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RESEARCH ARTICLE

OPTIMIZATION OF PROCESS PARAMETERS FOR THE PRODUCTION OF THERMOSTABLE LACCASE BY *PLEUROTUS FLABELLATUS* ATK-1 USING RESPONSE SURFACE METHODOLOGY

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ABSTRACT

Optimization of laccase production was carried out using a new strain, *Pleurotus flabellatus* ATK-1 with three independent process parameters viz glucose, incubation time and pH using full factorial central composite experimental design. The optimum values for the process parameters for the maximum laccase production were obtained glucose 2.8%, incubation time 12.6 days and pH 6.7. In the work, we have demonstrated the use of a central composite design for maximum enzyme production by optimizing the process parameters.

INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) are multi-copper containing enzymes which reduce molecular oxygen to water and simultaneously perform one electron oxidation of various aromatic substrates (diphenols, methoxy-substituted monophenols, aromatic amines). This rather broad substrate specificity of laccase has attracted great scientific interest for the biotechnological application of laccase. Currently laccases are used in pulp delignification, textile dye bleaching, effluent detoxification, washing powder components, removal of phenolics from wines and transformation of antibiotics and steroids as well as in biosensors (Bauer *et al.,* 1999; Nyanhongo *et al.,* 2002). Laccase production is a common feature of many basidiomycetes, particularly those associated with wood decay or terminal stages of decomposition. Fungal laccases participate in conidial pigmentation and in infection processes during phytopathogenesis, to overcome the reactions of the host organism by polymerizing endogenous plant phenols, thus rendering them non-toxic (Mayer, 1987). However, the major role attributed to laccases in fungal species lies in the degradation of lignin and in the humification process. *Pleurotus flabellatus* ATK-1, isolated from campus of Indian Institute of Technology, Chennai, India, is a fast growing white rot fungus which produces thermostable laccase (Arulmani, 2007). However, in contrast to several other *Pleurotus* sp, there is no much information available in the literature about laccase production by *P. flabellatus* and optimization of laccase production using response surface methodology.

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In this study we describe for the first time (central composite design) the optimization of process parameters glucose, incubation time and pH, which have been reported to play a very significant role in enhancing the production of laccase. *Pleurotus flabellatus* ATK-1 was used as producer of laccase enzyme, it was grown at 28ºC for a week and maintained on Potato dextrose agar slants at 4ºC and was subcultured at four week intervals. Identification of the fungal strain was done using morphological characters and molecular techniques (partial ITS (Internal Transcribed Spacer) sequences; GenBank accession number EU769554). Amplification of ITS gene was done using ITS 1 and ITS 4 primers (Pandey *et al.,* 2003).

Production of laccase was carried out in laccase production medium (Peptone 1.0g/l, glucose 10g/l, K_2HPO_4 0.4g/l, KH_2PO_4 0.6g/l, $ZnSO_4$ 0.001g/l, $FeSO_4.7H_2O$ 0.0005g/l, $MnSO_4$ 0.05g/l, $MgSO_4.7H_2O$ 0.5g/l, $CuSO_4.7H_2O$ 0.02g/l, Thiamine HCl 0.001g/l; pH 5.2; 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 30°C in a static condition. Fermented broth was filtered in filter paper at 4°C and the supernatant was analyzed for laccase activity. Laccase activity was determined as given in (Dhawan and Kuhad, 2002). One unit of laccase was defined as the change in absorbance of 0.1/ml/min at 470 nm.

The concentrations of glucose, incubation time and pH in the culture medium for enzyme production were carried out according to the experimental design shown in the Table 1. The pH

The quadratic model equation for predicting the response variable in enzyme production was expressed using second order polynomial according to Eq. 2.

Table 1. Central composite design consisting of 20 experiments for the study of 5 experimental factors in uncoded units and Observed responses and predicted values

Serial No	Glucose (%)	Time (days)	pH	Laccse yield (U/ml)	
				Observed response	Predicted value
1	2	6	4	12	20.29
2	5	6	4	1.4	-6.05
3	2	16	4	32	33.50
4	5	16	4	43.8	36.45
5	2	6	8	40.2	46.51
6	5	6	8	16.2	13.65
7	2	16	8	55.8	62.21
8	5	16	8	68	58.66
9	0.97	11	6	85	71.10
10	6.02	11	6	30.6	45.96
11	3.5	2.59	6	4.2	00.95
12	3.5	19.4	6	45.2	49.90
13	3.5	11	2.63	0.0	02.47
14	3.5	11	9.36	44.2	43.19
15	3.5	11	6	54	75.85
16	3.5	11	6	69.2	75.85
17	3.5	11	6	85.8	75.85
18	3.5	11	6	78.6	75.85
19	3.5	11	6	77.8	75.85
20	3.5	11	6	90	75.85

Table 2. Model coefficients estimated by multiples linear regression

Factor	Coefficient	Computed -t-value	P-value
Intercept	-233.0	-3.608	0.005
X_1	6.6	0.421	0.682
X_2	14.8	3.205	0.009
X_3	63.3	5.025	0.001
X_{12}	-2.7	-1.807	0.101
X_{13}	-0.7	-5.259	0.000
X_{23}	-4.7	-5.530	0.000
X_{11}	1.0	1.610	0.139
X_{22}	-0.5	-0.357	0.728
X_{33}	0.1	0.137	0.893

sterilization by adding 1N NaOH or 1N HCl. In the present work, 2^3 full factorial central composite design for three experimental variables, each at five levels were carried out with 20 experiments were carried out including six star points and six replicates at the center point to fit the response data into second order model (Table 1). Three independent variables were concentration of glucose (X_1), incubation time (X_2) and pH (X_3) and center values chosen for the experimental design area as follows: X_1= 3.5 %; X_2= 11 days and X_3= 6.0 these center points was obtained from conventional optimization data not shown). For statistical calculations, the variable X_i was coded as x_i according to the following relationship Eq. 1:

$$x_i = (X_i - X_0)/\delta X \quad \text{-----} \quad (1)$$

Where x_i is the coded value of the variable,

X_i is the actual value of the variable,

X_0 is the center point value and

δX is the step change between the levels.

$$\eta = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 \quad \text{-----} \quad (2)$$

where η = laccase enzyme activity;

X_1, X_2, X_3 = Variables;

β_0 = constant term;

β_1; β_2; β_3 = coefficients of the polynomial for linear effects;

β_{11}; β_{22}; β_{33} = coefficients of the polynomial for quadratic effects;

β_{12}; β_{13}; β_{23} = = coefficients of the polynomial for interaction effects.

The Minitab (Version 12.2, PA, USA) was used for regression and graphical analysis of the data obtained.

All 20 experiments were conducted in triplicate, and the average values of laccase yields were tabulated, as observed response (Table 1). The predicted values were calculated by using the regression coefficient model equation as given in equation 3

The goodness of fit of the model was checked by the regression coefficient (R^2). In this case the value of the regression coefficient (R^2 = 0.901) indicates that only 9.9% of the total variations are not explained by the

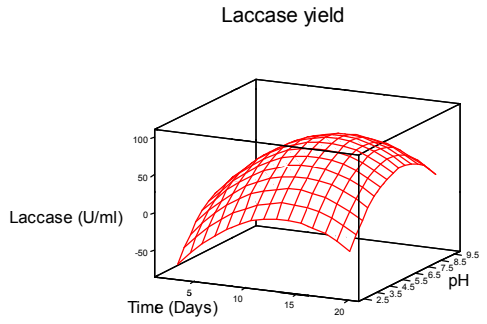

Fig. 1. Response surface plot showing the effect on medium pH, incubation time and their mutual effect on the production of laccase. Other variables are held at zero level

model. A higher value of the correlation coefficient (R = 90.1%) signifies an excellent correlation between the independent variables and the response (Box *et al.,* 1978).

The application of response surface methodology (Box and Wilson, 1951; Khuri and Cornell, 1987; Kenneth *et al.,* 1995) yielded the following regression equation which is an empirical relationship between the laccase enzyme yields and process parameters in uncoded unit:

$$Y = -233.049+6.580X_1+14.806X_2+63.504X_3-2.722X_1^2-0.713X_2^2-4.687X_3^2+0.977X_1X_2-0.542X_1X_3+0.062X_2X_3 \quad \text{----- (3)}$$

Where Y is the response, that is, the enzyme concentrations expressed and X_1, X_2 and X_3 are the coded values of the test parameters (glucose, incubation time and pH).

The significance of each coefficient was determined by student's t-test and p values (Table 2). The larger the magnitude of the t-value and the smaller the p- value, the more significant is the corresponding coefficient (Akhnazarova and Kafarov, 1982; Khuri and Cornell, 1987). This implies that the first order main effects of incubation time (P = 0.009) and pH (0.001) are having more significant effect on enzyme production where as glucose is having less significant effect in enzyme production (0.682). These values suggest that the pH and incubation time have a direct relationship on the production of the enzyme. The second order effect of glucose have the more effect than incubation time and pH, as is evident from their respective P-values (P X_{11} < 0.139, P X_{22} < 0.728 and P X_{33} <0.893).

The laccase production is predominantly influenced by incubation time and pH, are therefore the key factors which control the biosynthesis of the enzyme (Revankar *et al.,* 2006). Extended incubation time as well as higher pH may cause inhibition of enzyme synthesis. This fact was also reported by other investigators during other enzyme production studies on carbon repression effects (Arora and Gill, 2000; Gill and Arora, 2003).

Each of the observed values for Y is compared with the predicted values, from the regression model, using analysis of variables (ANOVA). The fisher F-test value from ANOVA with a very low probability value demonstrates a very high significance for the regression model (Akhnazarova and Kafarov, 1982). The regression equation was solved Monte-Carlo optimization technique and optimal values obtained for test variables in uncoded units are glucose 2.80%, incubation time, 12.6 days, medium pH 6.7. The modeled values predict that the maximum concentrations of enzyme that can be obtained by using the above optimized concentrations of the variables is 79.9 U/ml. The predicted results obtained from optimum were confirmed by carried out experiments optimized conditions. Enzyme production of about 76.4 U/ml was observed, which is with in 5% variation of the predicted values. This result therefore corroborates the predicted values, and the effectiveness of the regression model. From above, it indicates the excellent adequacy of the regression model choosen (Box and Willson, 1951; Cochran and Cox, 1957; Box *et al.,* 1978; Akhnazarova and Kafarov, 1982; Khuri and Cornell, 1987; Yee and Blanch, 1993; Kenneth *et al.,* 1995).

Response surface plots as a function of two parameters, maintaining all other parameters at fixed levels (zero, for instance), are more helpful in understanding both the main and the interaction effects of these two parameters. These plots can be easily obtained by calculating from the model. The yield values for different concentrations of the parameters can also be predicated from the respective response surface plots (Fig. 1). The maximum predicted yield is indicated by the surface confined in the response surface diagram.

Our recent fungal survey demonstrated laccase production by other mushroom species, such as *Trametes modesta* and several *Pleurotus* species investigated by others (Nyanhongo *et al.,* 2002; Stajic *et al.,* 2006). Among fungi tested in the present survey *Pleurotus* species was found to be promising candidate for large scale laccase production. We are particularly interested in the laccase produced by *Pleurotus flabellatus* (ATK-1), because this enzyme is apparently more stable at higher temperature than the enzyme produced by other fungal strains, although the enzyme productivity by the former fungi was lower. High thermal stability (up to 60-70˚C) of an enzyme is generally preferable and especially beneficial for its application to industrial waste water treatment, because the temperature of industrial waste water tends to be above ambient, which would accelerate enzyme inactivation.

Conclusion

This work has demonstrated the use of a central composite design for optimization of parameters leading to maximum yield of enzyme production. In this study maximum enzyme yield of 76.4 U/ml was obtained at glucose 2.8%, pH 6.7 and incubation time 12.6 days. Response surfaces obtained from the study are very helpful in visualizing the main effects and interaction of the factors. Thus, organism identified could be successfully used for production of laccase which has wide range of applications.

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REFERENCES

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

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

Arulmani M. 2007. Characterization of a thermostable laccase from *pleurotus* sp. MAK-11 and laccase gene diversity from basidiomycetes: application of laccase in effluent treatment and dye degradation. Ph.D Thesis, University of Madras.

Bauer CG, Kuhn A, Skorobogatko O, Holt P, Bruce NC, Makower A, Lowe Box GEP, Hunter WG and Hunter JS. 1978. Statistics for experiments. John Wiley and Sons, New York, pp 291-334

Box GEP and Wilson KB. 1951 On the experimental attainment of optimum conditions. J Roy Stat Soc B13**:**1-45

Cochran WG and Cox GM. 1957. Experimental designs, 2nd edn. John Wiley and Sons, New York, pp 346-354

CR, Scheller FW. 1999. New enzyme sensors for morphine and codeine based on morphine dehydrogenase and laccase. *Fresenius J. Anal. Chem.,* 364: 179-183.

Dhawan S, Kuhad RC. 2002. Effect of amino acids and vitamins on laccase production by the birds nest fungus, *Cyathus bulleri*. *Bioresource Technol*., 84:35-38

Gill PK, Arora DS. 2003. Effect of culture condition on manganese peroxidase production and activity by some white rot fungi. *J. Ind. Microbiol. Biotechnol.,* 30: 28-33.

Kenneth WY, Mak Miranda GS and Yap Wah Koon T 1995. Formulation and optimization of two culture media for the production of tumor necrosis factor-b in *Escherichia coli*. *J. Chem. Tech. Biotechnol.,* 62**:**289-294

Khuri AI and Cornell JA. 1987. Response surfaces: design and analysis. Marcel Dekker, New York.

Mayer AM. 1987. Polyphenol oxidases in plants-recent progress. *Phytochem.,* 26:11–20.

Nyanhongo GS, Gomes J, Gubitz G, Zvauya R, Read JS and Steiner W. 2002. Production of laccase by a newly isolated strain of *Trametes modesta*. *Biores. Technol.,* 42: 89–94.

Pandey AK, Reddy MS and Suryanarayanan TS. 2003. ITS-EFLP and ITS sequence analysis of a foliar endophytic *Phyllostricta* from different tropical trees. *Mycol Res.,* 107:439-444

Revankar MS and Lele SS. 2006. Enhanced production of laccase using a new isolate of white rot fungus WR-1. *Process Biochem* 41:581-588

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

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

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