# Determination of optimal conditions for laccase production by *Pleurotus ostreatus* using sawdust as solid medium and its use in phenol degradation

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## Abstract:

The ability of four local fungal isolates for extracellular laccase production has been tested with five grams 1:1(w/v) humidified sawdust as substrate in mineral salt medium. After 21 day of incubation at 25±1 ˚C and using one mycelial plug (5mm), higher level of laccase activity (0.15U/ml) and specific activity (15U/mg) were observed by *Pleurotus ostreatus* in comparison with other fungal isolates. The results of optimum conditions for laccase production from selected isolate showed that, the maximum laccase activity (0.55U/ml) and specific activity (55U/mg) were obtained at moisture ratio 1:3 (w/v), using 3 mycelial plugs (5 mm), after 15 days incubation period at 25±1 ˚C. The results of phenol degradation by crud laccase revealed that, 100% degradation of phenol occurred after 24 hr of incubation at 25 ˚C using shaking water bath except at 200mg/l, the remaining phenol was 10.13%.

**Key words: Sawdust, laccase, *Pleurotus ostreatus*, optimization, biodegradation.**

## Introduction:

Enzyme production is an important field in Biotechnology. Most enzyme manufacturers produce enzymes by submerged fermentation (SmF) techniques. However, in the last decades there has been an increasing trend towards the use of the solid-state fermentation (SSF) technique to produce several enzymes. A direct comparison between SSF and SmF cultivation techniques is difficult to make because the two processes are quite different. Studies on fungal enzyme production in SSF have shown that SSF, in comparison with SmF, provides higher volumetric productivities, is less prone to problems with substrate inhibition and yields enzymes at a higher temperature or pH stability [1].

Laccase (E.C. 1.10.3.2) is the most common ligninmodifying enzyme produced by the white-rot fungi belonging to the family Polyporaceae [2]. Among them, *Trametes versicolor* has extensively been used as the main experimental organism for laccase production studies [3]. Most studies dealing with ligninolytic enzyme production by white-rot fungi have been carried out using the liquid culture conditions, in spite of the fact that these organisms grow in nature in solid-state conditions. Recent reviews on solid-state fermentation (SSF) point out the enormous potential of this culture technique for the development of different bioprocesses [4]. The selection of a substrate for SSF processes depends upon several factors mainly related with cost and availability and thus may involve screening of several agro-industrial residues. Moreover, the utilization of this type of supports helps to solve the pollution problems caused by their

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disposal. The potential use of laccases in biotechnology has stimulated the need to discover suitable enzymes in large quantities. Laccase production may be affected by fermentation factors such as, fungal isolate, medium composition, pH, temperature and aeration. There have been reports describing increased production of extracellular laccases in many species of white rot fungi when grown on natural substrates, such as cotton stalk [5], molasses waste water [6], wheat bran [7] and barley bran [8]. Utilization of industrial and agricultural wastes for laccase production is an effective way to reduce production costs and also simultaneously utilize these substrates efficiently [9]. Laccase has been intensively investigated because of its ability to degrade biopolymeric structures and usefulness in the synthesis of organic compounds [10]. The current work aims to select the optimal conditions for laccase production from local fungal isolates using sawdust as substrate and its role in phenol degradation.

## Materials and methods :

### Fungal isolates

The isolates *Trichoderma* sp. and *Rhizoctonia solani* were obtained from Department of Biology, while *Fusarium* sp. and *Pleurotus ostreatus* were obtained from Department of Biotechnology, College of Science, Baghdad University. All fungal isolates were cultivated at 25±1°C on potato dextrose agar (PDA) and stored at 4°C.

### Laccase production on solid substrate

Solid-substrate fermentation (SSF) medium consists of 5gm sawdust has been used for producing the fungal enzymes. The substrate was humidified with a 5 ml w/v of mineral salt solution containing (0.2g $KH2PO4$, 0.1g $MgSO4.7H2O$, 0.3g $NH4Cl2$, 1.0g $CaCO3$ in 1L distilled water pH, 5.3). The humidified medium was placed in 250 ml Erlenmeyer flasks and autoclaved at (121°C, 20 min). The sterilized medium was inoculated with one mycelial plug (5 mm) from 7-day-old cultures of *Trichoderma* sp., *R. solani*, *Fusarium* sp. and *P. ostreatus* separately (two flasks for each isolate). Then the flasks were incubated for 21 days at 25±1° C [11]. Flasks without inoculation were used as control.

### Enzyme extraction:

Laccase was extracted from sawdust culture using 50ml (1:10 w/v), of 0.1M cooled citrate-phosphate buffer, pH 5.6. The contents of the flasks were grind in a mortar for 30 min in ice bath. The crud extracts were filtered through gauze, and then centrifuged at 6000 rpm for 15 min using cooled centrifuge [12].

### Enzyme and protein assays

Laccase activity was followed spectrophotometrically at 525 nm, through the oxidation of syringaldazine to its quinone form, using a molar absorptivity of 65,000 for the product [13]. The reaction mixture contained 2 ml citrate- phosphate buffer (0.1 M, pH 5.6), 0.2 ml syringaldazine (0.5 mM in methanol solution), and 1 ml of culture filtrates. The enzymatic activities were expressed as international units (U), defined as the amount of enzyme required to produce 1µmol product/min. Protein concentrations were determined using standard curve of bovine serum albumin [14].

**Effect of cultural conditions on laccase production:**

**- Moisture ratio:**

Mineral salt solution was added to the 5g sawdust at different ratio 1:1, 1:2, 1:3 and 1:4 w/v. The humidified medium was placed in 250ml Erlenmeyer flasks and autoclaved (121°C, 20 min). The autoclaved medium was inoculated with one mycelial plug (5 mm) from 7-day-old cultures of *P. ostreatus* (two flasks for each moisture ratio). The flasks were incubated for 21 days at 25±1° C. Flasks without inoculation were used as control.

**- Incubation period:**

Mineral salt solution has been added to the 5g sawdust at ratio 1:3 (w/v). The humidified medium was placed in 250ml Erlenmeyer flasks and autoclaved (121°C, 20 min). The sterilized medium was inoculated with one mycelial plug (5 mm) from 7-day-old cultures of *P. ostreatus* (two flasks for each incubation period). The flasks were incubated for 5, 8, 15, 18, 21, and 25 days at 25±1° C. Flasks without inoculation were used as control.

**- Inoculum size:**

Same medium above has been used with different inoculums size of 1, 2, 3, and 4 mycelial plugs (5 mm) from 7-day-old cultures of *P. ostreatus* (two flasks for each inoculum size). The flasks were incubated for 15 days at 25±1° C. Flasks without inoculation were used as control.

**- Incubation temperature:-**

To determine the effect of incubation temperatures in laccase production, 5g of sawdust with moisture ratio 1:3 have been inoculated with 3 mycelial plugs (5mm). The flasks were incubated for 15 day at 20, 25, 28, 30, and 37℃, (two flasks for each incubation temperatures). Flasks without inoculation were used as control.

- **Biodegradation of phenol by crud enzyme:**

The oxidation of standard phenol by crud enzyme has been performed by mixing 1ml enzyme filtrate with 2ml of 0.1M citrate-phosphate buffer (pH, 5.6) at different phenol concentrations (6.25, 12.5, 25, 50, 100, and 200 mg/l).The mixture was incubated at 25 ° C in shaking water bath. The percent (%) of phenol degradation was calculated after zero, 2, 4, 6, and 24 hr according to the following equation:

$$\text{Phenol degradation (\%)} = \frac{\text{Phenol concentration after incubation at different times} \times 100}{\text{Phenol concentration at zero time}}$$

## Results and discussion:

**- Laccase production from fungal isolates on solid state fermentation:**

In general five grams of sawdust humidified with mineral salt solution (1:1) (w/v) has been used as solid medium for laccase production from four fungal isolates. Results in (Tab.1) indicated that, the highest level of laccase production was obtained from *P. ostreatus*; laccase activity was 0.15 U/ml with specific activity 15U/mg. Other fungal isolates seem to be as a weak laccase producers, laccase activities were 0.02, 0.03 and 0.05 U/ml from *Trichoderma sp., Fusarium* sp., and *Rhizoctonia solani* respectively. However, [15] found that, a very low activity (0.005 U/ml) and high protein concentration (0.387 mg/ml) were observed when SmF was used to produce laccase from *P. ostreatus*. In current study, high level of laccase activity observed by using sawdust as substrate due to presence of

lignin as the main component of this waste which serve as enzyme inducer.

Solid state fermentation possesses several biological advantages as compared with submerged fermentations. Such advantages include higher fermentation productivity, less catabolic repression, low water demand and hence, lower sterility demand due to the low water activity [16]. However, the most suitable condition for maximum production of *Pleurotus pulmonarius* laccase (8,600 U/g substrate) was at initial moisture content of 75% in 5 days cultivation at 30 °C using syringaldazine as substrate [7].

The ability of various white-rot fungi for laccase production has been studied using different cereal crop wastes as substrate, for example, maximum laccase activity (68 U/ml) was obtained with *P. ostreatus* at 8th days of fermentation on wheat straw using (2,2_-azinodi-[3-ethyl-benzo-thiazolin-sulphonate]) ABTS as substrate for laccase activity assay [17] . On the other hand rice straw was identified as a suitable substrate for laccase production(17.3U/g)from *Streptomyces psammoticus* [18].

**Table 1: Laccase production from different fungal isolates on humidified sawdust Medium.**

| Fungal isolate | Laccase activity U/ml | Protein con. mg/ml | Specific activity U/mg |
|---|---|---|---|
| *Trichoderma sp.* | 0.02 | 0.015 | 1.33 |
| *Fusarium sp.* | 0.03 | 0.032 | 0.94 |
| *Rhizoctonia solani* | 0.05 | 0.017 | 2.94 |
| *Pleurotus ostreatus* | 0.15 | 0.010 | 15 |

## Effect of moisture ratio on *P. ostreatus* laccase production:

Moisture is another key parameter to control the growth of microorganism and metabolite production in SSF. In present work, Five grams of sawdust has been humidified by mineral solution with different moisture ratio (1:1, 1:2, 1:3, and 1:4 (w/v). Laccase production was increased with increasing the ratio of moisture reached up to 0.27 U/ml with 1:3 moisture ratios and then decreased (Fig, 1).

Higher initial moisture in SSF leads to suboptimal product formation due to reduced mass transfer, while decrease in initial moisture level results in reduced solubility and low availability of nutrients to the culture. [18] observed optimum laccase production by *S. psammoticus* at initial moisture content of 65%.

Sawdust is comparatively a dry substrate and hence a low initial moisture level was observed to be inadequate for moistening the substrate evenly. However increasing the initial moisture content above the optimum also resulted in decreased enzyme yield, and this may be due to the reduction in interparticle space and decreased porosity. Humidified grape seeds and barley bran with moisture ratios (1:3 and 1:6) were used to produce laccase from *Trametes hirsute*, barley bran led to the highest activity levels reaching maximum values of about 1.2kUl¯1 [19]. While, maximum laccase activity 40U/g obtained using wheat straw with moisture ratio 1:4 by *Fusarium incarnatum* LD-3, amongst the forty one isolates tested [20].

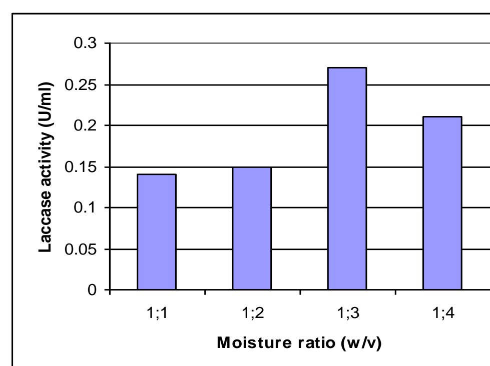


**Fig 1: Effect of moisture ratio (w/v) in laccase production by *Pleurotus ostreatus* on humidified sawdust medium.**

- **Effect of incubation period on *P. ostreatus* laccase production:**

The effect of incubation period on *P. ostreatus* laccase production has been studied. Maximum level of laccase activity (0.395 U/ml) was observed after 15 days of incubation (Fig,2) and specific activity was increased up to 39.5 U/mg (results are not showen). And no activity was observed even after 25 days of incubation. However, other investigator found high level of *P. ostreatus* laccase production (68U/ml) at 8th days of incubation, when wheat straw was used as substrate [17]. Different pattern in production of laccase was found with several white- and brown rot fungi cultivated on *Eucalyptus grandis* wood chips. *Trametes versicolor* produced the highest xylenase and cellulase from solid state culture at 15 days, while maximum laccase and peroxidase production were obtained until 60 days of incubation [21].

- **Effect of inoculm size on *P. ostreatus* laccase production:**

Different mycelial plugs (1, 2, 3, and 4 × 5mm) of *P. ostreatus* fungal isolate have been used. Laccase activity and protein concentration were determined after 15 day of incubation at 25±1°C. Highest level of laccase activity was obtained by using 3 mycelia plugs as inoculum, laccase activity was 0.55U/ml with specific activity 55U/mg (results are not shown). After the same incubation period (15 days) laccase activities were 0.390, 0.450, and 0.500 U/ml with using of 1, 2, and 4 mycelail plugs respectively (Fig. 3).

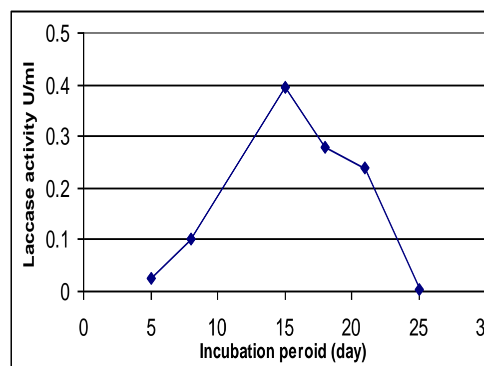


**Fig 2: Effect of incubation period (day) in laccase production by *Pleurotus ostreatus* on humidified sawdust medium.**

The enzyme yield has been reduced at lower and higher inoculum levels. A very low inoculum size was found to be inadequate for enzyme production while the inoculums above optimum level cause lowering in the yield probably due to the competition for nutrients.

In previous studies different inoculms size have been used for laccase production, four *p. pulmonarius* mycelial plugs measuring 10mm in diameter were used for inoculation of 5g of wheat bran, results showed higher laccase activity reached up to 8.600U/g substrate [7], while [19] have been used three *T. hirsute* agar plugs (diam., 3mm) for inoculated 2.5g barley bran or 5g grape seeds.

Among various substrates (wheat straw, corncobs, coconut coir, wheat bran, and rice bran) inoculated with five agar plugs (8mm) of white-rot fungi. Highest level of laccase production was observed with *P. ostreatus* at 8th day of fermentation using wheat straw as substrate [17]. Recent study with *F. incarnatum* LD3, four mycelial agar plugs of 8mm in diameter for inoculation of 5g wheat straw was used; laccase production was increased from 40U/g to 650U/g of substrate [20].

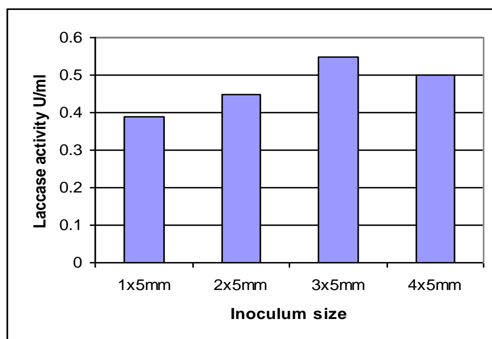


**Fig. 3: Effect of inoculum size (5mm mycelial plug) in laccase production by *Pleurotus ostreatus* on humidified sawdust medium.**

**Effect of incubation temperature on *P. ostreatus* laccase production:**

Temperature is much significance in the SSF systems because during fermentation there is a general increase in the temperature of the fermenting mass due to respiration [22]. Even though the impact of temperature is more prominent in the scale up processes it remains an inevitable factor in all fermentation systems due to its impact on microbial growth and metabolite production. Results of the present study (Fig. 4) suggested that an incubation temperature of 25ºC was the optimum for laccase production (0.69U/ml) and considerable specific activity was observed (69 U/mg) (results are not shown).

Although, the effect of temperature on the growth of *P. ostreatus* as well as laccase production by this organism has been studied extensively in earlier work [18], and it can be concluded that temperature exerts a similar effect on growth and enzyme production despite of the mode of fermentation. The data on effect of pH and temperature on laccase production was scarce, but most reports indicated that the initial pH between 4.5 and 6.0 was suitable for enzyme production [23]. [24] pointed that the optimum temperature for laccase production was between 25◦C and 30◦C, while the activity of laccase reduced when fungi were cultivated at temperatures higher than 30◦C the [25].

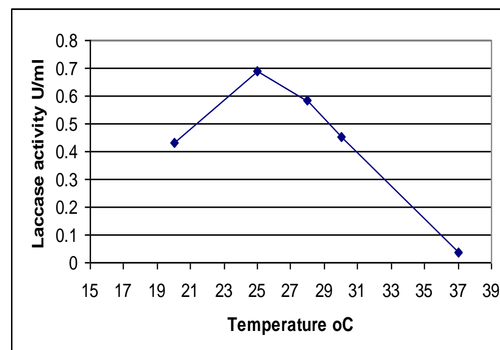


**Fig. 4: Effect of incubation temperature in laccase production by *Pleurotus ostreatus* on humidified sawdust medium.**

**Degradation of phenol by crud laccase:**

Phenol degradation by crud laccase has been investigated using enzyme filtrate with activity 0.55U/ml. The reaction mixture contains 1ml enzyme and 2ml phenol in citrate-phosphate buffer (0.1M, pH, 5.6) at different concentrations. The reaction carried out in shaking incubator at 25 ˚ C. About 50% of phenol was degrading after 4 hrs of incubation at all concentrations (Tab. 2), and 100% of phenol was degrading after 24 hr except at 200g/l, the remaining phenol was 10.13%.

Laccase is blue oxidase capable to oxidizing phenols and aromatic amines by reducing molecular oxygen to water by multicopper system [26]. Continuous elimination of 2,6-dimethoxyphenol by immobilized laccase has been carried out in a packed bed reactor. Gas chromatographical analysis showed that there was no 2,6-dimethoxyphenol dissolved in the filtrate after 20 hr [27].

**Table 2: Degradation (%) of phenol by crud laccase after different incubation time at 25 ◦C using shaking water bath.**

| Phenol con. mg/l | phenol degradation (%) | | | | |
|---|---|---|---|---|---|
| Incubation time (hr) | Zero time | 2 | 4 | 6 | 24 |
| 6.25 | 0 | 49.2 | 50.6 | 59.5 | 100 |
| 12.5 | 0 | 21.3 | 51.3 | 59.8 | 100 |
| 25 | 0 | 17.3 | 52.5 | 59.6 | 100 |
| 50 | 0 | 15 | 52.7 | 52.7 | 100 |
| 100 | 0 | 8.9 | 51.6 | 53.2 | 100 |
| 200 | 0 | 4.2 | 50.2 | 54.5 | 89.87 |

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# تحديد ألظروف المثلى لأنتاج اللاكتيز من الفطر *Pleurotus ostreatus* بأستخدام نشارة ألخشب كوسط صلب و دور ألانزيم في تفكيك الفينول

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**ألخلاصة :**

أختبرت قابلية أربعة عزلات فطرية محلية على انتاج اللاكيز بأستخدام 5غم من نشارة ألخشب التي تم ترطيبها بمحلول معدني بنسبة 1:1 (وزن/ حجم) كوسط للانتاج خلال 21 يوم من الحضانة بدرجة 25 ±1 مْ وبعد تلقيح الوسط بقطعة واحدة من النمو المايسيلي للفطر بحجم 5ملم. أعلى مستوى للفعالية والفعالية النوعية (0.15 وحدة/ملم) ( 15وحدة/ملغم) كانت من الفطر *Pleurotus ostreatus* تم تحديد الظروف المثلى لانتاج الانزيم من العزلة الفطرية المنتخبة وكانت : 1: 3 (وزن/حجم) نسبة الترطيب، ثلاثة قطع من النمو المايسيلي بحجم 5ملم، خلال 15 يوم من ألحضانة بدرجة 25 ±1 مْ حيث بلغت قيمة الفعالية والفعالية النوعية 0.55 وحدة/ملم 55 وحدة/ ملغم على التوالي.

أعطت نتائج تحلل الفينول بأستخدام مستخلص ألانزيم ألخام نسبة 100 % بعد 24 ساعة من الحضانة بدرجة حرارة 25 ْم بأستخدام الحمام المائي الهزاز، عدا عند ألتركيز 200 ملغم/لتر فأن نسبة الفينول المتبقي كانت .10.13%