.

Isolation of laccase gene

Genomic DNA was isolated from the mushroom *Hypsizygus ulmarius* as described Doyle and Doyle [27]. The 18S ribosomal RNA sequence of *Hypsizygus ulmarius* was retrieved from NCBI (GenBank AY265850.1). Since the laccase coding gene sequence for *Hypsizygus ulmarius* was not available, a similarity search using BLAST was done to identify the laccase coding gene from similar species. It was found that the laccase gene from *Hypsizygus marmoreus* showed 97% homology and it was retrieved from NCBI (GenBank EU375894.1).

Amplification of the laccase gene was performed by PCR using Taq polymerase with the forward primer (5'- GCGAATTCATGGGGTTCTTTCAAGCGAG -3') and reverse primer (5'- GCGGATCCCTATTTGTCAAGAGAGTAC-3'), with the recognition sites of *EcoR*I and *BamH*I, respectively and translation codon ATG and stop codon TAA of open reading frame (ORF) are shown in italics. The PCR was performed as follows: 95°C for 4 min, 94°C for 45 sec, 57°C for 1 min, 72°C for 45 sec, 30 cycles, and 72°C for 7 min. The amplified DNA was inserted in pET-29a(+). After the successful ligation of the laccase gene into the vector transformation was done in *E.coli* (BL-21). Clone confirmation of the gene was done with plamid DNA which was isolated from transformed colonies [28].

Production, purification and characterization of the recombinant laccase

The transformed colonies were transferred to the laccase producing medium and the produced recombinant laccase was purified and characterized.

Assay of laccase activity

Laccase activity was assayed spectrophotometrically by measuring the oxidation of ABTS at 420 nm [29]. The assay mixture in a total volume of 1 ml contained 0.1 ml enzyme extract and 1 mM ABTS in 100 mM citrate buffer (pH 3.4). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol ABTS per minute at 30^{o}C and the activities were expressed in units per liter.

Purification and characterization of laccase

Ammonium sulfate precipitation

The crude extract was precipitated by 70% saturation with ammonium sulfate. The precipitate obtained after centrifugation at 10,000 g for 30 min was suspended in 10 mM Tris HCl buffer, pH 7.5.

Dialysis

The enzyme precipitated in the previous step was dialyzed against the 100 mM Tris phosphate buffer (pH 7.5) for 24 hrs at 4^{o}C and then the buffer was changed twice. The dialyzed enzyme was assayed for the laccase activity. The amount of protein was estimated [30].

Ion-exchange chromatography

The enzyme preparation obtained from the above step was further purified by passing through a column (25 cm x 2.6 cm) of activated DEAE-cellulose previously equilibrated with Tris HCl, pH 6.0. The fractions of 3 ml each were eluted at the flow rate of 20 ml/hr with and linear gradient upto 0.4 M NaCl, analyzed for enzyme activity and protein content. The active fractions were pooled and concentrated.

Characterization of laccase

Optimum pH

For determination of optimum pH of the enzyme, the reaction buffer mixture was varied over the pH range of 3 - 8. The buffers were used 0.1 M acetate buffer (pH 3.0 - 5.6) and 0.1 M potassium phosphate buffer (pH 5.7 - 8.0). Enzyme activity was measured as described earlier.

Optimum temperature

The effect of temperature on the enzyme activity was assessed by carrying out the enzyme assay at various temperature intervals (20-60^{o} C). Enzyme activity was measured as described earlier.

Effect of metal ions

The effect of metal ions on enzyme activity was studied by supplementing different metal ions ($MnCl_2$, $FeCl_2$, NaCl, $CoCl_2$ and $CuCl_2$) at a concentration of 0.05M.

Molecular weight determination using SDS-PAGE

Molecular weight was determined by SDS – PAGE with proteins of known molecular weight along with the protein to be characterize [31] in 10% polyacrylamide gel.

RESULTS AND DISCUSSION

The cloning and expression of laccase gene from *Hypsizygus ulmarius* in *E coli* have been studied and the enzymatic properties of the recombinant laccase were also characterized.

Isolation of genomic DNA

The genomic DNA from *Hypsizygus ulmarius* was isolated and used for PCR amplification of the laccase gene. The genomic DNA was isolated from the mushroom. It was analyzed on 0.8% agarose gel (Figure 1). Gel analysis revealed that the DNA preparation from the mushroom was good. There was no shearing of DNA, since a single intact band (High molecular weight) was observed on gel. Also DNA was devoid of RNA contamination as no contaminating band was observed on gel. The quality of DNA was good as the 260/280 ratio was 1.5.

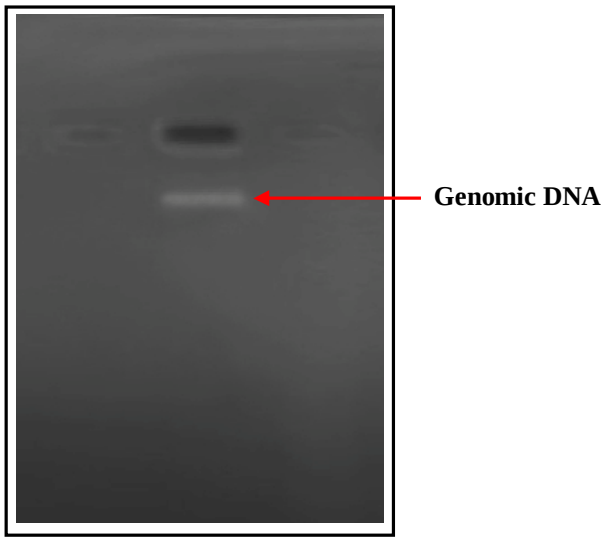


Figure 1. Genomic DNA from *Hypsizygus ulmarius*

Amplification of laccase gene by PCR

The laccase gene fragment was amplified from the mushroom *Hypsizygus ulmarius* using forward and reverse primers. A single band of approximately 1.6kb was amplified as shown in the figure 2. After confirmation with AGE it was digested with restriction enzymes and ligated into the vector and subjected for transformation.

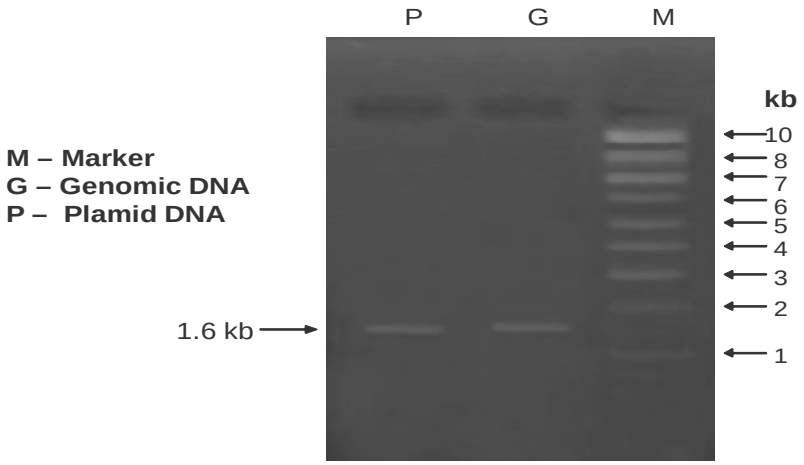


Figure 2. Amplification of laccase gene by PCR

Transformation

Before transformation of ligated product, transformation efficiency of competent cells was checked. For this supercoiled plasmid (10 ng) was used for transformation.

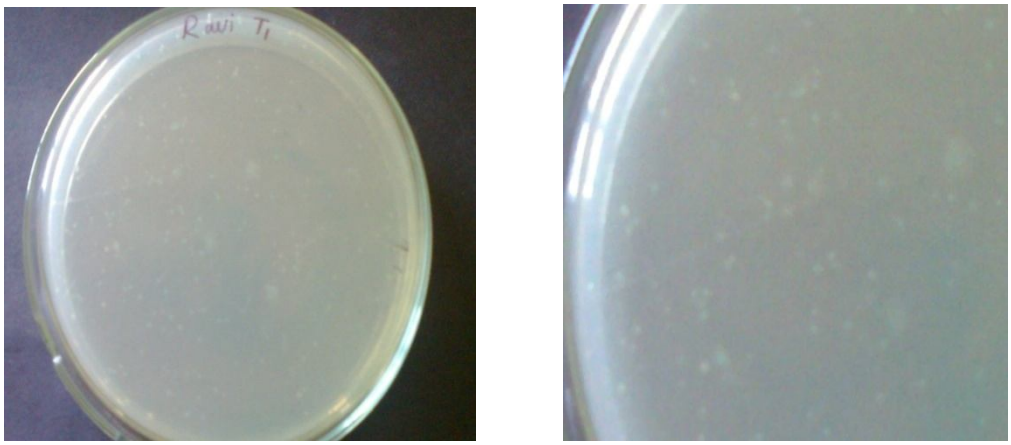


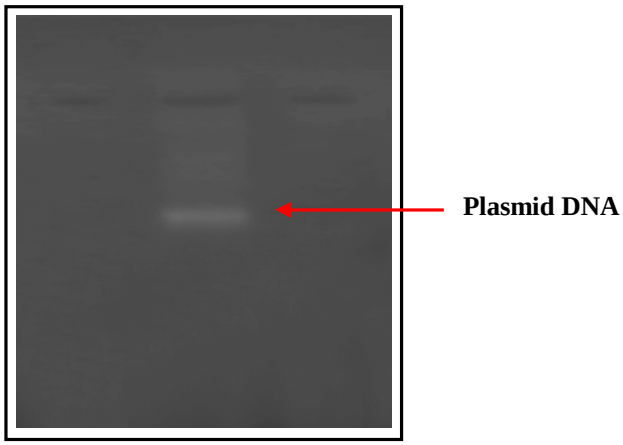
Figure 3 showing the growth of colonies

The efficiency of the competent cells was estimated to $6.1x10^5$ colonies/g plasmid. This transformation efficiency is good for regular transformation experiments. The selection of transformed colonies was based on the Blue-White selection. The transformed colonies were of white color (Figure 3). The white colored colonies were selected, subjected for plasmid DNA isolation and transferred to the fermentation medium for laccase production.

Plasmid isolation

The plasmid DNA was isolated from positive clones and analyzed on agarose gel. The plasmid was visualized on a UV transilluminator after electrophoretic separation (Figure 4).

Figure 4. Plasmid DNA



Clone confirmation by PCR

The expression of laccase gene in *E. coli* was confirmed by PCR using the plasmid DNA. As shown in figure 2, a single band showing approximately 1.6 kb was obtained which was similar to that of amplified gene with genomic DNA. This confirms the successful amplification and expression of laccase gene from *Hypsizygus ulmarius* in *E. coli*.

Confirmation of gene by sequencing

After the successful expression of laccase gene in *E. coli* it was sequenced and compared with other genes using BLAST (N) and the results showed 98% homology to that of the original sequence.

Purification and characterization of the recombinant laccase

The purification of crude enzyme through DEAE cellulose column chromatography gave 23.23 folds increase in purity with 52% recovery of the recombinant laccase from *Hypsizygus ulmarius* (Table 1). The molecular mass of the purified laccase was determined using SDS–PAGE. It was found to be 63 kDa by comparison of the single laccase protein band with the protein molecular weight standards (Figure 6). Our results were supported by Wu *et al* [32] who reported the purification of recombinant laccase from *Aeromonas hydrophila* WL-11.

Table 1. Purification of the recombinant laccase from *Hypsizygus ulmarius*

`Steps	Laccase production (IU)	Total protein (mg)	Specific activity (IU/mg)	Purification (fold)	Recovery (%)
Crude extract	121.32	60.35	2.01	1	100
70% Ammonium sulphate precipitation	98.13	7.75	12.67	6.30	80.89

Dialysis	75.78	2.22	34.12	16.97	62.46
DEAE Cellulose column chromatography	62.59	1.34	46.71	23.23	51.59

Generally, expression system in *E. coli* offers rapid and economical production of recombinant proteins compared to fungal expression system which is much more difficult to work with. However, overproduction of heterologous proteins in the cytoplasm of *E. coli* often results in the formation of insoluble and biologically inactive aggregates known as inclusion bodies [33]. The formation of inclusion bodies is a major obstacle for large-scale production.

It has been reported that overproduction of laccases from *B. subtilis* [34], *S. lavendulae* [35] and *Klebsiella* sp. 601 [36] also resulted in an extensive intracellular aggregation. Therefore, many attempts of avoiding the formation of inclusion bodies have so far focused on increasing the soluble expression of heterologous proteins by manipulating the cellular folding apparatus [37, 38]. In this study, we have succeeded in high-level production of recombinant laccase as soluble and active form in *E. coli*. This will enable structural studies by crystallization followed by X-ray diffraction and commercial application.

Characterization of the recombinant laccase from *Hypsizygus ulmarius*

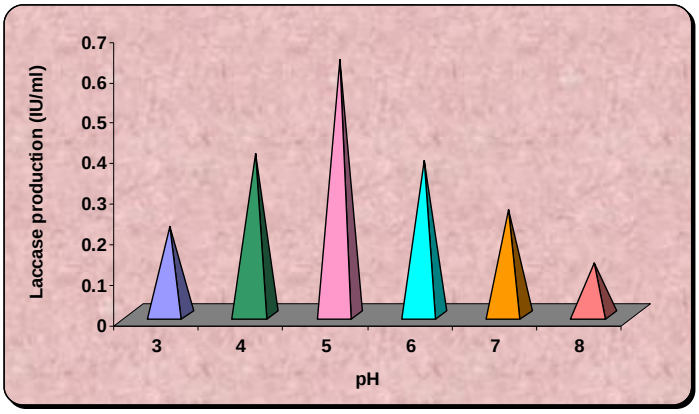


Figure 5 Effect of pH on recombinant laccase production

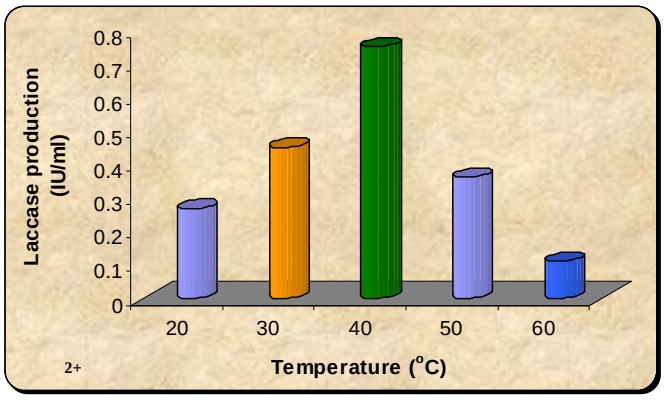


Figure 6 Effect of temperature on recombinant laccase production

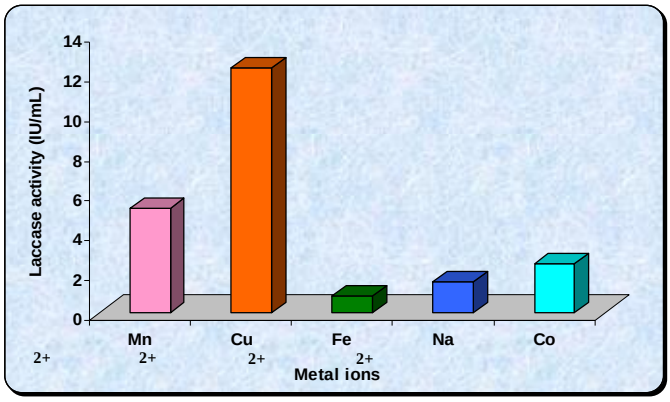
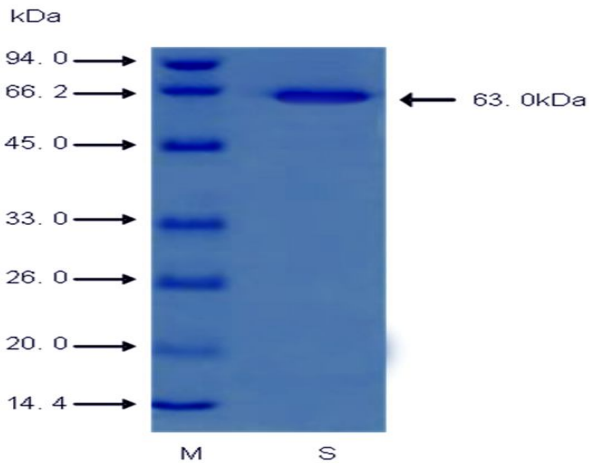


Figure 7 Effect of metal ions on recombinant laccase production

The enzymatic properties of the recombinant laccase was studied and it was observed that it was similar to that of original enzyme with optimum pH 6 (Figure 5), temperature 40°C (Figure 6) with a molecular weight of 63kDa (Figure 8). The enzyme activity was enhanced by the metal ions $Mn^{2+}$and Cu^{2+} whereas it was reduced by Fe^{2+}, Na^{2+} and Co^{2+} (Figure 7).



Lane 1 M – Marker

2 S – Laccase enzyme

Novelty of the recombinant laccase from *Hypsizygus ulmarius*

The recombinant laccase from the mushroom was compared (figure 9) with that of the laccase produced with optimum conditions. The results revealed that the recombinant laccase showed high level of purity (23.23 fold) when compared to the wild laccase (5.11 fold) [39].

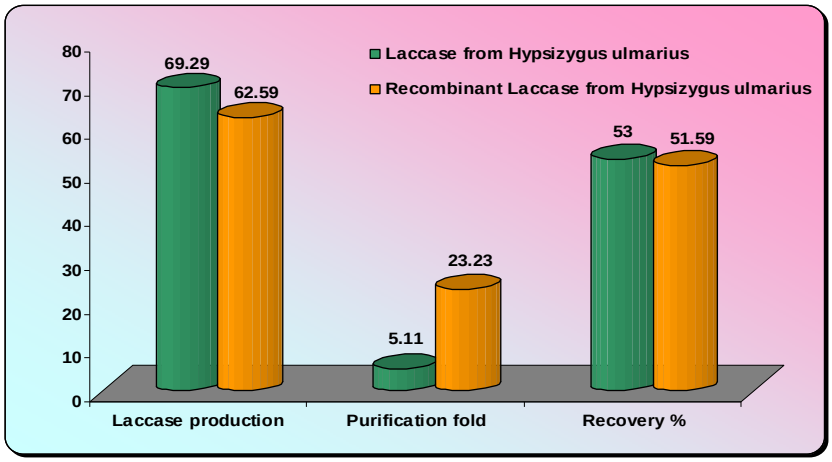


Figure 9 Novelty of the recombinant laccase from *Hypsizygus ulmarius*

CONCLUSION

The gene encoding laccase was isolated from *Hypsizygs ulmarius* and then successfully cloned and expressed in *E. coli* BL21 cells. The optimum production, recovery, high-level purity of the recombinant laccase suggests that this enzyme can be used in various applications.

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