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A study on the optimization of spirulina concentration on the lignolytic enzyme production by the newly isolated *Trametes hirsuta* under laboratory conditions

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Abstract

Lignin is the second most abundant aromatic polymer in nature with three-dimensional structure composed of phenyl propanoid units linked through several carbon-carbon and ether bonds. Such complex structure of lignin is designed in plant cell wall to protect plant cells from microbial attack. Degradation of recalcitrant lignin requires an oxidative process mediated by lignolytic enzymes. The lignolytic enzymes include three enzymes viz., Lignin peroxidase, Manganese peroxidase and Laccase. These enzymes have its usage in lignin degradation and other pollutant degradation. Several fungi are known to secrete these enzymes. *P.chrysosporium* and *T.versicolor* are some of the prominent fungi studied for the production of lignolytic enzymes. The lignolytic enzyme production by *T.hirsuta*, a newly isolated species, was studied under laboratory conditions. The lignolytic enzyme production was studied in semi-synthetic culture media namely GPB. The proportions of lignolytic enzymes produced were as follows: Laccase [0.72 ± 0.13 U/ml], Lignin peroxidase [0.98 ± 0.26 U/ml] and Manganese peroxidase [0.92 ± 0.18 U/ml]. The effect of addition of spirulina to the culture medium was also studied and it showed increased production of lignolytic enzymes by *T.hirsuta*.

Keywords: Lignolytic enzyme, spirulina, fungi, degradation

INTRODUCTION

Enzymes are protein biomolecules that serve as catalysts to speed up or slow down the chemical reactions. Laccases (benzenediol: oxygen oxidoreductases EC 1.10.3.2) are multi-copper enzymes belonging to the group of blue oxidases. Laccases catalyze the oxidation of a variety of phenolic compounds, as well as diamines and aromatic amines, with concomitant reduction of molecular oxygen to water (Thurston ,1994).

Ligninolytic enzymes of the basidiomycetes play a crucial role in the global carbon cycle. The demand for application of ligninolytic enzymes complexes of white-rot fungi in industry and biotechnology is ever increasing due to their use in a variety of processes. The enzymes responsible for lignin degradation are mainly: lignin peroxidase (LiP), manganese peroxidase (MnP) and a copper containing phenoloxidase, known as laccase

Laccase is the most widely distributed of all the large blue copper-containing proteins, as it is found in a wide range of higher plants and fungi (Leontievsky *et al.,* 1997; Kiiskinen. & Saloheimo, 2004) and recently some bacterial laccases have also been characterized.

Laccases in plants have been identified in trees, cabbages, turnips, beets, apples, asparagus, potatoes, pears, and various other vegetables (Levine, 1965). Laccases have been isolated from Ascomyceteous, Deuteromyceteous and Basidiomyceteous fungi (Assavanig *et al.,* 1992). The number of fungal laccase producers are immense (Rodríguez and Toca Herrera , 2006) and they belong to the Basidiomycetes and Ascomycetes. Laccase enzyme has been reported from the following bacteria viz., *Azospirillum lipoferum* (Diamantidis *et al.,* 2007), *Bacillus subtilis* (Martins *et al.,* 2002), *and Streptomyces lavendulae* (Suzuki *et al.,* 2003), *S. cyaneus* (Arias *et al.,* 2003), *Marinomonas mediterranea* and *Streptomyces griseus*, (Jimenez *et al.,* 2005; Alexandre and Bally, 1999; Hosono *et al.,* 2002; Givaudan *et al.,* 1993; Hullo et al.,2001; Sánchez-Amat et al.,2001; Solano *et al.,* 2001).

Lignin peroxidase [EC 1.11.1.14] is another lignolytic enzyme, produced by several white rot fungi. Lignin peroxidase (LiP) plays a central role in the biodegradation of the plant cell wall constituent lignin. The mechanism by which lignin peroxidase (Lip) interacts with the lignin polymer which involves Veratryl alcohol (Valc), a secondary metabolite of white rot fungi, acts as a cofactor for the enzyme. LiP is able to oxidize aromatic compounds with redox potentials higher than 1.4 V (NHE) by single electron abstraction, but the exact redox mechanism is still poorly understood. *Phanerochaete chrysosporium* is one of the main organisms which is known to secrete LiP. Due to their high redox potentials and their enlarged substrate range LiPs have great potential for application in various industrial processes (Erden *et al.,* 2009). LiP shows little substrate specificity, reacting with a wide variety of lignin model compounds and even unrelated molecules (Barr and Aust, 1994).

Manganese peroxidases (EC 1.11.1.13) belong to the family of oxidoreductases and it was also first discovered in *Phanerochaete chrysosporium* and subsequent investigations have shown that MnP is distributed in almost all white-rot fungi (Hofrichter, 2002). The redox potential of the Mn peroxidase system is lower than that of lignin peroxidase and it has shown capacity for preferable oxidize in vitro phenolic substrates.

Lignolytic enzyme production has been found to be highly dependent on the conditions for the fungus cultivation (Heinzkill et al.,1998) and media supporting high biomass did not necessarily support high laccase yields (Buswell and Odier, 1987). Ligninolytic systems of white-rot fungi were mainly activated during the secondary metabolic phase and were often triggered by nitrogen concentration (Buswell *et al.,* 1995) or when carbon or sulfur became limiting. Laccases were generally produced in low concentrations by laccase producing fungi (Vasconcelos *et al.,* 2000), but higher concentrations were obtainable with the addition of various supplements to media (Lee *et al.,* 1999).

In several organisms, laccases are constitutively produced in small amounts. However, their production can be considerably enhanced by a wide variety of substances, including aromatic or phenolic compounds, metal ions, alcohol, and detergents (Leonowicz *et al.,* 2001). The enhancing effect of various inducers (phenolics, alcohols, heavy metals, vitamins, amino acids, antibiotics) for laccase production were also reported (Baldrian , 2004).

Other alternatives options to increase laccase production have been the use to diverse food wastes as inducers, such as apple, orange and potato, which were screened for laccase production, under solid-state fermentation conditions, by the white-rot fungus *Trametes hirsuta*. Potato peelings gave the highest activity, reaching about 5000 U/L within 8 days. These values are higher than those reported to date (Castillo *et al.,* 1994).

The lignolytic enzyme production by *Trichoderma hirsuta*, a newly isolated species, was studied under laboratory conditions. The lignolytic enzyme production was studied in semi-synthetic culture media namely GPB. The effect of addition of spirulina to the culture medium was also studied.

MATERIALS and METHODS

Isolation, identification and maintenance of fungal strain:

Lignolytic enzyme producing *Trametes hirsuta* was isolated from decaying wood and was identified by morphological and cultural characteristics then they were maintained on Malt Extract agar (MEA). For usage the cultures were sub-cultured on SDA plates.

Media Preparation:

The Glucose –Peptone-Broth [GPB] used for the study has the composition as shown in table-1.

Table-1: GPB composition

Ingredient	Quantity(in g/l)
Glucose	10.0
Peptone	3.0
KH_2PO_4	0.6
$ZnSO_4$	0.001
$K2HPO_4$	0.4
$FeSO_4$	0.0005
$MnSO_4$	0.05
$MgSO_4$	0.5

Inoculum preparation & inoculation

The isolated fungi, which were maintained in the MEA slants, were transferred to SDA plates and were incubated at room temperature for 2 weeks. The well grown fungi were used for inoculating GPB media. The fungal mat on SDA plates were cut with 8mm cork borer and with the help of inoculation loop and 5 discs was transferred to GPB flasks aseptically.

Culture conditions

The pH of the above culture media was adjusted to 6.5 and the flasks were incubated at 30°C Temperature in shaking incubator with 150 rpm and in a dark for 4 weeks.

Preparation of crude extract:

The conical flasks, after incubation period, were kept in shaker for 20 minutes and were shaken at 150RPM, then they culture was filtered with coarse filter paper and the filtrate was collected. Culture filtrate was then centrifuged at 8,000 rpm for 10 min. and the supernatant was used for enzyme assay.

Tests for lignolytic enzymes:

Manganese peroxidase activity was determined by using Castillo procedure [29]. One ml reaction mixture contained 007mM 3-methyl 2- benzo thiazolinone hydrazone (MBTH), 0.99mM 3 dimethylamino benzoic acid (DMBA), 0.34mMMnsO4, 100mM sodium lactate/succinate buffer (pH5.0) and 100-200 µL culture fluid. The reaction was initiated by the addition of 0.05mM H2O2. Absorbance was measured at 590nm using UV-Vis spectrophotometer after 1min.

Lignin peroxidase activity was evaluated by UV spectrometry of the veratryl aldehyde produced (ε 310= 9300 M−1cm−1) during veratryl alcohol oxidation. The reactive mixture contained 375 µL sodium tartrate buffer 0.33 M pH 3.0, 125 µL veratryl alcohol 4 mM, 50 µL hydrogen peroxide 10 mM, 450 µL distilled water and 250 µL culture medium for a final volume of 1250 µL (Eichlerova *et al.*, 2006).

Laccase activity was assayed spectrophotometrically by measuring the oxidation of ABTS at 420 nm at 30°C. The assay mixture in a total volume of 1 ml contained 0.1 ml cell-free supernatants at various dilutions and 1 mM ABTS in 100 mM citrate buffer (pH 3.4). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 µmol ABTS per minute (Bourbonnais and Paice, 1990).

Effect of Spirulina on Lignolytic enzyme production:

Commercially available spirulina [Dabur] was used for this experiment. About 2.0 grams of spirulina was added to the GPB medium and was designated as Spirulina-GPB media. The media was prepared and sterilized by standard procedures and was inoculated with *T.hirsuta* and incubated as above. The enzyme Lignolytic enzyme production was assayed as above.

Effect of varying spirulina concentrations of Lignolytic enzyme production:

To study the effect of varying concentrations of spirulina on lignolytic enzyme production by *T.hirsuta*, the following concentrations of spirulina were added to spirulina-GPB media [Grams/100ml] : 0.01, 0.05, 0.1, 0.5, 1.0, 1.5, 2.0 and 2.5. The inoculation, incubation and enzyme assay were done as above.

RESULTS and DISCUSSION:

The lignolytic enzyme production by *T.hirsuta* in GPB media was given in table-2 (and figure-1). It was found the *T.hirsuta* produces 1.78± 0.009 U/ml of laccase, 0.71± 0.001 U/ml of Lignin Peroxidase and 0.89± 0.023 U/ml of Manganese Peroxidase .The table-3 gives the comparative production of lignolytic enzymes by *T.hirsuta* and the same is given in figure-2. It is evident that the quantity of all the three lignolytic enzymes production in spirulina supplemented media [spirulina-GPB media] is higher than that of GPB media alone. The effect of varying concentrations of spirulina on lignolytic enzyme production by *T.hirsuta* is depicted in table-4 and figure-3. It was found that there was an increase in for Laccase and Lignin peroxidase enzymes concentration with increase in spirulina concentration upto 1gms/100, beyond that there is no increase in enzyme yield with increase in spirulina concentrtion. Similarly, for Manganese peroxidase the increase in enzyme yield was seen upto 1.5 grams of spirulina/100ml of GPB media; beyond that, here also, there is no increase in enzyme yield with increase in spirulina concentration.

Table-2: Lignolytic enzyme production in GPB media by *T.hirsuta*

Fungi	**Enzyme produced (U/ml) in GPB media**
Laccase Enzyme	**1.78± 0.009**
Lignin Peroxidase	**0.71± 0.001**
Manganese Peroxidase	**0.89± 0.023**

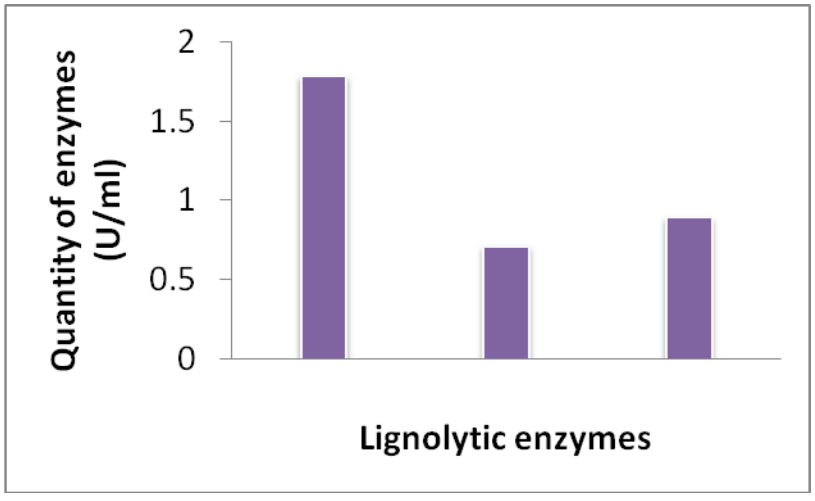

Figure-1: Lignolytic enzyme production in GPB media by *T.hirsuta*

Table-3: Comparison of Lignolytic enzyme production in GPB media and spirulina-GPB media by *T.hirsuta*

Fungi	Enzyme produced (U/ml) in	
	GPB media	GPB + Spirulina media
Laccase Enzyme	1.78 ± 0.009	2.58 ± 0.11
Lignin Peroxidase	0.71± 0.001	0.92 ± 0.006
Manganese Peroxidase	0.89 ± 0.023	1.02 ± 0.02

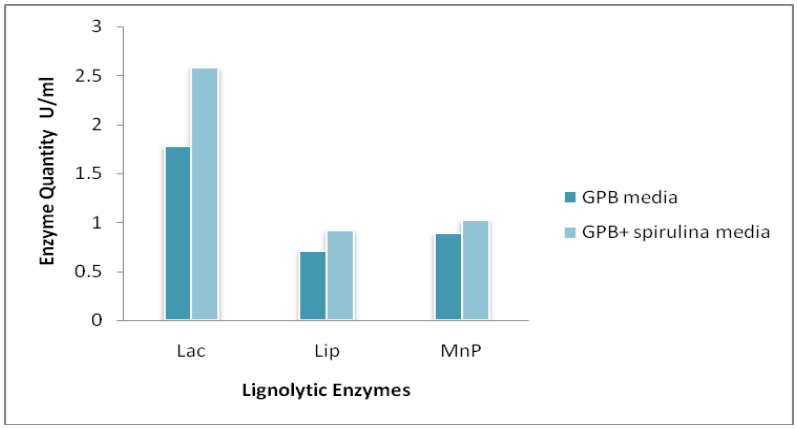

Figure-2: Comparison of Lignolytic enzyme production in GPB media and spirulina-GPB media by *T.hirsuta*

Table-4: Effect of spirulina concentration on Lignolytic enzyme production in GPB media by *T.hirsuta*

Conc. of Spirulina	Lignolytic Enzymes {U/ml}		
	Laccase	Lignin Peroxidase	Manganese Peroxidase
0.01	**0.21** ± 0.01	**0.01** ± 0.001	**0.12** ± 0.01
0.05	**0.56** ± 0.04	**0.23** ± 0.01	**0.36** ± 0.11
0.1	**1.64** ± 0.23	**0.38** ± 0.02	**0.59** ± 0.24
0.5	**2.11** ± 0.22	**0.61** ± 0.01	**0.78** ± 0.02
1.0	**2.58** ± 0.17	**0.92** ± 0.01	**1.02** ± 0.12
1.5	**2.58** ± 0.11	**0.92** ± 0.01	**1.05** ± 0.13
2.0	**2.58** ± 0.33	**0.92** ± 0.01	**1.05** ± 0.07
2.5	**2.58** ± 0.31	**0.92** ± 0.011	**1.05** ± 0.05

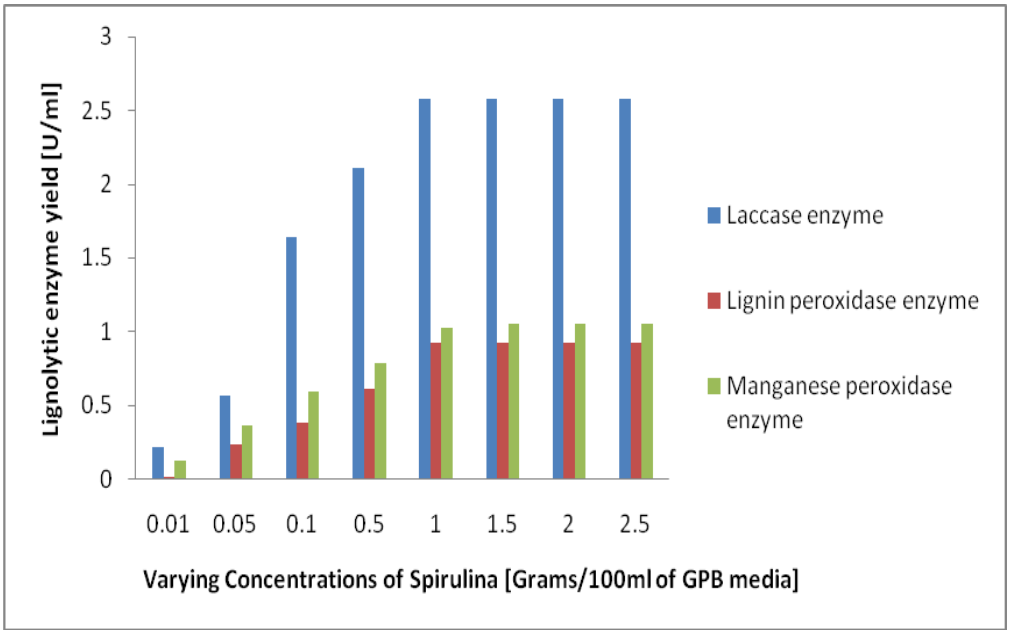

Figure-3: Effect of spirulina concentration on Lignolytic enzyme production in GPB media by *T.hirsuta*

Spirulina was shown to enhance the laccase production to a greater extent more than the aromatic inducers [(190 Ug-1 in the presence of Spirulina and (132 Ug^{-1}) in the presence of Tween 80] by Sampoorna Laxmi and Mazharuddin Khan (2010). Similarly, Laccase production was found to be 163.32 U/gds with medium supplemented with Cyano-bacterial biomass [CBM] 2.55g, when compared to the media without CBM (Abha Mishra *et al*., 2008).

SUMMARY & CONCLUSION

Thus, from the above experiment it is concluded that the addition of spirulina to the culture media increases the yield of all the three lignolytic enzymes. However, further experiments on statistical methods of media optimization are to be carried out to find the exact effect of media components and spirulina on lignolytic enzyme yield.

REFERENCES

Abha Mishra, Sunil Kumar and Sudhir Kumar.2008. Application of Box-Benhken solid experimental design for optimization of laccase production by Coriolus versicolor MTCC138 instate fermentation. Journal os scientific and industrial research, Vol.67, 1098-1107.

Alexandre G. and Bally R.1999. Emergence of laccase-positive variant of Azospirillum *lipoferum* occurs via a two-step phenotypic switching process. FEMS Microbiol Lett, 174: 371–378.

Assavanig, B. Amornkitticharoen, N. Ekpaisal, V. Meevootisom, and T. W. Flegel.1992. "Isolation, characterization and function of laccase from *Trichoderma* sp.Applied Microbiology and Biotechnology, vol. 38, no. 2, pp. 198–202, 1992.

Baldrian, P. 2004. Purification and characterization of laccase from the white-rot fungus Daedalea quercina and decolorization of synthetic dyes by the enzyme. Applied Microbiology Biotechnology 63: 560-563

Barr, D. P., and S. D. Aust. 1994. Mechanisms white rot fungi use to degrade environmental pollutants. Environ. Sci. Technol. 28:78A–87A.

Bourbonnais R, Paice MG .1990. Oxidation of non-phenolic substrates: an expanded role for laccase in lignin biodegradation. *FEBS Lett*.**267**:99–102.

Buswell JA, Cai Y, and Chang S. 1995. Effect of nutrient nitrogen and manganese on manganese peroxidase and laccase production by *Lentinela (Lentinus) edodes*. FEMS Microbiol. Lett. 128: 81-88

Buswell, J.A. and E. Odier, 1987. Lignin biodegradation. Crit. Rev. Biotechnol., 6: 1-60.

Castillo M. D., Stenstrom J. and Ander P.(1994). Determination of Manganese Peroxidase Activity with 3-Methyl-2-

benzothiazolinone Hydrazone and 3-(Dimethylamino) benzoic Acid. Analytical Biochemistry .Volume 218(2): 399-404.

Diamantidis G, Effosse A, Potier P, Bally R , 2000. Purification and characterization of the first bacterial laccase in the rhizospheric bacterium *Azospirillum lipoferum*. Soil Biol. Biochem. 32: 919-927.

Eichlerova, I., Homolka, L. and Nerud, F.: Ability of industrial dyes decolorization and ligninolytic enzymes production by different Pleurotus species with special attention on Pleurotus calyptratus, strain CCBAS 461. Process Biochem., 41: 941-946 (2006).

Endo K. Hosono K., Beppu T. and Ueda K. 2002. A novel extracytoplasmatic phenol oxidase of *Streptomyces*: its possible involvement in the onset of morphogenesis. Microbiology 148: 1767–1776.

Erden, E.; Ucar, C.M.; Gezer, T. and Pazarlioglu, N.K. (2009). Screening for ligninolytic enzymes from autochthonous fungi and applications for decolorization of Remazole Marine Blue. Brazilian Journal of Microbiology, vol. 40, no. 2, p. 346-353.

Givaudan A., Effosse A., Faure D., Potier P., Bouillant M.L. and Bally R. 1993. Polyphenol oxidase in *Azospirillum lipoferum* isolated from rice rhizosphere: evidence for laccase activity in non-motile strains of *Azospirillum lipoferum*. FEMS Microbiol Lett 108: 205–210.

Hofrichter, M. (2002). Review: Lignin conversion by manganese peroxidas (MnP). Enzyme and Microbial Technology, vol. 30, no. 4, p. 454-466.

Hullo M.F., Moszer I., Danchin A. and Martín-Verstraete I. 2001. CotA of *Bacillus1subtilis* is a copper-dependent laccase. J Bacteriol 183: 5426–5430.

Kiiskinen, L.-L. & Saloheimo, M.2004. Molecular cloning and expression in *Saccharomyces cerevisiae* of a laccase gene from the ascomycete *Melanocarpus albomyces*. Appl Environ Microbiol 70, 137–144

Lee IY, Jung KH, Lee CH, Park YH. Enhanced production of laccase in *Trametes versicolor* by the addition of ethanol. Biotechnol Lett 1999;21:965–8.

Leonowicz, A.; Cho, N. S.; Luterek, J.; Wilkolazka, A.; Wojtas-Wasilewska, M.; Matuszewska, A.; Hofrichter, M. and Wesenberg, D. ,2001. Fungal laccase: properties and activity on lignin. J. Bas. Microbiol., 41, 185-227.

Leontievsky A., Myasoedova N., Pozdnyakova N., Golovleva L.1997. 'Yellow' laccase of Panus *tigrinus* oxidizes non-phenolic substrates without electron-transfer mediators. FEBS Lett., 1997, 413(3):446-448.

M. E. Arias, M. Arenas, J. Rodr íguez, J. Soliveri, A. S. Ball, and M. Hernández.2003. "Kraftpulp biobleaching and mediated oxidation of a nonphenolic substrate by laccase from *Streptomyces cyaneus* CECT 3335," Applied and Environmental Microbiology, 69(4), pp. 1953–1958.

M. Heinzkill, L. Bech, T. Halkier, P. Schneider, and T. Anke,(1998). "Characterization of laccases and peroxidases from wood-rotting fungi (family Coprinaceae)," Applied and Environmental Microbiology, 64(5), pp. 1601–1606.

M.V.Sampoorna Laxmi and Mazharuddin Khan. 2010. Effect of Natural Phenolic and Lignin rich Inducers on the Production of Laccases by *Streptomyces griseus* MTCC 4734. International Journal of Engineering Science and Technology Vol. 2(6), 2010, 2130-2132.

Martins, L. O., C. M. Soares, M. M. Pereira, M. Teixeira, T. Costa, G. H. Jones, and A. O. Henriques. 2002. Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the *Bacillus subtilis* endospore coat. J. Biol. Chem. 277:18849-18859.

N. Jimenez-Juarez, R. Roman-Miranda, A. Baeza, A. Sánchez-Amat, R. Vazquez-Duhalt, and B. Valderrama,2005. "Alkali and halide-resistant catalysis by the multipotent oxidase from *Marinomonas mediterranea*," Journal of Biotechnology, 117(1), pp. 73–82, 2005.

Rodríguez Couto S, Toca Herrera JL.2006. Industrial and biotechnological applications of laccases: a review. Biotechnol Adv. 24(5):500-13.

Sánchez-Amat A., Lucas-Elío P., Fernández E., García-Borrón J.C. and Solano F.2001. Molecular cloning and functional characterization of a unique multipotent polyphenol oxidase from *Marinomonas mediterranea*. Biochim Biophys Acta, 1547: 104–116.

Solano F., Lucas-Elío P., López-Serrano D., Fernández E. and Sánchez-Amat A.2001. Dimethoxyphenol oxidase activity of different microbial blue multicopper proteins. FEMS Microbiol Lett, 204: 175–181.

Suzuki, T., K. Endo, M. Ito, H. Tsujibo, K. Miyamoto, and Y. Inamori. 2003. A thermostable laccase from *Streptomyces lavendulae* REN-7: purification, characterization, nucleotide sequence, and expression. Biosci. Biotechnol. Biochem. 67:2167-2175.

Thurston CF, (1994). The structure and function of fungal laccase. Microbiology 140: 19–26.

Vasconcelos, A. F. D. , A. M. Barbosa, R. F. H. Dekker, I. S. Scarminio, and M. I. Rezende.2000. "Optimization of laccase production by Botryosphaeria sp. in the presence of veratryl alcohol by the response-surface method," Process Biochemistry, 35(10), pp. 1131–1138.

W.G. Levine,1965. *Laccase, a review, In: The biochemistry of copper* (Academic Press Inc., New York, 1965), pp.371-385.