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Enhanced Laccase Production By Trametes hirusta Using Wheat Bran Under Submerged Fermentation

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Abstract: The productivity of laccaseusing white rot fungus *Trametes hirsuta* was observed in a shaking liquid culture. Gradual increments in laccase production were achieved via screening of the carbon, nitrogen and inducer by one factor at a time method. Time course of laccase production and time course of inducer additionwas monitored.Laccase production of around 9300 U/Lwas obtained with wheat bran as the carbon source, which is one of the lignocellulosic materialsabundantly found as an agro industrial waste, and the production was increased to 17390 U/L with wheat bran and peptone combination. Addition of 1mM 2, 5 xylidinein combination with 1mM copper sulphate on third day enhanced the laccase production to 23543U/L. Further enhancement in laccase production and the influence of significant media components wereaccomplishedusing plakettburmann design (PB) statistical method with the maximum laccase activity of 25889 U/L. The following components viz, wheat bran, peptone, magnesium sulphate, copper sulphate and 2, 5 xylidine were found to be the significant media components.
Key Words: Laccase, *Trameteshirsuta*, Wheat Bran, Peptone, 2, 5 Xylidine, PlakettBurmann Design.

Introduction

Laccase(benzenediol:oxygenoxidoreductases, (EC. 1.10.3.2), Phylogenitically an old enzyme of oxidoreductase family and major secretome of many of the white rot fungi than MnP and lip. Capable of oxidizing organic and inorganic compounds particularly phenols with formation of water[1].It comprises four catalytic copper atoms viz, one T1 copper, two T2 copper and a T3 copper, placed in different position according to their spectroscopic property[2]. It has widely been found in nature, such as plants[3, 4], fungi[5, 6], bacteria[7, 8] and insects[9, 10] respectively.Diverse group of fungi viz white rot, brown rot, and leaf litter fungi with different nutrition patterns have been found in the phylum basidiomycota and possess unique features like edible in nature, medicinal, biotechnological and environmental importance. Notwithstanding that the fungi from white rot basidiomycets have the potential to degrade completely all wood constituents such as cellulose, hemicellulose, and lignin by their enzymatic systems viz, ligninolytic and hydrolytic enzymes[11,12]. Ligninolytic enzyme production by the wood rotting fungi is a phenomenon that the combined interaction between the physiology of fungi and the

composition of essential media used for cultivation. There are some instances have distinctly proved the statement that in a synthetic medium, the white rot fugus *Phanerochaete chrysosporium* has expressed the both peroxidases (MnP,LiP) only in a nitrogen limited media[13]. In contrast both peroxidases and the oxidative enzyme laccase were produced when cultivated in a media containing lignocellulosic substrate as carbon source and a highly concentrated organic nitrogen source have led to the change[14].The pattern of laccase expression by the wood degrading fungi and species is a inconsistent process and vary one another, also its production can be influenced by different cultivation modes and conditions[15,16]. Almost, laccases from the majority of lignolytic fungi have been expressed in low quantities in the culture fluid[17], albeit the production has been induced with aid of compounds such as ferulic acid, 2,5-xylidine, p-anisidine or veratryl alcohol that belong to the aromatic or phenolic compounds related to lignin or lignin derivatives[18].The implementation of many applications attributed on laccase is possible only by increasing the production to larger amount as well as with low production cost. The utilization of lignocellulosic wastes and its byproducts facilitates the laccase production economical and high yield, due to the presence some naturally available inducers along with the soluble carbohydrates[19].This enzyme able to degrade lignin and hazardous compouds[1], oxidation of both phenolic and non-phenolic compounds, synthetic dyes respectively.Owing to versatile capability of this enzyme it has been utilized in various industrial applications viz biopulping and bleaching1, textile industry[21, 22,] food industry[23], synthesis of organic chemicals[24], biosensors[25]etc.Based on the striking biotechnological applications of laccase, extraordinary efforts have been devoted towards the optimization of process for improving the laccase production using various groups of microorganisms by different researchers so far. The problem ever associated with this enzyme is minimization of production cost. Searching of low cost, as well as easily available raw materials make the production process economical. Laccase production has been improved by the use of inducers for white fungi have frequently been performed especially the aromatic compounds[26].The present work is focused on the laccase production by the use of agro industry waste wheat bran as carbon source to achieve the economical and better production. Also several aromatic inducers were examined to increase laccase production. The statistical tool plakett-Burmann design implemented to screen the prominent process variables for laccase production by white rot fungus *T. hirsuta*.

Materials and methods

Chemicals

All the chemicals used in this experimentation were purchased from Hi-Media Limited, Mumbai, and were of the highest purity available. 2, 2'-Azinobis (3-ethylbenzthiazoline-6-sulfonic acid (ABTS) was obtained from Sigma.

Microorganism

The white rot fungus *T.Hirsuta* 141 was purchased from microbial type culture collection (MTCC), Chandigarh, and maintained in glucose yeast extract agar (YEA) and sub cultured every 30 days and maintained at 4^{o}C.

Inoculum preparation

The actively grown slant was used to prepare spore suspension by the addition of sterile water and 10 ml of the spore suspension was transferred to 100 ml of yeast extract broth (YEB) and incubated at 25^{o}C at 150 rpm. The three day old culture was used to inoculate the production media.

Growth media and culture conditions

A modified media was used for laccase production by *Trametes hirsuta* contained (g/l): glucose 2g; peptone (peptone from meat, bacteriological) 5g; yeast extract 5g, magnesium sulfate 5g; copper sulphate 1mM[6]. The experiments were conducted in 250-mL Erlenmeyer flasks containing 100 ml of the basal media was inoculated with three ml of inoculum and pH was adjusted to 5.8 and sterilized at 121^{o}C for 15 min and incubated at 150 rpm at 25^{o}C.

Experimental work

Time course of laccase production

In order to predict the optimum fermentation period of laccase production by the organism was observed with respect to the cell mass concentration. Samples were taken every 24 hour for analysis.

Time course of copper sulphate addition

To establish a precise time period to incorporate the copper sulphate in the production media for increasing the laccase production was studied. So the optimum concentration of copper sulphate was added with an interval of 24 hour till 5^{th} day.

Time course of 2, 5 xylidine addition

To establish the proper time period to add 2, 5 xylidine in the media for enhancing the laccase production was examined. So the optimum xylidine concentration was added, from the inoculation period to 5^{th} day of the fermentation.

Effect of lignocellulosic material on laccase production

In order to reduce the production cost as well as to achieve better laccase production different lignocellulosic materials were examined for laccase production. Various ligninocellulosic materials such as rice bran, wheat bran, elephant grass, waste paper, orange peeling, lemon peeling, water hyacinth and baggase, were collected from the local market and they were powdered and dried at 60^{o}C. The basal media cultivated with 20 g of each lignocellulosic material and flask with glucose was maintained as control.

Effect of nitrogen source on laccase production

To assess the impact of nitrogen source on laccase production was studied in single and mixed mode. Nitrogen sources such as peptone, urea, L-aspargine, yeast extract, malt extract,NH_4SO_4, tryptone, yeast extract + NH_2SO_4, yeast extract + L-Asparagine, yeast extract + malt extract, urea + peptone, Peptone + NH_2SO_4 and peptone + yeast extract maintained as control. All the nitrogen sources were taken in the range of 10 g/l of the basal media for single mode and 5 g/l for mixed mode.

Effect of inducers on laccase production

Different inducers such as 2,5 xylidine, 2,4 dimethoxy phenol, 1- napthol, veratryl alcohol, galic acid, Ferulic acid, hydroxybenzotriazole (HBT), were examined for enhancing the laccase production. Except guaiacol and vanilic acid, all the compounds were dissolved in 5% ethanol and sterilized by filtration. These inducers were added on the 3^{rd} of the fermentation.

Effect of various concentration of copper sulphate on laccase production

Various concentrations of copper sulphate were analyzed to enhance the laccase production. The following concentrations such 0.25mM, 0.5mM, 0.75 mM, 1mM, 1.5 mM, 1.75 mM, and 2 mM were added on 3^{rd} day of the fermentation. The flask without the addition of copper sulphate was maintained as control.

Effect various concentrations of 2, 5 xylidine

To optimize the suitable concentration of 2, 5 xylidine on laccase production by *T. hirsute* was performed. The concentrations such as 0.25 mM, 0.5 mM, 0.75 mM, 1mM, 1.5 mM, 1.75 mM and 2 mM were added on third day of fermentation. The flask without 2,5 xylidine was maintained as control.

Statistical method

The traditional method viz, one factor at a time method was used to screen the effect of media components such as, carbon, nitrogen and inducers respectively, nevertheless it misses to examine the interaction among the media components. The statistical tool Plakett- Burmann desing (PB) design was adapted to screen the essential media components as well as to improve the laccase production

Analytical Determinations

Laccase activity

Laccase activity was determined using 2, 2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) as substrate at room temperature. The reaction mixture contained 5 mM ABTS, 0.1M sodium acetate buffer (pH.5) and 0.17 ml of supernant was measured through rise of absorbance at 420nm (ε = 36000M−1 cm−1). One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1 micro mole of ABTS per minute. The laccase activities were expressed in U/L. Heat inactivated enzyme employed as control[27].

Biomass determination

The biomass measurement was determined by dry weight of fungal mycelium. The culture broth was filtered using Whatman No. 1 filter paper. The biomass retained was washed with distilled water and dried at 100°C to a constant weight.

Result and discussion:

Time course of laccase production

Many of the wood degrading white rot fungi of basidiomycetes are found to be the excellent producers of laccase[28,29]. But variations prevailed in relation to optimum expression period of this enzyme among the organisms. In the present sutdy the time of secretion of laccase by the white rot fungus *T. hirsuta* was evaluated using the glucose, yeast extract basal media. From the period of inoculation within 4 to 5 days it reaches the maximum cell mass concentration with very low laccase activity. Incontrast most of the white rot fungi produces laccase with respect to increase in mycelia growth in a gradual manner and attains maximum productivity while the growth reaches the stationary phase[30]. The morphology of cell was observed that pellet, dense s well as small in size.In addition there is a color change was identified (orange color), due to the secretion of pigment.Pigment synthesis one of biological functions of some white rot fungi. Maxima of laccase production was attained during the stationary phase on twentiethday of the fermentation with laccase activity of 7614 U/L with cell mass of 263.6 g/l. Figure.1 illustrates the laccase production in relation to cell mass concentrations.After twentieth day of fermentation a reduction in the production of laccase was observed. In addition the broth was changed as watery and decrease in the cell mass was also observed. This might be due to the digestion of cell pellets by autolysis caused by the protealytic enzymes in the native cell.

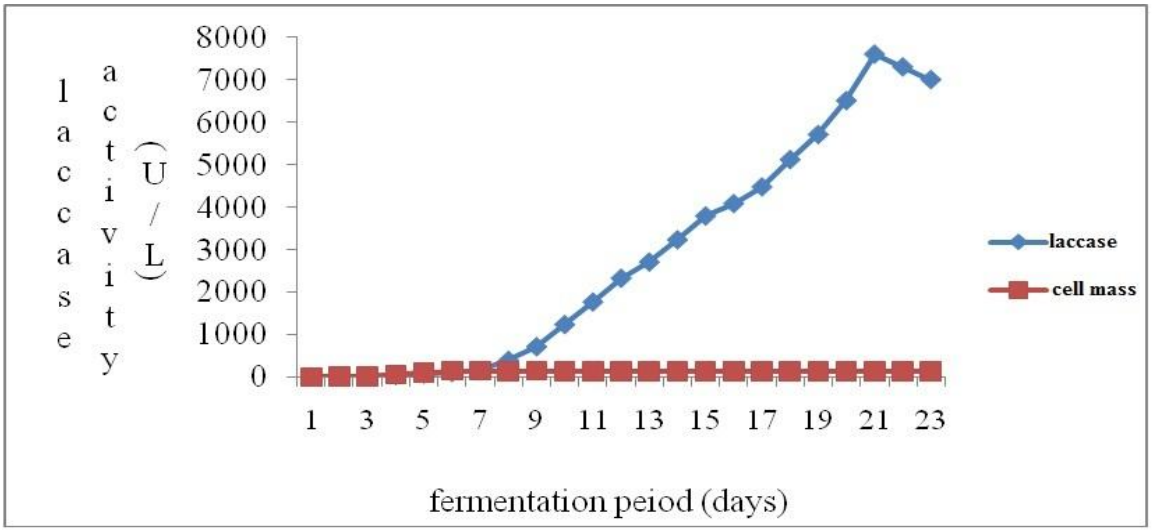

Figure.1 shows the time course of laccase production by *T. hirsita* with respect to cell mass concentrations

Time course of copper sulphate addition

Apart from the addition of inducers, laccase production can also be increased by the supplementation of sufficient concentration of copper sulphate to the culture medium. Copper sulphate is not inducer, a micronutrient but has the potential to raise the laccase production considerably. Nevertheless its addition period contributes important role in stimulation of laccase production. Addition of copper sulphate concentrations such as 0.1–2 mM after 8 day of fermentation with complete consumption of glucose in the medium by the organism *Trametes pubescens* MB 89 intensely failed to produce higher laccase activity. The effect of copper sulphate addition with respect to time has frequently been demonstrated for different white rot fungi. The concentration, 1mM copper sulphate was found as the optimum concentration based on the induction of laccase activity. In order to examine the exact time period of copper sulphate accommodation for laccase induction was studied by adding the copper sulphate from the period of inoculation to 5th day of the fermentation. Gradual increment in

laccase production was identified up to third day of fermentation, followed by a laccase production decreased slowly. Suppression of cell growth was found at during inoculation, first and second day of the process.In *Trametes pubescens* MB 89 addition of low concentration of copper sulphate (0.1–1 mM), during inoculation no growth inhibition was observed, while higher concentrations of copper sulphate (1.5–2 mM) partially repressed the growth[6]. Figure.2 illustrates the time course of copper sulphate addition for laccase production by *T.hirsuta.*

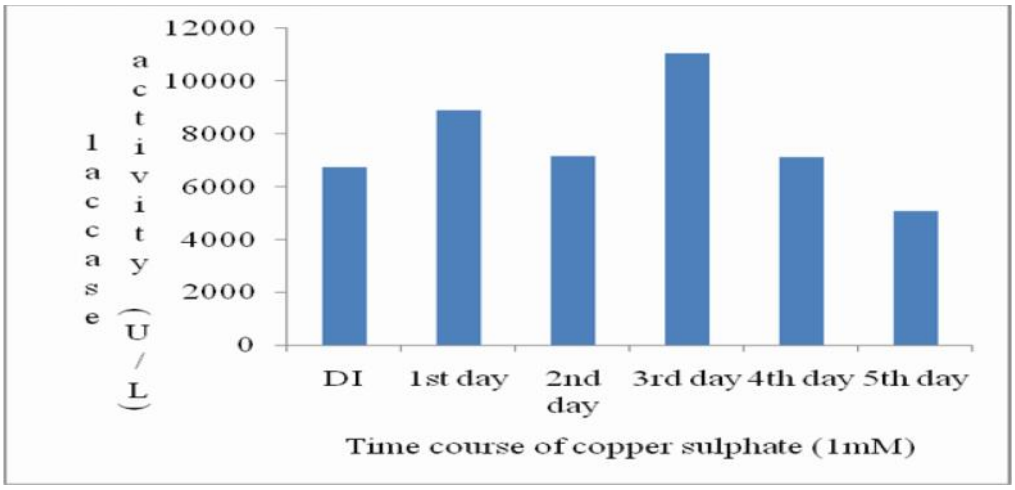

Figure.2 depicts the time course of copper sulphate addition on laccase production by *T. hirsuta*.

Effect of various concentration of copper sulphate on laccase production

An economical way to increase laccase production addition of copper sulphate to the culture medium and has widely been performed by different authors for different organisms to enhance the laccase production. In some white rot fungi viz *P. chrysosporium* ME446[31], *T. pubescens*[6]and *P.ostreatus*[32]addition of copper increases laccase production. The different concentration tested were 0.25 mM, 0.5 mM, 0.75 mM, 1 mM, 1.5 mM, 1.75 mM, and 2 mM. Among the various concentrations tested 1mM copper sulphate produced maximum laccase activity of 17074 U/L. Growth as well as laccase production was suppressed due to addition of concentrations above 1mM. It has been corroborated that addition of above1.5 mM concentration of copper sulphate to the cultures of *Lentinula edodes has* inhibited the total growth[33]. Figure.3 depicts the effect various concentrations of copper sulphate on laccase production by *T. Hirsuta*. Low production of laccase activity around 4236 U/L was monitored from the control flask cultivated without copper sulphate.

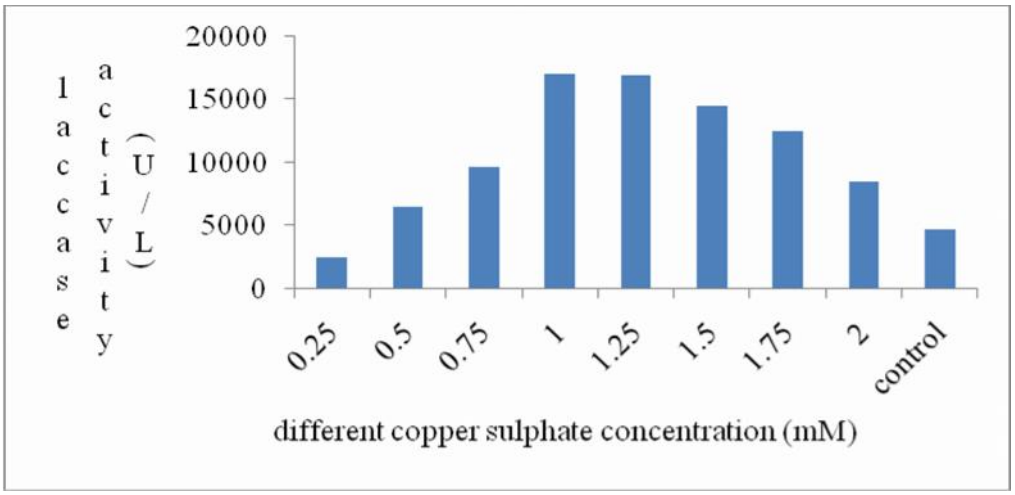

Figure.3 indicates the influence different copper sulphate concentration on laccase production by T. hirsute.

Time course of 2, 5 xylidine addition

To investigate the time at which the incorporation of 2, 5 xylidine influences the laccase production was examined. There are plenty of inducers recommended for laccase induction especially aromatic inducers have been widely used to enhance the laccase production. However their supplementation into the production medium in accurate period plays important role. It has been elaborately studied that laccase production has been governed by inducers with respect to the time of addition and appropriate concentration to the culture medium. To enhance the laccase production in *Trametes pubescens* MB 89 the aromatic inducer 2, 5 xylidine with concentration of 1mM was added on fourth day of the fermentation led to laccase activity of 8.6 U/ml[6]. The optimum concentration of 1mM xylidine was supplemented every 24 hours including the time of inoculation. Laccase production was gradually increased from the day of supplementation to third day of fermentation with high laccase titer of 23543 U/l. followed by the production decreased gradually on 4th and 5th day of the process. Inhibition in cell growth was found in during inoculation, first day and second day respectively compared to the control culture. Figure.4 shows the deviation in laccase production by *T. hirsuta* using 2, 5 xylidine addition in terms of days.

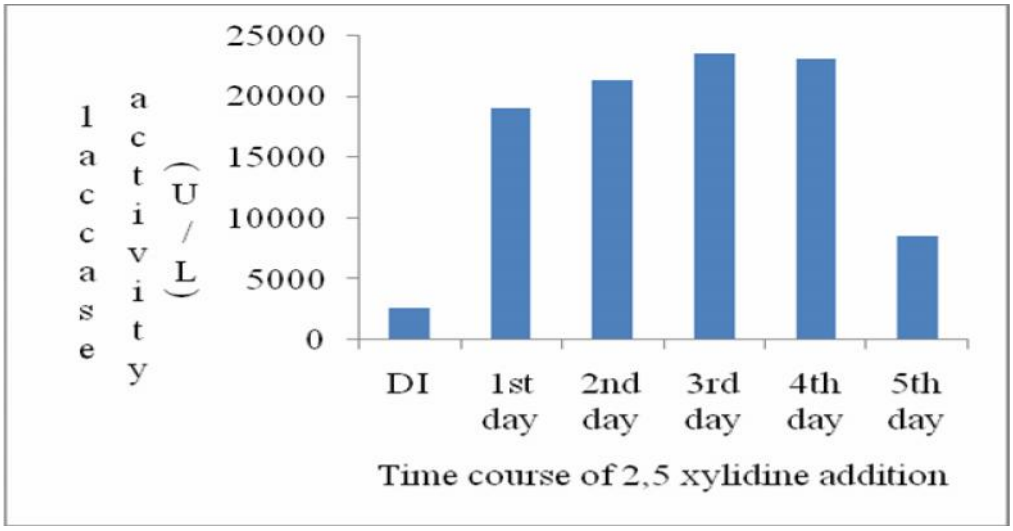

Figure.4 shows the deviation in laccase production by *T. hirsuta* 2, 5 xylidine addition in terms of days .

Effect of various 2, 5 xylidine concentrations on laccase production

Though the addition of aromatic inducers has the ability to increase the laccase production, unless find the impact of optimum concentration on laccase production is not a successful one. Several concentrations of 2,5 xylidine such as 0.25 mM, 0.5 mM, 0.75 mM, 1 mM, 1.5 mM, 1.75 mM, and 2 mM were analyzed in order to find the impact of the relevant concentration on laccase production. In *P. cinnabarinus* 0.1 mM 2, 5-xylidine has been found to be a good laccase inducer[34]. The laccase production was slowly increased by the concentrations 0.25 mM-1mM. Maxima of laccase production were expressed from the concentration of 1mM 2, 5 xylidine with laccase activity of 23543 U/L. Many reports have been reported in relation to the effect appropriate concentration of 2, 5 xylidine on laccase production by white rot fungi. The higher concentrations 1.5 - 2mM caused gradual reduction in laccase production. Incorporation of higher concentration of 2, 5 xylidine reduces laccase production, it may be due to toxicity of the compound[34]. Figure.5 depicts the various 2, 5 xylidine concentrations on laccase production by *T.Hirsuta.*

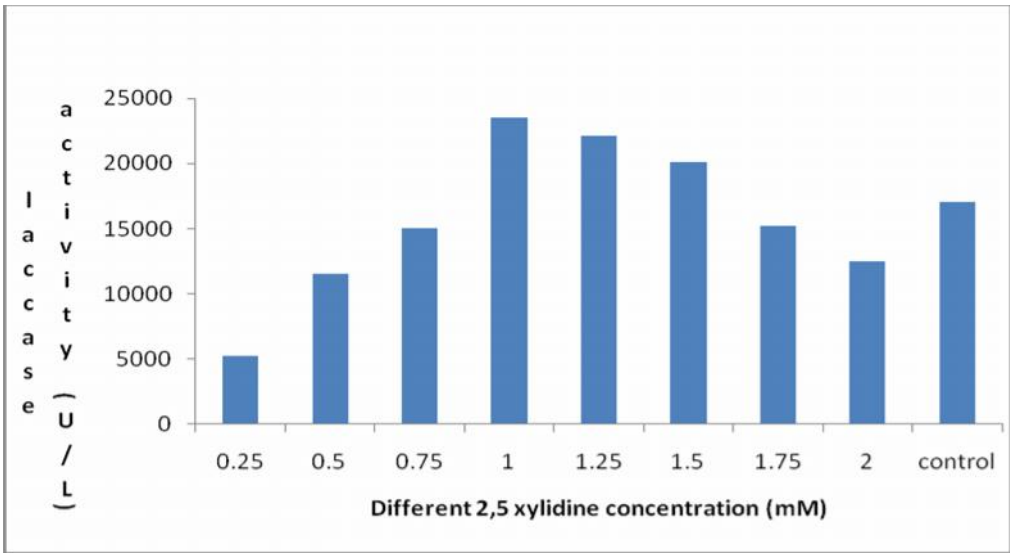

Figure.5 depicts the various 2, 5 xylidine concentrations on laccase production by *T.Hirsuta.*

Effect of lignocellulosic material on laccase production

In general laccase production was achieved either limited carbon and nitrogen ratio or higher carbon and nitrogen ratio in the cultivation medium. Also laccase production was successfully performed in a carbon and nitrogen sufficient medium. Different lignocellulosic materials viz, rice bran, wheat bran, elephant grass, waste paper, orange peeling, lemon peeling, water hyacinth, baggase respectively. Of all tested lignocellulosic substrates wheat bran showed maximum laccase activity of 9300 U/L followed by lemon peeling (8512 U/L) and bagasse (6543 U/L) showed maximum activity.Wheat bran a byproduct received from the milling industry of wheat and constituted by 58% nonstarch polysaccharides, 3% lignin, 19% starch and 18% crude protein. The nonstarch part composed by 70% α - arabinoxylans, 24% cellulose and 6% β-(1,3) 1,4 - glucan respectively[35]. Figure.6 depicts the effect of laccase production by *T.hirsuta* using various lignocellulosic materials.

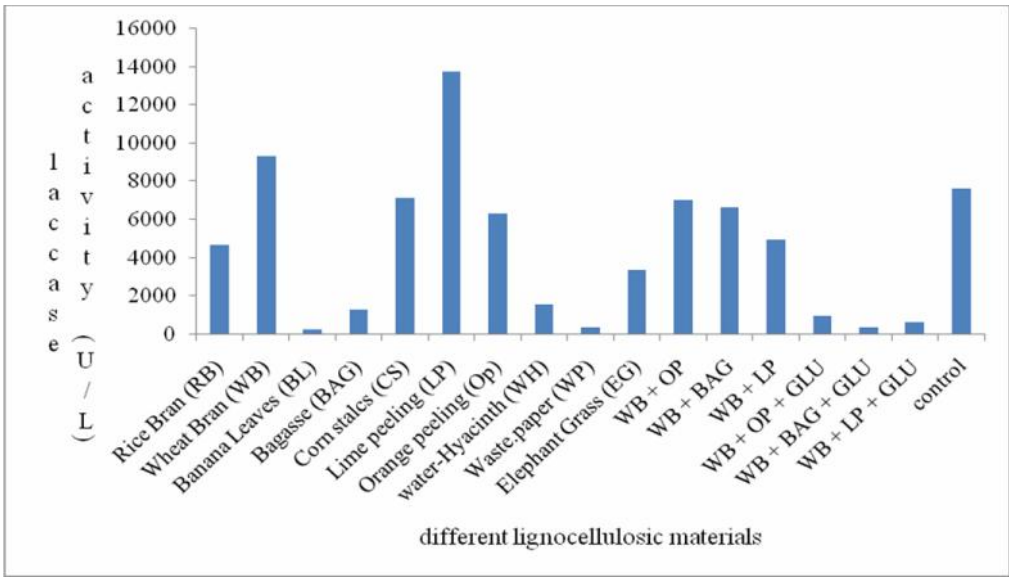

Figure.6 depicts the effect of laccase production by *T.hirsuta* using various lignocellulosic materials.

In the past decades the various agricultural substrates and by-products have widely been exploited for laccase production via white rot fungi under submerge and solid state cultivation. More over the nature of lignocellulosic substrate determine the type and amount of enzyme formation during the process.[36, 37, 38.] Plenty of data are reported in relation to laccase production by white rot fungi using lignocellulosic material so far. For

instance, mandarin peels.[39], grape seeds[40], *Eucalyptus grandis*wood chips[41]respectively. In addition the influence of mixed lignocellulosic substrates and mixed lignocellulosic substrates in combination with glucose was studied for laccase production. From the experimental results of screening of the best lignocellulosic residue indicated that wheat bran, baggase and lime peeling showed appreciable laccase activity. Table.1 shows the effect lignocellulosic materials on laccase production by *T. hirsute.*Three combinations were made to explore the interaction between the two agricultural residue such as WB + OP, WB + BAG, WB + LP and these three combinations were further examined with glucose for laccase production viz, WB+ OP + GLU, WB + BAG + GLU, WB + LP + GLU respectively. All three combinations of mixed lignocellulosic substrates tested for increasing laccase production showed, moderate laccase activity of 7032 U/L, 6624 U/Land 4917 U/L. On the other hand mixed lignocellulosic residue with glucose combination gave low production (917 U/L, 331U/L, 586 U/L). It is noteworthy that all these six interactions failed to produce high laccase activity than the better laccase yield giving agro residuewheat bran. This might have happened due to the accumulation of higher glucose concentration liberated from lignocellulosic substrate to the medium. Control flask which was cultivated with glucose alone gave a laccase activity of 7614 U/L.

Table.1 shows the effect of different lignocellulosic materials on laccase production by *T. hirsuta*

Table.1-

s.o	Different lignocellulosic material	Laccase (U/L)
1	Rice Bran (RB)	4643
2	Wheat Bran (WB)	9300
3	Banana Leaves (BL)	229.3
4	Bagasse (BAG)	6543
5	Corn stalcs (CS)	7130
6	Lime peeling (LP)	13733
7	Orange peeling (Op)	6284
8	water-Hyacinth (WH)	1508.2
9	Waste paper (WP)	305.8
10	Elephant Grass (EG)	3320
11	WB + OP	7032
12	WB + BAG	6624
13	WB + LP	4917
14	WB + OP + GLU	917
15	WB + BAG + GLU	331
16	WB + LP + GLU	586
17	control	7614

Effect nitrogen source on laccase production

One of the important and easiest way to increase laccase production is to screen the best nitrogen source with appropriate concentration. Ligninolytic enzyme productions by the wood-rotting basidiomycetes have been influenced by the both nature and concentration of nitrogen sources and they are the important factors for regulation of ligninolytic enzyme production and this has formerly been demonstrated.[42,43,44]. According to one factor at a time method the dominant nitrogen source was screened using wheat bran as the carbon source with concentration of 20g/l. The effect of single and combined nitrogen source on laccase production by *T.hirsuta* was studied. The nitrogen source such as yeast extract ,urea, peptone, L- Aspargine, ammonium sulphate, malt extract and ammonium tartarate were examined with concentration of 10 g/l and the combined nitrogen source viz, ME + NHSO4, YE + Urea, YE + ME, ME + PEP, YE + $NHSO_4$, YE + TRY were assessed with concentration of 5 g/l. Of all the above tested nitrogen source peptone produced maximum laccase activity of 17390U/L, after that yeast extract and L- aspargine provided 7038 U/L and 8665 U/L respectively. Likewise the mixed nitrogen sources viz, ME + NHSO4 and YE + ME gave somewhat average laccase production (12059 U/L, 10226 U/L). Very low laccase activity was detected from urea and ammonium sulphate around 16.66 U/L and 1759 U/L was obtained in a single nitrogen mode performance, in addition, the cell growth of these cultures were found to be suppressed. Figure.6 illustrates the impact of single and mixed nitrogen source on laccase production by T. hirsuta. It is well noted that nature of the nitrogen source affected laccase production in this

fungus. Particularly the organic nitrogen source viz, peptone and yeast extract gave maximum laccase activity than the inorganic nitrogen source like Urea, ammonium sulphate. The inorganic nitrogen sources urea and ammonium sulphate have failed to produce high laccase titters when using them as a sole nitrogen source, but in the combined form with malt extract, yeast extract viz, malt extract + NHSO4, yeast extract + Urea produced greater laccase activity (12059, 965 U/L), conversely, it was already mentioned that yeast extract exhibited high laccase activity when cultivated as sole nitrogen source. Figure.7 indicates the effect different nitrogen source on laccase production by T. Hirsuta. Likewise the organic nitrogen sources peptone and yeast extract produced high laccase activity in a single mode, but unable to produce large amount in the combined mode. Which clearly indicates that the nature of the nitrogen source strongly influence the laccase production by *T.hirsuta*. Table indicates the effect of single and mixed nitrogen source on laccase production by *T. hirsuta*.

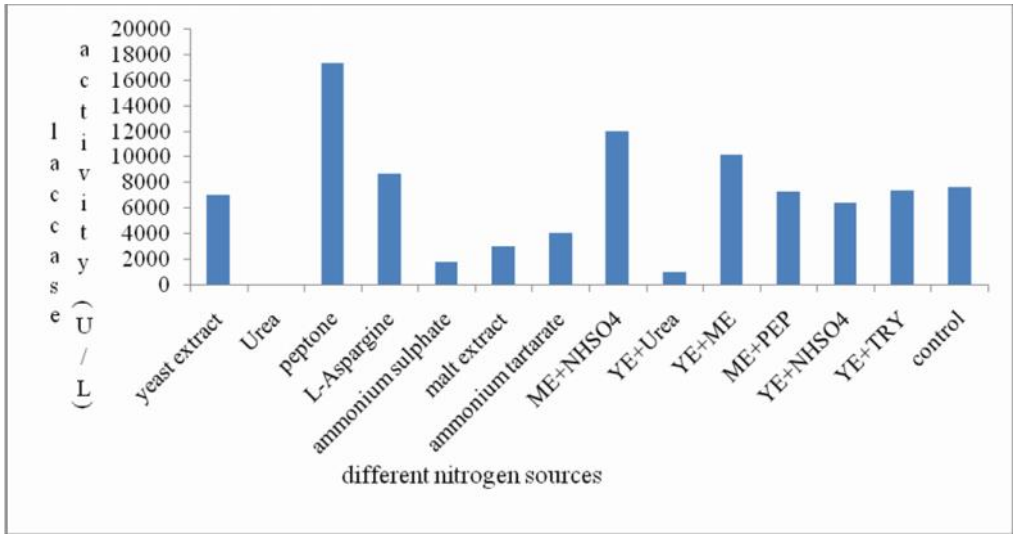

Figure.7 indicates the effect different nitrogen source on laccase production by T. Hirsuta.

Effect inducers on laccase production

In general extracellular laccase productions by white rot fungi are constitutively produced in small amounts in the broth[17], hence their expression can be enhanced by the addition of different inducers viz aromatic or phenoilic compounds related to lignin or lignin derivatives.[45].There are plenty of studies have been focused on the type, concentration, and the feeding time of inducers on laccase production so far[5, 46].Several aromatic inducers were explored in order to stimulate the laccase production such as 2,5 xylidine, guaiacol, catcechol, veratryl alcohol, hydroxybenzotryazole (HBT), p-anisidine, 2,6 dimethoxy phenol (DMP), vanilic acid, ferulic acid, gallic acid respectively. All these inducers were prepared with 5% ethanol, and sterilized by filtration. They are added into the medium in the range of 1mM concentration on third day of the fermentation. The inducers assessed for laccase induction by this fungus displays different observation in regarding to cell growth and laccase production. Based on the study conducted for time course of addition of inducers suggested the optimum time period to add the inducers in the medium. Accordingly all the inducers were incorporated on third day of the fermentation in order to avoid the repression of cell growth as well as to acclimatize the environment by the organism in relation to laccase production. This fungus produced substantial laccase activity with all inducers according to their nature of structure. Among the inducers verified for laccase induction by *T.Hirsuta*, 2, 5xylidine gave maximum laccase activity of 23456 U/L also it was detected that cell mass formation was high. In contrast, growth inhibition as well as small laccase titre was obtained in p-anisidine and 1-napthol. After 2, 5 xylidine, better laccase activity was observed from guaiacol (6847U/L), vanilic acid (6347 U/L), 2, 6 dimethoxy phenol (5447 U/L), ferulic acid (4598 U/L) and Gallic acid (4048 U/L). On the contrary the other inducers revealed no appreciable inducement in laccase production than the control culture viz, p-anisidine (2499 U/L), 1-Napthol, (2948 U/L), hydroxybenzotryazole (HBT) (2898 U/L), veratryl alcohol (VA) (3798 U/L). Figure.8 depict the effect of different aromatic inducers on laccase production by *T. Hirsuta*. The findings of our study coincided with previous results that The white rot fungus Coriolus versicolor MTCC 138 has produced of 820 U/ml with the inducing effect by 1mM of copper sulphate and 2,5-xylidine[47]. One of the most

common and most frequently used inducer for laccase production by diverse fungal strains is 2, 5xylidine, induced laccase synthesis in *Fomesannosus*, *Pholiotamutabilis*, *Pleurotusostreatus* and *T. versicolor*[17].

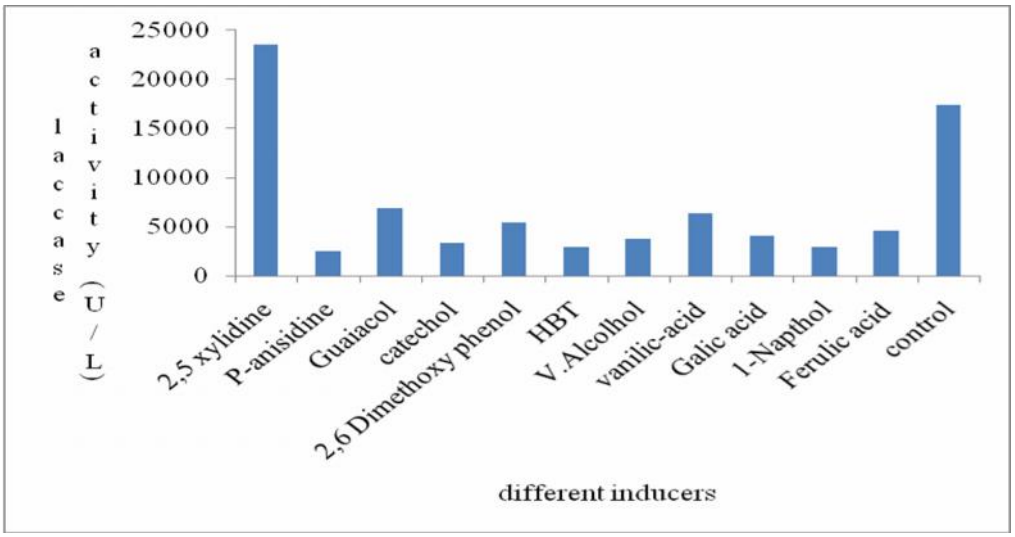

Figure.8 depict the effect of different aromatic inducers on laccase production by *T. Hirsuta*.

Plakett-burmann design

The system governed by multiple variables like biochemical process, where the number of effective variables influencing the system should be examined before optimization using an initial screening design[48,49]. The Plackett–Burman[50] experimental design was adapted to assess the relative importance different nutrients on laccase production under submerge fermentation by the white rot fungus T.hirsuta. This design presumes that there are no interactions among the different media components, xi, in the range of variables under consideration. A linear approach is considered to be sufficient for screening.

$$Y= \beta_0 + \sum \beta_{i\, xi\, (i=1,\cdots,k)}$$

where Y is the estimated target function and β_i are the regression coefficients. The PB design is a fractional factorial design and the main effect (the contrast coefficient, b) of such a design may be simply computed as the variations between the average of measurements made at the high level (+1) of the factor and the average of measurements made at the low level (-1) of the varibles used laccase production in terms of (g/l) as follows: A(Wheat Bran) 10,20 , B (peptone) 5,10; C ($MgSO_4$) 5,10; D (KCl) 5, 10; E (KH_2PO_4) 0.1, 0.5; F (K_2HPO_4) 0.1 0.5; G ($CuSO_4$) 0.1 0.24g, H (Xylidine) 0.06, 0.121; I ($CaCl_2$) 0.01, 0.1; J ($MnSO_4$) 0.0001, 0.0005, K ($ZnSO_4$) 0.0001,0.0005; L ($FeSO_4$) 0.0001, 0.0005. Contrast coefficients allow the determination of the effect of each constituent. A large contrast coefficient either positive or negative indicates that a factor has a large impact on titre; while a coefficient close to zero means that a factor has little or no effect. Table.2 indicates 12 medium components with high (+) and low (-) levels for screening of the essential media components. In Table.3 the column shows the different variables and rows mention the 12 trials were performed for laccase production.

Table.2

Variables	Media components	minimal value (-1) (g/l)	maximal value (+1) (g/l)
A	Wheat bran	10	20
B	Peptone	5	10
C	$MgSO_4$	5	10
D	Kcl	5	10
E	KH_2PO_4	0.1	0.5
F	K_2HPO_4	0.1	0.5
G	$CuSO_4$	0.1	0.24
H	2,5 Xylidine	0.06	0.121
I	$CaCl_2$	0.01	0.1
J	$MnSO_4$.	0.0001	0.0005
k	$ZnSO_4$	0.0001	0.0005
l	$FeSO_4$	0.0001	0.0005

Table.2 shows the 12 media components generated for plakett-Burmann design to produce laccase by *T. hirsuta*.

According to the design the experimentation conducted revealed that the media 2, 3 and 10 produced laccase activity of 24156 (U/L), 24785 (U/L) and 25899(U/L) respectively.

Table.3 Plackett-Burman design generated for 12 medium components for *T.Hirsuta* with laccase activity.

Run	A	B	C	D	E	F	G	H	I	J	K	L	Laccase U/L
													Experimental
1	+	+	+	+	-	+	-	+	+	-	-	+	21443
2	+	+	+	-	+	-	+	+	-	-	+	-	24156
3	+	+	-	+	-	+	+	-	-	+	-	-	24785
4	+	-	+	-	+	+	-	-	+	-	-	-	14562
5	-	+	-	+	+	-	-	+	-	-	-	+	10230
6	+	-	+	+	-	-	+	-	-	-	+	+	16523
7	-	+	+	-	-	+	-	-	-	+	+	+	4521
8	+	+	-	-	+	-	-	-	+	+	+	+	14756
9	+	-	-	+	-	-	-	+	+	+	+	-	10254
10	-	-	+	-	-	-	+	+	+	+	-	+	25889
11	-	+	-	-	-	+	+	+	+	-	+	-	20147
12	+	-	-	-	+	+	+	+	-	+	-	+	13522
13	-	-	-	+	+	+	+	-	+	-	+	+	9754
14	-	-	+	+	+	+	-	+	-	+	+	-	14523
15	-	+	+	+	+	-	+	-	+	+	-	-	5421
16	-	-	-	-	-	-	-	-	-	-	-	-	4522

*Predicted by PB model: A (Wheat Bran), B (peptone), C ($MgSO_4$), D (KCl), E (KH_2PO_4); F (K_2HPO_4); G ($CuSO_4$), H (2,5 Xylidine), I ($CaCl_2$), J ($MnSO_4$), K ($ZnSO_4$), L ($FeSO_4$).

Similarly the Figure.9 depicts the main effect and negative effects of the variables for laccase production by *T.hirsuta*. The positive effect indicates that additional concentration is required than the used amount for the production of laccase. Similarly the negative effect indicates that the concentration used in the production is to be reduced than the used amount.

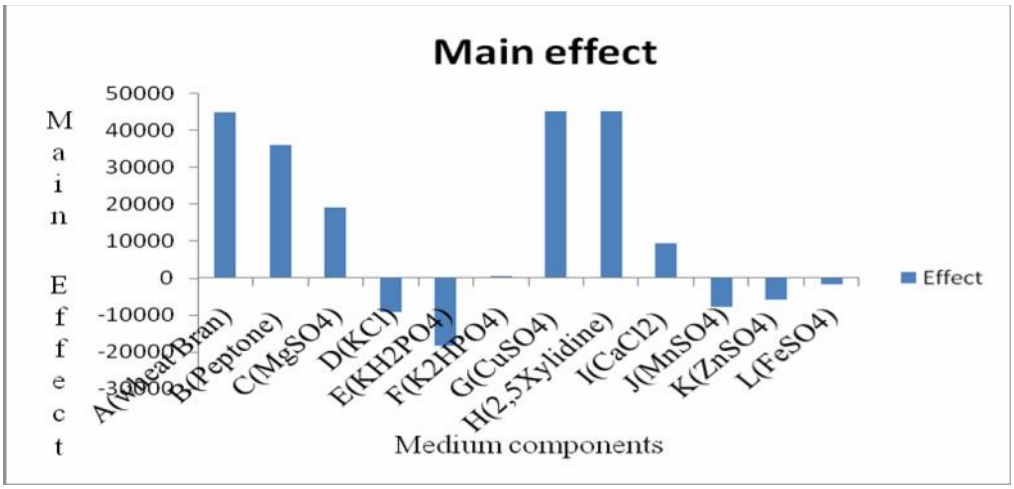

Figure.9 depicts the main effect and negative effects of the variables for laccase production by *T.hirsuta*.

The figure.10 Pareto plot sequentially illustrates the significant media components with respect to their percentage effect on laccase production by *T. hirsuta*.

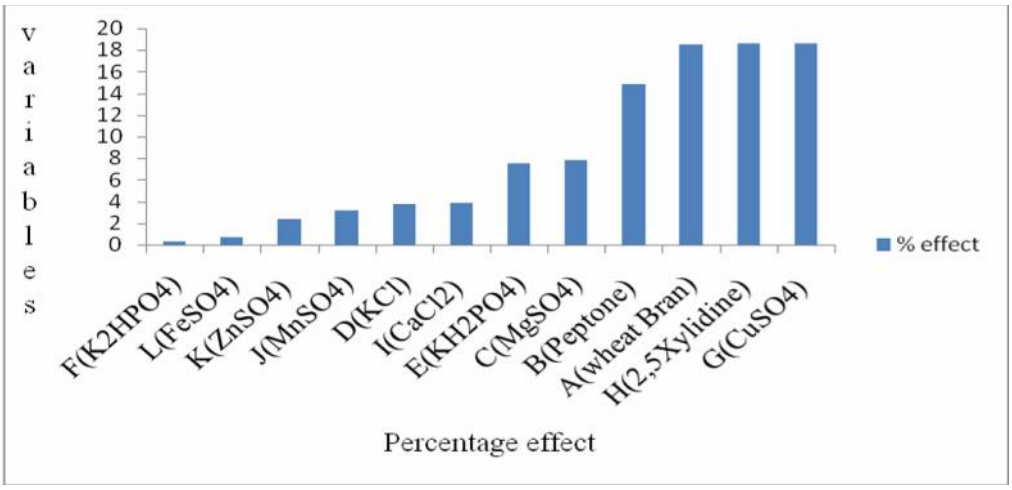

Figure.10 Pareto plot sequentialy illustrates the significant media components

Conclusion

The white rot fungus *Trametes hirsuta* was examined to find its capability of laccase production under submerged fermentation. The time period of appearance of laccase by the organism was observed on 20th day of the process with maximum laccase activity of 7614 U/L. Similarly the optimum concentration of copper sulphate and 2, 5 xylidine were optimized. According to one factor at a time-method the effect the primary process constituents viz carbon as agro industry waste, nitrogen and inducers on laccase production were performed. The lignocellulosic material wheat bran gave maximum laccase activity of 9300 U/L. The combined action of both wheat bran and peptone with supplementation of 1mM copper sulphate induced the laccase activity of 17390 U/L. production of laccase was further stimulated to 23543U/L by 1mM 2, 5 xylidine incorporation. With the use of statistical method viz, Plakett - Burmann design enhanced laccase production to 25889 U/L and the influence of significant media components were screened.

References:

1. 1.Thurston CF (1994) the structure and function of fungal laccases. Microbiol (UK) 140:19–26
2. Messerschmidt A, Huber R (1990). The blue oxidases, ascorbate oxidase, laccase and ceruloplasmin—modeling and structural relationships. Eur J Biochem 187:341–352
3. 3.De Marco A, Roubelakis-Angelakis KA (1997) Laccase activity could contribute to cell-wall reconstitution in regenerating protoplasts. Phytochemistry 46:421–425
4. 4.Ranocha P, McDougall G, Hawkins S, Sterjiades R, Borderies G, Stewart D, Cabanes-Macheteau M, Boudet AM, Goffner D (1999) Biochemical characterization, molecular cloning and expression of laccases—a divergent gene family in poplar. Eur J Biochem 259:485–495
5. Arora, D. S., & Gill, P. K. (2001). Effects of various media and supplements on laccase production by some white rot fungi. Bioresource Technology, 77, 89–91.
6. 6.C. Galhaup · D. Haltrich(2001)**,** Enhanced formation of laccase activity by the white-rot fungusTrametespubescens in the presence of copper.ApplMicrobiolBiotechnol 56:225–232,DOI 10.1007/s002530100636.
7. 7.Givaudan A, Effosse A, Faure D, Potier P, Bouillant ML, Bally R (1993) Polyphenol oxidase in Azospirillumlipoferum isolated from rice rhizosphere: evidence for laccase activity in nonmotile strains of Azospirillumlipoferum. FEMS MicrobiolLett 108:205–210.
8. 8.Martins LO, Soares CM, Pereira MM, Teixeira M, Costa T, Jones GH, Henriques AO (2002) Molecular and biochemical characterization of a highly stable bacterial laccase that occurs as a structural component of the Bacillus subtilis endospore coat. J BiolChem 277:18849–18859
9. 9.Dittmer NT, Suderman RJ, Jiang H, Zhu YC, Gorman MJ, Kramer KJ, Kanost MR: Characterization of cDNAs encoding putative laccase- like multicopper oxidases and developmental expression in the tobacco hornworm, *Manducasexta*, and the malaria mosquito, *Anopheles gambiae*. *Insect BiochemMolBiol*2004, 34:29-41.
10. 10. Kramer KJ, Kanost MR, Hopkins TL, Jing H, Zhu YC, Xhu R, Kerwin JL, Turecek F: Oxidative conjugation of catechols with proteins in insect skeletal systems. *Tetrahedron* 2001, 57:385-392.
11. 11.Kirk TK, Farrell RL. Enzymatic combustion: the microbial degradation of lignin. Annu Rev Microbiol 1987; 41:465–505.
12. 12. Eriksson K-EL, Blanchette RA, Ander P. Microbial and enzymatic degradation of wood and wood components. Berlin: Springer-Verlag; 1990
13. 12.Reddy CA, D'Souza TM. Physiology and molecular biology of the lignin peroxidases of *Phanerochaetechrysosporium*. FEMS Microbiol Rev 1994;13:137–52. (Reddy and D'Souza1994)
14. 14.Kapich AN, Prior BA, Botha A, Galkin S, Lundell T, Hatakka A. Effect of lignocellulose-containing substrate on production of ligninolytic peroxidases in submerged cultures of *Phanerochaetechrysosporium*ME-446. Enzyme MicrobTechnol 2004; 34:187–95. (kapich et al 2004)
15. 15.Jang YM, Ryu RW, Cho HM. Laccase production from repeated batch cultures using free mycelia of *Trametes*sp. Enzyme MicrobTechnol 2002;30:741–6. (Jang et al 2002) (Jiang-Ping et al 2001)
16. 16.Jiang-Ping L, Wei L, Li-Ming X, Pei-Lin C. Production of laccase by *Coriolusversicolor*and its application in decolorization of dyestuffs. I. Production of laccase by batch and repeated-batch processes. J Environ Sci 2001; 15:1–4.
17. 17.Bollag JM, Leonowicz A. Comparative studies of extracellular fungal laccases. Appl Environ Microbiol 1984; 48 (4): 849 – 54.
18. 18. Hess J, Leitner C, Galhaup C, Kulbe K, Hinterstoisser B, Steinwender M, Haltrich D. Enhanced formation of extracellular laccase activity by the white-rot fungus Trametes multicolor. ApplBiochem Biotech 2002; 98–100:229–41.
19. 19. GiorgiSongulashvilia,b, Vladimir Elisashvilib,*****,Soloman P. Wassera, EviatarNevoa,Yitzhak Hadarc(2007),Basidiomyceteslaccase and manganese peroxidase activity in submerged fermentation of food industry wastes.Enzyme and Microbial Technology 41 (2007) 57–61
20. 20.Bourbonnais R, Paice MG, Freiermuth B, Bodie E, Borneman S. Reactivities of various mediators and laccases with kraft pulp and lignin model compounds. Appl Environ Microbiol 1997; 63:4627–32.
21. 21.Setti L, Giuliani S, Spinozzi G, Pifferi PG. Laccasecatalyzedoxidative coupling of 3-methyl 2-benzothiazolinone hydrazine and methoxyphenols. Enzyme MicrobTechnol 1999; 25:285–9
22. 22.Harazono K, Nakamura K (2005) Decolorization of mixtures of different reactive textile dyes by the white-rot basidiomycetePhanerochaetesordida and inhibitory effect of polyvinyl alcohol. Chemosphere 59:63–68

23. 23.selinheimo E, Kruus K, Buchert J, Hopia A, Autio K. Effects of laccase, xylanase and their combination on the rheological properties of wheat doughs. J Cereal Sci 2006; 43:152–9.
24. 24.Karamyshev AV, Shleev SV, Koroleva OV, Yaropolov AI, Sakharov IY. Laccase-catalyzed synthesis of conducting polyaniline. Enzyme MicrobTechnol 2003;33:556–64.
25. 25.Jarosz-Wilkołazka A, Ruzgas T, Gorton L. Amperometric detection of mono- and diphenols at Cerrena unicolor laccase-modified graphite electrode: correlation between sensitivity and substrate structure. Talanta 2005; 66:1219–24.
26. 26.Terron, M.C., Gonzalez, T., Carbajo, J.M., Yagu e, S., Cuenca, A.A., Tellez, A., Dobson, A.D.W., Gonzalez, A.E., 2004. Structural closerelated aromatic compounds have different effects on laccase activity and on lcc gene expression in the ligninolytic fungus Trametes sp. I-62. Fungal.Genet. Biol. 41, 954–962.
27. 27.Wolfenden, B.S., Willson, R.L., 1982. Radical-cations as reference chromogens in kinetic studies of one electron transfer reactions: pulse radiolysis studies of 2, 2-azinobis-(3-ethylbenzothiazoline-6-sulphonate). J.chem. soc. Perkin Trans.II. 805-812. ()
28. 28.Bourbonnais R, Paice MG, Reid ID, Lanthier P, Yaguchi M (1995) Lignin oxidation by laccaseisozymes from *Trametesversicolor*and role of the mediator 2, 2'-azinobis (3-ethylbenzthiazoline-6-sulfonate) in kraft lignin depolymerization. Appl Environ Microbiol 61:1876–1880
29. 29.Leontievsky A, Myasoedova N, Pozdnyakova N, Golovleva L (1997) 'Yellow' laccase of *Panustigrinus*oxidizes non-phenolic substrates without electron-transfer mediators. FEBS Lett 413:446–448
30. 30.Freitag, M. and Morrell, J.J. (1992) Changes in selected enzyme activities during growth of pure and mixed culture of the white-rot decay fungus Trametesversicolor and the potential biocontrol fungus Trichodermaharzianun. Can. J. Microbiol.38, 317^323.
31. 31.Dittmer JK, Patel NJ, Dhawale SW, Dhawale SS (1997) Production of multiple laccase isoforms by *Phanerochaetechrysosporium*grown under nutrient sufficiency. FEMS MicrobiolLett 149:65–70.
32. 32.Baldrian P, Gabriel J (2002) Copper and cadmium increase laccase activity in Pleurotusostreatus. FEMS MicrobiolLett 206: 69–74.
33. 33.Hatvani N, Mecs I. Effects of certain heavy metals on the growth, dye decolorization and enzyme activity of *Lentinulaedodes*. Ecotoxicol Environ Safety 2003;55:199–203.
34. 34.Eggert, C.; Temp, U.; Eriksson, K.E.L. The ligninolytic system of white rot fungus *Pycnoporuscinnabarinus*: Purification and characterization of the laccase. *Appl. Environ. Microbiol.*, 62, 1151- 1158, 1996.
35. 35.Songulashvili G, Jimenéz-Tobón G, Jaspers C, Penninckx MJ. 2011 – High production of laccase by *Ganodermalucidum*447 in submerged cultivation on ethanol production residue supplemented with Cu2+. Mycosphere 2(4), 507–513.
36. 36.D'Souza TM, Merritt CS, Reddy CA. Lignin-modifying enzymes of the white rot basidiomycetes*Ganodermalucidum*. Appl Environ Microbiol 1999; 65:5307–13.
37. 37. Moldes D, Lorenzo M, Sanrom´an MA. Different proportion of laccaseisoenzymes produced by submerged cultures of *Trametesversicolor*grown on lignocellulosic wastes. BiotechnolLett 2004; 26:327 – 30.
38. 38.Mikiashvili N, Wasser S, Nevo E, Chichua D, Elisashvili V. Lignocellulolytic enzyme activities of medicinally important basidiomycetes from different ecological niches. Int J Med Mushr 2004; 6: 63 – 71.
39. 39.J. F. Osma, V. Saravia, J. L. T. Herrera, and S. R. Couto, "Mandarin peelings: the best carbon source to produce laccase by static cultures of Trametespubescens," *Chemosphere*, vol. 67, no. 8, pp. 1677–1680, 2007.
40. 40.E. Rosales, S. R. Couto, andM.A. Sanrom´an, "Reutilisation of food processing wastes for production of relevant metabolites: application to laccase production by Trameteshirsuta," *Journal of Food Engineering*, vol. 66, no. 4, pp. 419–423, 2005.
41. 41.Esposito E, Innocentini-Mei LH, Ferraz A, Canhos \'P, Duran N. 1993. Phenoloxidases and hydrolases from Pjcnoporussanguineus (EUC-2050 strain) : applications. J Biotechnol 29:219-228.
42. 42.Galhaup C, Wagner H, Hinterstoisser B, Haltrich D. Increased production of laccase by the wood-degrading basidiomycete*Trametespubescens*. Enzyme MicrobTechnol 2002;30:529–36
43. 43. Zakariashvili NG, Elisashvili VI. (1992) Regulation of *Cerrena unicolor* lignocellulolytic activity by a nitrogen source in culture medium.Microbiology (Moscow) 1992; 62:525–8.

44. 44. Sun X, Zhang R, Zhang Y. Production of lignocellulolytic enzymes by *Trametesgallica*and detection of polysaccharide hydrolase and laccase activities in polyacrylamide gels. J Basic Microbiol 2004; 44:220–31.
45. 45.Farnet A-M, Tagger S, LePetit J (1999) Effects of copper and aromatic inducers on the laccases of the white-rot fungus Marasmiusquercophilus. C.R. Academy Science Paris, Sciences de la vie/Life Sciences 322:499–503.
46. 46. Hou H, Zhou J, Wang J, Du C, Yan B (2004) Enhancement of laccase production by *Pleurotusostreatus*and its use for the decolorization of anthraquinone dye. Process Biochem 39:1415–1419.
47. 47.Madhavi S. Revankar and S.S. Lele*; (2006) Increased production of extracellular laccase by the white rot fungus Coriolusversicolor MTCC 138; World Journal of Microbiology & Biotechnology (2006) 22:921-926; DOI 10.1007/s11274-006-9136-2
48. 48.Montgomery, D.C., (2001). *Design and Analysis of Experiments*. (John Wiley and Sons, New York).
49. 49.Box, G.E.P., Hunter, W.G. and Hunter, J.S., (1978). *Statistics for Experimenters: An Introduction to Design, Data Analysis, and Model Building*. (John Wiley and Sons, New york).
50. 50.Plackett, R.L. and Burman, J.P., 1946, The design of optimum multifactorial experiments. Biometrika, 33: 305–325.

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