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STATISTICAL DESIGNS AND RESPONSE SURFACE TECHNIQUE FOR THE OPTIMIZATION OF EXTRA CELLULAR LACCASE ENZYME PRODUCTION BY USING *PLEUROTUS* SP.

K. Saravanakumar[1*], R. Saranya [2], Sankaranarayana Arathi[3] and V. Kaviyarasan[1]

[1]*Centre for Advanced studies in Botany, Mycology lab, University of Madras, Chennai, Tamil Nadu, India,*
[2]*Department of Biotechnology, JJ College of Arts and Science, Pudukkottai, Trichy, India*
[3]*School of chemical and Biotechnology, Sastra University, Thanjavur, Tamil Nadu, India*

Abstract

A response surface methodology (RSM) was used for the optimization of extra cellular laccase production using *Pleurotus* sp. Central composite experimental design was applied in the analysis of results and this procedure required limited number of experiments performed while providing possible interactions of selected components. Optimum values of tested variables for the maximum laccase production were; mannitol 4.8gm/l, beef extract 3gm/l and pH 6.2 and copper sulphate 4 mg/l, the maximum laccase production was 90.861U/ml. Only 27 experiments were attained this optimum conditions for enzyme production and the coefficient of determination was 89.0%. In the present study, we have demonstrated that the use of central composite factorial design for high yield of enzymes.

Keywords: ABTS, *Pleurotus* sp., Laccase, Response surface methodology

Introduction

Lignin and cellulose are both rather rigid organic polymers (Tuor *et al*., 1995), which have developed during evolution for construction and preservation purposes (Call & Mücke, 1997). The degradation of lignin in the pulping and bleaching processes is essential for the manufacturing of paper products. These compounds have to be exposed to harsh physiochemical conditions to modify or degrade their structure for utilization in the pulp and paper industry (Coll *et al*., 1993). The problems caused by chemicals used in bleaching forced industry to consider alternative, more environmental friendly methods (Yang & Eriksson, 1992). Such a biological alternative to traditional bleaching was provided through the discovery of oxidative enzymes (Poppius-Levlin et al., 1997). Laccase is a polyphenol oxidase that contains four coppers, and is able to oxidize its substrates by using molecular oxygen as an electron acceptor. Laccase can be found in plants, insects and bacteria, but its major sources from fungi. Fungi associated with many biological functions such as lignin degradation, removal of potentially toxic phenols in addition to paper industry (Argyero Poulos, D.S., 2001.). The oxidative enzymes can be used for biodecolorization and detoxification of industrial effluent waste water (Feng Xu, 2005). Laccase and peroxidase enxymes are generally produced by submerged fermentation in commercial practice; the medium composition play a significant role in the enzyme production. Research efforts have been pointed towards evaluating the effect of various organic and inorganic nutrients, metal ions, pH, temperature, aeration, and agitation on the yield of enzymes. From the conventional method the following parameters were selected for the Response Surface Methodological optimization. Mannitol, beef extract, pH and ferrous sulphate were found to be important factors in enhancing the laccase production. The conventional method of optimization involves varying one parameter at a time and keeping the others are invariant.

Response surface methodology (RSM) is a simple model to analyze the effect of various factors influencing the responses by varying them simultaneously. Carbon and nitrogen sources have been promised to play a significant role in enhancing the production of fungal laccase. The central composite design used to analyze the interaction effects of variables on production of laccase enzymes (Fannin *et al*., 1981;Deshayes 1980; Matthews *et al*., 1981) (Box and Wilson 1951; Cochran and Cox, 1957; Box *et al*., 1978; Akhnazarova and Kafarov, 1982; Khuri and Cornell, 1987; Yee and Blanch, 1993; Mak *et al*., 1995). Conventional method of optimization involves changing one independent variable at a time (vanillin, glycerol and mineral salt solution) and maintaining all the others at a constant.

* Corresponding Author, *Email*: subhulakshmisaravanan@gmail.com

Materials and Methods

Isolation and identification

Laccase-producing fungi were screened from Dry Evergreen forest of Guindy National Park and IIT Madras, Chennai. Fungi were isolated by placing pieces of unexposed tissue of fruit body on MEA (Malt Extract Agar) and PDB (Potato dextrose Broth) plates containing different indicator compounds. The plates were incubated at 27^{o}C for at least a week and strains were sub cultured when clear positive reactions were visible. Production of laccase was carried out in production medium and these medium were sterilized by autoclaving at 121°C for 15 min. The above medium (50ml in 250ml Erlenmeyer flasks) was inoculated with two mycelial discs (8mm) and maintained at 27°C in a static condition. Fermented broth was filtered in filter paper at 4°C and the supernatant was recovered and used as enzyme source. The range of test variables are given in table 1. The concentration of mannitol, beef extract, pH and ferrous sulphate were varied according to the experimental design (table 2). The pH value of the medium was adjusted before sterilization.

Enzymatic assay

Extra cellular laccase activity of cell free filtrate was assayed spectrophotometrically on 9th day, 10 mM of ABTS as substrates in 100 mM sodium acetate buffer (pH 5.0), 0.1 ml of the culture filtrate (assay for 1ml of reaction mixture) and absorbance was measured at 436 nm. (Mansur *et al*., 1997). 0.1 ml of distilled water, 0.9 ml of sodium acetate buffer was kept as blank.

Optimization by RSM

Box-Behnken design consists of a group of controlled experimental factors and measured responses, according to one or more selected criteria. A prior knowledge to understanding the process and process variables under investigation are necessary for achieving a more realistic model. Among the different components of the media, carbon, nitrogen, pH and metal ion were the major variables were selected to find optimal conditions for higher enzyme production using Response Surface Methodology. The range of experimental variables was investigated in this study (Table 1). Several experimental design have been selected the Box-Behnken design proposed by Box *et al*., (1978). For this study, 4 factorial design were employed to fit the second order polynomial model which indicates 27 experiments were required for this procedure. The mid values (zero level) chosen for experimental design comprised mannitol, beef extract, pH and ferrous sulphate. The three significant independent variables X1, X2, X3 and X4 and the mathematical relationship of response Y on these variables can be approximated by quadratic / (Second degree) polynomial equation as shown below:

$$Y=b_o+b_1X_1+b_2X_2+b_3X_3+b_4X_4+b_{11}X_1^2+b_{22}X_2^2+b_{33}X_3^2+b_{44}X_4^2+b_{12}X_1X_2+b_{13}X_1X_3+b_{14}X_1X_4+b_{23}X_2X_3+b_{34}X_3X_4$$

Where

Y is the predicted response,

b_o is the constant,

b_1, b_2, b_3 &b_4 are the linear co-efficients,

b_{11}, b_{22}, b_{33} & b_{44} are the quadratic co-efficients and

b_{12}, b_{13}, b_{23},b_{24}& b_{34} are the cross-product co-efficients. The optimum values of the selected variables were obtained by solving the regression equation (Cornell 1987). In that, the 27 fermentation experiments were conducted in triplicate.

Results and discussion

Pleurous sp. was grown in CDB at different temperatures ranging from 20 and 40˚C at 5°c interval. Maximum production of 44 U/ml was recorded at 25˚C. The temperature above and below 25˚C did not enhance the Laccase production (fig.2). Saravanakumar and Kaviyarasan (2010) studied the optimal parameter of maximum laccase enzyme production was found to be pH 8.0, temperature 25°C, glycerol, vanillin and CuSo4, but the laccase production was decreased at 30°C (Thurston, 1994). In general, the fungi were cultivated at temperatures between 25°C and 30°C for optimal laccase production (Arora & Gill, 2000; Fahreus & Reinhammar, 1967; Pointing *et al*., 2000; Vasconcelos *et al*., 2000). When the temperatures higher than 30°C the activity of ligninolytic enzymes was reduced (Zadrazil *et al*., 1999).

The pH showed a significant influence on the production of extra cellular Laccase, the test fungus produced Laccase between the pH of 5.0 to 9.0. Maximum Laccase production of 94.3 and 94.0 U/ml was recorded at pH 5.5 and 7.5 respectively, and the moderate activity was observed in pH 9.0 and then declined (fig.1). The optimal pH for laccase production was found to be at pH 6.5 in submerged culture of *Chaetomium globosum* (El-Zayat, 2008). The laccase production was 25-fold higher in *Botryosphaeria rhodina* showed a significant effect with increased pH of 3.5 - 7.5 for both induced and noninduced cultures (Dekker *et al*., 2007). Among the different carbon sources tested namely Glucose, Sucrose, Mannitol, Maltose, Glycerol and Fructose at 1% concentration, the level of Laccase production was maximum at 161.1 U/ml, in mannitol amended medium. The enzyme production of 149 U/ml and 90.9 U/ml was recorded with Fructose and Glycerol on 15th day of incubation. The carbon sources like glucose and maltose showed decreased effect on Laccase production from *pleurotus* sp., (fig.3). (Dekker *et al*., 2007)

Fig.1. Effect of different pH on extra cellular laccase production

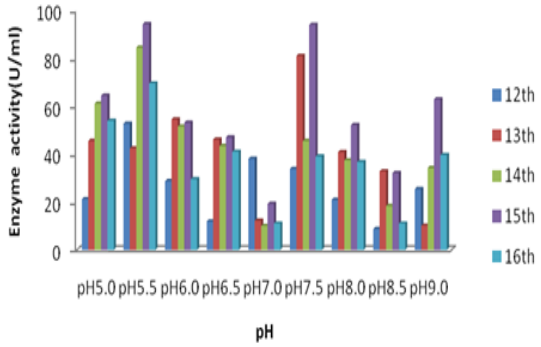


Fig.2. Effect of temperature on extra cellular laccase production

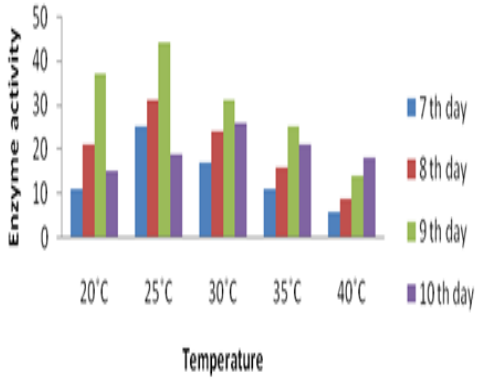
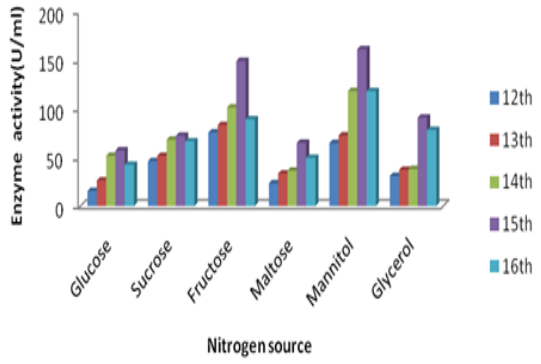
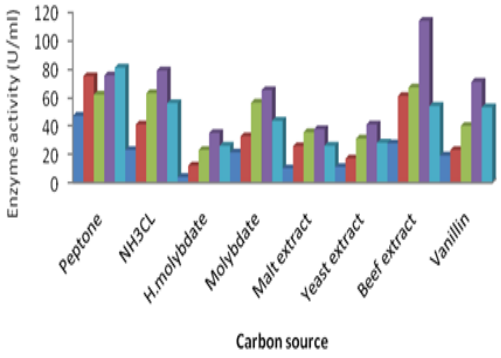


Fig.3. Effect of different carbon source on extra cellular laccase production



Churapa Teerapatsakul *et al*., (2007) showed that *Ganoderma* sp. produced maximum laccase activity in the presence of glycerol as a carbon source in liquid medium. In addition, Rodriguez Couto *et al*.,(2006) reported glycerol as a sole carbon source in *Trametes hirsuta*, which showed a maximum laccase activity of 19,400 U/L, which was reported the highest from the fungus. *Pleurotus* sp. produced maximum laccase activity when beef extract was sole nitrogen source (114 U/ml) as shown in fig.4. However, most of the fungal laccases were stimulated by organic nitrogen than inorganic nitrogen source. Kirk and Farrell (1987), Higuchi (1990), Cullen (1997) reported Laccase and other ligninolytic enzyme activity was increased due to carbon and organic nitrogen. Saravanakumar and Kaviyarasan (2010) reported maximum laccase production (84 U/ml) by using vanillin. Monteiro and De Carvalho (1998) reported high laccase activity with semi-continuous production in shake-flasks using a low carbon to nitrogen ratio (7: 8 g.g). Buswell *et al.,* (1995) found that laccases were produced at high nitrogen concentrations, although it is generally accepted that a high carbon to nitrogen ratio is required for laccase production. Laccase was also produced earlier when the fungus was cultivated in a substrate with a high nitrogen concentration and these changes did not reflect differences in biomass. Heinzkill *et al.,* (1998) also reported a higher yield of laccase using nitrogen rich media rather than nitrogen limited media usually employed for production of oxido reductase.

Fig.4. Effect of different nitrogen source on extra cellular laccase production



Response surface methodology (RSM)

A response surface methodology allowed calculation of maximum production based on a few sets of experiments in which all the factors were varied within chosen ranges. This method has been successfully applied in the optimization of medium compositions (Lee and Chen, 1997) conditions of enzymatic hydrolysis (Ma and Ooraikul, (1986) and fermentation processes (Rosi *et al*., (1987) and Sonia (2005). In optimizing the effect of carbon and nitrogen sources on laccase production from *Pleurotus* sp., 4 factorial designs were applied using mannitol, peptone, pH and metal ion. Box-Behnken design aims to select most important variables in the system that influence over all enzyme productivity. Each variable varies for a desired response represented at high and low levels. Generally calculated F values should be several times more than tabulated value, if the model was a good prediction of experimental results and estimated factors effects are real. Also high F value and a very low probability (P> F = 0.0001) indicate that present model is in a good prediction of experimental results. The F

value of model implies that model was significant. (Akhnazarova and Kafarov, 1982; Cornell, 1987). The goodness of fit was checked by determination coefficient (R2), and value of the determination coefficient (R2 = 89.5%) indicates that only 10.5% was not explained by the model. The adjusted coefficient (Adj. R2 = 75 %) was also very high, which indicates a high significance of the experiment (Box and Wilson 1951; Cochran and Cox 1957; Akhnazarova and Kafarov 1982; Khuri and Cornell 1987; Yee and Blanch 1993; Mak *et al*., 1995). Box *et al*., 1978 reported a higher value of the correlation coefficient (R = 98.1%) signifies an excellent correlation between the independent variables (Box and Wilson 1951; Cochran and Cox 1957; khnazarova and Kafarov 1982; Yee and Blanch 1993; Mak *et al*., 1995). The application of Response Surface Methodology (Box and Wilson 1951; Khuri and Cornell 1987; Mak *et al*., 1995) was yielded higher enzyme production. The logarithmic values of enzyme yields and test variables in uncoded unit as follows:

$Y=50.33+b_1(-3.44)+b_2(1.22)+b_3(16.29)+b_4(-3.44)+b_{11}(-0.95)+b_{22}(-14.23)+b_{33}(-12.27)+b_{12}(-3.44)(1.24)+b_{13}(-3.44)(16.29)+b_{23}(1.24)(16.29)+b_{14}(-3.44)(-3.44)+b_{24}(16.29)+(-3.44)$

The enzyme concentrations expressed in logarithmic values, and X1, X2, X3 and X4 were the coded values of test variables (mannitol, beef extract, pH and copper sulphate respectively). The significance of each coefficient was determined by t- value and p values (Table 2). The larger t- value and smaller the p-value indicates the significance of the model. This implies the quadratic main effects of vanillin, glycerol and mineral salt solution of the medium are more significant (Akhnazarova and Kafarov 1982; Khuri and Cornell 1987). The carbon and nitrogen source support the maximum enzyme activity and their respective P-values were P X1 2 > 0.021, P X2 2 < 0.166 and P *X3* 2 <0.022. The mannitol and beef extract have a direct relationship on the production of the enzyme. From the model each observed values (Y f (0) was compared with the predicted values (Y f (P) (table 2). Perturbation plot is an important diagrammatic representation to compare effect of all variable at a particular point in design space; real benefits from this plot were selecting axes and constants in wire frame. Response surface plots showed two variables at a time and maintaining others were fixed level, it was more helpful to understanding the interaction effects of these two factors. A circle in the square shows that response was sensitive to that factor (fig.5,8,9,10). A relatively other showed insensitiveness to the enzyme yields (fig.6,7). If there are more than two factors, perturbation plot could be used to find effect of variables on enzyme production. These influential factors were good choices for axes on counter plots and can be easily obtained by calculating from model. The yield values of different variables can also predicated from the respective response surface plots (fig. 5 to 10). The laccase production was predominantly influenced by mannitol and peptone. Mannitol and peptone were the key factors which control the biosynthesis of laccase enzyme. At lower concentration of beef extract as well as higher metal ion may not cause inhibition of enzyme synthesis. (Ikehata *et al*., 2004; Sakurai *et al*., 2002; Ragalski *et al*., 2006; Stajic *et al*., 2006). The regression equation solve the optimal values of test variables in uncoded units and the values were beef extract 4.8 gm/l, mannitol 3gm/l, pH 6.2 and copper sulphate 4mg/l. Monteiro and De Carvalho (1998) reported high laccase activity with semi-continuous production in shake-flasks using a low carbon to nitrogen ratio (7: 8 g.g). Buswell *et al.,* (1995) found that laccases were produced at high nitrogen concentrations, although it is generally accepted that a high carbon to nitrogen ratio is required for laccase production. This model predicts maximum production of enzyme that can be obtained by using the above RSM and the production was increased up to 2-3 folds.

Fig.5. Optimization of extra cellular laccase

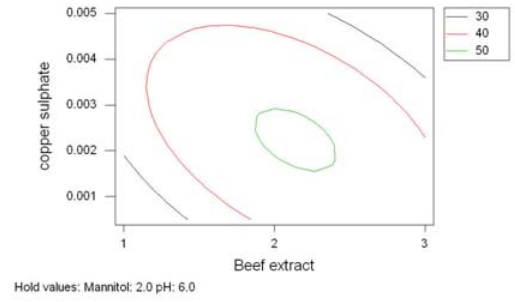


Fig.6. Optimization of extra cellular laccase

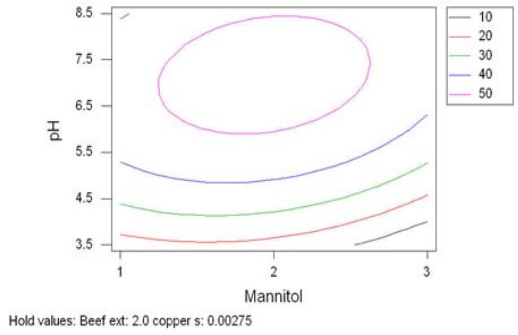


Fig.7. Optimization of extra cellular laccase

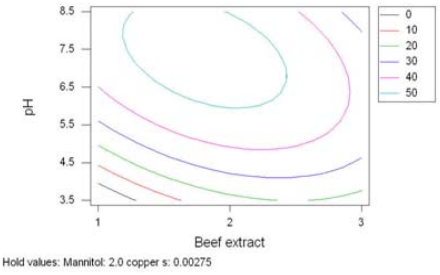


Fig.8. Optimization of extra cellular laccase

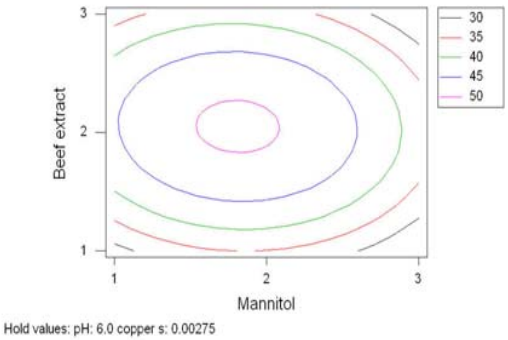


Fig.9. Optimization of extra cellular laccase

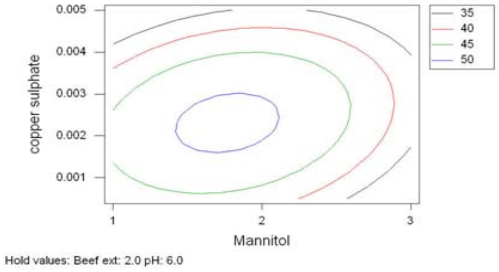


Fig.10. Optimization of extra cellular laccase

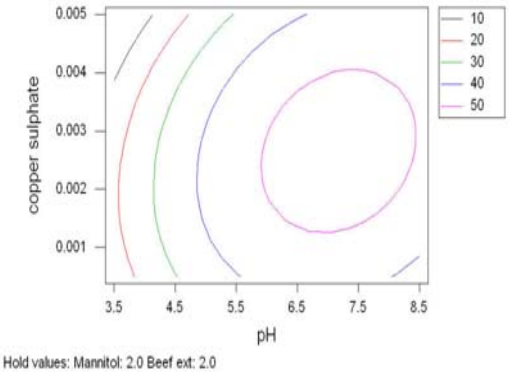


Table 1 Observed responses and predicted values

S.No	Mannitol	Beef extract	pH	Ferous sulphate	Peroxidase Yield (U/ml)		Residual value
					Observed Value	Predicted value	
1	1	1	6.0	0.00275	33.3	28.1333	5.1667
2	3	1	6.0	0.00275	10.7	23.1500	-12.4500
3	1	3	6.0	0.00275	37.5	32.5167	4.9833
4	3	3	6.0	0.00275	11.1	23.7333	-12.6333
5	2	2	3.5	0.00050	10.7	14.3333	-3.6333
6	2	2	8.5	0.00050	25.1	36.4667	-11.3667
7	2	2	3.5	0.00500	0.9	-3.0000	3.9000
8	2	2	8.5	0.00500	36.2	40.0333	-3.8333
9	1	2	3.5	0.00275	1.7	16.0833	-14.3833
10	3	2	3.5	0.00275	1.4	-0.5500	1.9500
11	1	2	8.5	0.00275	36.5	38.9167	-2.4167
12	3	2	8.5	0.00275	55.7	41.7833	13.9167
13	2	1	6.0	0.00050	21.6	14.9333	6.6667
14	2	3	6.0	0.00050	60.7	41.5167	19.1833
15	2	1	6.0	0.00500	12.5	32.1500	-19.6500
16	2	3	6.0	0.00500	3.4	10.5333	-7.1333
17	1	2	6.0	0.00050	34.9	40.9667	-6.0667
18	3	2	6.0	0.00050	20.7	25.4833	-4.7833
19	1	2	6.0	0.00500	38.2	25.4833	12.7167
20	3	2	6.0	0.00500	41.2	27.2000	14.0000
21	2	1	3.5	0.00275	1.5	-10.7500	12.2500
22	2	3	3.5	0.00275	16.2	16.2833	-0.0833
23	2	1	8.5	0.00275	54.4	46.3833	8.0167
24	2	3	8.5	0.00275	20.0	24.3167	-4.3167
25	2	2	6.0	0.00275	24.6	50.3333	-25.7333
26	2	2	6.0	0.00275	56.5	50.3333	6.1667
27	2	2	6.0	0.00275	69.9	50.3333	19.5667

Table 2 Model coefficients estimated by multiples linear regression

	Coefficient	t- value	p- value
Constant	50.33	5.082	0.000
X_1 (C)	-3.44	-0.695	0.500
X_2 (N)	1.24	0.251	0.806
X3(pH)	16.29	3.290	0.006
X4(M)	-3.44	-0.695	0.500
X11(C+C)	-9.22	-1.242	0.238
X22(N+N)	-14.23	-1.915	0.080
X_{33} (pH+pH)	-17.05	-2.295	0.041
X44(M+M)	-11.32	-1.525	0.153
X_{12} (C+N	-0.95	-0.111	0.914
X_{13} (C+pH)	4.88	0.568	0.580
X_{14} (C+M	4.30	0.501	0.625
X_{23} (B+pH)	-12.27	-1.431	0.178
X24 (B+M)	-12.05	-1.405	0.185
X_{34} (pH+M)	5.23	0.609	0.554

Table.3 Analysis of variance (ANOVA) for the factorial design

Source of variation	Sum of squares	Degree of freedom	Mean square	F- value	Probe (P) >F
Regression	7023	14	501.7	1.70	0.180
Residual error	1465	12	3532		
Total	10555	26			

R= 89%; Adjusted R^2= 75%

Conclusion

Box- Behnken experimental design for process optimization involves a study of specific region of individual factors. Yield of laccase increased by supplementation of medium. In this study, response surface analysis and central composite design were used for optimizing culture media for maximizing laccase production by using *Pleurotus* sp. in submerged fermentation. The optimized fermentation medium composition increased 2 to 3 fold of enzyme production.

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Reference

Akhnazarova S and Kafarov V. (1982). Experiment optimization in chemistry and chemical engineering, Mir publications, Moscow.

Argyeropoulos DS. (2001). Oxidative delignification chemistry AGS symposium series 785. American chemical society, Washington,D.C.

Arora DS and Gill PK. (2000). Laccase production by some white rot fungi under different nutritional conditions. *Bioresource Technol*. 73: 283-285.

Box GEP and Wilson KB. (1951). On the experimental attainment of optimum conditions. *J.Roy.Stat.Soc* . 13:1-45.

Box GEP, Hunter WG and Hunter JS. (1978). Statistics for experiments. John Wiley and Sons New York. 291-334.

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.

Cai W, Martin R, Lemaure B, Leuba JL and Petiard V. (1993). Hydroxy indoles: a new class of laccase substrates. *Plant Physiol. Biochem*. 31:441-445.

Call HP and Mucke I. (1997). Minireview. History, overview and applications of mediated lignolytic systems, especially laccase-mediator-systems (Lignozymprocess). *J. Biotechnol*. 53: 163-202.

Cochran WG and Cox GM. (1957). Experimental designs, 2nd edn. John wiley and sons, New York. 346-354.

Cochran WG and Cox GM. (1992). Experimental designs, 2nd edn. John wiley and sons, New York. 335-375.

Coll PM, Fernandez-Albalos JM, Villanueva RS and Peres P. (1993). Purification and characterization of phenoloxidase (laccase) from the lignin-degrading Basidiomycete PM1 (CECT 2971). *Appl. Environ. Microbiol*. 59 (8): 2607-2613.

Cullen D. (1997). Recent advances on the molecular genetics of ligninolytic fungi. *J. Biotechnol* 53273–289.

Deshayes CMP. (1980). Utilisation de modeles mathematiques pour optimization en fermentation, applications aux transformations par les micro-organisms. *Bull. Soc .Chim. Fr.* 124- 34.

Eggert C, Temp U and Eriksson KEL. (1996). The lignolytic system of the white rot fungus Pycnoporus cinnabarinus: Purification and characterization of laccase. *Appl. Environ. Microbiol*. 62:1152-1158.

Eggert C, Temp U, Dean JFD and Eriksson KEL. (1996). A fungal metabolite mediates degradation of non-phenolic lignin structures and synthetic lignin by laccase. *FEBS Lett*. 391:144-148.

El-zayat SA (2008). Preliminary studies on laccase production by *Chaetomium globosum* and endophytic fungus in *Glinus lotoides*. *American-Eurasian J.Agric. & Environ. Sci*., 3(1) 86-90.

Fahraeus G and Reinhammar B. (1967). Large scale production and purification of laccase from cultures of the fungus Polyporus and some properties of Laccase A. *Acta Chemica Scandinavica* 21: 2367-2378.

Fannin TE, Marcus MD, Anderson DA and Bergman HL (1981). Use of a fractional design to evaluate interactions of environmental factors affecting biodegradation rates. *Appl.Environ.Microbiol*. 42936-943.

Feng Xu. (2005). Applications of oxidoreductases: Recent progress. *Industrial Biotechnology*. 1: 38-50.

Heinzkill M, Bech L, Halkier T, Schneider P and Anke T. (1998). Characterization of laccase and peroxidase from woodrotting fungi (family Coprinaceae). *Appl. Environ. Microbiol*. 64(5): 1601-1606.

Hela Zouari-Mechichi, Tahar Mechichi, Abdelhafidh Dhouib, Sami Sayadi Angel T. martinez and maria jesus martinez (2005). Laccase purification and characterization from *Trametes trogii* isolated in Tunisia: decolorization of textile dyes by the purified enzyme. *Enzyme and microbial technology*. 39:141-148.

Higuchi T. (1990). Lignin biochemistry: biosynthesis and biodegradation. *Wood Sci Technol* 2423–63.

Keisuke Ikehata, Michael A. Pickard, Ian D. Buchanan, and Daniel W. Smith (2004). Optimization of extracellular fungal peroxidase production by 2

Coprinus species. *Can. J. Microbiol.* 50(12): 1033–1040.

Khuri AI. and Cornell JA. (1987). Response surfaces Design and analysis. Marcel Dekker,Inc, New York.

Kirk KT and Farrell RL. (1987). Enzymatic ‘‘combustion’’: the microbial degradation of lignin. *Annu Rev Microbiol*. 41465–505.

Lee, S.L. and W.C. Chen. (1997). Optimization of medium composition for the production of glucosyltransferase by *Aspergillus niger* with response surface methology, *Enzyme Microb. Technol*. 21 436–440.

Ma AYM and Ooraikul B. (1986). Optimization of enzymatic hydrolysis of canla meal with response surface methology, *J. Food Process. Preservation.* 10 (99–113).

Mansur M., Suarez T., Fernandez-Larrea J.B., Brizuela M.A. and Gonzalez A.E. (1997). Identification of a Laccase gene family in the New Lignin-Degrading Basidiomycete CECT 20197. *Appl Environ Microbiol* 632637–2646.

Matthews R.J., Scott R.G. and Morgan S.L. (1981). Characterization of an enzymatic determination of arsenic (v) based on response surface methodology. *Anal. Chim. Acta* 133. 169-182.

Monteiro, M.C. & De Carvalho, M.E.A (1998). Pulp bleaching using laccase from *Trametes versicolor* under high temperature and alkaline conditions. *Appl. Biochem. Biotechnol.* 70-72: 983-993

Pointing, S.B., Jones, E.B.G., Vrijmoed, L.L.P. (2000). Optimization of laccase production by Pycnoporus sanguineus in submerged liquid culture. *Mycologia* 92: 139-144.

Ragakski J, Szczodrak J and Janusz G (2006). Manganese peroxide production in the submerged cultures by free and immobilized mycelia of *Nemataloma frowardii. Biores. Technol.* 97, 469-476.

Robert F.H. Dekker, Aneli M. Barbosa, Ellen C. Giese, Saulo D.S. Godoy and Luiz G. Covizzi. (2007). Influence of nutrients on enhancing laccase production by *Botryosphaeria rhodina* MAMB-05. *International microbiology* 10:177-185.

Rodriguez Couto, S., A. Roudriguez, R.R.M. Paterson, N. Limba and J.A.Teixeira (2006). Laccase activities from the fungus *Trametes hirsuta* using an air lift bioreactor. The Society for Applied Microbiology, *Letters in Applied Microbiology* 42: 612-616.

Rosi, L. Costamagna, M. Bertuccioli, S. Clement, G. Cruciani (1987).Wine fermentation by immobilized yeast: an optimization study, in: M. Martens, G.A. Dalen Jr., H. Russwurm (Eds.), Flavor Science and Technology, John Wiley and Sons, New York. 239.

Sakurai A, Kawamoto S, Abarca JF and Sakakibara M (2002). Peroxidase production by *Coprinus oinereus* using rotating disk contactor. *Biochem. Enginee. J.* 10, 47-53.

Sonia KG, Chadha, H.S (2005). Saini, Sorghum straw for xylanase hyperproduction by *Thermomyces lanuginosus* (D2W) under solid-state fermentation, *Bioresour.Technol*. 96 1561–1569.

Stajic M, Persky L, Friesem D, Hadar Y, Wasser SP, Nevo E and Vukojevic J (2006). Effect different carbon and nitrogen sources on laccase and peroxidase production by selected *Pleurotus* species. *Enzyme. Microb. Technol.* 38, 65-73.

Thurston CF (1994). The structure and function of fungal Laccase. *Microbiol*. 140: 19-26.

Tuor U, Winterhalter K and Fiechter A (1995). Enzymes of white-rot fungi involved in lignin degradation and ecological determinants for wood decay. *J. Biotech*. 41: 1-17.

Vasconcelos AF, Barbosa AM, Dekker RFH, Scarminio IS and Rezende MI (2000). Optimization of laccase production by Botryospaeria sp. in the presence of veratryl alcohol by the response-surface method. *Process Biochem*. 35: 1131-1138.

Yang JL and Eriksson KEL (1992). Use of hemicellulytic enzymes as one stage in bleaching of kraft pulps. Holzforschung. 46: 481-488.

Yee L. and Blanch H.W (1993). Defined media optimization for the growth of recombinant *Escherichia coli* x90. *Biotechnol. Bioeng* 41:221-227.

Zadrazil, F., Gonser. A. and Lang, E (1999). Influence of incubation temperature on the secretion of extracellular ligninolytic enzymes of *Pleurotus* sp. and *Dichomitus squalens* into oil. Proceedings of the conference on Enzymes in the environment: Activity, *Ecology and Applications*, 12-16 July, Granada,Spain.