OPTIMIZATION OF LACCASE PRODUCTION FROM *BACILLUS SP*. MSK-01 USING SWEET LIME PEELS AS AN EFFECTIVE SUBSTRATE

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Abstract

Laccases are multicopper oxidases containing four copper atoms per monomer distributed in three redox sites. Laccases are miraculous enzymes as they are found uses in a number of applications including biobleaching, xenobiotic bioremediation, dye synthesis, bread baking and even in reducing the cell growth in cancer. Because of its tremendous applications, research on laccase is growing in past few years with the discovery of novel laccase having wide range of applications. Besides being applied in industry, the yield of laccase is also an important issue for its applicability in the industry as there is a great demand of low cost high value proteins in market. In the present study, the extracellular laccase from *Bacillus sp.* MSK-01 was produced in large quantity by solid state fermentation using sweet lime peels as substrate laccase activity were found to increase from 120 IUg^{-1} to 687 IUg^{-1} with sweet lime peels under optimized conditions which lead to 3.51 fold increase in the laccase yield. The result of this study is highly useful in increasing the amount of laccase using no cost substrate sweet lime peels.

Keywords: *Bacillus* sp. MSK-01, laccase, solid state fermentation, Sweet lime peels, response surface methodology

I. INTRODUCTION

Laccases (benzenediol: oxygen oxidoreductases; EC 1.10.3.2) are multicopper oxidases (MCOs) which catalyze the oxidation of a wide variety of organic and inorganic compounds with concomitant four electron reduction of molecular oxygen to water. They catalyze the oxidation of both phenolic and non-phenolic substrates [1]. Laccases are very useful enzymes with respect to their applications in industry. They have found uses in biotechnological applications such as biobleaching, xenobiotics bioremediation, decolourization of textile dyes, biosensors, food industry, plastic degradation etc. [2]. Besides stability of an enzyme at industrial conditions, major obstacle in the path of the actual use of an enzyme at industrial level is its production cost. The development of procedures for the production of highly efficient, environment friendly and cost effective enzyme is a primary approach [3]. Solid state fermentation (SSF) is an cost effective method for increasing the enzyme production and is defined as any fermentation process occurring in the absence or near absence of free liquid, using an inert or a natural substrate as a solid support [4]. Although most of the times SSF has been observed to be successful for filamentous organisms such as fungi, actinomycetes etc. [4, 5] but recently there are reports where SSF has been found to be useful for the production of enzymes from the bacteria also [6, 7].

However, there are very few reports on the production of bacterial laccases by SSF [8]. This is probably because bacterial laccases reported so far are mostly intracellular or spore bound [1]. Recently, it has been found that laccases are also secreted extracellularly by bacteria [9]. The searches to isolate new bacteria for laccases that meet up the requirement of the industries are still on. In this regard, in the present study, a new *Bacillus* sp. MSK-01 producing laccase was isolated and laccase production has been optimized through SSF using statistical software's i.e. Plackett-Burman (PB) and Response Surface Methodology (RSM). RSM is a useful tool for increasing the enzyme yield as it involves minimum number of experiments and thus is time saving approach [5].

As laccases have many important roles to play industrially, the analysis of cost of MSK-01 laccase production is an important feature for its industrial exploitation. Therefore, in this paper the cost of producing MSK-01 laccase from *Bacillus sp.* MSK-01 was also studied in detail.

II. MATERIALS AND METHODS

MATERIALS

Organism

The organism *Bacillus* species used in this study was previously isolated in our lab.

Substrates and chemicals
Guaiacol was taken from Sigma Aldrich (USA). All other routine chemicals and media components were of analytical grade from HI Media (India) and Sisco Research Laboratory (SRL) India.
Sweet lime peels, pineapple peels, pomegranate peels and orange peels were taken from local juice vendors in Chandigarh, India.
Instruments, Glassware and Plastic ware
Borosil and Schott Duran glassware was used throughout the study, which were washed twice with the detergent with final rinsingin distilled water. It was then dried in hot air oven at 70°C and then used.

Methods

A. *Chemicals*

Guaiacol used in the present study was purchased from Sigma (USA). Other chemicals were obtained from Hi-media (India) and were of an analytical grade. Fruit juice waste was obtained from a local vendor of fruit juices.

B. *Laccase assay*

Laccase assay was performed at 75ºC for 10 min in 0.1 M Tris-HCl buffer (pH 8.0) using guaiacol (2 mM) as substrate. The change in absorbance due to the oxidation of guaiacol was monitored at 465 nm (ε = 12,000 M^{-1} cm^{-1}) in a UV–Visible spectrophotometer. The enzyme unit was expressed in IU g^{-1}. One unit of laccase (IU g^{-1}) was defined as the amount of the enzyme required to transform 1 µmol substrate per min per gram of substrate.

C. *Preparation of substrate for the production of laccase*

Sweet lime peel was taken from local juice vendors and dried in oven at 55°C for 48h. The dried peels were ground in the grinder and sieved through 0.5 mm mesh size sieve to obtain fine particles of equal size. 5 g of each substrate was autoclaved and inoculate with 10% of overnight grown culture of *Bacillus sp.* MSK-01. The medium was moistened with 1x M162, 500 µM $CuSO_4.5H_2O$ and 0.1% Tween 80. Moisture content of 1:1 was maintained by using 0.1 M Tris-HCl buffer pH 8.0. The flask was thoroughly shaken to mix the contents appropriately and incubated at 37°C for 48 h at steady state. The flask was shaken after specific interval of time each day for even growth of culture.

D. *Extraction of enzyme*

After 48 h, 25 ml of 0.1M Tris-HCl buffer (pH 8.0) was added to the flask for the extraction of enzymes. The flask was kept at 150 rpm for 15 min for proper extraction of enzymes. The entire content was sieved through muslin cloth and the filtrate was centrifuged at 10,000 rpm for 10 min. The supernatant was diluted accordingly and laccase activity was assayed.

E. *Standardization of laccase production in Solid State fermentation using Sweet Lime peels as substrate*

Plackett-Burman design

On the basis of the available literature, the variables that significantly influence the laccase production were screened using Plackett Burman (PB) design of Design Expert 10.0.3 (Stat-Ease, Inc., Minneapolis, USA). The parameters evaluated were as follows:

A- pH, B- Temperature, C-pH, D-Inoculum Percentage, E-Moistening Solution, G-Tween 80, H-Copper sulphate , I-Magnesium Sulphate, J- Manganese Sulphate, K-Yeast Extract, L-Tryptone

11 independent medium compositions were evaluated at two levels (high and low). The significant factors were screened in 12 combinations, according to the design matrix and the responses were determined. All the experiments were carried out in triplicates and average of laccase activity was taken as response. The factors showing highest positive effects were further optimized by response surface methodology.

Optimization using Response Surface Methodology
Response Surface Methodology (RSM) was employed to optimize the nutritional factors having significant positive influence on laccase production as obtained from PB design .It was studied at five different levels. A 23- factorial central composite design (CCD), with eight axial points (α = 2) and six replications at the centre points (no. = 8) led to a total number of 30 experiments. The statistical software

Design Expert Version 10.0.3 was used to analyze the results of the experimental design.

Prediction of optimum values

After getting the model equation that explains the process, it was used for optimization of laccase production using numerical optimization method of Design Expert 10.0.3. Criteria were set for each independent variable and the response (dependent variable). The independent variables were kept in range used by the experimental set up and the response was set to maximum. A solution was generated with predicted levels of the independent variables and a predicted maximum response.

Validation experiments

To validate the statistical model, the fermentation was carried out using the optimal conditions predicted by the model and response (enzyme yield) was measured as described earlier and compared to the predicted values. Each experiment was done in triplicates and data presented as mean ± SD.

III. RESULTS AND DISCUSSION

Laccase is known for its wide range of industrial applications. For an enzyme to be industrially applicable, the yield of enzyme and cost of its production are the two important parameters for consideration besides the novel properties an enzyme have. The hindrance in the use of bacterial laccase at industrial level is intracellular/spore bound nature of most of the bacterial laccases reported so far [11]. But in the last few years, increasing reports are on the extracellular secretion of laccase have open up the production and optimization of laccase at large scale [8, 11]. Earlier we have isolated an extracellular laccase producing bacteria Bacillus sp. MSK-01 [12]. In this study, the production of laccase from MSK-01 was increased using solid state fermentation technology.

Selection of significant parameters for optimum MSK laccase production

A larger PB design is necessary for getting higher quality information on the significance of each real factor [13].To check the pattern of influence of different factors on enzyme activity, pareto charts were analyzed. Out of 11 factors, 7 factors viz., time, moistening solution, tryptone, yeast extract, temperature, tween 80 and CuSO4 showed positive effect on laccase production in sweet lime peels (Fig. 1).The significance of the model and the influence of parameters were checked by ANOVA (Table 1). The results confirmed that the model was significant. Out of the 7 factors showing positive effects on laccase production, 4 factors showing positive effect on the production of enzyme viz. yeast extract, time, tryptone and copper sulphate were further.

Response surface design

In order to increase the production, factors selected from PB were analyzed by Response Surface Methodology (RSM). RSM is an experimental tool by which the optimal conditions of multiple variables can be determined. Response surface methodology is a collection of statistical tools and techniques for constructing and exploring an approximate functional relationship between a response variable and a set of design variables. It is possible to derive an expression for the performance measurement based on the responded values obtained from experiments at some particular combination of the input variables [14].

Central Composite design (CCD)

CCD of RSM ofwas employed to study the combined effect of variables on sweet lime peels for laccase production .The design resulted in a total of 30 experiments. The actual responses for laccase activity with the residuals are presented in Table 2. On analysis of results by ANOVA, it was found that the model is significant for sweet lime peels for the production of laccase .

Analysis of Response surface results

Explanatory models were devised by software using results in (Table 3). The p-values of <0.0245 indicated that the quadratic model have significant effect on production of laccase. The lack of fit is non-significant for the enzyme. The p value for the lack of fit was 0.4867for laccase, indicating that this quadratic model adequately fit into the data. The determination coefficient R^2 (0.7305 respectively) indicated that the predicted and experimental values had perfect coherence with each other.

The behaviour of the system in case of sweet lime peels is calculated by the following equation:

$$Y=+146.53-5.69* A-27.38* B-5.44 *C-6.93* D+14.81* AB+17.58* AC+9.39* AD+16.06* BC-8.51* BD+11.66* CD-0.22* A2+21.22* B2-15.19* C2-18.00* D2 \quad \ldots.Eq\ (1)$$

Where Y represents response variable (enzyme activity), β0 is the interception coefficient, βi is the coefficient of the linear effect, βii is the coefficient of quadratic effect and βij is the coefficient of interaction effect.

The significance of each coefficient was determined by p values. The statistical software was used for multiple regression analysis and to construct the plots of the obtained data. The coded variables of $CuSO_4$,

tryptone, yeast extract and time were constructed into their actual values to find out the optimum range of the variables for the production of laccase in sweet lime peels.

Interactive effect between the variables

The interactive effect of process parameters on laccase production was studied by three dimensional graphs. The interaction between term AB where A is time and B is tryptone is shown in fig. 2A. If time is increased then laccase yield increases and in case of tryptone it does not increases much. This response surface graph implies that if these two factors are taken in combination then laccase yield increases. The interaction between term AC where A is time and C is copper sulphate is shown in Fig. 2B. Copper sulphate resulted in decrease in laccase yield with increasing concentration but when both the factors are implied together, there is little increase in laccase yield.

Validation of predicted variables for maximum laccase production

The value of parameters for optimum laccase production was determined using software's numerical optimization option. The solid state medium showing maximum laccase yield was selected and validation experiments were conducted using those values. The experimental value was found to be close to the predicted value and hence, the model was successfully validated (Table 4). Thus, the results of optimization studies are useful in substantial economization of laccase production by *Bacillus species MSK -01*. There was only 3.51 fold increase in the laccase yield after the optimization process which involved the use of sweet lime peel as a substrate.

Conclusion

Laccase is an important multi copper oxidase with a great potential in biotechnology applications in various fields like cpsmetics, bio bleaching, bioremediation, food and paper industry etc. Introduction of laccase using low cost substrate can be important for their application in industry as the industries usually prefer low cost enzymes to replace the conventional, biological methods. In this study laccase from *Bacillus* sp. MSK-01 was produced in higher yield sweet lime peels as substrates. Sweet lime peels are a waste product of juice industry and are of no importance. It is usually discarded as waste. We have employed this waste for increasing the laccase yield. As the substrate employed causes no cost therefore, laccase produced can have applications in industry.

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AUTHOR'S CONTRIBUTION

Dr. Sonica Sondhi is the main and the corresponding author of the manuscript. Kiranjot Saini has carried out the research work. Mrs. Palki Sahib Kaur have supported in providing necessary facilities for the research work undertaken.

CONFLICT OF INTEREST

The author states that there is no conflict of interest.

ETHICAL APPROVAL

This article does not contain any studies with human participants or animals performed by any of the authors.

REFERENCES

RSM – response surface methodology; CCD – central composite design; PB – Plackett-Burman; SSF – solid state fermentation; 3D- three dimensional

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Table 1: Analysis of Variance (ANOVA) of PB analysis for laccase in Sweet lime Peels.

Analysis of variance table [Partial sum of squares - Type III]						
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	3.179E+005	9	35322.15	1229.63	0.0008	significant
A-pH	*10883.62*	*1*	*10883.62*	*378.88*	*0.0026*	
B-Temperature	*30573.21*	*1*	*30573.21*	*1064.31*	*0.0009*	
C-Time	*27300.71*	*1*	*27300.71*	*950.39*	*0.0011*	
E-Moisting solution	*1.096E+005*	*1*	*1.096E+005*	*3816.37*	*0.0003*	
F-Tween-80	*31421.55*	*1*	*31421.55*	*1093.84*	*0.0009*	
G-Copper sulphate	*90153.61*	*1*	*90153.61*	*3138.42*	*0.0003*	
J-Manganese sulphate	*5994.49*	*1*	*5994.49*	*208.68*	*0.0048*	
K-Yeast extract	*5692.64*	*1*	*5692.64*	*198.17*	*0.0050*	
L-Tryptone	*6251.36*	*1*	*6251.36*	*217.62*	*0.0046*	
Residual	57.45	2	28.73			
Cor Total	3.180E+005	11				

Table 2: Central composite design matrix with predicted values of laccase in Sweet lime Peels

RUN	FACTOR 1 A:Time h	FACTOR 2 B:Tryptone %	FACTOR 3 C:Copper Sulphte Micro molar	FACTOR 4 D:Yeast extract %	Response 1 Laccase Activity U/g
1	72	0.4	350	0.4	197.916
2	72	0	350	0.4	333.333
3	96	0.6	500	0.2	136.09
4	72	0.4	350	0.4	170.833
5	72	0.4	350	0.4	107.917
6	48	0.2	200	0.2	243.75
7	72	0.4	50	0.4	129.583
8	24	0.4	350	0.4	191.666
9	72	0.4	350	0	115.417
10	96	0.2	500	0.6	183.07
11	120	0.4	350	0.4	141
12	96	0.2	500	0.2	95.833
13	72	0.4	350	0.4	118.75
14	96	0.6	200	0.2	108.072
15	48	0.2	200	0.6	195.833
16	48	0.2	500	0.6	111.666
17	72	0.8	350	0.4	170.833
18	72	0.4	350	0.4	177.083
19	48	0.6	200	0.2	109.583
20	72	0.4	350	0.6	75
21	48	0.6	500	0.2	114.583
22	48	0.6	200	0.6	35.411
23	96	0.2	500	0.4	114.7
24	96	0.6	200	0.6	105.076
25	72	0.4	350	0.4	106.66
26	96	0.6	500	0.6	114.09
27	96	0.2	200	0.2	112.09
28	48	0.6	500	0.6	102.916
29	72	0.4	650	0.4	83.333
30	96	0.2	200	0.6	101.09

Table 3: Analysis of Variance (ANOVA) of PB analysis for laccase in Sweet lime peels

	Sum of		Mean	F	p-value	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Model	69648.02	14	4974.86	2.90	0.0245	significant
A-time	*777.82*	*1*	*777.82*	*0.45*	*0.5107*	
B-tryptone	*17996.91*	*1*	*17996.91*	*10.51*	*0.0055*	
C-Copper Sulphate	*709.12*	*1*	*709.12*	*0.41*	*0.5297*	
D-yeast extract	*1153.48*	*1*	*1153.48*	*0.67*	*0.4247*	

AB	*3509.58*	*1*	*3509.58*	*2.05*	*0.1728*	
AC	*4942.11*	*1*	*4942.11*	*2.89*	*0.1100*	
AD	*1411.83*	*1*	*1411.83*	*0.82*	*0.3783*	
BC	*4129.07*	*1*	*4129.07*	*2.41*	*0.1414*	
BD	*1158.06*	*1*	*1158.06*	*0.68*	*0.4238*	
CD	*2176.71*	*1*	*2176.71*	*1.27*	*0.2773*	
A^2	*1.33*	*1*	*1.33*	*7.752E-004*	*0.9782*	
B^2	*12347.83*	*1*	*12347.83*	*7.21*	*0.0170*	
C^2	*6327.74*	*1*	*6327.74*	*3.69*	*0.0738*	
D^2	*8887.99*	*1*	*8887.99*	*5.19*	*0.0378*	
Residual	25693.71	15	1712.91			
Lack of Fit	*17676.74*	*10*	*1767.67*	*1.10*	*0.4867*	*not significant*
Pure Error	*8016.97*	*5*	*1603.39*			
Cor Total	95341.72	29				
Std.Dev.	41.39		R-Squared	0.7305		
Mean	136.77		Adj R-Squared	0.4790		
C.V%	30.26		Pred R- Square	-0.1890		
Press	1.134E+005		Adeq Precisior	7.706		
-2 Log Likeliho	287.72		BIC	338.74		
			AICc	352.01		

Table 4: Actual V/s predicted yield of laccase

Variables				**Response (LACCASE Yield)**		
Time h	**Tryptone %**	**Copper sulphate Micro molar**	**Yeast extract %**	**Actual (IU ml^{-1})**	**Predicted (IU ml^{-1})**	**Difference (%)**
92.800	1.193	413.982	0.209	461	455.656	1.15921

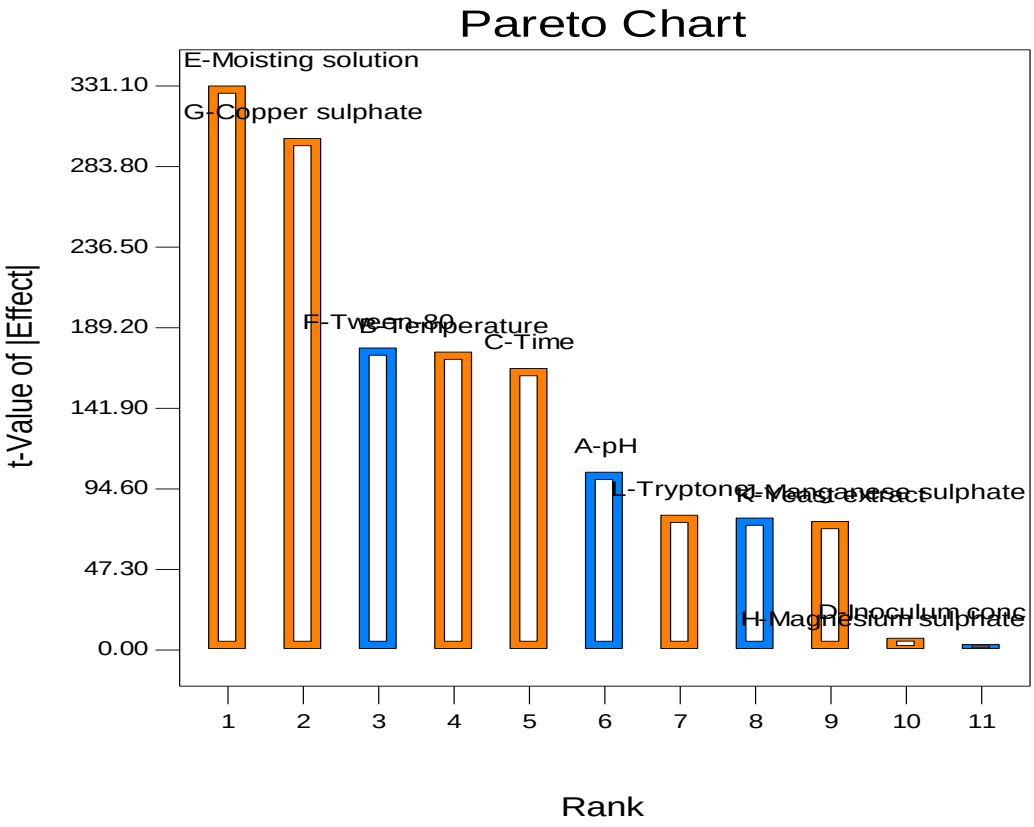
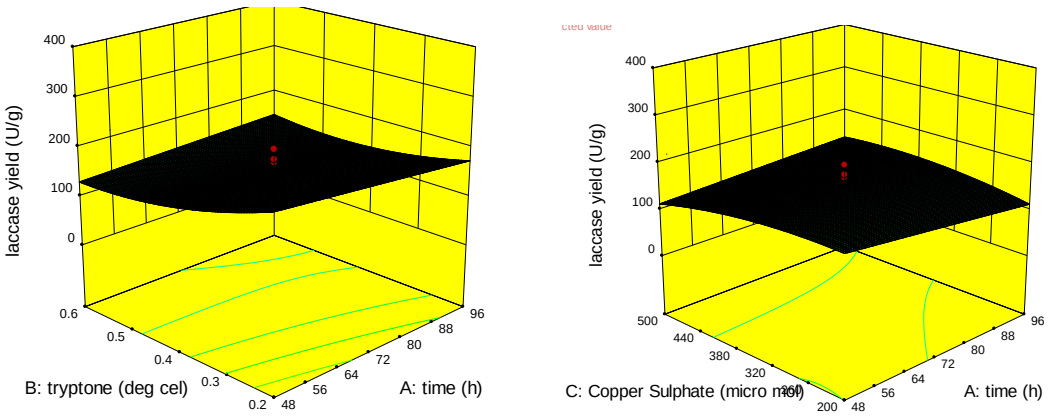


Fig. 1 Pareto chart showing the influence of various factors on laccase production from Bacillus species MSK-01 in Sweet lime peels. The positive factors are in orange color and negative factors are in blue colour



(A) (B)

Fig. 2 Interactive effect of parameters on laccase yield