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Production of laccase by Resinicium bicolor in submerged cultures: application of the Plackett-Burman experimental design to screen major factors

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Abstract—The present work was aimed towards the evaluation of effects of the physical and chemical factors on laccase production by white rot fungus, Resinicium bicolor ATCC 64897 under submerged condition in shake flask using Plackett-Burman design. R.bicolor potentially detoxifies ground tire rubber (GTR) and facilitates growth of Thiobacillus ferrooxidans, a bacterium that performs devulcanization of GTR but is sensitive towards rubber additives present in GTR. In this experiment out of 11 factors screened, copper sulphate and yellow flame bark powder were found to be the most significant factors affecting the laccase production. The study indicated that the screened factors can be optimized for enhanced laccase production and can potentially be utilized for attaining maximum detoxification of GTR and hence further devulcanization.

Keywords— white-rot fungus, Resinicium bicolor, laccase, inducers

I. Introduction

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belong to the copper metalloenzymes and to the blue oxidase sub-group that catalyzes reduction of molecular oxygen to water (1,2,3,4). White rot fungi (basidiomycetes) have a natural mechanism to decompose lignocellulosic materials and xenobiotics (5) with the help of their extracellular enzymes viz., lignin peroxidase, manganese peroxidase, versatile peroxidases, and laccases which allows them to decompose

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organic matter esp. lignin and wood components and xenobiotic compounds (6,7,8). The production of laccase under submerged condition and the involvement of lignocellulosic materials and inducers in enhancing laccase production has been widely documented (9,10,11, 12,13,14,15,16,17). The ability of white rot fungus in synthetic dye decolorization and degradation of organo-pollutants and recalcitrant compounds have been extensively studied (18,19, 20, 21,22,23,24,25).

The devulcanization potential of Thiobacillus ferrooxidans has been well proven by several research groups (26,27,28,29) but in most cases T. ferroxidans was found sensitive towards additives like accelerants, stabilizers etc added during rubber vulcanization process (30,31). In a study it was demonstrated that the microbial toxicity towards rubber constituents have profound effect on devulcanization process whereby a white rot fungus Resinicium bicolor has been proven to be very effective in detoxifying ground tyre rubber under submerged culture condition with its extracellur ligninolytic enzymes (32). Thus the objective of this study was to screen out the major factors affecting laccase production in R. bicolor under submerged culture using plackett-burman design (PBD). Thus, this work is an initiative towards the detoxification of ground tire rubber using the laccase mediator system (LMS) of R. bicolour to facilitate the devulcanization process by bacteria T. ferroxidans.

II Materials and Methods

A. Fungal strain

The fungus Resinicium bicolor strain 64897 used in this study was obtained from ATCC (American Type Culture Collection, Manassas, USA). Stock cultures were grown at room temperature (26-28ºC) for 3-4 days, maintained on malt agar (ATCC® Medium 323) plates and stored at 4°C with subsequent transfers every 4-5 weeks.

B. Substrate

The lignocellulosic substrate consisted Cassava peel, Yellow flower tree bark and Rubber tree bark. These were crushed in a mechanical crusher (HITOP-SY-20) and ground to 0.5 mm in a rotomill- Pulverisette 14 (Fritsch, Germany) and sieved through 200 mesh to get powder with uniform particle size to be used as carbon source in fermentation medium.

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C. Chemicals

p-Coumaric acid, 1-Hydroxybenzotriazole, Syringaldazine and 2,5-Xylidine of analytical grade were purchased from Sigma-Aldrich (St. Louis, MO). Copper sulphate and buffer salts were from HmbG (Germany). Malt extract (LP0039) was from Oxoid (England) and malt extract broth was from Merck Darmastadt (Germany). Bacto^{TM} Agar was from Difco (USA).

D. Fermentation medium

The malt extract broth 1.7% (w/v) was used both as nitrogen source and fermentation medium. The fermentation medium was supplemented by inducers and substrates in the concentration as per values from plackett-burman design (PBD) and was autoclaved at 1.5 atmosphere and 121ºC for 15 minutes.

E. Inoculum preparation and submerged cultivation

Inocula were prepared as spore suspensions by scraping a 4 days old malt agar slant with 20 ml sterile distilled water. Each 250 ml shaker flask containing 100 ml culture medium received 10 ml of this inoculum and pH was adjusted using 1M HCl. All incubations were performed in an incubator shaker in the dark with three replicates under conditions according to PBD.

F. Plate assay

To determine the presence of extracellular laccase, a plate assay using method of (33) was performed. The petri plates containing 20 ml of 4.5% malt agar was supplemented with 0.08% (w/v) of bromophenol blue. The fungal discs of 7 mm were incised from the periphery of actively growing plate of R. bicolor and were acseptically transferred to plates containing bromophenol blue. The plates were incubated for 1 day at room temperature. The formation of clear halo around mycelia was considered to be a positive test for extracellular laccase.

G. Estimation of laccase activity

Laccase assay was performed by using the method of (34). The reaction mixture contained 0.3 ml of 0.216 mM syringaldazine, 2.2 ml of 100 mM citrate- phosphate buffer; pH 6.5 (35) and 0.5 ml of crude enzyme sample obtained by centrifuging culture broth at 10,000 rpm for 10 minutes. Oxidation of syringaldazine was followed by increase in absorbance at 530 nm. Enzyme activity was expressed in units/ml (U/ml). One unit produced a ΔA_{530} of 0.001 per minute at pH 6.5 at 30ºC in a 3 ml reaction volume using syringaldazine as substrate. The change in absorbance was recorded by a spectrophotometer (ViVa spec. LS, Sartorius stedim, Germany).

H. Screening using Plackett-Burman design

The Plackett-Burman method is an effective two-level factorial design, which identifies the crucial factors required for enhanced enzyme production by screening N variables in N+1 experiments (36) whereby interaction between various factors is not taken into account. The screened variables (factors) that are significant and influential can further be optimized using response surface methodology. To determine the major factors significantly influencing the laccase activity in Resinicium bicolour strain ATCC 64897, a set of 12 runs were conducted using 11 independent variables or factors that included 8 chemical factors and 3 physical factors wherein each factor was performed at two levels i.e; low and high as shown in table I. Each run was performed in triplicate and mean value and standard deviation (Mean ± S.D) of response (laccase activity) was measured.

The complete experimental design with different combination of factors in each run has been depicted in table II.

TABLE I. LEVELS OF THE VARIABLES IN PLACKETT-BURMAN DESIGN

Variables	**Level**		
	Factors	***Low***	***High***
Chemical factor (X_1)	Copper sulphate (mM)	0	5
Chemical factor (X_2)	p-Coumaric Acid (mM)	0	1
Chemical factor (X_3)	1-Hydroxybenzotriazole (mM)	0	1
Chemical factor (X_4)	2,5-Xylidine (mM)	0	1
Chemical factor (X_5)	Temperature (ºC)	24	28
Chemical factor (X_6)	Cassava peel powder (g)	1	5
Chemical factor (X_7)	Yellow flower tree bark powder (g)	1	5
Chemical factor (X_8)	Rubber tree bark powder (g)	1	5
Physical factor (X_9)	Agitation (rpm)	140	180
Physical factor (X_{10})	pH	2.5	5.5
Physical factor (X_{11})	Incubation period (days)	1	4

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TABLE II. PLACKETT-BURMAN DESIGN FOR LACCASE PRODUCTION

Run	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}
1	5	0	0	0	28	5	5	1	180	5.5	1
2	0	0	1	1	28	1	5	5	140	5.5	1
3	0	0	0	0	24	1	1	1	140	2.5	1
4	0	1	0	0	24	5	5	5	140	5.5	4
5	5	0	1	0	24	1	5	5	180	2.5	4
6	5	1	0	1	28	1	5	1	140	2.5	4
7	5	1	1	0	28	5	1	5	140	2.5	1
8	0	1	1	1	24	5	5	1	180	2.5	1
9	5	1	0	1	24	1	1	5	180	5.5	1
10	5	0	1	1	24	5	1	1	140	5.5	4
11	0	0	0	1	28	5	1	5	180	2.5	4
12	0	1	1	0	28	1	1	1	180	5.5	4

III Results and Discussion

A. Plate assay

Plate assay is rapid technique of identifying the enzyme of concern in a particular strain and is reported by several groups using substrates like syringaldazine, guaiacol and 2, 2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (37,38,39,40). The strain *R. bicolour* gave positive reaction in plate assay using the azo dye, bromophenol blue as substrate for extracellular laccase which was visualized by the appearance of halo around mycelia as shown in figure 1. The halo appeared after 24 hours of incubation in the absence of any mediators which shows that the laccase secreted by *R. bicolour* can mediate the decolorization of synthetic dyes even in absence of any mediator in a short period as decolorization of azo dyes were performed in the presence of mediators (41).

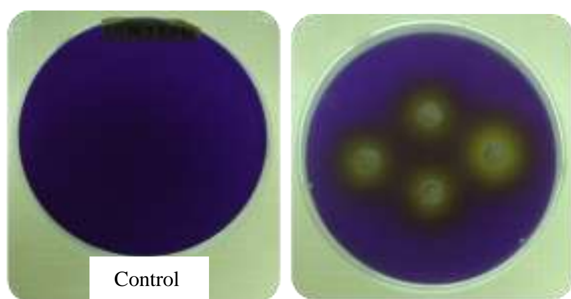

Figure 1. Plate assay method for laccase test showed the yellowish halo developed by *R. bicolour* using bromophenol blue as substrate after 24 hours of incubation.

B. Screening of factors using plackett-burman design

To screen out the major factors responsible for increased laccase production, 11 factors comprising chemical inducers, lignocellulosic materials, agitation, pH and incubation period were compared at two levels. The results of screening experiment has been presented in figure 2. On the basis of these results, the main effect of each factor on laccase activity was calculated using (1). The contribution of different factors towards main effect has been depicted in figure 3. A broad variation in the laccase activity ranging from 0.53 to 6.8 (U/ml) was found. This variation is an indication for optimization of screened factors for enhanced laccase productivity.

$$\text{Main effect} = \frac{\Sigma\,\text{Response value from high level} - \Sigma\,\text{Response value from low level}}{\Sigma\,\text{Number of runs}}$$

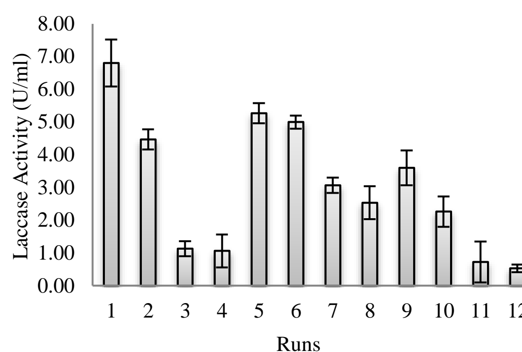

Figure 2. Laccase activity as response for plackett-burman screening.

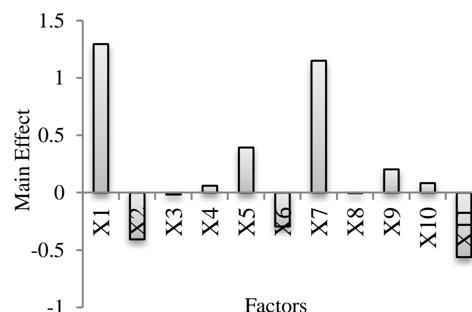

Figure 3. Different factors and their contribution towards main effect.

As from figure 3, it is clear that the activity of laccase in case of *R. bicolor* is primarily influenced by three major factors which were copper sulphate (X_1), yellow flame bark (X_7) and incubation period (X_{11}). 2,5-xylidine (X_4) and pH (X_{10}) have little positive effect on laccase activity and p-coumaric acid (X_2) and cassava peel (X_6) gave negative effect on laccase activity while effect of 1-hydroxybenzotriazole (X_3) and rubber bark powder (X_8) were negligible. Thus X_1, X_5, X_7, X_9 and X_{11} were selected for analysis. The analysis of variance of experimental design showed that X_1, X_7 and X_{11} are significant model terms (table III). The final regression equation developed by placket-burman design in terms of coded factors was as follows:

$$R_1 = 3.04+1.30X_1+0.39X_5+1.15X_7+0.20X_9-0.56X_{11} \quad (2)$$

Where 'R_1' is the laccase enzyme activity (U/ml) and X_1, X_5, X_7, X_9 and X_{11} are coded factors.

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TABLE III. Analysis of variance (ANOVA) for selected factorial model.

Source	Sum of Squares	DF	Mean Square	F Value	Prob > F	
Model	42.18	5	8.44	16.09	0.0020	significant
X_1	20.13	1	20.13	38.41	0.0008	significant
X_5	1.86	1	1.86	3.55	0.1087	
X_7	15.89	1	15.89	30.32	0.0015	significant
X_9	0.50	1	0.50	0.96	0.3652	
X_{11}	3.79	1	3.79	7.23	0.0361	significant
Residual	3.15	6	0.52			
Cor Total	45.32	11				
Std. Dev.	0.72				R^2	0.9306
Mean	3.04				Adj R^2	0.8728
C.V.	23.83				Pred R^2	0.7224
PRESS	12.58				Adeq Precision	11.8932

The Model F-value of 16.09 implied that the model is significant. Values of "Prob>F" and less than 0.05 indicated that model terms are significant. In this case copper sulphate (X_1) , yellow flame bark powder (X_7) and incubation period (X_{11}) were considered as significant model terms (factors) with confidence level greater than 95% ($P<0.05$). The "Predicted R-Squared" of 0.7224 is in reasonable agreement with the "Adjusted R-Squared" of 0.8728. The coefficient of determination (R^2) of the model was calculated to be 0.9306, which indicated that 93.06% of the variability in the response could be explained by the model.

C. Copper/lignocellulose synergistic induction of laccase

The inducers employed in this experiment consisted of copper sulphate, p-coumaric acid, 1-hydroxybenzotriazole and 2,5-xylidine whereas the lignocellulosic materials used in the current study were: cassava peel powder, yellow flame tree bark powder and rubber tree bark powder. Out of these two categories, the copper sulphate and yellow flame bark powder was found to be significant in enhancing the laccase activity. The highest laccase activity was 6.80 U/ml. The increment in the laccase production using lignocellulosic materials and copper sulphate as inducer has already been established by several research groups including (42, 43). To our knowledge this is the first attempt to determine the effect of Yellow flame bark powder as lignocellulosic material for laccase production in strain *R. bicolor*.

The lignocellulosic material being as rich carbohydrate source increases laccase activity (44) and the major portion of total phenolic content of yellow flame bark consist of tannins (45) especially tannic acid which has been shown to act as a good substrate as well as an inducer for laccase production in different white rot fungi (46, 47).

D. Incubation period and its effect on laccase production

The strain exhibited laccase activity at the end of first day of incubation. Thus, it can be said that availability of fresh culture medium supplemented with lignocellulosic substrates and inducers under agitation condition supported the growth and metabolic reaction in fungal species which resulted in laccase production.

Conclusion

The white rot fungal strain *R. bicolor* has the ability to produce laccase under submerged culture condition using cheap substrates and inducers. The placket-burman screening showed three significant factors affecting laccase production viz., copper sulphate, yellow flame bark powder and incubation period which can be further optimized to get maximum laccase production and can be utilized for optimum detoxification ground tire rubber. Till date there is no data available for the detection of laccase in *R. bicolor*.

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