ISSN: 0975-833X

Available online at http://www.journalcra.com

International Journal of Current Research
Vol. 6, Issue, 11, pp.9517-9522, November, 2014

INTERNATIONAL JOURNAL OF CURRENT RESEARCH

RESEARCH ARTICLE

OPTIMIZATION OF CULTURE CONDITIONS FOR IMPROVED LACCASE PRODUCTION BY *AGARICUS* SP. LCJ262

Jose Lincy, J. and *Joel Gnanadoss, J.

Microbial and Environmental Biotechnology Research Centre, Department of Plant Biology and Biotechnology, Loyola College (Autonomous), Chennai – 600 034, Tamil Nadu, India

ARTICLE INFO

Article History:
Received 11th August, 2014
Received in revised form
25th September, 2014
Accepted 04th October, 2014
Published online 18th November, 2014

Key words:
Laccase,
White rot fungi,
Agaricus sp.,
Submerged fermentation

ABSTRACT

Five different white rot fungi were isolated and screened for laccase production. The white rot fungus LCJ262 was found to exhibit high activity of laccase and was identified as *Agaricus* sp. according to the morphological characteristics. The culture parameters influencing the production of laccase by *Agaricus* sp. LCJ262 were optimized using one factor at a time method. Initially, five different basal media were tested and one best medium was selected for laccase production. Sucrose (20 g/L) and yeast extract (20 g/L) were the best carbon and nitrogen source, respectively for laccase production. Additional supplementation of 0.6 mM of copper sulphate and 0.2 mM of ferulic acid proved to be the best inducers for laccase production. An improved laccase production was also obtained with the addition of 0.2 g/L of powdered paddy straw (natural inducer) in the medium. An initial medium pH of 6.0, inoculum size of 5 g/L and shaking condition also augmented laccase production in *Agaricus* sp LCJ262. Isoenzyme determination of extracellular laccase from *Agaricus* sp. LCJ262 showed single laccase isoform.

INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductase E.C 1.10.3.2) are multi-copper enzymes belonging to the group of blue oxidases. Laccases catalyze the oxidation of a wide variety of organic and inorganic substrates including mono-, di-, and poly phenols, amino phenols, methoxy phenols, aromatic amines and ascorbate with the simultaneous four electron reduction of oxygen to water (Galhaup *et al*., 2002). Laccase enzymes are widely distributed in plants, bacteria and fungi. However, many researchers have reported that laccases are mostly of fungal origin especially from white rot fungi (Kiiskinen *et al*., 2004a; b). Fungal laccases play an important role in food industry, paper and pulp, textile, synthetic chemistry, cosmetics, soil bioremediation and biodegradation of environmental pollutant (Couto and Toca, 2006; Baldrian, 2006). These potentials of laccases in biotechnological and environmental applications have stimulated the need to discover promising laccases in huge amount and the demand for this enzyme requires the production process to be economical. Submerged fermentation is widely employed for laccase production and for other industrial enzyme production. This process of submerged fermentation involves the growth of microorganisms in a liquid media (Baldrian, 2006).

Corresponding author: Joel Gnanadoss, J.
Microbial and Environmental Biotechnology Research Centre, Department of Plant Biology and Biotechnology, Loyola College (Autonomous), Chennai – 600 034, Tamil Nadu, India

Maximizing laccase production can be achieved by optimizing nutritional conditions which includes carbon, nitrogen and inducer sources and physical conditions such as pH, agitation and inoculum size (Dong *et al*., 2005; Baldrian, 2006). The utilization of agro-wastes as natural inducer for laccase production is an efficient way to reduce production cost and replace aromatic chemical inducers (Lorenzo *et al*., 2002). The objective of the present study was to isolate laccase producing white rot fungi and optimization of culture conditions to enhance maximum laccase production under submerged fermentation.

MATERIALS AND METHODS

Isolation and culture maintenance

The cultures used in this study were isolated from fresh fruiting body collected from the Loyola College Campus, Chennai, India. The cultures were maintained on Potato Dextrose Agar (PDA) plates and sub-cultured every 2 weeks.

Screening for lacccase producing fungi

Qualitative assay for laccase activity

The isolates were screened for selecting the best laccase producing strain by growing them on PDA plates containing 4 mM guaiacol. The fungal isolates were inoculated and

incubated at 30 °C. The intense brown colour was formed under and around the fungal colony in guaiacol supplemented agar was considered as a positive reaction. The strain that exhibited maximum clear zone was selected for laccase production.

Quantitative assay for laccase activity

About 100 mL of Potato Dextrose Broth (PDB) was dispensed into 250 mL conical flask and autoclaved at 121˚C for 15 min. Then three fungal mycelial discs (6 mm) were inoculated into the conical flask under sterile condition. The flasks were then incubated on a rotary shaker at 120 rpm. Culture filtrate (5 mL) was taken on every alternate day and centrifuged at 10,000 rpm for 10 min. The clear supernatant was used as a crude enzyme source for determining laccase activity.

Production of laccase

Selection of liquid medium for laccase production

Laccase production by submerged fermentation was studied in five different basal media: Medium 1 (Jonathan and Fasidi, 2001) g/L: Glucose - 10, Yeast extract - 1.0, KH_2PO_4 - 1.0, $MgSO_4.7H_2O$ - 0.5, $CaCl_2$ - 0.14, thiamine - 0.0025; Medium 2 (Annuar, 2009) g/L: Glucose - 20, Yeast extract -2, Malt extrate - 2, Peptone - 2, KH_2PO_4 -0.46, $MgSO_4$ - 0.5, K_2HPO_4 - 1; Medium 3 (Couto *et al.,* 2006) g/L: Glucose - 20, Yeast extract - 15, $(NH_4)_2SO_4$ - 2, KH_2PO_4 -2, $MgSO_4$ - 0.5, $CaCl_2$ - 0.1, KCl - 0.05; Medium 4 (Tellez-Tellez *et al*., 2008) g/L: Glucose - 10, Yeast extract - 5, KH_2PO_4 - 0.6, $MgSO_4$ - 0.5, K_2HPO_4 - 0.4, $CuSO_4$ - 0.25, $MnSO_4$ - 0.05, $ZnSO_4$ -0.001; Medium 5 (Kalarani *et al.,* 2011) g/L: Glucose - 20, KH_2PO_4 - 2, $MgSO_4$ - 0.5, $CaCl_2$ -0.1, $CuSO_4$ - 0.1, Malt extract - 4, $(NH_4)_2SO_4$ - 2, KCl - 0.05. Each medium (100 mL) was dispensed into 250 mL conical flask and autoclaved at 121˚C for 15 min. Three fungal mycelia discs (6 mm) were inoculated into each of the conical flask, under sterile condition and they were then incubated on a rotary shaker at 120 rpm. 5 mL of the culture filtrate was taken every alternate day and centrifuged at 10,000 rpm for 10 min. The clear supernatant was used as a crude enzyme source for determining laccase activity.

Optimization of laccase production under submerged fermentation

Effect of carbon and nitrogen sources on laccase production

The laccase production by the selected isolate was optimized by supplementing different carbon sources such as glucose, sucrose, starch, maltose and fructose at 10 g/L concentration. The original medium was maintained as control. After screening for the suitable carbon source, its concentration range from 5 to 30 g/L was optimized. Similarly, the suitable nitrogen source for laccase production by the selected isolate was studied using organic nitrogen (yeast extract, peptone, and urea) and inorganic nitrogen (sodium nitrate, potassium chloride and sodium sulphate) sources. The original medium was maintained as control. The concentration of the optimum nitrogen source in the range of 5 to 30 g/L was also studied.

Effect of inducers on laccase production

The effect of copper concentration on laccase production was studied. Different concentration of copper sulphate ranging from 0.2 to 1.0 mM was added on the 4th day of inoculation and laccase activity was determined at interval of 2 days. The effect of inducer on laccase production was studied using a chemical inducer (ferulic acid) and natural inducer (paddy straw). Different concentration of ferulic acid (0.2 to 1 mM) was amended into the liquid medium on the 4th day of inoculation. The optimal concentration of finely powdered paddy straw for the production of laccase was studied by adding the different concentration (0.2 to 1.0 g/L) into the liquid medium on the day of inoculation. The basal medium without inducer was maintained as control and laccase activity was determined every alternate day of incubation.

Effect of physical factors on laccase production

The effect of optimum pH on laccase production was carried out by incubating the cultures in medium with different initial pH. The experiments were carried out individually at various pH ranging from 4 to 8. The enzyme assay was carried out individually every alternate day of incubation. Effect of inoculum size (1 to 5 g/L) on laccase production was carried out. The flasks after inoculation were incubated at 30 °C and laccase activity was determined. The influence of static and shaking condition on laccase production was also studied. One set of flask after inoculation was incubated at static condition and another set of flask was incubated on an orbital shaker at 120 rpm and the laccase activity was determined on every alternate day.

Analytical methods

Laccase assay

Laccase activity was assayed using 3 mL of 10 mM guaiacol in 100 mM acetone buffer (pH5.0) containing 10% (V/V) acetone and 1 mL culture filtrate was added then incubated for 15 min. The enzymatic activity was assayed by measuring oxidation of guaiacol at 470 nm (Collins and Dobson, 1997).

Calculation

$$\text{Laccase (U/mL)} = \frac{(\Delta A_{470} \times \text{total volume} \times \text{dilution factor} \times 10^6) / 1000}{\epsilon_{\text{Guaiacol}} \times \text{sample volume}}$$

ΔA_{470} = rate of reaction i.e. final absorbance – initial absorbance ÷ time (min)
$\epsilon_{Guaiacol}$ = molar extinction coefficient of the product (27.75)
Total vol = total volume of reaction mixture (mL)
Sample vol = volume of enzyme used (mL)

Protein assay

The protein content of the crude enzyme was estimated by using the method described by Lowry *et al*. (1951) with bovine serum albumin as the standard.

Zymogram analysis for laccase activity

Zymogram analysis for laccase activity was performed on Native-PAGE using modified SDS-PAGE technique

(Laemmli, 1970). Native PAGE was carried out under non-denaturating conditions. After separation, the gel was stained with 10 mM guaiacol in 100 mM sodium acetate buffer at pH 5.0.

RESULTS

Screening for laccase positive fungi

The fungi used for this study were isolated from different natural habitats and the isolated fungal strains were labeled as LCJ260 to LCJ264. Qualitative analysis for laccase activity was carried out in PDA plates supplemented with guaiacol. After the 3rd day of incubation, all the five isolates exhibited intense brown colour zone under and around the fungal colony. All the five isolates were found to be laccase producers. The results of the qualitative analysis are presented in Table. 1. Quantitative assay for laccase activity was carried out spectrophotometrically using guaiacol as the substrate. Among all the isolates tested, high amount of laccase was secreted by LCJ262 (3.89 U/mL) on the 8th day (Table 1) and hence this fungi were selected for further studies. Based on the macro-morphological characters of fruiting bodies and the culture characteristics, the isolate LCJ262 was identified as *Agaricus* sp.

Table 1. Qualitative and quantitative screening for laccase activity

Fungal isolates	Laccase activity	
	Qualitative screening	Quantitative screening Laccase activity (U/mL)
LCJ260	++	2.66
LCJ261	+++	3.04
LCJ262	+++	3.89
LCJ263	++	0.69
LCJ264	++	0.98

(Oxidation scale: 1-2 cm - +, 2-3 cm - + +, 3-4 cm - + + +)

Production of laccase

The production of laccase by *Agaricus* sp. LCJ262 was tested using five different culture media. Among all the culture media tested, *Agaricus* sp. LCJ262 produced maximum laccase (25.1 U/mL) in Medium 3 on the 8th day (Fig. 1).

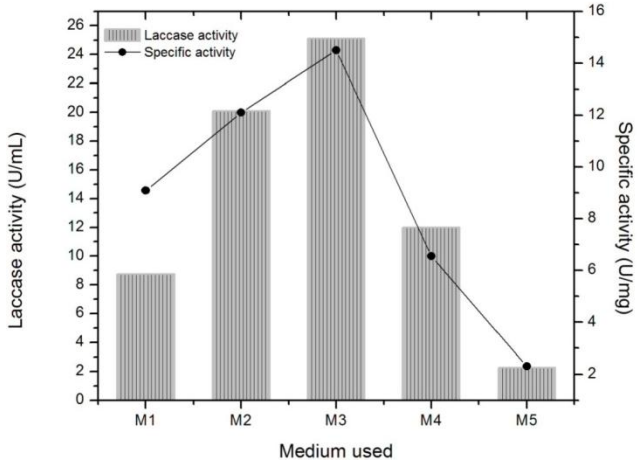

Fig. 1. Effect of different culture media on laccase production by *Agaricus* sp. LCJ262 on different days

(M1- Medium 1, M2- Medium 2, M3- Medium 3, M4- Medium 4, M5- Medium 5)

Therefore, Medium 3 was selected for further studies. The growth of *Agaricus* sp. LCJ262 and its laccase production in Medium 3 was also studied on different days after inoculation. The results showed that the laccase activity was the maximum on the 8th day of incubation (16.5 U/mL) and thereafter declined.

Optimization of laccase production

Effect of carbon and nitrogen sources on laccase production

Effect of different carbon sources on the laccase production by *Agaricus* sp. LCJ262 was studied using carbon sources such as glucose, sucrose, fructose, maltose and starch. The results showed that *Agaricus* sp. LCJ262 produced more laccase in the liquid medium containing sucrose (23.2 U/mL) when compared to the other carbon sources (Table 2).

Table 2. Effect of carbon and nitrogen sources on the laccase production by *Agaricus* sp. LCJ262 on the 8th day of incubation

Factors	Laccase activity (U/mL)
Carbon sources	
Glucose	14.9
Fructose	12.3
Sucrose	23.2
Starch	20.3
Maltose	15.1
Nitrogen sources	
Control	19.8
Potassium nitrate	15.3
Sodium sulphate	0.16
Sodium nitrate	1.82
Peptone	18.2
Urea	0.20
Yeast extract	25.4

This was closely followed by starch (20.3 U/mL). The influence of different concentrations of sucrose was tested and it was observed that the addition of 20 g/L sucrose enhanced maximum laccase production (26.1 U/mL). It was initially observed that the production of laccase increased with increasing concentration of sucrose but declined when the concentration increased above 20g/L (Table 3).

Table 3. Effect of sucrose and and yeast extract concentration on the laccase production by *Agaricus* sp. LCJ262 on the 8th day of incubation

Factors	Laccase activity (U/mL)
Sucrose concentration (g/L)	
Control	15.3
5	11.3
10	23.4
15	19.7
20	26.1
25	18.7
30	9.11
Yeast Extract concentration (g/L)	
Control	19.8
5	16.9
10	18.1
15	19.0
20	20.3
25	20.2
30	20.2

The influence of different nitrogen sources on the laccase production by *Agaricus* sp. LCJ262 was studied using organic (yeast extract, peptone, and urea) and inorganic nitrogen sources (sodium nitrate, potassium chloride and sodium sulphate). Results showed that the cultures grew better and also secreted high laccase when they were grown in medium containing organic nitrogen sources. A maximum laccase production of 25.4 U/mL on the 8th day was recorded in the medium amended with yeast extract. In medium containing peptone, the laccase activity of 18.2 U/mL was recorded (Table 2). The effect of varying concentration of yeast extract showed that in *Agaricus* sp. LCJ262, the maximum laccase activity of 20.3 U/mL was observed when the yeast extract concentration was 20 to 30 g/L. However due to cost consideration 20 g/L of yeast extract was chosen for the production of laccase (Table 3).

Effect of inducers on laccase production

The effect of different concentrations of copper sulphate on laccase activity was studied. The addition of 0.6 mM of copper sulphate induced a maximum laccase activity of 23.6 U/mL (Table 4). The effect of chemical inducer on laccase production by *Agaricus* sp. LCJ262 showed that the addition of 0.2 mM of ferulic acid enhanced the production of laccase (23.9 U/mL) (Table 3). The effect of addition of natural inducer to the medium on laccase production by *Agaricus* sp. LCJ262, showed that addition of 0.2 g/L of paddy straw was the best for the production of laccase (21.4 U/mL) as shown in Table 4.

Table 4. Effect of inducer on the laccase production by *Agaricus* sp. LCJ262 on 8th day of incubation

Factors	Laccase activity (U/mL)
Copper sulphate concentration (mM)	
Control	17.8
0.2	13.7
0.4	21.6
0.6	23.6
0.8	22.2
1.0	13.3
Chemical inducer (ferulic acid) concentration (mM)	
Control	9.76
0.2	23.9
0.4	21.5
0.6	20.7
0.8	16.5
1.0	14.8
Natural inducer (paddy straw) concentration (g/L)	
Control	10.8
0.2	21.4
0.4	14.8
0.6	20.8
0.8	20.3
1.0	14.0

Effect of physical factors on laccase production

The effect of initial medium pH ranging from 4 to 8 on laccase production by *Agaricus* sp. LCJ262 demonstrated that the initial pH of 6 was the best pH for the production of laccase (24.9 U/mL). Considerable laccase activity was observed at all pH evaluated in this study, however pH 6 was the optimum pH for maximizing laccase production (Table 5). The effect of different inoculum size on laccase production by *Agaricus* sp. LCJ262 was studied. Results showed that the laccase production increased with increase in inoculum size (Table 5). The maximum laccase production of 23.1 U/mL was observed when the inoculum size was 5 g/L. It was also found in that shaking condition favoured maximum laccase production of 13.6 U/mL on the 8th day of incubation when compared to static condition (Table 5).

Table 5. Effect of physical factors on the laccase production by *Agaricus* sp. LCJ262 on the 8th day of incubation

Factors	Laccase activity (U/mL)
pH	
4	11.3
5	10.2
6	24.9
7	10.5
8	10.4
Inoculum size	
1	3.65
2	6.62
3	18.6
4	21.2
5	23.1
Static and shaking	
Static	11.5
Shaking	13.6

Zymogram analysis for laccase activity

Zymogram analysis for laccase activity using extracellular laccase from *Agaricus* sp. LCJ262 produced in the optimized medium was carried out. Staining of the polyacrilamide gel with 10 mM guaiacol resulted in a single band as seen in Fig. 2.

Fig. 2. Zymogram analysis of laccase from *Agaricus* sp. LCJ262 produced in optimized culture medium by native PAGE stained by a 10 mM guaiacol

DISCUSSION

Laccase producing fungi are generally selected based on the primary screening in agar plate containing a suitable substrate. Laccase producing organism showed a brown coloured zone on the PDA plate amended with guaiacol. Alfarra *et al.* (2013) and Gnanasalomi and Gnanadoss (2013) also used guaiacol as an indicator for identification of laccase positive isolates. Among the five white rot fungi tested LCJ262 was found to be the most potent laccase producer and was identified as *Agaricus* sp. The production of laccases under submerged fermentation was evaluated using five different basal media and enzyme activity was observed to be different in each of the media used indicating the importance of the nutrient composition in enhancing the enzyme production by specific organisms. Similarly, Elshafei *et al.* (2012) evaluated eight different media for the production of laccase by fungal isolates. Radhika *et al.* (2013) also studied laccase production using five different liquid media. Each fungi has its own requirements for production of laccase under submerged fermentation and hence have to be standardized.

In this study using *Agaricus* sp. LCJ262, the carbon source sucrose enhanced maximum laccase production. Similarly, Bettin *et al*. (2009) demonstrated that sucrose is relatively a cheaper and most suitable carbon source for the growth and laccase production by *P. sajor-caju* PS-2001.

In the present study, inorganic and organic nitrogen substances were incorporated in the basal medium and their effect on the production of the enzyme was studied. Yeast extract enhanced maximum laccase production by *Agaricus* sp. LCJ262. Manimozhi and Kaviyarasan (2012) earlier reported that the addition of yeast extract enhanced laccase production in *Agaricus heterocystis*. The study also showed that the addition of copper sulphate (0.6 mM) favoured maximum laccase production by *Agaricus* sp. LCJ262. Palmieri *et al.* (2000) showed that the addition of 150 µM copper sulphate to the culture medium resulted in a fifty-fold increase in laccase production compared to the original medium. Addition of ferulic acid (synthetic inducer) also enhanced laccase production. Previous reports demonstrated that ferulic acid induced synthesis of laccase in white rot fungi (Herpoel *et al*., 2000). In the present study, finely powered paddy straw (natural inducer) enhanced maximum laccase production by *Agaricus* sp. LCJ262. Lorenzo *et al*. (2002) reported that agro-wastes which are rich in lignin can replace the aromatic compounds to enhance laccase production. They can be preferred due to their availability and low cost.

The present study showed that an initial pH of 6 favoured maximum laccase production by *Agaricus* sp. LCJ262. Most fungal cultures prefer a slight acidic pH in the medium for growth and enzyme production (Haltrich *et al*., 1996). Sivakumar *et al*. (2010) reported that *Ganoderma* sp. exhibited optimum laccase production at a pH of 6.0. The influence of inoculum size on laccase production by *Agaricus* sp. LCJ262 showed that 5 g/L inoculum of *Agaricus* sp. LCJ262 favoured maximum laccase production. Earlier, Sabu *et al*. (2005) stated that lower level of inoculum may not be sufficient to initiate growth and enzyme production. Hence the selection of a suitable inoculum size is considered important in augmenting the enzyme production. The present study showed that the production of laccase was higher in shaking condition than the static conditions. This is because shaking increases the oxygen transfer from the culture medium to cells. This facilitates optimum fungal growth and also the enzyme production (Swamy and Ramsay, 1999). The findings of the present study clearly indicate that *Agaricus* sp. LCJ262 is an efficient producer of laccase under submerged fermentation condition. The conventional one factor at a time method of optimization of various parameters for the production of laccase are governed by parameters such as carbon source, nitrogen source, copper and inducer concentration, pH, inoculum size and other parameters. A two fold increase in laccase production was obtained after optimization of culture conditions when compared to the original medium. The optimized medium components under submerged fermentation will help in maximizing laccase production by *Agaricus* sp. LCJ262 under large scale processes.

REFERENCES

Alfarra, H.Y., Hasali, N.H.M. and Omar, M.N. 2013. A ligninolytic Fungi with Laccase Activity Isolated from Malaysian local Environment for Phytochemical Transformation Purposes. *Int. Res. J. Biological Sci*. 2(2): 51-54.

Annuar, M.S.M. 2009. Optimization of nutrient levels for laccase fermentation using statistical techniques. *Asia-Pac. J. Mol. Biol.* 19 (2): 73- 81.

Baldrian, P. 2006. Fungal laccases - occurrence and properties. *FEMS Microbiol Rev.* 30: 215 - 242.

Bettin F., Montanari Q., Calloni R., Gaio T.A., Silveira M.M. and Dillon A.J.P. 2009. Production of laccases in submerged process by *Pleurotus sajor-caju* PS-2001 in relation to carbon and organic nitrogen sources, antifoams and Tween 80. *J. Ind. Microbiol. Biotechnol.* 36: 1-9.

Collins, P.J. and Dobson, A.D.W. 1997. Regulation of laccase gene transcription in *Trametes versicolor*. *Appl Environ Microbiol* 63: 3444-3450.

Couto, S.R. and Toca Herrera, J. L. 2006. Industrial and biotechnological applications of laccases: a review. *Biotech. Adv*. 24(5): 500–513.

Dong, J.L., Zhang, Y.W., Zhang, R.H., Huang, W.Z. and Zhang, Y.Z. 2005. Influence of culture conditions on laccase production and isozyme patterns in the white rot fungus *Trametes gallica. J. Basic Microbio* l45: 190-198.

Elshafei, A.M., Elsayed, M.A., Hassan, M.M., Haroun, B.M. and Othman, A.M. 2012. Optimization of cultural and nutritional parameters for the production of laccase by *Pleurotus ostreatus* ARC280. *Br. Biotechnol. J* 2(3): 115-132.

Galhaup, C., Goller, S., Peterbauer, C.K., Strauss, J. and Haltrich, D. 2002. Characterization of the major laccase isozyme from *Trametes pubsescens* and regulation of it's synthesis by metal ions. *Microbiology*. 148: 2159-2169.

Gnanasalomi, V.D.V., and Gnanadoss, J.J. 2013. Molecular characterization and phylogenetic analysis of laccase producing fungal isolates with dye decolourizing potential. *Res. Biotechnol*. 4 (5): 1-8.

Haltrich, D., Nidetzky, B. and Kulbe, K.D. 1996. Production of fungal xylanases. *Biores. Technol*. 58: 137-161.

Herpoel, I., Moukha, S., Lesage – Meessen, L., Sigoillot, J.C. and Aster, M. 2000. Selection of *Pycnoporus cinnabarinus* strains for laccase production. *FEMS Microbiol. Lett*. 183: 301-306.

Jonathan, S.G. and Fasidi, I.O. 2001. Effect of carbon, nitrogen and mineral sources on growth of *Psathyerella atroumbonata* (Pegler), a Nigeria edible mushroom. *Food Chem*. 72: 479-483.

Kalarani, R., Mohamad, S.M.A. and Muhd, A.A.K. 2011. Optimization of nutrient levels for laccase fermentation using statistical techniques. *Asia-Pac J. Mol. Biol.* 19 (2): 73- 81.

Kiiskinen, L.L., Ratto, M. and Kruus, K. 2004b. Screening for novel laccase-producing microbes. *J. Appl. Microbiol*. 97(3): 640–646.

Kiiskinen, L.L., Kruus, K., Bailey, M., Yiosmaki, E., Siika-aho, M. and Saloheimo, M. 2004a. Expression of *Melanocarpus albomyces* laccase in *Trichoderma reesei* and characterization of the purified enzyme. *Microbiology*. 150: 3065-3074.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227: 680-685.

Lorenzo, M., Moldes, D., Couto, R. and Sanroman, A. 2002. Improving laccase production by employing different lignocellulosic wastes in submerged cultures of *Trametes versicolor*. *Bioresour Technol*. 82: 109-113.

Lowry, O.H, Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with folin phenol reagent. *J. Biol. Chem*. 193: 265-275.

Manimozhi, M., and Kaviyarasan, V. 2012. Screening the effect of nutritional parameters on biomass and laccase production in submerged medium by litter decomposing basidiomycetes *Agaricus heterocyctis*. *Int. J. Pharm. Pharm. Sci.* 4 (3): 592-599.

Palmieri, G., Giardina, P., Bianco, C., Fontallella, B. and Sannina, G. 2000. Copper induction of laccase isoenzyme in the ligninolytic fungus *Pleurotus ostreatus*. *Appl. Microbiol. Biotechnol.,* 66: 920–924.

Radhika, R., Jebapriya, G.R. and Gnanadoss, J.J. 2013a. Production of cellulase and laccase using *Pleurotus* sp. under submerged and solid state fermentation. *Int J. Curr. Sci.* 6: E 7-13.

Rodriguez Couto, S., Lopez, E., and Sanroman, M.A. 2006. Utilisation of grape seeds for laccase production in solid state fermenters. *J. Food Eng*. 74: 263-267.

Sivakumar, R., Rajendran, R., Balakumar, C. and Tamilvendan, M. 2010. Isolation, Screening and Optimization of Production Medium for Thermostable Laccase Production from *Ganoderma* sp. *Int. J. Eng. Sci. Technol.* 2(12): 7133-7141.

Swamy, J. and Ramsay, J.A. 1999. Effects of glucose and NH_4 concentrations on sequential dye decoloration by *Trametes versicolor*. *Enzyme Microb. Tech.* 25: 278–284.

Tellez-Tellez, M., Fernandez, F.J. and Montiel - Gonzalez, A.M. 2008. Growth and laccase production by *Pleurotus ostreatus* in submerged and solid state fermentation. *Appl. Microbiol. Biotechnol*. 81: 675.

****