RESPONSE SURFACE METHODOLOGY FOR THE OPTIMIZED PRODUCTION OF AN ALKALOPHILIC LACCASE FROM γ-PROTEOBACTERIUM JB

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γ-Proteobacterium JB, an alkali-tolerant soil isolate, produced laccase ($8X10^3$ nkat/L) in M162 medium. The optimization of process conditions (pH, incubation time, agitation, and $CuSO_4$ concentration) for laccase production during submerged fermentation was carried out using response surface methodology (RSM) based on a central composite design (CCD). Maximum laccase production achieved was $7.4 X 10^4$ nkat/L at pH 8.0, 210 rpm, 100 µM of $CuSO_4$ after 60 h of incubation. This design of experiment methodology increased laccase production by 9.3 fold over the control. Experimental findings were in close agreement with the model predictions.

Keywords: Alkalophilic laccase; Production; RSM; γ-proteobacterium JB

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INTRODUCTION

Laccases (benzenediol oxygen oxidoreductases, EC 1.10.3.2) are polyphenol oxidases that require molecular oxygen to oxidize phenols, polyphenols, aromatic amines and different non-phenolic substrates by one-electron transfer, resulting in the formation of reactive radicals. They are members of the multicopper protein family that has developed from small sized prokaryotic azurins to eukaryotic plasma proteins. Laccases are widely distributed in plants and fungi, where they are involved in melanin formation. Their use in a variety of different physiological functions, such as fungal morphogenesis, plant pathogenesis, and fungal virulence has been frequently proposed. They also occur in prokaryotes viz. *Azospirillum lipoferum* , *Marinomonas mediterranea*, *Bacillus subtilis* spore and γ-proteobacterium JB (Bains et al. 2003). A laccase-type phenol oxidase activity has also been detected in *Streptomyces galbus* and multicopper oxidase in *Escherichia coli*. Laccase production in fungi with respect to culture medium composition has been widely reported (Palmer et al. 2001; Xiao et al. 2004). Very few workers have investigated laccase production in bacteria (Sharma et al. 2007), although this system offers many advantage over the fungal system. The faster multiplication rates result in early enzyme production. Laccase is a potentially important industrial enzyme that can be applied extensively in many fields which include waste detoxification and textile dye transformation, delignification of lignocellulosic material and cross linking of polysaccharides, upgrading of wine quality and removal of fermentation inhibitors to increase the yield of ethanol, improvement of drug analysis as well as construction of

new energy-producing devices and enzyme sensors (Couto and Herrera 2006). Laccase from γ-proteobacterium JB decolorized indigo carmine dye maximally at pH 9 in the presence of syringaldehyde (Singh et al. 2007) and delignified wheat straw rich soda pulp at pH 8 in the presence of ABTS (Singh et al. 2008). Its action was similar to other fungal and bacterial laccases which perform in the presence of mediators like ABTS, syringaldehyde and HBT, which increase their substrate specificity and spectrum for industrial applications. However lower yields of laccase in bacteria are not sufficient for successful commercial applications. So the present work was taken up to enhance the bacterial laccase production. Also, there are no reports available on laccase production from bacteria by employing statistical experimental design. It is well known that laccase production in fungi and bacteria is greatly influenced by a myriad of factors such as media components (Palmer et al. 2001; Xiao et al. 2004), metal ions (Malhotra et al. 2004) organic molecules and aromatic-phenolic compounds (Sheel et al. 2000).

Response surface methodology (RSM) is widely applied in the optimization of various industrially important microbiological, biochemical, and biotechnological products such as chemicals and enzymes. Based on the principle of design of experiment (DoE), the methodology encompasses the use of various types of experimental designs, generation of polynomial equations, and mapping of the response over the experimental domain to determine the optimum product (Box and Draper 1987). The technique requires minimum experimentation and time, thus proving to be far more effective than the conventional methods of developing such products. The present work is aimed at statistically optimizing four variables *viz.,* pH, time, agitation, and $CuSO_4$ for enhanced production of alkalophilic laccase by γ-proteobacterium JB.

EXPERIMENTAL

Microorganism and Growth Conditions

γ-Proteobacterium JB, isolated and identified previously in our laboratory (Bains et al. 2003) was maintained as a suspension in 20% glycerol at -70°C and was routinely cultured on M162 medium (g/L): $CaSO_4.2H_2O$, 0.4; $MgCl_2.6H_2O$, 2.0; nitrilotriacetic acid, 1.0; 0.01M ferric citrate solution, 5 mL; micronutrient solution (g/L: H_2SO_4, 0.5 mL; $MnSO_4H_2O$, 2.28; $ZnSO_47.H_2O$, 0.5; H_3BO_3, 0.5; $CuSO_4.5H_2O$, 0.025; $Na_2MO_4.2H_2O$, 0.025; $CoCl_26H_2O$, 0.045), 10 mL; yeast extract, 3 and tryptone, 3 (Degryse et al. 1978). One mL of overnight culture was used to inoculate 100 mL of M162 medium and incubated at 37°C and 150 rpm for 24 h. The culture supernatant was obtained by centrifugation at 10,000 x *g*, 4°C for 10 min and was used as crude extracellular enzyme.

Enzyme Assay

Laccase activity was determined using 2 mM guaiacol as substrate, at 55°C in 0.1 M phosphate buffer (pH 6.5). The change in absorbance due to oxidation of guaiacol in the reaction mixture was monitored at 465 nm (ε = 48,000 M^{-1} cm^{-1}) for 15 min of incubation. Enzyme units were expressed in nkat (nmoles of substrate converted sec^{-1}). Protease activity was determined as described by (Dutta et al. (2004).

Experimental Design

Second-order experimental design, *i.e.,* Central Composite Design (CCD) with four factors at five levels was employed to investigate the first- and higher-order main effects of each factor and interactions amongst them. The design involved 16 factorial, 8 star and 6 center design points with α value being ± 2 . The five coded levels investigated in the current study are - 2, -1,0,+1 and + 2. The statistical software package Design Expert® version 7.0 (Stat ease, Inc, Minneapolis, USA) was used to generate polynomials and the contour plots. All experiments were carried out in triplicate. For a four-factor system the model equation generated was

$$1/Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_4 D + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{44} D^2 + \beta_{12} AB + \beta_{13} AC + \beta_{14} AD + \beta_{23} BC + \beta_{24} BD + \beta_{34} CD + \beta_{123} ABC + \beta_{124} ABD + \beta_{134} ACD + \beta_{234} BCD + \beta_{112} A^2 B + \beta_{113} A^2 C + \beta_{124} A^2 D + \beta_{122} AB^2 ,$$

where Y is the predicted response *i.e*. laccase production (nkat/L); β_0 is the intercept; β_1, β_2, β_3, and β_4 are the linear coefficients of factors A,B,C,D respectively; β_{11}, β_{22}, β_{33}, and β_{44} are the respective quadratic coefficients; β_{12}, β_{13}, β_{14}, β_{23}, β_{24}, and β_{34} are the second-order interaction coefficients; and $\beta_{123} + \beta_{124} + \beta_{134} + \beta_{234} + \beta_{112} + \beta_{113} + \beta_{124} + \beta_{122}$ are the higher-order interaction coefficients.

Model Validation

The mathematical model generated during RSM implementation was validated by conducting three check point studies. The experimentally obtained data were compared with the predicted data, and the prediction error was calculated.

RESULTS

γ-Proteobacterium JB is a Gram-negative, non-sporulating, non-hemolytic, short rod occurring singly. It did not grow on McConkey and cetrimide agar and was oxidase positive but catalase, indole, methyl red, Voges-Proskauer, and citrate negative. The glucose reaction was positive with no production of gas and acid. The 16S rDNA sequencing of γ-proteobacterium JB was carried out previously in our laboratory (Bains et al. 2003). The BLASTn algorithm downloaded from GenBank database (http://www.ncbi.nlm.nih/BLAST) exhibited 98% identity with the closest match γ-proteobacterium F8, so the present organism was named γ-proteobacterium JB.

Experimental Design

Preliminary studies with several variables (Table 1) using OVAT (one-variable-at-a-time) method revealed several of them to be important, and pH, time of incubation, agitation and concentration of $CuSO_4$ were found to be highly influential in regulating the production of laccase.

Table 1. Parameters Examined for Conventional (one variable at a time, OVAT) Optimization of Laccase Production by γ-Proteobacterium JB

Parameter (range)	Optimum
• pH (6-9)	7.0
• Metal salts (10-100 µM): $CuSO_4$,$LiCl_2$,$MnCl_2$,$CaCl_2$	$CuSO_4$ (100 µM)
• Aeration (0-200 rpm)	150 rpm
• Incubation time (12-72 h)	48 h
• Carbon source (0.5-1%): Glucose,Xylose, Maltose, Sucrose	None
• Pesticides (0-100 µg/ml)	Atrazine (100 µg/ml)
• Inducers (10-100 µM)	
*Dyes	Ethidiumbromide (10 µM)
*Aromatic compounds	*p*-Toluidine (10 µM)
*Substrates	Anthracine (10 µM)
• Temperature (15-40ºC)	37ºC
• Inoculum size (1-5 %)	1%

RSM was subsequently applied on these factors for response surface mapping and the optimization of enzyme production.

Prior to the RSM study, various parameters were optimized by conventional methodology (Table 1). Maximum production of laccase was approx. $8x10^3$ nkat/L by applying the conventional way of optimization at pH 7.0, temperature 37ºC and 150 rpm. The bacterium attained maximum growth after 12 h of incubation and its lysis started thereafter. The pH of the medium showed a significant role in laccase production by γ-proteobacterium JB. The organism produced maximum laccase only when pH shifted from initial 7 to ~8 at 48 h of growth in M162 medium (Bains et al. 2003)

In case of optimization by the RSM application pH, $CuSO_4$ agitation and time of incubation were found to be the highly influential variables identified for the production of laccase. Thereafter, CCD was employed to investigate the effect of these four factors to improve laccase production. Real factor values for the coded -2, -1, 0, +1, and +2 levels are presented in Table 2.

Table 2. Experimental Range and Levels of Independent Variables in Terms of Coded Factors

Variable	Range and Levels				
	-2	-1	0	+1	+2
pH	6	7	8	9	10
Time (h)	20	40	60	80	100
Agitation (rpm)	90	120	150	180	210
$CuSO_4$ (µM)	0	50	100	150	200

The results of first-order factorial design points in the experimental domain showed that all the four independent variables had significant effects individually on laccase production. Amongst the 30 experimental runs, maximum laccase production was $7.4X10^4$ nkat/L at pH 8.0, agitation 210 rpm, 100 μM concentration of $CuSO_4$ after 60 h of incubation time (Table 3).

Table 3. Design Layout as per CCD along with Actual and Coded Levels of Four Factors

Run	Factors				Response (Y)
	pH	Time (h)	Agitation (rpm)	$CuSO_4$ (μM)	Laccase production (nkat/L) $x10^3$
1	7	40	180	50	29.7
2	7	40	120	150	3.8
3	7	40	120	50	4.5
4	8	60	150	100	38.5
5	9	80	180	50	19.6
6	9	80	180	150	18.7
7	9	80	120	150	18.9
8	8	60	90	100	46.2
9	8	60	150	100	38.5
10	9	80	120	50	18.4
11	6	60	150	100	1
12	9	40	180	50	24.2
13	7	80	120	150	8.8
14	7	80	180	150	22.1
15	8	100	150	100	22.1
16	8	60	210	100	74
17	7	40	180	150	27.6
18	9	40	120	50	23.3
19	7	80	120	50	7.2
20	8	60	150	100	38.5
21	8	60	150	100	38.5
22	9	40	120	150	36.3
23	8	20	150	100	37.3
24	8	60	150	100	38.5
25	10	60	150	100	43.6
26	9	40	180	150	22.8
27	7	80	180	50	18.8
28	8	60	150	0	8.3
29	8	60	150	200	51.9
30	8	60	150	100	38.5

The response Y was the mean values of at least three experiments, with ±1.3-5.3 % standard deviation range.

Contour plots were drawn for the production as a function of concentrations of two variables while keeping the other two factors constant at their central levels (Fig. 1, a-f). The optimum concentration of $CuSO_4$, pH, agitation, and time were identified based on numerical optimization (undesirability function). Overall increase of ~ 9.3 fold in laccase production was observed when all the factors were investigated together by RSM. Experimental findings were in close agreement with the model predictions. The regression equation obtained after ANOVA indicated R^2 value of 0.99, which is in reasonable agreement with the adjusted R^2 of 0.98. This ensured a satisfactory adjustment of higher order polynomial model to the experimental data. The predicted sum of squares (PRESS), which is a measure of how a particular model fits each point in the design, was 0.42. An adequate precision value of 49.7 obtained by ANOVA (Table 4) indicates an adequate signal-to-noise ratio (S/N > 4 is desirable). This model can therefore be used to navigate the design space. Table 5 shows the results obtained from the three check-point studies. The observed and the predicted values were quite close and therefore show good predictability of the model.

Table 4. Analysis of Variance (ANOVA) Table for Response Surface

Standard deviation	0.020
Mean	0.25
R^2	0.99
Adjusted R^2	0.98
Predicted R^2	0.5
PRESS	0.42
Adequate precision	49.7

Table 5. Comparison of Predicted and Observed Values of Response Variables Obtained during Model Validation

pH	Time (h)	$CuSO_4$ (µM)	Agitation (rpm)	Laccase production (nkat/L x 10^3)	
				predicted	obtained
8	60	100	150	38	42
8	60	100	200	71	77
7.5	21.5	120	180	75	71

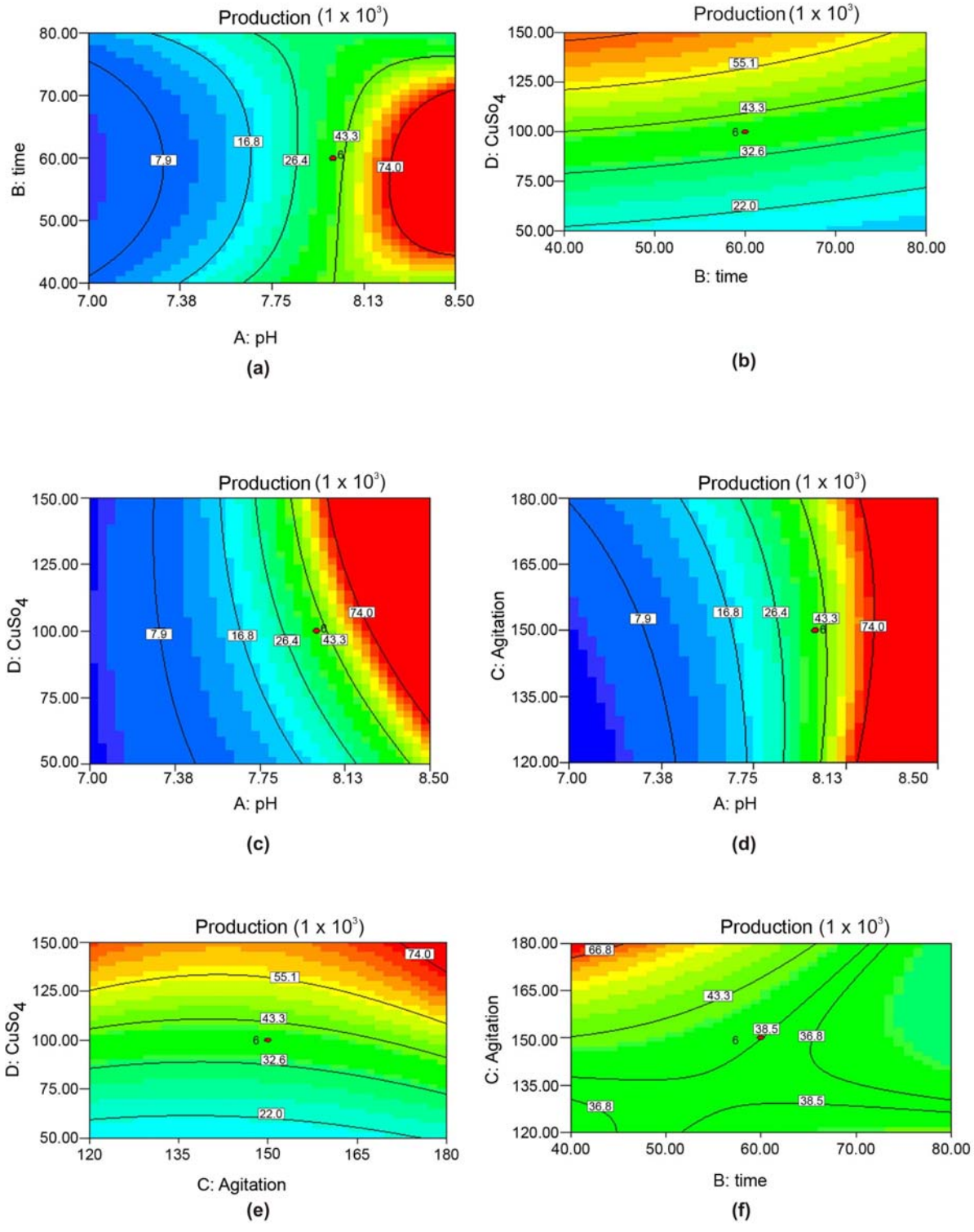

Fig.1 (a-f). Contour plot of laccase production (nkat), effect of different variables: a, time (h) and pH ; b, $CuSO_4$ concentration (μM) and time ; c, $CuSO_4$ and pH ; d, agitation (rpm) and pH; e, $CuSO_4$ and agitation ; f, agitation and time, and their mutual interaction on enzyme production

In order to investigate whether these culture conditions (pH, time, agitation, and $CuSO_4$ concentration) influenced the synthesis of new laccase isozymes, a native PAGE was carried out. Protein bands exhibiting laccase activity were stained red with guaiacol (2 mM) in 50 mM phosphate buffer, (pH 6.5). Native PAGE analysis of γ-proteobacterium JB culture media revealed only one band in all the 30 experiments (data not shown), which suggests that the effect on laccase production was not due to new isozymes expression. Also γ-proteobacterium JB did not produce proteases.

DISCUSSION

Results of this study show that culture conditions can increase laccase production in γ-proteobacterium JB. Otherwise enhanced laccase production in fungi and bacteria occurs only by mutagenic and harmful aromatic compounds e.g. ethidium bromide, malachite green, pesticides *etc*. (Dhawan et al. 2003; Malhotra et al. 2004). Arockiasamy et al. (2008) applied Plackett and Burman design criterion and central composite design for enhanced (7.6 fold) production of laccase from *Coriolus versicolor* NCIM996 in 9 days. But in the present study, the increase (9.3 fold) in laccase production was achieved in just 60 h. The high specific activity ($1.414x10^6$ nkat or $8.4x10^4$ μmol min^{-1} mg^{-1} protein) of purified γ-proteobacterium laccase (Singh et al., 2007) makes it distinct from other fungal and bacterial laccases. *Streptomyces cyaneus* laccase had very low (6.3 U mg^{-1} protein) specific activity in comparison to the total (51.6 U) activity (Arias et al. 2003). *Cerrena unicolor* laccase too showed low (10.6 nkat mg^{-1} protein) specific activity in comparison to the total (83.7 U) activity (Seok et al. 2008). γ-proteobacterium JB produced laccase only in less enriched media (M162 optimized) but not in highly enriched Luria or nutrient broth or glucose media, despite confluent cell growth (data not shown).

Induction of laccase seems to be greatly affected by culture conditions. Copper regulated *lcc* transcription in *T. versicolor*. As the copper concentration in growth medium increased, an enhanced level of *lcc* mRNA transcripts with concomitant increase in laccase production (Reinhammar et al. 1984) was observed. Froehner and Eriksson (1974) also observed decreased laccase production by the ascomycete *Neurospora crassa* when copper was removed from its growth medium. Addition of inducers reduced cell growth and enhanced laccase production in γ - proteobacterium JB (Malhotra et al. 2004). Induction of ligninolytic enzymes' expression and their increased activity have been reported for copper , veratryl alcohol and a phenolic mixture (Dekker et al. 2002).

In the present work aeration, time, and pH have also played an important role in enhanced laccase production. The ascomycete, *Botryosphaeria* sp. produced two extracellular constitutive laccases (PPO-I and PPO-II). Aeration of the cultures increased the production of both enzymes 4-5 fold in the presence of veratryl alcohol (Dekker et al. 2002). Response surface analysis showed that pH in the range 3.0-5.0 significantly enhanced the laccase production by *T. versicolor* (Tavares et al. 2006). γ-Proteobacterium JB was able to grow and produce laccase under alkaline (7-10 pH) conditions (Bains et al. 2003). The enzyme showed different pH optima for different substrates and was 100, 60, and 49% stable at pH 9.0 (Tris–HCl, 0.1 M), 10.6 (glycine–NaOH, 0.1 M) and 4.0

(citrate, 0.1 M), respectively after 60 days at 4 ºC. Stability of the enzyme can be because the organism did not produce proteases (Singh et al. 2007). Our previous work has shown that laccase has good potential for new environmental friendly technologies for denim bleaching and pulp and paper industry. Commercial application, however, requires its production in high yields.

CONCLUSION

This work is the first report on optimization of alkalophilic laccase production from a prokaryotic source by application of RSM. Enhanced production of laccase by using optimized factors will help in various biotechnological applications at industrial levels, e.g. biobleaching of pulp and denim (Singh et al. 2008). Attempts to clone this laccase gene in order to understand the regulatory control of production in γ-proteobacterium JB are being carried out.

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