

# FINAL REPORT

**PROJECT TITLE:** "Operational strategies to improve laccase production from white rot fungi and its application for the degradation of polycyclic aromatic hydrocarbons."

**PROJECT NO:** F.47-052/06.

**DURATION:** 15.01.2007-15.01.2009.

**PRINCIPAL INVESTIGATOR:** Urvish Chhaya  
Lecturer in Microbiology  
Natubhai V.Patel College of  
Pure & applied Sciences.  
Vallabh-Vidyanagar,  
Gujarat, India.

PROGRESS REPORT (2007-2009)  
**OPERATIONAL STRATEGIES FOR IMPROVED LACCASE  
PRODUCTION AND DEGRADATION OF POLYCYCLIC  
AROMATIC HYDROCARBONS.**

Introduction

Laccase is a copper containing polyphenol oxidase (EC 1.10.3.2) catalyzing the oxidation of broad no of aromatic amines by using molecular oxygen as the electron acceptor which is letter reduced to water. Recent studies have proved that this enzyme has been used in the development of biosensors which has open new area of application such as immunoprobe and drug development.

Litter production in the forest ranges from around 1.5-1.8 per hectare per year and reaches up to 15 tons per hectare per year in tropical rain forest, without the activity of Litter decomposing fungi we and forest would in time be buried by the cast of leaves and branches. Litter is often colonized by LDF during the final stage of decay and therefore the accumulation of recalcitrant material like lignin is minimized.

Fungi that colonize soil litter in particular litter decomposing fungi (LDF) include basidiomycetes and ascomycetes living in the uppermost portion of the soil and in the humus layer of forest and grassland. Here LDF are particularly important because of their production of wide range of ligninocellulolytic enzymes.

There are various operational strategies which can be applied to enhance ligninolytic enzyme production as they are constitutively produced in the in small amounts. One of the strategy is to enhance the enzyme production by a wide variety of substances like o-dianisidine, veratryl alcohol, catechol, vanillin, gallic acid, guaiacol as they have similar structure with lignin and its derivatives. Another approach is to optimize carbon source nitrogen source and trace elements by employing statistical screening design like Plackett Burman Design followed by Response Surface Methodology to hunt the optimum concentration of selected components.

LDF because of their ability to synthesize ligninolytic enzymes has capacity to degrade certain recalcitrants like synthetic dyes and polycyclic aromatic hydrocarbons. Certain LDF like *C.dryophila*, *S.rugosomulata*, are known to decolorize azo dyes while *Stropharia coronilla* is known to degrade Benzo{a} pyrene .

## Result and Discussion

Following objectives have been achieved over a time span of two years.

1. Collection of soil samples from decomposed litter.
2. Isolation of fungal isolates from the soil samples.
3. Primary screening of fungal isolates for ligninocellulolytic activity.
4. Secondary screening of fungal isolates for ligninocellulolytic activity.
5. Production profile study of selected fungal isolates using agricultural waste like wheat straw and sugarcane baggasse by solid state fermentation.
6. Effect of various inducers on the production of laccase and Mn-peroxidase.
7. Screening of medium components by Plackett Burman design.
8. Optimization of selected components by response surface methodology using Central Composite Design.
9. Identification of novel laccase and Mn-peroxidase producing litter decomposing fungal isolate LD-3.
10. Effect of moisture content on the ligninolytic enzyme production.
11. Effect of various agricultural waste as a substrate for ligninolytic enzyme production under solid state fermentation.
12. Strain improvement and rational selection for hyper ligninolytic enzyme producer.
13. Optimization of minimum lethal dose of U.V. radiation as a physical mutagen.
14. Isolation, screening and selection of hyper laccase producing mutants.
15. Isolation of naphthalene resistant, anthracene resistant and copper resistant mutants, their screening and selection of hyper laccase producing mutants.
16. Effect of ethidium bromide on the production of laccase
17. Application of hyper laccase producer for the degradation of naphthalene.

18. High Performance Thin Layer Chromatographic analysis of the naphthalene degradation.

➤ **Collection of soil sample from decomposed litter:**

Soil samples for desired isolates were collected during the period of September 2006 to December 2006. Twelve soil samples were collected from the local region of Vallabh Vidyanagar like Shastri Maidan and the region near Home Science College and Pharmacy College. Out of these twelve samples six samples were screened. Forty soil samples were collected from the temperate deciduous rain forest of Panchmarhi, Madhya Pradesh. Out of forty soil samples, eighteen soil samples were screened.

➤ **Isolation of fungal isolates:**

Soil samples collected as mentioned above are suspended in the phosphate buffer pH 7.0 and they are kept on a shaker for 120 mins followed by plating of various dilutions on the SDA plates which were incubated at 28-30°C for 8-10 days. Isolated fungal colonies were purified and subcultured on SDA plates. Out of six samples obtained from the local region of V.V. Nagar, twelve isolates were selected and out of eighteen soil samples obtained from Panchmarhi forest region, twenty-nine fungal isolates were selected for the primary screening.

➤ **Primary screening:**

Primary screening of total 41 fungal isolates was subjected for the detection of phenol oxidase activity which was performed on SDA plates containing 0.1% o-Dianisidine. Out of 41 isolates, eighteen isolates showing positive phenol oxidase activity (Bavandam reaction) were short-listed for the quantitative or secondary screening.

➤ **Secondary screening:**

Secondary screening of eighteen isolates were performed for the quantitative detection of lignino cellulolytic enzymes under solid state fermentation using wheat straw as a solid substrate and Asther medium as a moistening agent in the ratio of 1:4. Out of these eighteen isolates LD-3 and LD-15 showing highest laccase activity and LD-8 showing highest cellulolytic activity were short listed for the further studies.

➤ **A. Study of production profile on wheat straw:**

Production profile of LD-3 was studied for the 408 hrs (17days). It was found that LD-3 exhibits maximum Endo1, 4 $\beta$ glucanase activity (Carboxymethyl cellulose) at 4<sup>th</sup>day, maximum filter paper activity at 13<sup>th</sup>day, maximum Exo1, 4 $\beta$  glucanase (cotton activity) at 10<sup>th</sup>day hrs while maximum laccase and Mn dependent peroxidase were obtained at 8<sup>th</sup>day and 7<sup>th</sup>day respectively under solid state fermentation.

**B. Study of production profile on sugarcane baggasse:**

Production profile of LD-3 was studied for the 408 hrs (17days). It was found that LD-3 exhibits maximum Endo1, 4 $\beta$ glucanase activity (Carboxymethyl cellulose) at 14<sup>th</sup> day, maximum filter paper activity at 6<sup>th</sup>day, Exo1, 4 $\beta$  glucanase(cotton activity) was not detected during the complete course of fermentation, while maximum laccase and Mn dependent peroxidase were obtained at 6<sup>th</sup> day under solid state fermentation.

➤ **Effect of various inducers on the production of laccase and Mn-peroxidase:**

Ligninolytic enzyme activity Laccase and Mn-peroxidase respectively was evaluated in the presence of seven different inducers under solid state fermentation using wheat straw as a substrate. Enzyme activity in units per gram of substrate was compared in the presence of galic acid, veratryl alcohol vanillin, catechol, guiacol, ortho dianisidine and ethanol respectively, and o-dianisidine was found to be the best inducer exhibited 3.12 fold increase in the Laccase activity and 2.03 fold increase in the Mn-peroxidase activity. Thus o-dianisidine was selected further for the screening of medium component by Plackett Burman design.

➤ **Screening of medium components by Plackett Burman design:**

Screening of fourteen different medium components was performed and out of fourteen three of them i.e .ortho-dianisidine, thiamine hydrochloride and  $\text{CuSO}_4$  showing 'p' value of 0.01,0.03,0.04 for laccase and 0.05,0.16,0.2 for Mn-eroxidase respectively were selected for the response surface methodology by Central Composite Design.

➤ **Optimization of selected components by response surface methodology using Central Composite Design:**

Further optimization of selected medium components was performed using response surface methodology by Central composite design using design expert 1.3.7 software. The 'p' value of the model was found to be 0.001, while the lack of fit was found not significant. From the response surface plots following concentrations of the selected medium components were obtained, ortho-dianisidine (0.388mM), thiamine hydrochloride (0.0135g/l) and  $\text{CuSO}_4$  (0.01g/l).which is expected to give 16 fold increase in the laccase and Mn-peroxidase .

➤ **Identification of novel laccase and Mn-peroxidase producer, litter decomposing fungal isolate LD-3:**

Litter decomposing fungal isolate producing laccase and Mn-peroxidase was sent for the identification to the Bangalore Genie Bangalore for full length 18s r-RNA sequencing and it is found to be *Fusarium incarnatum*.

➤ **Effect of moisture content on the ligninolytic enzyme production.**

Effect of moisture content in terms of different ratio of dry substrate and moistening agent has been evaluated on the laccase and Mn-peroxidase production, and 1:4 ratio corresponds to 5gms of wheat straw and 20ml of Asther's medium as a

moistening agent is found to be the best as it has shown maximum laccase and Mn-peroxidase activity i.e. 392units per gram and 153.98 units per gram respectively.

➤ **Effect of various agricultural wastes as a substrate for ligninolytic enzyme production under solid state fermentation.**

Effect of different agricultural waste has been evaluated on the production of laccase and Mn-peroxidase production namely wheat straw, sugarcane baggasse, saw dust, rice bran and wheat bran. Rice bran is found to be the best agricultural waste for laccase production as it produced 2611.74 units per gram of laccase which is found to be highest among all other agricultural waste tested, however there is no production of Mn-peroxidase on rice bran. Since wheat straw is showing both laccase and Mn-peroxidase production, it has been selected for the further studies.

➤ **Strain improvement and rational selection for hyper ligninolytic enzyme producer.**

Efforts are initiated in the direction of strain improvement of *Fusarium incarnatum* LD-3 followed by rational selection of hyper producers applying different selection pressures. The first round of random mutagenesis has been conducted using Ultraviolet radiation as a physical mutagen and 27 different mutants were obtained after 10 minutes exposure. The quantitative analysis for laccase and Mn-peroxidase is under progress. The minimum inhibitory concentration for  $\text{CuCl}_2$ , Naphthalene and Anthracene has been found out and it is 600ppm, 300ppm and 200ppm respectively. All these selection pressures at their minimum inhibitory concentrations will be used as a selection pressure for the next round of mutation.

➤ **Optimization of minimum lethal dose of U.V. radiation as a mutagen.**

Laccase producing litter decomposing fungi *Fusarium incarnatum* LD-3 was exposed to U.V. radiation for the different time intervals i.e. 2mins, 5mins, 7mins,

10mins, 15mins, 20mins. And on the basis of 0.1% survival exposure of 10 mins has been selected as a minimum lethal dose.

➤ **Isolation screening and selection of hyper laccase producing mutants.**

Fungal isolate was exposed to U.V. radiation for 10mins after plating the suspension of mycelia on modified SDA plates containing 0.1% ortho dianisidine and incubated at  $28\pm 2^{\circ}\text{C}$  for three to four days and depending upon the 0.1% survival compare to the control plates, 27 mutants are selected and tested for the laccase production. After random mutagenesis two hyper laccase producers are short listed namely UC-11 and UC-14 which are showing laccase activity of 1021 units per gram and 1433 units per gram respectively as compared to control isolate which is showing 769 units per gram of laccase activity this indicate the rise of 32.75% and 86% respectively in the laccase production.

➤ **Isolation of naphthalene resistant, anthracene resistant and copper resistant mutants, their screening and selection of hyper laccase producing mutants.**

Mutant isolate UC-14 was further subjected to U.V. mutagenesis and plated on the SDA plates containing different selection pressures like anthracene 200ppm, Napthalene 300ppm and Copper 600 ppm as mentioned above. After the incubation 10 naphthalene resistant 13 anthracene resistant and 4 copper resistant mutants are selected and screened for their laccase activity. Out of these 27 mutants mutant no UC-34 and UC-36 (200ppm anthracene resistant) are selected for the further studies as they have shown laccase activity of 3036units per gram and 3210units per gram of wheat straw with compare to control which has shown 1800units per gram of laccase activity.

➤ **Effect of ethidium bromide on the production of laccase**

Effect of ethidium bromide (EtBr) in different concentrations (0.5  $\mu\text{g/ml}$ , 1.0  $\mu\text{g/ml}$ , 1.5  $\mu\text{g/ml}$ , 5.0  $\mu\text{g/ml}$ , 7.5  $\mu\text{g/ml}$ , 10  $\mu\text{g/ml}$ ) has been evaluated on the laccase production with mutant isolate UC-14 and 7.5  $\mu\text{g/ml}$  concentration of ethidium



bromide concentration was found to be optimum for the laccase production. In the second round of the treatment mutant was transferred from EtBr containing plates to control ME plates and ME plates containing 7.5 µg/ml of EtBr and evaluated for the laccase production and it has been found that fungal blocks transferred from Etbr containing plates give more laccase production than control ME plates.

➤ **Application of hyper laccase producer for the degradation of naphthalene.**

Hyper laccase producing mutant UC-36 which was found to be resistant to the 200ppm of anthracene and 300ppm of naphthalene was subjected for the degradation study, and the degradation product of naphthalene were analysed by HPTLC analysis followed by its densitometry. It was found that naphthalene degradation begins at 5<sup>th</sup> day of incubation and it is degraded completely after 15<sup>th</sup> day. Which can be observed in HPTLC in terms of two distinct band showing different Rf value that the naphthalene standard extracted from the abiotic control.

### Conclusion

1. Fungal isolate LD-3(*Fusarium incarnatum*) is a potential ligninolytic enzyme producer.
2. Production of these enzymes can be enhanced by employing operational strategies like application of inducers and statistical medium optimization and selection of hyper laccase producing mutant by rational approach.
3. Thus fungal isolate LD-3(*Fusarium incarnatum*) offers strong candidature as an agent for bioremediation i.e. degradation of polycyclic aromatic hydrocarbons and synthetic dyes.

