Enzymes exhibit a number of features that make their use advantageous as compared to conventional chemical or microbial catalysts. The search for efficient and green oxidation technologies has increased the interest in the use of enzymes to replace the conventional non-biological methods. Lignolytic fungi secrete one or more of the extracellular lignin modifying enzymes (LMEs) as lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) that are essential in xenobiotic degradation. Because of their nonspecificity towards substrates, they are also capable of mineralizing a wide range of highly recalcitrant xenobiotics with structural similarities to lignin (Veignie et al., 2004). LMEs production occurs during secondary metabolism and their synthesis and secretion is induced by limited nutrient levels, oxygen tensions, mediator concentrations etc.

Real textile effluents are extremely variable in composition as they contain not only dyes but also salts at very high ionic strength and extreme pH values, chelating agents, precursors, by-products and surfactants that can inhibit enzyme activity and decolorization. So decolorization requires an appropriate choice of enzyme as well as of reactor environment (Wesenberg et al., 2003). Recently, increasing attention in decolorization of textile dye effluents has been paid to laccases because of their ability to act on chromophore group compounds such as azo, reactive dyes which leads to the suggestion that they can be applied in industrial decolorization processes. It has been proved that the addition of small molecules, generally known as redox mediators such as verartyl alcohol, guaiacol, ethanol enhance the production of laccase and therby facilitate the decolorization process.
The present study thus relates to (i) the detection of extracellular LMEs as lignin peroxidases (LiP), manganese peroxidases (MnP) and laccase (Lac), (ii) to examine the effect of pH, temperature, inoculum size (iii) concentrations of Asther’s media components such as glucose, ammonium nitrate, CuSO$_4$ (iv) concentrations of guaiacol, ethanol, glycerol, veratryl alcohol as inducers and (v) natural lignocellulosic substrates as saw dust, neem hulls, rice bran and wheat straw on the extracellular laccase activity by R. oryzae.

**Materials and Methods**

$10^6$ spores/ ml of R. oryzae were inoculated in Asther’s medium (Kasinath, 2002) comprising (g/L) KH$_2$PO$_4$ 0.2, CaCl$_2$ 0.132, MgSO$_4$ 7H$_2$O 0.05, ammonium ferric citrate 0.085, ZnSO$_4$. 7H$_2$O 0.0462, MnSO$_4$.4H$_2$O 0.035, COCl$_2$.6H$_2$O 0.007, CuSO$_4$. 5H$_2$O 0.007, L-asparagine 1, NH$_4$NO$_3$ 0.5, Thiamin-HCl 0.0025, yeast extract 0.2, glucose 10. The medium was buffered with 20 mM Phthalate buffer pH 5 and incubated at 30°C on a rotary shaker at 200 rpm. The culture filtrate was examined for detection of extracellular LMEs activity.

The effect of various pH (3 - 9), temperatures (25°C - 45°C), inoculum size ($10^4$ - $10^{10}$ spores/ml), concentrations of Asther’s medium components as glucose (0 – 4 g/L), NH$_4$NO$_3$ (0 – 24mM), CuSO$_4$ (0 – 2mM), glycerol (0 – 4%), veratryl alcohol (0 – 1mM), guaiacol (0 – 2mM), ethanol (0 – 10%) as inducers and natural lignocellulosic substrates as neem hulls, saw dust, wheat straw and rice husk were examined on extracellular laccase activity (maximally produced enzyme) at every alternate day, from day “0” upto 14 days. The experiments were conducted in triplicates.
Pretreatment of lignocellulosic substrates

5 g each of lignocellulosic substrate as neem hulls, saw dust, wheat straw and rice husk (coarsely ground) were taken in 250 ml Erlenmeyer flasks, wetted with distilled water and autoclaved for 30 minutes, after which distilled water was decanted off and 20 ml of Asther’s salt medium was added to it. This was again autoclaved for 15 minutes. Humidity was maintained by wetting the substrates with Asther’s mineral salt medium at every 3 – 4 days. Enzyme assay were performed by addition of 30 ml of 20mM Phthalate buffer (pH 5) and shaken for 1 h at 200 rpm for release of enzyme bound to the substrates, filtered through cheesecloth and centrifuged at 4°C for 20 minutes at 6000 rpm. The culture filtrate was used for the assay of LMEs.

Detection of extracellular LMEs (LiP, MnP, Lac) in R. oryzae

Lignin Peroxidase (LiP) (Tien and Kirk, 1984)

LiP was assayed by the oxidation of veratryl alcohol to veratraldehyde, as determined spectrophotometrically. The reaction mixture (2 ml) contained 1000 µL of 0.2 M sodium – tartarate buffer (STB) pH 3, 200 µL of 20 mM VA in STB, 720 µL of enzyme sample to which 80 µL of 10 mM H₂O₂ was added to initiate the reaction. The reaction was monitored at 310 nm against uninoculated medium as blank and the value was expressed as EU/ml. The extinction coefficient for veratraldehyde at 310 nm is 9300 -1 cm⁻¹.

One unit of LiP activity represents the quantity of enzyme producing 1 µmol of product per minute upon oxidation of veratryl alcohol to veratraldehyde in the above reaction mixture. LiP activity was calculated by the increase in A₃₁₀ using molar coefficient of 9300⁻¹ cm⁻¹ for the oxidation product.

\[
\text{EU/ml} = \frac{A_{310} \times 2}{9.3 \times t \times V}
\]

Where,
A_{310} = \text{change in absorbance at 310 nm, } t = \text{time (minute), } V = \text{ml of enzyme sample used in assay.}

**Manganese Peroxidase (MnP) (Vyas et al., 1994)**

MnP activity was determined spectrophotometrically by oxidation of 3-methyl-2-benzothiazoline hydrazone hydrochloride (MBTH) with 3-dimethylaminobenzoic acid (DMAB). The reaction mixture (2 ml) contained 1000 µL of 100 µM succinate lactate buffer (SLB) pH 4.5, 200 µL of 5 µM DMAB in SLB, 100 µL of 0.1 µM MBTH (in distilled water), 10 µL of 0.2 µM MnSO_4·H_2O, 10 µL of 0.1 µL H_2O_2 and 670 µL of crude enzyme extract and 10 µL of 10 mM H_2O_2 was added to initiate the reaction. The reaction was monitored at 590 nm against uninoculated medium as blank and value was expressed as EU/ml.

One unit of MnP activity is defined as the activity that produces one µmol of the colored product per minute upon oxidation of MBTH-DMAB in the above reaction mixture. MnP activity was calculated by the increase in A_{590} using molar absorption coefficient of 329000 M^{-1} cm^{-1} for the oxidation product.

$$\text{EU/ml} = A_{590} \times \frac{2}{32.9} \times t \times V$$

Where,

A_{590} = \text{change in absorbance at 590 nm, } t = \text{time (minute), } V= \text{ml of enzyme sample used in assay.}

**Laccase (Lac) (d’ Acunzo et al., 2002)**

Laccase activity was measured by following the rate of oxidation of 2,2’- azinobis (3-ethylbenzothiazoline-6-sulphonate) (ABTS) to ABTS radical cation (ABTS •⁺). The reaction mixture (3 ml) contained 2050 µl of 0.1M citrate buffer (CB), pH 5, 200 µl of 0.5mM ABTS in CB and 750 µl of enzyme sample. The reaction mixture was monitored at 414 nm against uninoculated media as blank and the value was
expressed as EU/ml. The extinction coefficient of ABTS \( \cdot^+ \) at 414 nm is \( 3.15 \times 10^4 \) M\(^{-1}\) cm\(^{-1}\).

One unit of Lac activity represents the quantity of enzyme producing 1 µmol of product per minute upon oxidation of ABTS to ABTS radical cation (ABTS \( \cdot^+ \)) in the above reaction mixture. Lac activity was calculated by the increase in \( A_{414} \) using molar coefficient of \( 3.15 \times 10^4 \) M\(^{-1}\) cm\(^{-1}\) for the oxidation product.

\[
\text{EU/ ml} = \frac{A_{414} \times 3}{31.5 \times t \times V}
\]

Where,

\( A_{414} = \) change in absorbance at 414 nm, \( t = \) time (minute), \( V = \) ml of enzyme sample used in assay.

**Plate assay for laccase (Sivakumar et al., 2010)**

*R. oryzae* was further examined for the presence of Lignolytic enzyme – laccase as a further confirmatory test on Czapke-Dox agar plate comprising (g/L) sucrose 30, NaNO\(_3\) 3, K\(_2\)HPO\(_4\) 1, MgSO\(_4\) 0.5, KCl 0.5, FeSO\(_4\) 0.01. ABTS (1mM) was amended with the basal medium comprising of g/L of peptone 1, KH\(_2\)PO\(_4\) 1, NaNO\(_3\) 4, MgSO\(_4\) 0.1, Na\(_2\)HPO\(_4\) 2, sucrose 20 and added to the above plate. The plates was inoculated with *R. oryzae* and incubated at 37°C for five days.

**Statistical Analyses**

Data were analysed by one-sample T-test for the effect of various pH, temperature and inoculum size. Paired T-test was performed for the effect of glucose, glycerol, VA, ethanol, guaiacol, CuSO\(_4\) as an inducer, concentrations of ammonium nitrate, lignocellulosic substrates and the laccase activity after optimization of cultural conditions using SPSS 17.0. Standard deviation was calculated by Microsoft Excel 2007.
Results and Discussion

Table 5.1 shows the detection of extracellular LMEs activity by *R. oryzae* under standard assay conditions using four day old cultural filtrate. The isolate showed LiP (0.0111 EU/ml) and Lac (0.0213 EU/ml) activities. MnP activity could not be detected.

**Table 5.1 LMEs activities by *R. oryzae***

<table>
<thead>
<tr>
<th>LMEs (EU/ml) →</th>
<th>LiP</th>
<th>MnP</th>
<th>Lac</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isolate ↓</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>R. oryzae</em></td>
<td>0.0111</td>
<td>-</td>
<td>0.0213</td>
</tr>
</tbody>
</table>

From the above enzymes detected, it was laccase that was produced in higher amount by *R. oryzae*. Hence, further enzymatic studies on decolorization and degradation were performed with laccase.

**Laccase plate assay**

The production of laccase by *R. oryzae* was further confirmed by the plate assay. The oxidation zone of green color around the fungal mycelial colony indicates the production of extracellular enzyme laccase by *R. oryzae* (Fig. 5.1).

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![Fig. 5.1 Production of laccase by *R. oryzae*](image)
Appended Tables 5.2 – 5.12 shows the effect of various parameters on growth and laccase activities and Table 5.14 shows the effect of optimized conditions on laccase activity by *R. oryzae*. Table 5.2 and Fig. 5.2 show the effect of various pH (3 - 9) on growth and extracellular laccase activity by *R. oryzae*.

Maximum growth and laccase activity were at pH 5 (109 mg/50 ml, 0.0140 EU/ml), followed by pH 4 (79 mg/50 ml, 0.0128 EU/ml) and pH 6 (31 mg/50 ml, 0.0118 EU/ml) respectively on 8th day. At further increase in pH i.e. pH 7 and 8, there was decline in growth as well as enzyme activity, ceasing after 10th day. The activity decreased with decreasing pH (3 - 4) as well as with increasing pH (6 - 8). However, at pH 9 there was complete suppression of growth and laccase
activity. The Fig and Table 5.2 clearly shows the delayed appearance of enzyme at decreasing pH i.e. 3 and 4 and at increasing pH i.e. 7 and 8. Thus, acidic conditions i.e. pH 5 proved to be favorable for laccase activity rather than neutral or alkaline conditions. Extreme pH i.e. 9 suppressed growth and enzyme activity. It was also observed that maximum enzyme was produced only after achieving maximum growth.

The pH of culture medium is too critical for growth, lignolytic enzyme production and xenobiotic degradation. The optimum pH for laccase production, as reported in many white rot fungi, falls between 4.5 – 6 (Sivakumar et al., 2010). Li et al., 2010 have reported pH 4 to be optimum for degradation of benzo pyrene by laccase and the activity rapidly decreases with either increasing or decreasing pH value and was almost lost at pH 7. Schliephake et al., 2000, have reported maximum laccase activity between pH 4.4 – 5 for Pycnoporus cinnabarinus and Polyporus sanguineus. The present results are in agreement to the above reports as most of the fungal enzymes especially laccases have maximum activity when the pH of the medium ranges between 4 – 6 (Galhaup et al., 2002; Jang et al., 2002; Chen et al., 2003) The results are also in accordance to that of Bhatt, 2008 reporting maximum laccase activity at pH 5 during degradation of paper pulp mill effluent by A.niger. Further, Patel et al., 2009, have reported that an exponential increase in laccase activity was observed from pH 3 to 5, with maximum at pH 5 and thereafter decreasing with increasing pH, the observation in accordance to the present results. However, Patrick et al., 2011 have reported highest laccase activity by Pleurotus sajor – caju at pH 6 and 5.5. This may be attributed to the fact that change in pH may alter the three dimensional structure of the enzymes (Shulter and Kargi et al., 2000), the electrostatic properties of the protein surface and the reaction centre or by influencing the stability of the enzyme (Patrick et al., 2011).
Table 5.3 and Fig. 5.3 shows the effect of various temperatures (25°C - 45°C) on growth and laccase activity by *R. oryzae*.

Maximum growth and laccase activity (136 mg/50 ml, 0.0070 EU/ml) were at 37°C, followed by 30°C (111 mg/50 ml, 0.0038 EU/ml) and 45°C (80 mg/50 ml, 0.0021 EU/ml) respectively. There was marginal growth at 25°C with complete suppression of enzyme activity. However at 45°C, growth was evident but the enzyme activity was suppressed ranging between 0.0013 – 0.0025 EU/ml for a brief period i.e. in between 4th – 6th day. Thus, lowest temperature of 25°C completely suppressed enzyme activity with marginal growth and highest temperature i.e. 45°C supported growth with loss of enzyme activity. Also maximum enzyme activity was after a period of maximum growth.

The time course activities of laccase were affected by changes in the temperature and organisms. It has been demonstrated that incubation temperature influences the activity pattern of LMEs and that the individual enzyme of a strain may be affected differently by temperatures, as temperature directly influences the growth and enzyme production (Lang *et al.*, 2000).

Zadrazil *et al.*, 1999 have reported maximum laccase activity at 30°C for *Pleurotus* and *Dichomitus squalens*. Similar results have been reported by Nakamura *et al.*, 1999 where maximum laccase activity from *Bjerkenda adusta* were attained at 30°C but above 37°C there was no activity observed. Li *et al.*, 2010 have reported 40°C temperature to be optimum for degradation of benzo pyrene indicating, higher temperature to be favourable for laccase activity. However, Lang *et al.*, 2000 have reported temperatures of 25 – 30°C to be optimum for LMEs with delayed appearance at 34°C, with no change in activity and suppression of enzyme activity at 45°C. Nyanhongo *et al.*, 2002, obtained highest laccase production at 30°C for *Trametes modesta*. Iqbal *et al.*, 2011, found substantial decrease in laccase activity of *Trametes versicolor* IBL-04 when cultivated at
temperature higher than 30°C. Declined growth and enzyme activity at high temperatures would lead to the loss of enzyme activity faster and the reduction of dissolved oxygen in the reaction system is adverse for enzyme catalysis. Thus, the present results indicate 37°C to be optimum for laccase with loss of activity at 45°C and complete suppression at 25°C.

Table 5.4 and Fig. 5.4 shows the effect of various inoculum sizes ($10^4 - 10^{10}$ spores/ml) on growth and laccase activity by *R. oryzae*.

Maximum growth and laccase activity were at $10^8$ spores/ml (123 mg/50 ml, 0.010 EU/ml), followed by $10^7$ spores/ml (111 mg/50 ml, 0.0076 EU/ml) respectively. Least growth and laccase activity were at $10^{10}$ spores/ml (60 mg/50 ml, 0.0038 EU/ml). There was a progressive increase in growth and laccase activity from $10^4$ spores/ml reaching maxima at $10^8$ spores/ml and decreasing therafter. However, maximum enzyme activity was after a period of maximum growth.

**Fig. 5.3 Laccase activity at temperatures (25 – 45 °C) by *R. oryzae***
Too high or too low inoculum concentrations cause low growth and productivity. Inoculum concentrations are important in fungal growth and productivity. A large inoculum in culture will rapidly lead to crowded growth and nutritional deficiency, poor aeration and low substrate availability resulting in decreased enzyme activity. Decrease in enzyme activity with either decrease or increase in inoculum size with maximum enzyme activity at 10⁸ spores/ml has been reported by Bhatt, 2008 which is in agreement to the present observations.

![Graph](image.png)

**Fig. 5.4 Laccase activity at inoculum size (10⁴ – 10¹⁰ spores/ml) by R. oryzae**

Table 5.5 and Fig. 5.5 shows the effect of glucose concentrations (0 – 4g/L) as C source on growth and laccase activity by R. oryzae.

To investigate the effect of C source on growth and laccase production by R. oryzae, glucose was added at the concentrations 1 – 4g/L replacing 1 g/L glucose in Asther’s medium. When glucose was used as a C source, maximum biomass
and laccase production were at 2g/L (180 mg/50 ml, 0.0125 EU/ml), followed by 1g/L glucose (118 mg/50 ml, 0.0100 EU/ml) respectively on 8th day. Least growth and enzyme activity were at 4g/L glucose (87 mg/50 ml, 0.0050 EU/ml). However, growth and laccase production were completely suppressed in absence of glucose indicating importance of carbon and its concentration in the medium.

Table 5.5 also suggests that in contrast to all previous parameters examined, maximum enzyme activity was during the period of maximum growth.

The results suggest that laccase synthesis is inhibited when glucose concentration is above or below certain level in medium. The same phenomenon has been reported for laccase production in *Trametes pubescens* and *T. versicolor* (Tavares et al., 2005). The results are also in accordance to Stajic et al., 2006 and Quaratino et al., 2008 suggesting glucose as optimum carbon source and have a significant effect on laccase production by *Pleurotus tigrinus* and *T. pubescens*. On contrary,
Hess et al., 2002 have reported that increasing glucose concentration from 10 – 40 g/L resulted in elevated biomass and laccase production. Patrick et al., 2011 have reported 10g/L glucose to be optimum for maximum laccase production by Plerotus sajor-caju. Galhaup et al., 2002 have reported 4 g/L glucose to be optimum for laccase. Thus, the present results suggest higher glucose concentration to be repressive for the production of laccase. Glucose is often found to repress genes that are used in the metabolism of alternative carbon sources. Glucose repression is widely known in fungi and yeasts and is thought to be an energy saving response (Ronne, 1995).

Reports of various Basidiomycetes grown in sugar–rich liquid medium were compared for laccase–producing ability. It was found that an excess of glucose or saccharose in the liquid medium eliminated the induction of laccases, where the constitutive production of laccase is maintained but the biosynthesis of induced laccase is repressed (Bollag and Leonowicz, 1984).

Table 5.6 and Fig. 5.6 show the effect of various NH₄NO₃ concentrations (0 – 24mM) as N source on growth and laccase activity by R. oryzae.

Maximum growth and laccase activity (121 mg/50 ml, 0.0120 EU/ ml) were at 24mM concentration, followed by 12mM concentration (113 mg/50 ml, 0.0085 EU/ml) and 6mM concentration (78 mg/50 ml, 0.0078 EU/ml). Least growth and laccase activity were in the absence of NH₄NO₃ in the medium. The results thus suggest that high nitrogen level of 24mM leads to enhanced laccase production indicating the importance of high nitrogen level for laccase production. Also, maximum enzyme activity was during the period of maximum growth.

Sivakumar et al., 2010 reported that the most widely used nitrogen source for fungal lignolytic enzyme production is ammonium nitrate in many white rot
fungi, namely *Basidiomycete* PM1, *Lentinula edodes*. Moreover, high nitrogen levels are usually required for greater amount of laccase to be produced, an observation in accordance to the present findings. However, as a deviation in some fungi, nitrogen limitation does not affect laccase production (Patrick *et al.*, 2011).

It is also observed that laccase production not only depends on the nitrogen source but also on the nitrogen concentration. Lignolytic systems of white – rot fungi get activated mainly during the secondary metabolite production and are often stimulated by nitrogen depletion as seen in *P. cinnabarinus* or *P. sanguineus* (Pointing *et al.*, 2000). However it is also found that the regulation of lignolytic activity by nitrogen source limitation is not a universal phenomenon among white rot *Basidiomycetes* (Leatham and Kirk, 1983). High nitrogen content of the medium has been shown to induce transcription of laccase genes in *Basidiomycete* I-62 (CECT 20197) and *P. sajor – caju* (Soden and Dobson, 2001). High levels of

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**Fig. 5.6 Laccase activity at NH₄NO₃ concentration (0 – 24mM) by *R. oryzae***
laccase were observed when *G. lucidum* (D’Souza *et al.*, 1999), *C. stercoreus* (Sethuraman *et al*; 1999) and *C. subvermispora* (Lobos *et al*., 1994) were grown in media with high nitrogen. However, Raghukumar *et al*., 1999, have shown *Flavodon flavus*, a Basidiomycete from coastal marine environment that was able to produce extracellular LMEs at low N levels.

Table 5.7 and Fig. 5.7 show the effect of various CuSO₄ concentrations (0 – 2 mM) on growth and laccase activity by *R. oryzae*.

Maximum growth and laccase activity were at 0.1 mM CuSO₄ concentration (134 mg/50 ml, 0.020 EU/mL), followed by 0.5 mM concentration (92 mg/ml, 0.0170 EU/ml) and 0.01 mM (118 mg/50 ml, 0.012 EU/ml) respectively on 8th day. Least growth and laccase activity were at 2mM concentration and in absence of CuSO₄. Thus, higher concentration of CuSO₄ decreased both growth and laccase activity indicating it as a potent inhibitor of fungal growth and laccase production. However, at highest concentration of CuSO₄, there was delayed appearance of laccase and in its absence, growth was evident with appearance of enzyme for a brief period. Also, maximum enzyme production was during the period of maximum growth.

A possible explanation for this stimulatory effect of Cu²⁺ on laccase biosynthesis could be a role of this enzyme in terms of defense mechanism against oxidative stress, with laccase involved in the synthesis of pigments to protect the uptake of metals. (Trupkin *et al*., 2003; Papinutti, 2009). Copper is an essential micro-nutrient for most living organisms and copper requirements by microorganisms are usually satisfied by very low concentrations of the metal. Copper is a cofactor of fungal laccase (Hoshida *et al*., 2005) and the ion has been reported to be a transcriptional regulator of laccase activity (Litvintseva and Henson, 2002).
However copper present at higher concentrations is extremely toxic to cells, though copper tolerant fungi have been reported, in agreement to the present findings suggesting inhibition of laccase at 2% CuSO$_4$ concentration. The induction of laccase synthesis by copper is widespread among fungi.

The amount of copper required to enhance laccase production varies greatly among different fungi – generally within the higher range of 1 – 25mM. Experimental results by Prasad et al., 2005 revealed that the presence of Cu$^{2+}$ significantly influenced laccase synthesis from 0.5mM – 1.5mM which are in agreement to the present result. Dekker et al., 2007 proved that the concentration of 0.1 - 1.0mM was reported to be optimum for laccase production and fungal growth, but at higher concentration the metal becomes toxic as demonstrated in growing cultures of $H$. acidophila which is totally inhibited at 10mM CuSO$_4$ which is again in agreement to the above results.

![Fig 5.7 Laccase activity at CuSO$_4$ concentration (0 – 2 mM) by R. oryzae](image-url)
Table 5.8 and Fig. 5.8 shows the effect of various concentrations of glycerol (0 – 4%) as sole C source on growth and laccase activity by *R. oryzae*.

Glycerol as a carbon source influenced the growth morphology of *R. oryzae* and laccase production. Glycerol proved to be a better source for both growth and laccase production as compared to glucose as a carbon source.

Maximum growth and laccase activity were at 3% glycerol concentration (198 mg/50 mL, 0.0215 EU/ml), followed by 4% (194 mg/50 ml, 0.0201 EU/ml) and 2% (168 mg/50 ml, 0.0191 EU/ml) respectively on 8th day. Least growth and laccase activity were at 1% (121 mg/50 ml, 0.0179 EU/ml). Fig. 5.8 shows that growth and laccase activity were slightly evident in absence of glycerol.

Gaulhaup *et al.*, 2002; Kasinath, 2002 have reported similar activity levels of laccase with both glucose and glycerol contrary to the present findings. Hess *et al.*, 2002, reported enhanced growth and laccase activities using glycerol than glucose by *T. multicolor* in accordance to the present observation. However, they also reported that raising the concentration from 20 to 40 g/L almost doubled the yield of laccase which did not prove to be the case with *R. oryzae*.

As is obvious from Fig. 5.8, laccase production started from 2nd day onwards utilizing glycerol from the beginning. As reported by Hess *et al.*, 2002, the production of laccase was prolonged by 5th day in *T. multicolor* indicating laccase production when glycerol was consumed in the medium, an observation contrary to the present findings suggesting production of laccase utilizing glycerol from 2nd day onwards. The results indicate glycerol to be favorable as a carbon source for *R. oryzae* as compared to glucose, even though the enzymes for glucose utilization are constitutive.
On contrary Hou et al., 2004 demonstrated that glycerol and glucose led to similar levels of laccase activity in static cultures of *P. ostreatus*. Undoubtedly, laccase production is dependent on the microbial taxa employed (Couto et al., 2005; Rodriguez et al., 2006).

Table 5.9 and Fig. 5.9 shows the effect of various concentrations of veratryl alcohol - VA (0.2 – 1 mM) as an inducer on growth and laccase activity by *R. oryzae*.

Maximum growth and laccase activity were at concentration of 0.4 mM (134 mg/50 ml, 0.0140 EU/ml), followed by 0.6 mM (121 mg/50 ml, 0.0126 EU/ml) and 0.2 mM (103 mg/50 ml, 0.0111EU/ml) on 8th day. Least growth and laccase activity were in absence of VA (66 mg/50 ml and 0.0040 EU/ml). Also, maximum enzyme activity was during the period of maximum growth. Thus, addition of VA at concentration of 0.4mM as an inducer led to 3.5 fold increase in laccase activity. The presence of aromatic compounds in production media has
significant influence on laccase yield. In the present study alcohols as VA and ethanol were examined to understand their relative influences on laccase production.

Inducers play an important role in affecting the ability of fungus on laccase production. VA can act either as a charge transfer mediator (Harvey et al., 1986), a stabilizer (Cancel et al., 1993), an inducer or a substrate for H$_2$O$_2$ producing enzyme or a source of reactive oxygen (De Jong et al., 1994). The addition of inducer during the exponential phase can generally reduce undesirable effects of toxic compounds on fungal growth.

Laccase production can be considerably stimulated by the presence of a wide variety of inducing substances, mainly aromatic or phenolic compounds related to lignin or lignin derivatives, such as ferulic acid, 2, 5 – xyldine and veratryl

![Fig. 5.9 Laccase activity at veratryl alcohol (VA) concentration (0 – 1 mM) by R. oryzae](image.png)
alcohol. Veratryl alcohol is a aromatic compound known to play an important role in enzymatic degradation of lignin (Zapanta and Tien, 1997). VA has been demonstrated to be a powerful inducer of laccase in Basidiomycete when added in low concentrations (Dekker et al., 2007). VA has been shown to enhance laccase production in several Ascomycota and WRF (Dekker et al., 2007). The addition of VA to cultivation medium of a lignin – degrading Basidiomyctes I – 62 resulted in a 10 fold increase in laccase activity (Schlosser et al., 1997). The inducing effect of laccase by VA has recently been reported in six wild isolates of B. rhodina from Brazil (Saldanha et al., 2007).

On contrary, Arora and Gill 2001 have reported inductive effect of VA on laccase production by Dichomitus squalens, G. lucidum and T. versicolor when various concentrations of 4, 16, and 20 mM were taken. Also, 40mM concentration led to 4 – 5 fold increase in laccase activity by Ascomycete Botryosphaeria sp (Barbosa, 2001).

Table 5.10 and Fig. 5.10 shows effect of various concentrations of guaiacol (0 – 2 mM) as an inducer on growth and laccase activity.

Maximum growth and laccase activity were at 2mM concentration (127 mg/50 ml, 0.0167 EU/ml), followed by 1 mM (98 mg/50 ml, 0.0135 EU/ml) and 0.5 mM (72 mg/50 ml, 0.0086 EU/ml) respectively on 8th day. Least growth and laccase activity (50 mg/50 ml, 0.0050 EU/ml) were in the absence of guaiacol supplementation suggesting the importance of an inducer. Thus, addition of guaiacol as an inducer led to 1.336 fold increase in laccase activity.

Patel et al., 2009 reported 1mM concentration to be optimum for P. ostreatus HP-1. Reports say that guaiacol enhanced laccase production in P. cinnabarinus, Phlebia radiata and Daedalea flavida (Herpoel et al., 2000). Similar results have been
obtained by Koroljova et al., 1998 in a comparative study on induction of laccase by a Basidiomycete Coriolus hirsutus.

Table 5.11 and Fig. 5.11 shows the effect of various concentrations of ethanol (0 – 10%) on growth and laccase activity.

Maximum growth and laccase activity were at 2% ethanol concentration (257 mg/50 ml, 0.191 EU/ml), followed by 1% (187 mg/50ml, 0.0153 EU/ml) and 5% (107 mg/50 ml, 0.0114 EU/ml) respectively on 8th day. Going to the extremes of concentration i.e. 0.5% and 10%, there was a steep decline in both growth and laccase production as shown in Fig. 5.11. Least growth and laccase production were in the absence of ethanol. Fig. 5.11 also indicates a peak in the laccase activity at only 2% ethanol concentration. Rest of the concentrations suppressed enzyme activity. Addition of ethanol thus, led to 25.13 fold increase in laccase activity.
The high yield of laccase may be attributed due to the inducible mechanism of ethanol at 2% concentration, however, the yield was less at higher concentration i.e. at 5 – 10% which may be attributed to its toxicity for fungal growth (Lomascolo, 2002).

Thus, amongst VA, guaiacol and ethanol as inducers, maximum fold increase (25.13) in laccase activity was with supplementing the medium with ethanol as evident from Fig. 5.11. The results are in accordance to other reports that indicate the addition of ethanol in the culture medium of a Basidiomycete stimulate laccase production to a great extent. Dekker et al., 2001 commented that ethanol stimulated the production of laccase in MAMB – 05 isolate of B.rhodina. With the addition of ethanol there was approximately 10 times increase in strain BM – 1 and 36.5 times increase in BG – 2. Also an increment of 11.37 times compared to control was obtained in T. versivolor after addition of 20 g/L ethanol, while it was 155 times increment in P. cinnabarinus after adding 35 g/L ethanol (Lomoscolo et al., 2003). Ethanol, at concentration of 40 g/L has been found to enhance laccase

![Fig. 5.11 Laccase activity at ethanol concentration (0 – 10%) by R. oryzae](image-url)
activity in *Trametes versicolor* ATCC 20869 submerged cultures, but the level was quite similar to that obtained with well-known inducers such as VA, Xylidine and Guaiacol (Lee et al., 1999). They reported that production of laccase in the production medium was 20 times higher with the addition of ethanol to the culture, the effect of which can be comparable to those well-known inducers such as veratryl alcohol, as well as xylidine and guaiacol. Vazquez - Gasciduenas *et al.*, 2011 reported that extracellular laccase activity showed a significant increase with addition of ethanol than with supplementation of Cu²⁺.

On the contrary, the presence of ethanol in semi solid-state cultures of *T. versicolor* CBS 100.29 led to laccase activities 40% lower than those achieved in control cultures. Meza *et al.*, 2007 demonstrated that ethanol acts as an inducer for laccase – gene expression in the case of the white rot fungus *P. cinnabarinus*.

It has been reported that ethanol may indirectly induce the synthesis of laccase through oxidative stress in cellular membranes brought about by alcohol (Crowe and Olsson, 2001). However it is not known if the molecular mechanism of induction by ethanol is similar to that of the induction by oxidative stress.

Table 5.12 and Fig. 5.12 show the effect of various natural lignocellulosic substrates (neem hulls, saw dust, rice husk and wheat straw) on growth and laccase activity.

Of the four different agro – residues (neem hulls, saw dust, rice husk and wheat straw) that were examined, all supported growth and laccase activity. Among the various substrates, saw dust was found to be the best substrate for both growth (127 mg/50 ml) and laccase production (0.266 EU/ml) on 6th day by *R. oryzae*. The next was rice husk showing maximum growth (72 mg/50 ml) and laccase activity (0.0135 EU/ml) on 8th day. Wheat straw supported least growth (98 mg/50 ml) as well as enzyme activity (0.0017 EU/ml). Also, there was no
further increase in fungal biomass after maximum laccase activity. The present study has proved the utility of saw dust, which is cost effective and easily available raw material as a suitable substrate for laccase production in solid state fermentation.

The purpose of supplementing glucose with lignocellulosics in the medium has been for two reasons: first it promotes the growth and rapid establishment of fungus within soild raw material. Second, the fungi needs additional easily metabolized carbon source to degrade lignin from lignocellulosic substrates (Kaal et al., 1995).

Lignocellulose is the major component of plant biomass, which represents the most abundant renewable organic resource. Agro residues contain significant amount of soluble carbohydrates and inducers for enzyme synthesis and therefore appear as prospective substrates for bioconversion into fungal biomass and lignolytic enzymes.

In recent years there has been significant interest in efficient use of agricultural waste as substrates in bioprocess for production of organic acids, ethanol, enzymes and other important secondary metabolites. Since the biotechnological applications require large amounts of low cost enzymes, one of the appropriate approaches for this purpose is to utilize the potential of lignocellulosic wastes, some of which may contain significant concentrations of soluble carbohydrates and inducers of enzymes synthesis ensuring efficient production of lignolytic enzymes.
Addition of different insoluble lignocellulosic materials including saw dust, rice husk, wheat straw, neem hulls, grape seeds, grape stalks, coconut husks, barley grain etc. into the culture medium were studied in order to enhance laccase production by white rot fungi. The agro-industrial wastes are abundant in most countries and are very likely to replace the aromatic compounds, such as ferulic acid, 2, 5-xylidine, veratryl alcohol, because of their availability and low cost. (Lorenzo et al., 2002). Pointing et al., 2000 suggested that *Pyc. sanguineus* grows effectively on agricultural lignocellulosic residues as laccase production increased 12 fold by the addition of wood fibres to liquid cultures without inducers. There was 3 fold increase in laccase production when *Pyc. Sanguineus* was grown on *Eucalyptus grandis* wood chips. Also laccase activity was higher with palm tree than with saw dust.

**Fig. 5.12 Laccase activity by various natural lignocellulosic substrates as source of ‘C’ by *R. oryzae***

Addition of different insoluble lignocellulosic materials including saw dust, rice husk, wheat straw, neem hulls, grape seeds, grape stalks, coconut husks, barley grain etc. into the culture medium were studied in order to enhance laccase production by white rot fungi. The agro-industrial wastes are abundant in most countries and are very likely to replace the aromatic compounds, such as ferulic acid, 2, 5-xylidine, veratryl alcohol, because of their availability and low cost. (Lorenzo et al., 2002). Pointing et al., 2000 suggested that *Pyc. sanguineus* grows effectively on agricultural lignocellulosic residues as laccase production increased 12 fold by the addition of wood fibres to liquid cultures without inducers. There was 3 fold increase in laccase production when *Pyc. Sanguineus* was grown on *Eucalyptus grandis* wood chips. Also laccase activity was higher with palm tree than with saw dust.
Simonic et al., 2010 have reported corn stem to be optimum for laccase production, whereas Balaraju et al., 2010 reported rice bran to be optimum for laccase. Schlosser et al., 1997 have reported high levels of laccase on wheat straw by *T. versicolor* and *T. tigrinus*. On the contrary, the above results indicate saw dust to be the best potential natural source for laccase activity.

*Ascomycetes* and *Basidiomycetes* spent their evolutionary history as terrestrials, with only some species adapting to water later in their evolution. Fungal products of biotechnological interest i.e. organic acids, ethanol, enzymes, spores and other important secondary metabolites were developed for use in solid substrates but not in liquids. Consequently, the cultivation of microorganisms in aqueous suspension may rather impair their metabolic efficiency (Holker et al., 2004).

Table 5.13 shows the optimized conditions that have been obtained for the production of laccase by *R. oryzae*.

Table 5.14 indicates the effect of optimized conditions (T) on laccase production by *R. oryzae* as compared to unoptimized conditions (C).

The above optimized conditions led to enhanced laccase production i.e. 0.266 EU/ml on 6th day as compared to unoptimized conditions, indicating laccase to be maximally produced on 8th day (Fig. 5.13). The optimized condition led to 22.16 fold increase in laccase activity as compared to control i.e. unoptimized conditions by *R. oryzae* as evident from Table 5.14. Thus, optimized conditions led to both enhanced laccase activity (22.16 fold) and decrease in time from 8th day to 6th day.
Table 5.13 Optimized conditions obtained for laccase activity by *R. oryzae*

<table>
<thead>
<tr>
<th>Sr No</th>
<th>Cultural Conditions</th>
<th>Optimized Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pH</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>Temperature</td>
<td>37°C</td>
</tr>
<tr>
<td>3</td>
<td>Inoculum size</td>
<td>$10^8$</td>
</tr>
<tr>
<td>4</td>
<td>Glucose (g/L)</td>
<td>2 %</td>
</tr>
<tr>
<td>5</td>
<td>Glycerol</td>
<td>3 %</td>
</tr>
<tr>
<td>6</td>
<td>Cu$^{2+}$</td>
<td>0.1 mM</td>
</tr>
<tr>
<td>7</td>
<td>Ethanol</td>
<td>2 %</td>
</tr>
<tr>
<td>5</td>
<td>Ammonium nitrate (mM)</td>
<td>1.2 mM</td>
</tr>
<tr>
<td>6</td>
<td>Lignocellulosic substrates (neem hulls, saw dust, wheat straw, rice husk)</td>
<td>Saw dust</td>
</tr>
</tbody>
</table>

Laccase formation is strongly influenced by the concentration of substrate used in the production medium. In general, substrates that are efficiently and rapidly
utilized by the organism result in high levels of laccase activity. It was also noted that the presence of aromatic compounds in production media had significant positive influence on the yield. However, this phenomenon is found to mainly depend on the type of aromatic compound present, the concentration and the time that substrate was added to the production medium. But at higher concentrations however, the yield was less indicating its toxicity towards fungal growth.

Extracellular laccases produced by filamentous fungi are part of the multienzymatic complex in charge of the degradation of lignocellulosic residues in nature. Enzyme activity has been widely studied in Basidiomycete, but has also been reported in Ascomycetes and their anamorphs. (Martinez et al., 2005). In white rot fungi, laccases are typically produced as multiple isoenzymes. Owing to its vivid biotechnological applications, studies on laccase producing organisms have been intensified in the recent years and optimization of laccase production from different microorganisms is being carried out by several groups. The ever-increasing demand for this enzyme requires the production process to be economical, identifying inexpensive raw materials for enzyme production could be viewed as a solution to make the entire process cost effective and further enhancement using inducers may add to the benefit. The use of inducers to enhance laccase production has been widely practiced in fungi especially white rots where laccase induction by aromatic compounds is well established (Sivakumar et al., 2010.)

In several organisms, extracellular laccases are constitutively produced in small amounts. However, their production can be considerably stimulated by the presence of wide variety of inducing substances, mainly aromatic or phenolic compounds related to lignin or lignin derivatives such as ferulic acid, 2, 5 –
xylidine, \( p \)-anisidine or veratryl alcohol, aliphatic alcohols, polysaccharides as well as by aqueous plant extracts.

Laccase expression in fungi is influenced by culture conditions such as nature and concentration of carbon and nitrogen sources, media composition, pH, temperature, presence of inducers, etc. The nutritive substances employed in the culture medium contribute significantly to the total production costs. Hence, it has been a matter of concern to find environmentally sound and economically feasible media constituents for laccase production. The biotechnological applications of laccase require large quantities of enzyme which can be obtained by optimization of culture conditions.

The present study shows that the addition of \( \text{CuSO}_4 \), ethanol, with a lignocellulosic substrate i.e. saw dust in the culture medium of \( R. \text{ oryzae} \) (Zygomycete) stimulates laccase production to a very high extent. The addition of milimolar amounts of \( \text{Cu}^{2+} \) is sufficient to boost the laccase production. Therefore, this strain can be an attractive and alternative source of laccase, an enzyme that has recently found widespread industrial applications.