The Bioscan
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
20(1): 619-632, 2025 www.thebioscan.com

Optimisation of Lignin Peroxidase Production Using Locally Isolated *Purpureocillium lilacinum* Through Factorial Design

S.Y. More[1*], A. M. Datir[2], Y. R. Baste[3], G. S. Phad[4]

[1,2]Department of Environmental Sciences, M.V.P. Samaj's K.T.H.M College, Gangapur Road, Nashik, Maharashtra. Affiliated to Savitribai Phule Pune University, Pune, Maharashtra.

[3]Department of Chemistry, M.V.P. Samaj's K.S.K.W A.S.C College, CIDCO, Nashik, Maharashtra.

[4]Department of Statistics, M.V.P. Samaj's K.T.H.M College, Gangapur Road, Nashik, Maharashtra. Affiliated to Savitribai Phule Pune University, Pune, Maharashtra.

Corresponding Author Email: sadhanaevs21@gmail.com

DOI: https://doi.org/10.63001/tbs.2025.v20.i01.pp619-632

KEYWORDS
Lignin peroxidase,
Purpureocillium lilacinum,
White rot fungi,
BBD, PBD,
Enzyme activity.
Received on:

18-01-2025

Accepted on:

15-02-2025

Published on:

20-03-2025

ABSTRACT

The lignin peroxidase enzyme is produced by the ligninolytic white rot fungus *Purpureocillium lilacinum*, which makes it a promising biological treatment tool. Numerous dangerous substances may be broken down by lignin peroxidase. For this technique to be applied effectively, process variables must be optimized to maximize the production of enzymes. An initial screening of medium components was conducted using a Plackett-Burman Design (PBD) with 11 factors. Among these, 8 factors were identified as statistically significant for Lignin Peroxidase production: incubation period, temperature, veratryl alcohol, H_2O_2, $FeSO_4$, $MgSO_4$, $CuSO_4$, and peptone. The maximum influential factors for production were found to be $CuSO_4$ and temperature. These 8 factors identified by PBD were then selected for further optimization using response surface methodology, specifically the Box-Behnken Design (BBD). The optimized conditions for producing Lignin Peroxidase were: an 11-day incubation period, a temperature of 18°C, 150mM veratryl alcohol, 0.148mM H_2O_2, 0.1 g/L $FeSO_4$, 0.1 g/L $MgSO_4$, 0.001 g/L $CuSO_4$, and 3.00 g/L peptone. The maximum enzyme activity achieved under these optimized conditions was 115.8. The close agreement between the actual and predicted values of enzyme activity confirms the reliability of the model.

INTRODUCTION

The lignin peroxidase, an extracellular heme peroxidase, is part of the ligninolytic system of the white rot fungus under circumstances involving carbon and nitrogen limitation, ligninolytic enzyme expression is idiopathic (Falade et al.,2016). The conditions of cultivation have a significant impact on the enzyme's expression. This fungus has a lot of potential as a biological treatment tool because it can break down a wide range of resistant substances (Falade et al.,2016, Sharma et al., 2017). The low levels of lignin peroxidase productivity have made it difficult to use the enzyme for practical waste treatment applications (Janusz et al., 2017). The use of various media for the production of lignin peroxidase has been the subject of multiple reports. The Lignin peroxidase activity of *Purpureocillium lilacinum* is enhanced by the addition of veratryl alcohol, a secondary metabolite, and an excess of trace elements. For solid-state fermentation, lignin was obtained from wheat straw (Suryadi et al., 2022). Optimized medium using Response Surface Methodology for solid-state fermentation produced a good yield of crude lignin peroxidase enzyme. Under nitrogen limiting, sufficient, and excess conditions, the time course of lignin peroxidase production was examined (Jadhav et al., 2023).

A recent study shows optimization of parameters for the synthesis of organic compounds and a comparative study of RSM-CCD models (Jadhav et al., 2023, Rath et al .,2022).

In this study, the Placket Burman Design was employed to screen a large number of factors and identify the most crucial ones influencing the enzyme activity of Lignin Peroxidase production. Response surface methodology (RSM) using the Box-Behnken Design was then utilized for optimization and to determine the optimal levels of each factor (Evi Susanti et. al 2016). We have successfully utilized such a large number of factors at once to enhance lignin peroxidase production using RSM. In the present report, we have successfully utilized several factors to enhance lignin peroxidase production using RSM.

Materials and methods

Organism:

Various wild white rot fungal isolates were gathered from the Nashik district's forest areas and rainy places as well as decomposed agro-waste. The isolates were brought to the laboratory after being gathered in sterile polythene covers. These fungal isolates of fleshy tissue were cut, sterilized with a 1% mercuric chloride solution, repeatedly cleaned with sterile distilled water, and then inoculated into media that contained

alkali lignin and minimal salt medium. At 30°C, the inoculated cultures were incubated. The cultures were stored at 4°C, with subculturing occurring every 15 days (Asgher, 2006).

Isolate Lp8i, the fungus that causes white rot, was isolated from a portion of degraded wood and identified by the ITS region. After identification, strain LP8i is found as *Purpureocillium lilacinum*. It was cultivated on potato dextrose agar slants at room temperature until sporulation (Janusz et al., 2017).

Medium composition: The medium used in this study was modified from that described by Tien and Kirk. A minimal salt basal medium gm/lit was prepared with a trace element solution. MSBM-L contain 6.00 $NaNO_3$, 0.8 KH_2PO_4,0.52 $MgSO_4$ $7H_2O$, 0.52 KCl, 30ml Alkali lignin, Trace element solution 1000 ml. 0.01 $CuSO_4$, 2.5 $NaNO_2$, 0.18 NaCl, 0.1 KH_2PO_4, 0.05 $FeSO_4.H_2O$, $MgSO_4$ $7H_2O$, 0.1mM H_2O_2, 100mM Veratryl alcohol (Thammaiah Vandana et.al 2018, Martina Vrsanskae et.al 2016). One litre of the culture medium was prepared by mixing 100 ml MSBM-L medium, 60 ml trace element solution with required concentrations of glucose, and glycerol as per the experimental design (Sudha Hariharan et.al 2013). The mixture was diluted to one litre with distilled water and autoclaved (Zahangir et al., 2009).

Statistical tool:

Minitab® 20 statistical software was utilized to create the Plackett-Burman Design (PBD) for screening independent factors and to design the Response Surface Methodology-Box-Behnken Design (RSM-BBD) for optimizing the selected factors identified during screening (Vandana et al., 2018, Zanirun et al., 2009).

Experimental design:

Plackett-Burman Design (PBD):

The Plackett-Burman Design (PBD) is an efficient screening method for evaluating independent factors with minimal experimental runs. Developed by Robert L. Plackett and J. P. Burman in 1946, this design focuses on the effective screening of multiple variables to identify those that have a significant impact on the desired outcome. The approximate function used in PBD with 'k' factors is the first-order polynomial model, as shown in the following equation:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \cdots + \beta_k X_k + \epsilon$$

Where Y is the response; β_0 is the intercept term; $\beta_i, (i = 1,2,\ldots,k)$ represents the estimated effect of the i-th factor; X_i is the level of i-th factor; ϵ is the error term.

The PBD was used to screen eleven factors, and eight factors were selected for further analysis.

Response Surface Methodology (RSM) with Box-Behnken Design (BBD):

In this study, Response Surface Methodology (RSM) using the Box-Behnken Design (BBD) was employed to optimize and analyze the selected eight factors for Lignin Peroxidase production. The effect of each independent factor on the response and model accuracy was assessed using analysis of variance (ANOVA) with Fisher's statistical analysis and p-values (Jadhav et al., 2023, Rath et al., 2022). The relationship between Lignin Peroxidase enzyme activity (Y) and the selected eight independent factors is represented by the following quadratic polynomial equation:

$$Y = \beta_0 + \sum_{i=1}^{8} \beta_i X_i + \sum_{i=1}^{8} \beta_{ii} X_i^2 + \sum_{i=1}^{8} \sum_{j=1(i<j)}^{8} \beta_{ij} X_i X_j + \in$$

Where Y is the response variable; β_0 is the intercept; β_i is the linear coefficient for the factor X_i, representing the effect of a single factor; β_{ii} is the quadratic coefficient for the factor X_i, representing the curvature of the response with respect to X_i; β_{ij} is the interaction coefficient between factors X_i and X_j, representing the combined effect of two factors on the response; ϵ is the error term, capturing random variation in the response. Furthermore, the enzyme activity of Lignin Peroxidase production was maximized, and the optimum conditions for the eight factors were obtained.

Enzyme production:

The selected factor was included in the control and test medium for maximizing the production of Lignin Peroxidase of *Purpureocillium lilacinum*. Five flasks were inoculated with the appropriate amount of wheat straw containing minimal salt solid medium and 10^6 spores/ml suspension and incubated for eleven days. Control flask was also incubated without inoculum with same cultural conditions (Asgher et al., 2009).

Lignin Peroxidase activity was measured using Azure B as the substrate (Tein and Kirk, 1983), where one unit of enzyme activity was calculated using a formula.

$$\text{Enzyme activity} = \frac{\text{Consumed substrate (µmol/ml) x Total Reaction Volume}}{\text{Reaction Time x Enzyme volume}}$$

Enzyme assay:

Lignin peroxidase activity was determined spectrophotometrically **(ShimadzuUV-1601PC)** at 644nm. One unit (U) of the enzyme activity is defined as one µ-mole of Azure B substrate oxidised per unit time. Enzyme activity was calculated using above-stated formula (Arora et al., 2001)

Results and Discussion:

Plackett-Burman Design (PBD) for screening independent factors:

In the present study, PBD was used to screen eleven factors in twelve randomized experimental runs with triplicates, as shown in table 2. The 11 factors, each at two levels (low and high) as shown in Table 1, selected for PBD were incubation period, temperature, pH, veratryl alcohol, H_2O_2, $FeSO_4$, $MgSO_4$, $CuSO_4$, yeast extract, peptone, and L-Histidine. These factors were investigated to identify the statistically significant ones. The enzyme activity of Lignin Peroxidase was used as the response variable.

Table 1: Level of independent factors used in PBD for screening

Sr. No.	Independent Factors	Unit	Experimental Value	
			Low (-1)	High (+1)
1	Incubation Period	days	9	11
2	pH	pH	3.5	4.5
3	Temperature	^{0}C	15	25
4	Veratryl alcohol	mM	50	150
5	H2O2	mM	0.05	0.15
6	FeSO4	g/l	0.01	0.1
7	MgSO4	g/l	0.01	0.1
8	CuSO4	g/l	0.001	0.1
9	Yeast Extract	g/l	2	4
10	Peptone	g/l	1	3
11	L-Histidine	g/l	0.1	0.01

Table 2: Plackett-Burman design matrix with Lignin Peroxidase production in uncoded units

Run	Incubation Period	pH	Temperature	Veratryl alcohol	H_2O_2	$FeSO_4$	$MgSO_4$	$CuSO_4$	Yeast Extract	Peptone	L-Histidine	Enzyme Activity
1	11	3.5	25	50	0.05	0.01	0.1	0.1	4	1	0.1	31.2
2	11	4.5	15	150	0.05	0.01	0.01	0.1	4	3	0.01	32.8
3	9	4.5	25	50	0.15	0.01	0.01	0.001	4	3	0.1	42.8
4	11	3.5	25	150	0.05	0.1	0.01	0.001	2	3	0.1	26.4
5	11	4.5	15	150	0.15	0.01	0.1	0.001	2	1	0.1	21.2
6	11	4.5	25	50	0.15	0.1	0.01	0.1	2	1	0.01	33.5
7	9	4.5	25	150	0.05	0.1	0.1	0.001	4	1	0.01	28.0
8	9	3.5	25	150	0.15	0.01	0.1	0.1	2	3	0.01	74.4
9	9	3.5	15	150	0.15	0.1	0.01	0.1	4	1	0.1	39.0
10	11	3.5	15	50	0.15	0.1	0.1	0.001	4	3	0.01	6.1
11	9	4.5	15	50	0.05	0.1	0.1	0.1	2	3	0.1	27.7
12	9	3.5	15	50	0.05	0.01	0.01	0.001	2	1	0.01	4.1
13	11	3.5	25	50	0.05	0.01	0.1	0.1	4	1	0.1	30.6
14	11	4.5	15	150	0.05	0.01	0.01	0.1	4	3	0.01	31.5
15	9	4.5	25	50	0.15	0.01	0.01	0.001	4	3	0.1	42.2
16	11	3.5	25	150	0.05	0.1	0.01	0.001	2	3	0.1	25.7
17	11	4.5	15	150	0.15	0.01	0.1	0.001	2	1	0.1	20.6
18	11	4.5	25	50	0.15	0.1	0.01	0.1	2	1	0.01	33.8
19	9	4.5	25	150	0.05	0.1	0.1	0.001	4	1	0.01	26.7
20	9	3.5	25	150	0.15	0.01	0.1	0.1	2	3	0.01	73.8
21	9	3.5	15	150	0.15	0.1	0.01	0.1	4	1	0.1	40.2
22	11	3.5	15	50	0.15	0.1	0.1	0.001	4	3	0.01	16.1
23	9	4.5	15	50	0.05	0.1	0.1	0.1	2	3	0.1	28.3
24	9	3.5	15	50	0.05	0.01	0.01	0.001	2	1	0.01	3.8
25	11	3.5	25	50	0.05	0.01	0.1	0.1	4	1	0.1	30.3
26	11	4.5	15	150	0.05	0.01	0.01	0.1	4	3	0.01	31.9
27	9	4.5	25	50	0.15	0.01	0.01	0.001	4	3	0.1	41.9
28	11	3.5	25	150	0.05	0.1	0.01	0.001	2	3	0.1	25.4
29	11	4.5	15	150	0.15	0.01	0.1	0.001	2	1	0.1	20.3
30	11	4.5	25	50	0.15	0.1	0.01	0.1	2	1	0.01	34.4
31	9	4.5	25	150	0.05	0.1	0.1	0.001	4	1	0.01	27.4
32	9	3.5	25	150	0.15	0.01	0.1	0.1	2	3	0.01	74.4
33	9	3.5	15	150	0.15	0.1	0.01	0.1	4	1	0.1	39.3
34	11	3.5	15	50	0.15	0.1	0.1	0.001	4	3	0.01	16.4
35	9	4.5	15	50	0.05	0.1	0.1	0.1	2	3	0.1	28.0
36	9	3.5	15	50	0.05	0.01	0.01	0.001	2	1	0.01	4.5

By looking at P-value of ANOVA table as shown in Table 3, the Normal plot and Pareto plot of the standardized effects for enzyme activity of Lignin Peroxidase production as shown in figure 1, indicate that the independent factors incubation period, temperature, veratryl alcohol, H_2O_2, $FeSO_4$, $MgSO_4$, $CuSO_4$, and peptone were significant, whereas the factors pH, yeast extract, and L-Histidine were not significant.

Table 3: Analysis of Variance (ANOVA) for enzyme activity of Lignin Peroxidase production Using Plackett-Burman design

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	11	9842.55	894.78	288.17	0.000
Linear	11	9842.55	894.78	288.17	0.000
Incubation Period	1	882.49	882.49	284.21	0.000
pH	1	2.10	2.10	0.68	0.419
Temperature	1	2353.08	2353.08	757.82	0.000
Veratryl alcohol	1	1149	1149	370.04	0.000
H2O2	1	1418.07	1418.07	456.69	0.000
FeSO4	1	335.56	335.56	108.07	0.000
MgSO4	1	64.07	64.07	20.63	0.000
CuSO4	1	2765.84	2765.84	890.74	0.000
Yeast Extract	1	1.04	1.04	0.34	0.568
Peptone	1	869.77	869.77	280.11	0.000
L-Histidine	1	1.53	1.53	0.49	0.490
Error	24	74.52	3.11	-	-
Total	35	9917.07	-	-	-

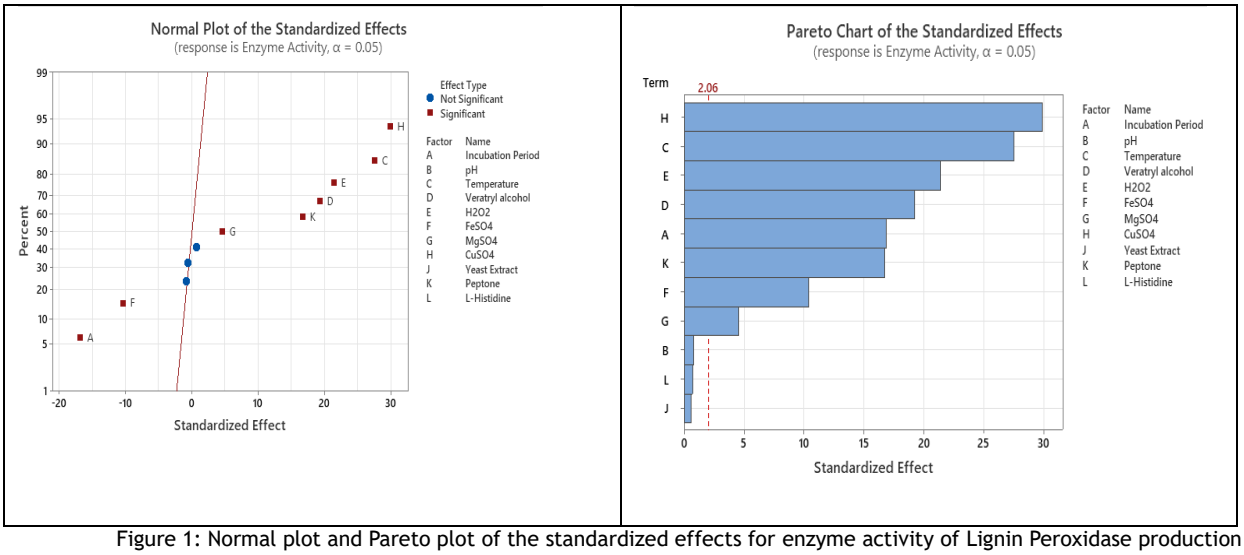


Figure 1: Normal plot and Pareto plot of the standardized effects for enzyme activity of Lignin Peroxidase production

The first order polynomial model equation for enzyme activity of Lignin Peroxidase production can be written as:

Enzyme Activity = 9.81 - 4.951 (Incubation Period) - 0.483 (pH) + 1.6170 (Temperature) + 0.11299 (Veratryl alcohol) + 125.52 (H_2O_2) - 67.85 ($FeSO_4$) + 29.65 ($MgSO_4$) + 177.07 ($CuSO_4$) - 0.170 (Yeast Extract) + 4.915 (Peptone) + 4.58 (L-Histidine)

Optimization using Response Surface Methodology (RSM) with Box-Behnken Design (BBD):

Following the Plackett-Burman Design (PBD) screening of independent factors, the eight statistically significant factors were selected for further optimization to identify their ideal values for maximizing Lignin Peroxidase enzyme activity. The eight selected independent factors using PBD are the incubation period, temperature, veratryl alcohol, H_2O_2, $FeSO_4$, $MgSO_4$, $CuSO_4$, and peptone, which were used for further optimization using RSM-BBD. The BBD matrix generated 81 experimental runs, as detailed in Table 4.

Table 4: Box-Behnken Design (BBD) based on selected independent factors with actual and predicted enzyme activity of Lignin Peroxidase production

Run	Incubation Period	Temperature	Veratryl alcohol	H_2O_2	$FeSO_4$	$MgSO_4$	$CuSO_4$	Peptone	Actual Enzyme Activity	Predicted Enzyme Activity
1	10	20	100	0.15	0.01	0.01	0.0505	2	63.46	53.67
2	11	25	150	0.1	0.055	0.055	0.0505	2	32.84	48.49
3	9	25	150	0.1	0.055	0.055	0.0505	2	42.83	26.16
4	9	15	150	0.1	0.055	0.055	0.0505	2	58.62	51.69
5	9	20	100	0.1	0.1	0.055	0.0505	2	104.71	98.04
6	10	20	100	0.15	0.1	0.01	0.0505	2	91.50	79.97
7	10	20	50	0.1	0.1	0.055	0.001	2	53.79	53.32
8	10	20	50	0.05	0.055	0.055	0.0505	1	42.18	35.79
9	10	20	150	0.1	0.01	0.055	0.1	2	69.26	71.25
10	10	20	100	0.1	0.055	0.055	0.001	3	73.77	61.14
11	9	20	100	0.1	0.055	0.01	0.0505	1	64.74	77.84
12	11	20	100	0.1	0.055	0.1	0.0505	1	69.90	67.75
13	11	25	50	0.1	0.055	0.055	0.0505	2	30.58	20.02
14	10	20	150	0.05	0.055	0.055	0.0505	1	70.22	52.18
15	10	15	100	0.1	0.01	0.055	0.0505	1	42.18	35.05
16	10	15	100	0.1	0.1	0.055	0.0505	3	57.98	56.92
17	11	20	100	0.1	0.055	0.01	0.0505	3	85.05	83.18
18	10	20	150	0.1	0.055	0.01	0.0505	2	66.03	68.59
19	10	20	150	0.1	0.055	0.1	0.0505	2	48.31	74.70
20	10	25	100	0.1	0.055	0.01	0.1	2	38.31	52.70
21	11	20	100	0.1	0.01	0.055	0.0505	2	72.48	89.57
22	10	20	50	0.15	0.055	0.055	0.0505	3	16.07	34.29
23	11	20	100	0.1	0.055	0.1	0.0505	3	92.79	81.22
24	11	15	50	0.1	0.055	0.055	0.0505	2	3.83	3.00
25	10	20	150	0.15	0.055	0.055	0.0505	1	49.60	47.69
26	10	25	100	0.1	0.055	0.1	0.001	2	31.87	31.13
27	9	15	50	0.1	0.055	0.055	0.0505	2	57.98	24.83
28	10	20	50	0.1	0.1	0.055	0.1	2	25.42	34.74
29	10	15	100	0.1	0.055	0.1	0.1	2	20.26	35.66
30	10	20	150	0.15	0.055	0.055	0.0505	3	66.68	73.25
31	9	20	100	0.05	0.055	0.055	0.001	2	27.36	48.49
32	10	15	100	0.1	0.055	0.1	0.001	2	71.19	56.98
33	10	20	150	0.1	0.1	0.055	0.1	2	69.26	67.25
34	11	20	100	0.15	0.055	0.055	0.001	2	80.86	81.64

35	10	20	100	0.1	0.055	0.055	0.1	3	92.46	78.18
36	9	20	100	0.1	0.055	0.01	0.0505	3	68.93	72.61
37	11	15	150	0.1	0.055	0.055	0.0505	2	63.46	69.19
38	10	20	100	0.05	0.1	0.01	0.0505	2	65.07	57.86
39	11	20	100	0.1	0.055	0.01	0.0505	1	75.06	73.74
40	11	20	100	0.05	0.055	0.055	0.001	2	58.62	68.08
41	10	20	100	0.1	0.055	0.055	0.001	1	85.70	75.07
42	10	25	100	0.1	0.055	0.01	0.001	2	48.63	33.41
43	10	25	100	0.1	0.01	0.055	0.0505	1	28.00	39.81
44	9	20	100	0.1	0.055	0.1	0.0505	1	74.41	77.81
45	9	20	100	0.15	0.055	0.055	0.1	2	71.19	72.48
46	10	20	100	0.05	0.01	0.1	0.0505	2	70.55	64.59
47	10	15	100	0.15	0.055	0.055	0.0505	2	27.68	35.66
48	11	20	100	0.1	0.1	0.055	0.0505	2	79.57	78.80
49	10	15	100	0.1	0.055	0.01	0.001	2	30.58	39.60
50	11	20	100	0.15	0.055	0.055	0.1	2	63.78	53.39
51	10	20	50	0.1	0.01	0.055	0.1	2	42.18	46.00
52	10	25	100	0.1	0.1	0.055	0.0505	3	25.74	43.63
53	10	25	100	0.05	0.055	0.055	0.0505	2	20.59	25.73
54	10	20	100	0.15	0.1	0.1	0.0505	2	98.27	82.68
55	10	20	50	0.15	0.055	0.055	0.0505	1	26.71	30.17
56	9	20	100	0.1	0.055	0.1	0.0505	3	73.77	76.62
57	10	25	100	0.15	0.055	0.055	0.0505	2	40.25	41.39
58	11	20	100	0.05	0.055	0.055	0.1	2	80.54	71.26
59	10	20	50	0.1	0.01	0.055	0.001	2	28.32	31.86
60	10	20	150	0.1	0.01	0.055	0.001	2	62.49	54.70
61	10	25	100	0.1	0.01	0.055	0.0505	3	30.26	26.21
62	10	20	100	0.15	0.01	0.1	0.0505	2	64.10	53.81
63	10	15	100	0.1	0.01	0.055	0.0505	3	48.31	45.77
64	9	20	100	0.15	0.055	0.055	0.001	2	57.65	77.68
65	10	15	100	0.1	0.1	0.055	0.0505	1	20.26	35.07
66	10	15	100	0.1	0.055	0.01	0.1	2	34.45	35.36
67	10	20	150	0.05	0.055	0.055	0.0505	3	59.59	56.30
68	10	25	100	0.1	0.055	0.1	0.1	2	42.18	33.34
69	10	20	100	0.1	0.055	0.055	0.1	1	68.29	56.01
70	10	20	50	0.05	0.055	0.055	0.0505	3	16.40	18.48
71	10	15	100	0.05	0.055	0.055	0.0505	2	28.00	39.98
72	10	20	50	0.1	0.055	0.1	0.0505	2	10.92	39.93
73	10	20	100	0.05	0.1	0.1	0.0505	2	63.46	55.75
74	10	25	100	0.1	0.1	0.055	0.0505	1	32.84	46.12
75	10	20	50	0.1	0.055	0.01	0.0505	2	42.83	48.01
76	9	20	100	0.1	0.01	0.055	0.0505	2	58.62	69.82
77	10	20	150	0.1	0.1	0.055	0.001	2	85.70	83.41
78	10	20	100	0.1	0.055	0.055	0.0505	2	97.94	77.80
79	9	25	50	0.1	0.055	0.055	0.0505	2	60.23	37.01
80	9	20	100	0.05	0.055	0.055	0.1	2	64.74	74.71
81	10	20	100	0.05	0.01	0.01	0.0505	2	71.19	69.28

We fit second order response surface model using BBD for enzyme activity. The second order quadratic models for enzyme activity as shown in following equation:

Enzyme Activity = 74 - 129 Incubation Period + 50.9 Temperature + 0.12 Veratryl alcohol + 987 H_2O_2 + 1669 $FeSO_4$ + 699 $MgSO_4$
+ 1106 $CuSO_4$ - 23.4 Peptone + 6.26 Incubation Period*Incubation Period - 1.248 Temperature*Temperature
- 0.00713 Veratryl alcohol*Veratryl alcohol - 4370 H_2O_2*H_2O_2 - 1 $FeSO_4$*$FeSO_4$ - 1074 $MgSO_4$*$MgSO_4$
- 1904 $CuSO_4$*$CuSO_4$ - 5.54 Peptone*Peptone + 0.24 Incubation Period*Temperature
+ 0.197 Incubation Period*Veratryl alcohol - 78 Incubation Period*H_2O_2 - 217 Incubation Period*$FeSO_4$
- 33 Incubation Period*$MgSO_4$
- 116 Incubation Period*$CuSO_4$ + 3.67 Incubation Period*Peptone
- 0.0377 Temperature*Veratryl alcohol + 20.0 Temperature*H_2O_2
+ 7.0 Temperature*$FeSO_4$ - 21.8 Temperature*$MgSO_4$ + 23.8 Temperature*$CuSO_4$
- 1.22 Temperature*Peptone + 0.11 Veratryl alcohol*H_2O_2 + 0.81 Veratryl alcohol*$FeSO_4$
+ 1.58 Veratryl alcohol*$MgSO_4$ + 0.24 Veratryl alcohol*$CuSO_4$ + 0.107 Veratryl alcohol*Peptone + 4190 H_2O_2*$FeSO_4$
+ 537 H_2O_2*$MgSO_4$ - 3174 H_2O_2*$CuSO_4$ + 107 H_2O_2*Peptone + 318 $FeSO_4$*$MgSO_4$ - 3672 $FeSO_4$*$CuSO_4$
+ 62 $FeSO_4$*Peptone - 1917 $MgSO_4$*$CuSO_4$ + 22 $MgSO_4$*Peptone + 182 $CuSO_4$*Peptone

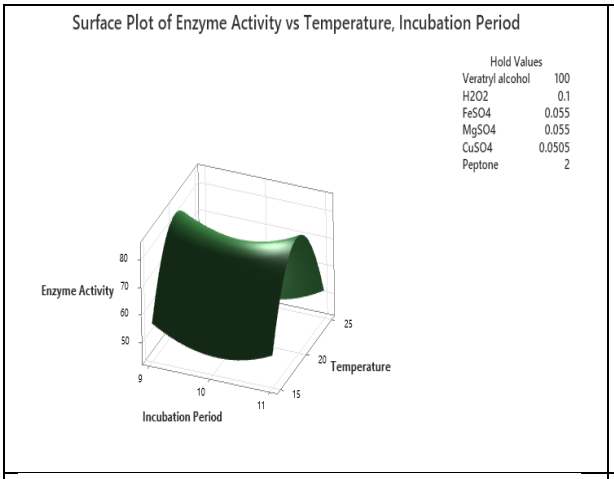
The ANOVA tables for the second-order response surface model using BBD are presented in table 5. The P-value for the regression model is 0.004 (< 0.05), indicating that the fitted second-order quadratic regression model using BBD is significant and does not show a lack of fit at the 5% significance level.

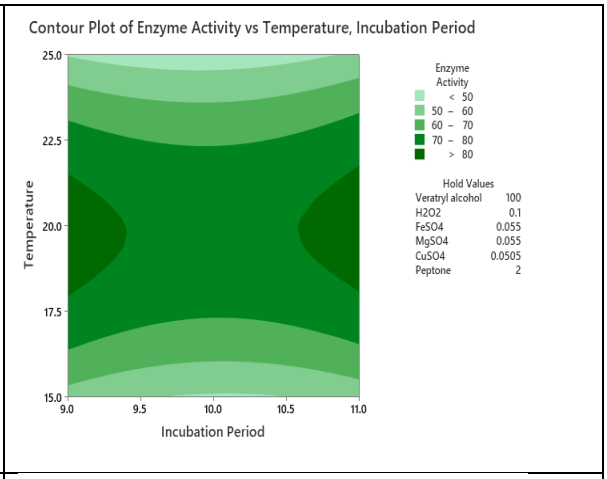
Table 5: Analysis of Variance (ANOVA) for enzyme activity of Lignin Peroxidase production Using Box-Behnken design

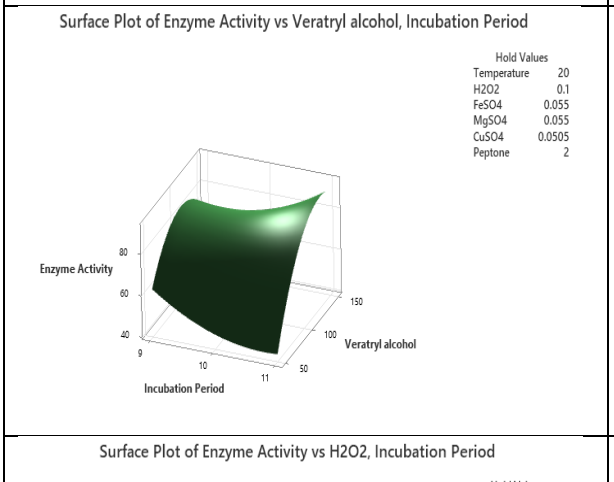
Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	44	32040.7	728.2	2.41	0.004

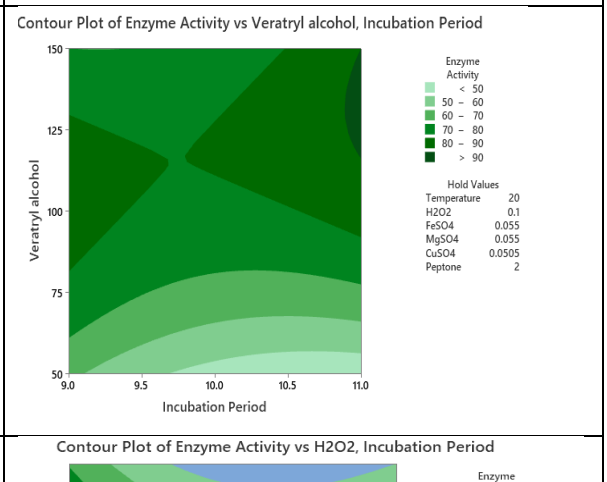
Linear	8	6378.4	797.3	2.63	0.022
Incubation Period	1	0.4	0.4	0.00	0.969
Temperature	1	127.0	127.0	0.42	0.521
Veratryl alcohol	1	5360.6	5360.6	17.71	0.000
H_2O_2	1	224.5	224.5	0.74	0.395
$FeSO_4$	1	532.9	532.9	1.76	0.193
$MgSO_4$	1	6.9	6.9	0.02	0.881
$CuSO_4$	1	7.2	7.2	0.02	0.878
Peptone	1	118.9	118.9	0.39	0.535
Square	8	19551.1	2443.9	8.08	0.000
Incubation Period*Incubation Period	1	421.0	421.0	1.39	0.246
Temperature*Temperature	1	10466.3	10466.3	34.59	0.000
Veratryl alcohol*Veratryl alcohol	1	3416.8	3416.8	11.29	0.002
H_2O_2*H_2O_2	1	1284.0	1284.0	4.24	0.047
$FeSO_4$*$FeSO_4$	1	0.0	0.0	0.00	1.000
$MgSO_4$*$MgSO_4$	1	50.9	50.9	0.17	0.684
$CuSO_4$*$CuSO_4$	1	234.3	234.3	0.77	0.385
Peptone*Peptone	1	330.1	330.1	1.09	0.303
2-Way Interaction	28	6111.1	218.3	0.72	0.812
Incubation Period*Temperature	1	11.7	11.7	0.04	0.845
Incubation Period*Veratryl alcohol	1	773.1	773.1	2.55	0.119
Incubation Period*H_2O_2	1	122.2	122.2	0.40	0.529
Incubation Period*$FeSO_4$	1	380.3	380.3	1.26	0.270
Incubation Period*$MgSO_4$	1	17.8	17.8	0.06	0.810
Incubation Period*$CuSO_4$	1	265.5	265.5	0.88	0.355
Incubation Period*Peptone	1	107.5	107.5	0.36	0.555
Temperature*Veratryl alcohol	1	711.1	711.1	2.35	0.134
Temperature*H_2O_2	1	99.8	99.8	0.33	0.569
Temperature*$FeSO_4$	1	19.8	19.8	0.07	0.800
Temperature*$MgSO_4$	1	193.3	193.3	0.64	0.429
Temperature*$CuSO_4$	1	276.8	276.8	0.91	0.345
Temperature*Peptone	1	296.1	296.1	0.98	0.329
Veratryl alcohol*H_2O_2	1	0.6	0.6	0.00	0.964
Veratryl alcohol*$FeSO_4$	1	26.3	26.3	0.09	0.770
Veratryl alcohol*$MgSO_4$	1	50.3	50.3	0.17	0.686
Veratryl alcohol*$CuSO_4$	1	2.9	2.9	0.01	0.922
Veratryl alcohol*Peptone	1	229.7	229.7	0.76	0.389
H_2O_2*$FeSO_4$	1	711.1	711.1	2.35	0.134
H_2O_2*$MgSO_4$	1	11.7	11.7	0.04	0.845
H_2O_2*$CuSO_4$	1	493.8	493.8	1.63	0.210
H_2O_2*Peptone	1	229.7	229.7	0.76	0.389
$FeSO_4$*$MgSO_4$	1	3.3	3.3	0.01	0.917
$FeSO_4$*$CuSO_4$	1	535.1	535.1	1.77	0.192
$FeSO_4$*Peptone	1	61.8	61.8	0.20	0.654
$MgSO_4$*$CuSO_4$	1	145.9	145.9	0.48	0.492
$MgSO_4$*Peptone	1	8.1	8.1	0.03	0.871
$CuSO_4$*Peptone	1	325.8	325.8	1.08	0.306
Error	36	10894.2	302.6	-	-
Total	80	42934.9	-	-	-

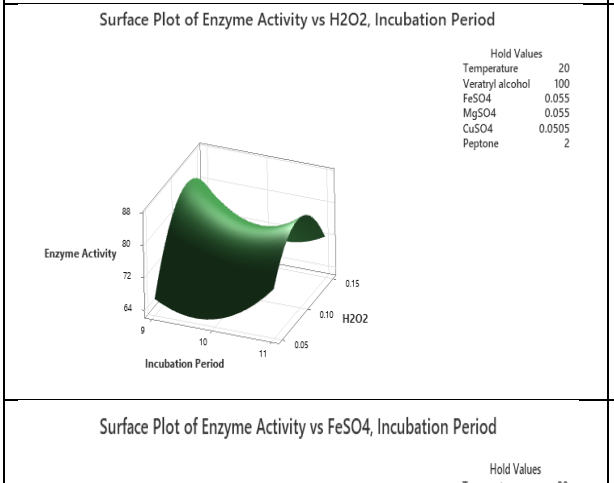
Response surface plot of enzyme activity *versus* Incubation Period, Temperature, Veratryl alcohol, H_2O_2, $FeSO_4$, $MgSO_4$, $CuSO_4$ and Peptone are given in Figure 2.

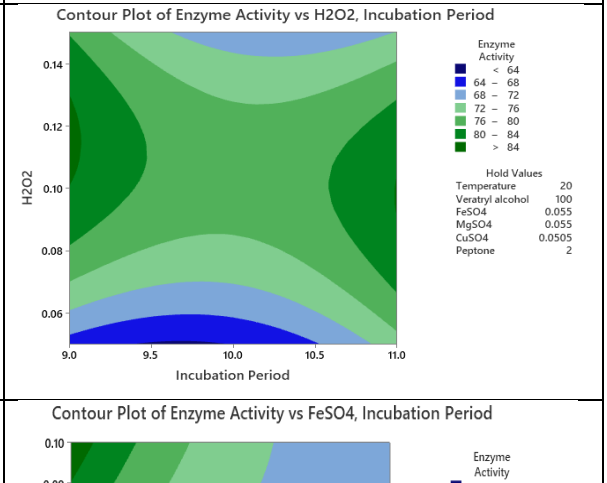

Surface Plot of Enzyme Activity vs Temperature, Incubation Period
Hold Values
Veratryl alcohol 100
H2O2 0.1
FeSO4 0.055
MgSO4 0.055
CuSO4 0.0505
Peptone 2
Enzyme Activity
Temperature
Incubation Period

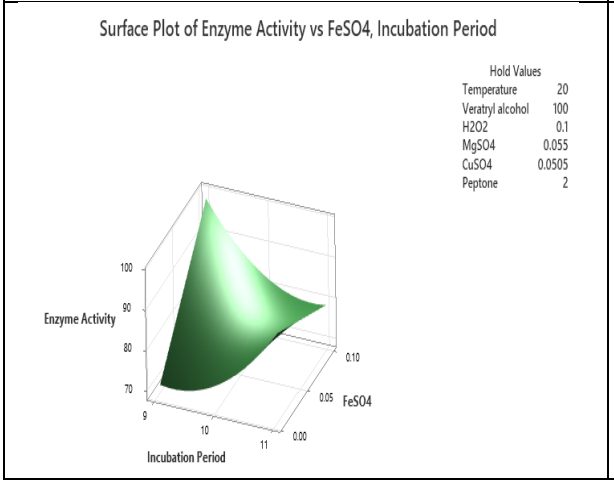

Contour Plot of Enzyme Activity vs Temperature, Incubation Period
Enzyme Activity
< 50
50 – 60
60 – 70
70 – 80
> 80
Hold Values
Veratryl alcohol 100
H2O2 0.1
FeSO4 0.055
MgSO4 0.055
CuSO4 0.0505
Peptone 2
Temperature
Incubation Period

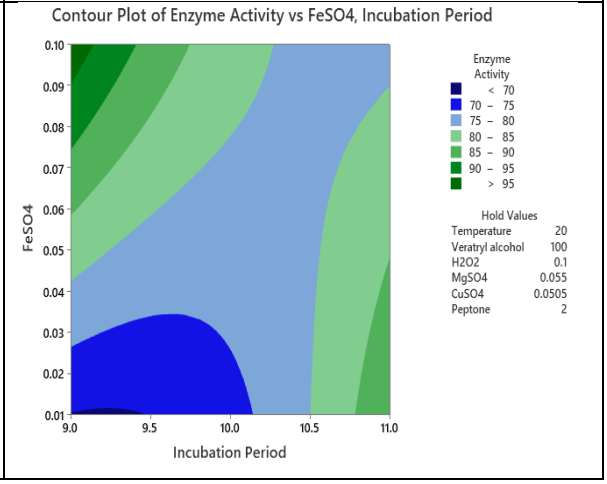

Surface Plot of Enzyme Activity vs Veratryl alcohol, Incubation Period
Hold Values
Temperature 20
H2O2 0.1
FeSO4 0.055
MgSO4 0.055
CuSO4 0.0505
Peptone 2
Enzyme Activity
Veratryl alcohol
Incubation Period

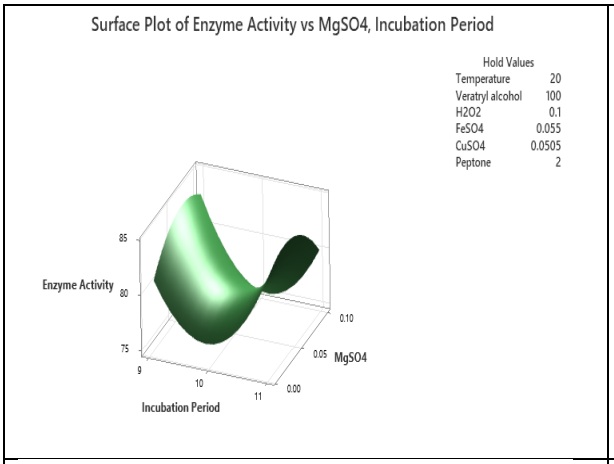

Contour Plot of Enzyme Activity vs Veratryl alcohol, Incubation Period
Enzyme Activity
< 50
50 – 60
60 – 70
70 – 80
80 – 90
> 90
Hold Values
Temperature 20
H2O2 0.1
FeSO4 0.055
MgSO4 0.055
CuSO4 0.0505
Peptone 2
Veratryl alcohol
Incubation Period

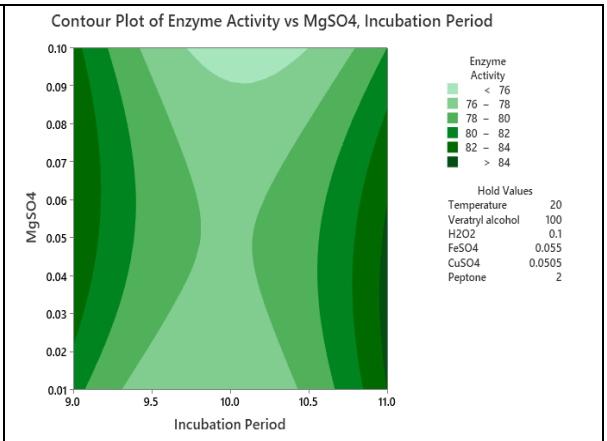

Surface Plot of Enzyme Activity vs H2O2, Incubation Period
Hold Values
Temperature 20
Veratryl alcohol 100
FeSO4 0.055
MgSO4 0.055
CuSO4 0.0505
Peptone 2
Enzyme Activity
H2O2
Incubation Period

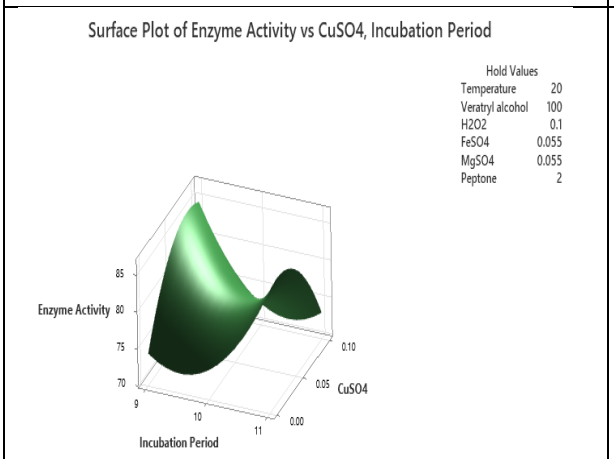

Contour Plot of Enzyme Activity vs H2O2, Incubation Period
Enzyme Activity
< 64
64 – 68
68 – 72
72 – 76
76 – 80
80 – 84
> 84
Hold Values
Temperature 20
Veratryl alcohol 100
FeSO4 0.055
MgSO4 0.055
CuSO4 0.0505
Peptone 2
H2O2
Incubation Period

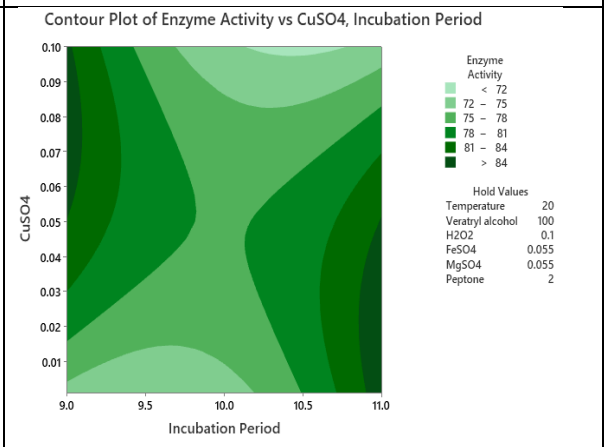

Surface Plot of Enzyme Activity vs FeSO4, Incubation Period
Hold Values
Temperature 20
Veratryl alcohol 100
H2O2 0.1
MgSO4 0.055
CuSO4 0.0505
Peptone 2
Enzyme Activity
FeSO4
Incubation Period

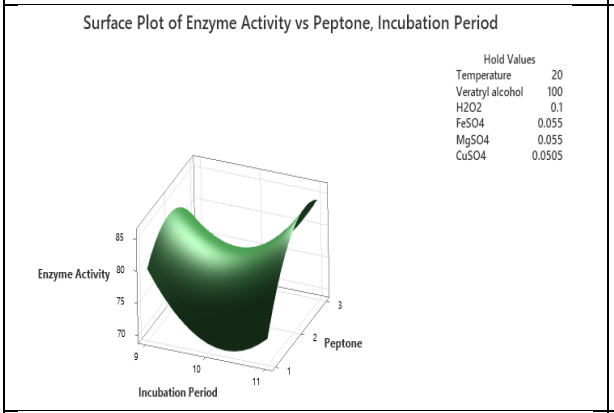

Contour Plot of Enzyme Activity vs FeSO4, Incubation Period
Enzyme Activity
< 70
70 – 75
75 – 80
80 – 85
85 – 90
90 – 95
> 95
Hold Values
Temperature 20
Veratryl alcohol 100
H2O2 0.1
MgSO4 0.055
CuSO4 0.0505
Peptone 2
FeSO4
Incubation Period

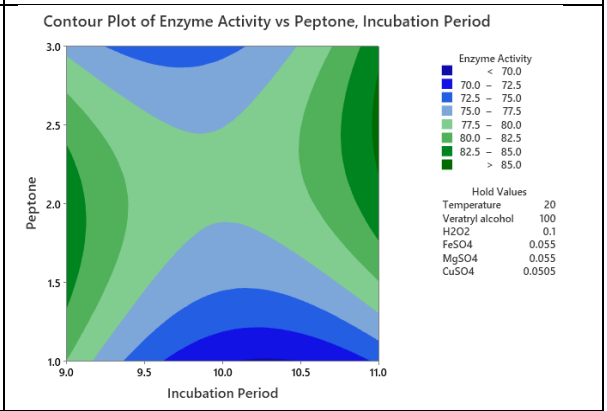

Surface Plot of Enzyme Activity vs MgSO4, Incubation Period
Hold Values
Temperature 20
Veratryl alcohol 100
H2O2 0.1
FeSO4 0.055
CuSO4 0.0505
Peptone 2
Enzyme Activity
Incubation Period
MgSO4

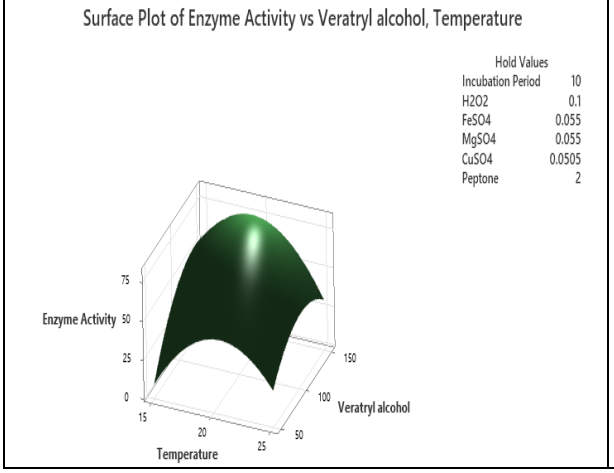

Contour Plot of Enzyme Activity vs MgSO4, Incubation Period
Enzyme Activity
< 76
76 – 78
78 – 80
80 – 82
82 – 84
> 84
Hold Values
Temperature 20
Veratryl alcohol 100
H2O2 0.1
FeSO4 0.055
CuSO4 0.0505
Peptone 2
MgSO4
Incubation Period

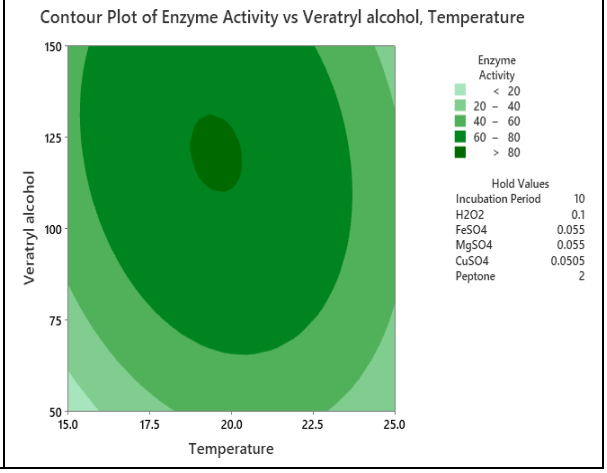

Surface Plot of Enzyme Activity vs CuSO4, Incubation Period
Hold Values
Temperature 20
Veratryl alcohol 100
H2O2 0.1
FeSO4 0.055
MgSO4 0.055
Peptone 2
Enzyme Activity
Incubation Period
CuSO4

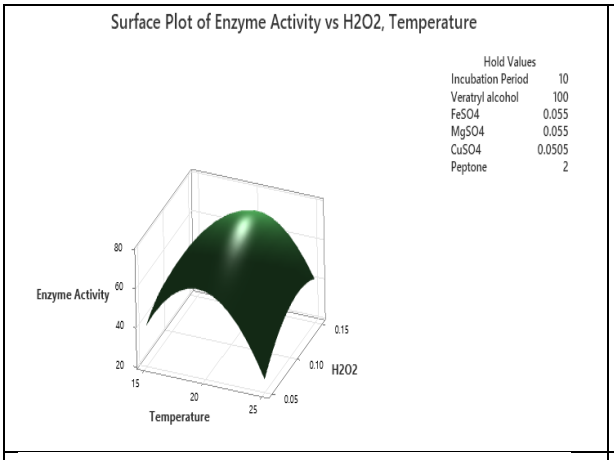

Contour Plot of Enzyme Activity vs CuSO4, Incubation Period
Enzyme Activity
< 72
72 – 75
75 – 78
78 – 81
81 – 84
> 84
Hold Values
Temperature 20
Veratryl alcohol 100
H2O2 0.1
FeSO4 0.055
MgSO4 0.055
Peptone 2
CuSO4
Incubation Period

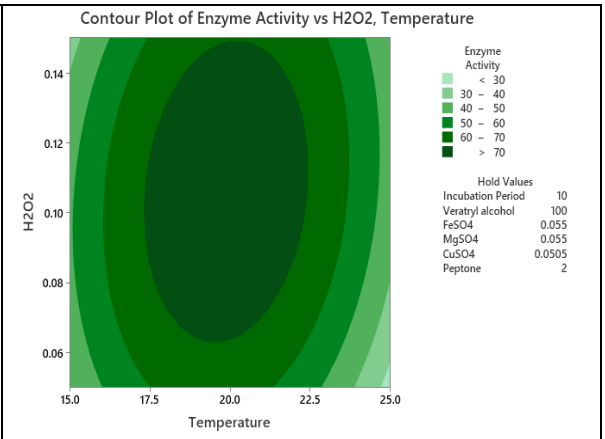

Surface Plot of Enzyme Activity vs Peptone, Incubation Period
Hold Values
Temperature 20
Veratryl alcohol 100
H2O2 0.1
FeSO4 0.055
MgSO4 0.055
CuSO4 0.0505
Enzyme Activity
Incubation Period
Peptone

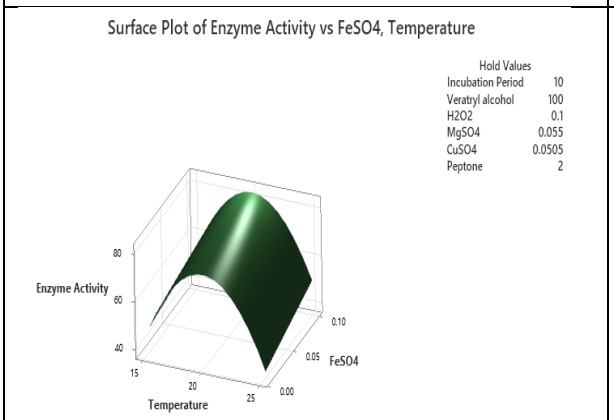

Contour Plot of Enzyme Activity vs Peptone, Incubation Period
Enzyme Activity
< 70.0
70.0 – 72.5
72.5 – 75.0
75.0 – 77.5
77.5 – 80.0
80.0 – 82.5
82.5 – 85.0
> 85.0
Hold Values
Temperature 20
Veratryl alcohol 100
H2O2 0.1
FeSO4 0.055
MgSO4 0.055
CuSO4 0.0505
Peptone
Incubation Period

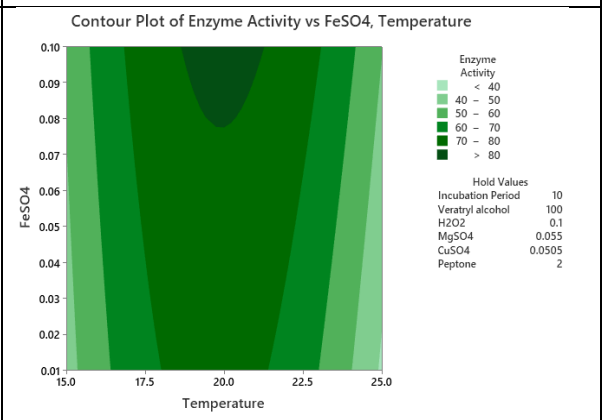

Surface Plot of Enzyme Activity vs Veratryl alcohol, Temperature
Hold Values
Incubation Period 10
H2O2 0.1
FeSO4 0.055
MgSO4 0.055
CuSO4 0.0505
Peptone 2
Enzyme Activity
Temperature
Veratryl alcohol

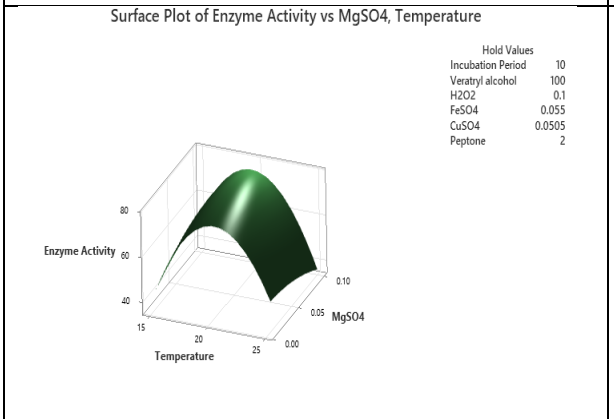

Contour Plot of Enzyme Activity vs Veratryl alcohol, Temperature
Enzyme Activity
< 20
20 – 40
40 – 60
60 – 80
> 80
Hold Values
Incubation Period 10
H2O2 0.1
FeSO4 0.055
MgSO4 0.055
CuSO4 0.0505
Peptone 2
Veratryl alcohol
Temperature

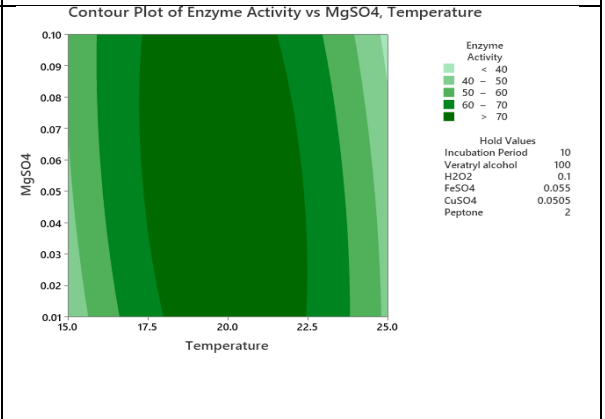

Surface Plot of Enzyme Activity vs H2O2, Temperature
Hold Values
Incubation Period 10
Veratryl alcohol 100
FeSO4 0.055
MgSO4 0.055
CuSO4 0.0505
Peptone 2
Enzyme Activity
Temperature
H2O2

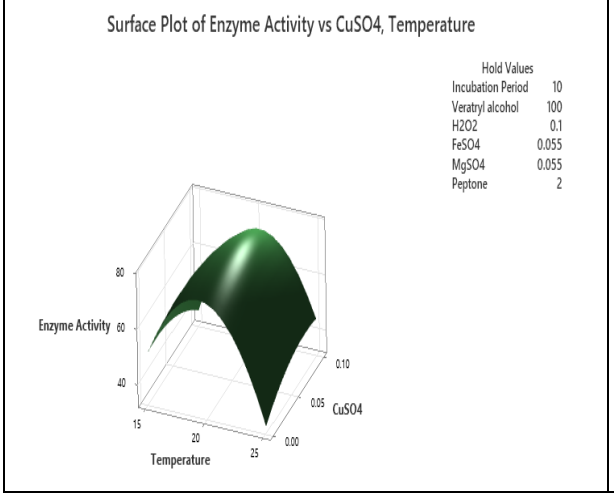

Contour Plot of Enzyme Activity vs H2O2, Temperature
Enzyme Activity
< 30
30 – 40
40 – 50
50 – 60
60 – 70
> 70
Hold Values
Incubation Period 10
Veratryl alcohol 100
FeSO4 0.055
MgSO4 0.055
CuSO4 0.0505
Peptone 2
H2O2
Temperature

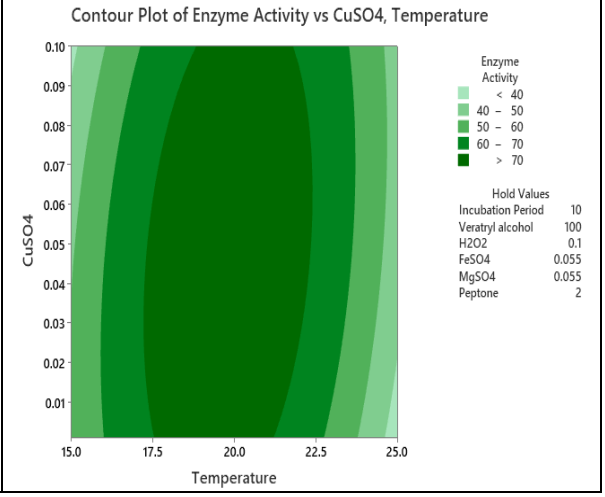

Surface Plot of Enzyme Activity vs FeSO4, Temperature
Hold Values
Incubation Period 10
Veratryl alcohol 100
H2O2 0.1
MgSO4 0.055
CuSO4 0.0505
Peptone 2
Enzyme Activity
Temperature
FeSO4

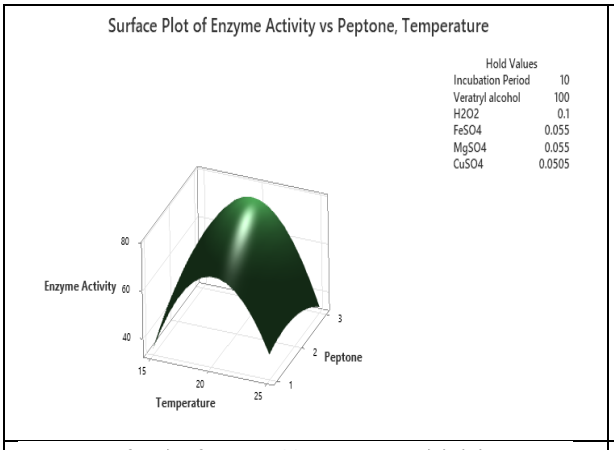

Contour Plot of Enzyme Activity vs FeSO4, Temperature
Enzyme Activity
< 40
40 – 50
50 – 60
60 – 70
70 – 80
> 80
Hold Values
Incubation Period 10
Veratryl alcohol 100
H2O2 0.1
MgSO4 0.055
CuSO4 0.0505
Peptone 2
FeSO4
Temperature

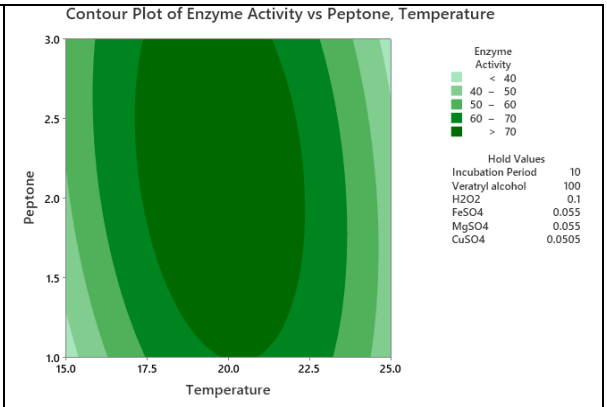

Surface Plot of Enzyme Activity vs MgSO4, Temperature
Hold Values
Incubation Period 10
Veratryl alcohol 100
H2O2 0.1
FeSO4 0.055
CuSO4 0.0505
Peptone 2
Enzyme Activity
Temperature
MgSO4

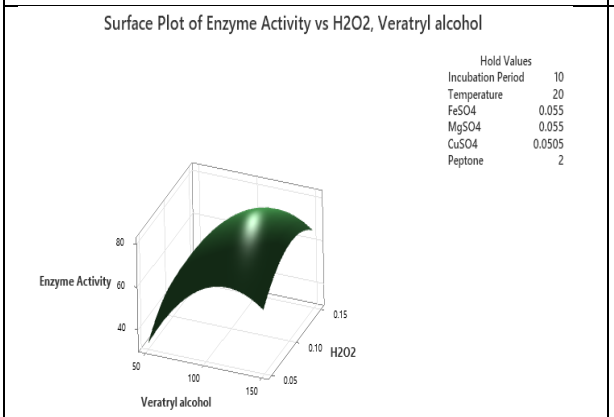

Contour Plot of Enzyme Activity vs MgSO4, Temperature
Enzyme Activity
< 40
40 – 50
50 – 60
60 – 70
> 70
Hold Values
Incubation Period 10
Veratryl alcohol 100
H2O2 0.1
FeSO4 0.055
CuSO4 0.0505
Peptone 2
MgSO4
Temperature

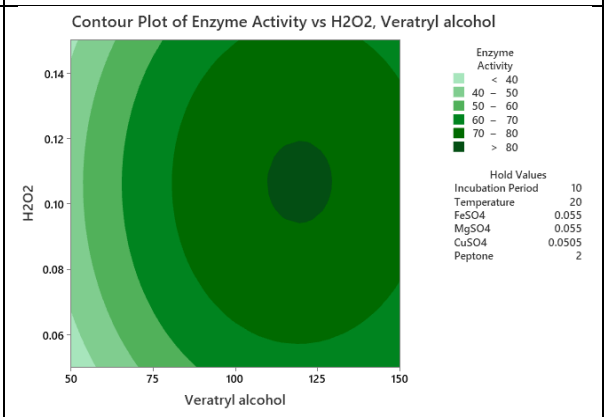

Surface Plot of Enzyme Activity vs CuSO4, Temperature
Hold Values
Incubation Period 10
Veratryl alcohol 100
H2O2 0.1
FeSO4 0.055
MgSO4 0.055
Peptone 2
Enzyme Activity
Temperature
CuSO4

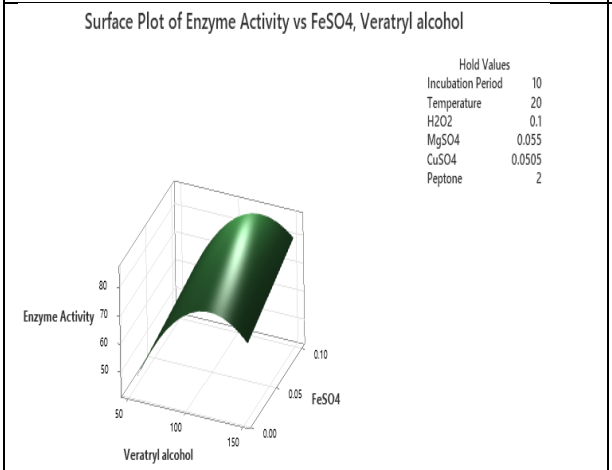

Contour Plot of Enzyme Activity vs CuSO4, Temperature
Enzyme Activity
< 40
40 – 50
50 – 60
60 – 70
> 70
Hold Values
Incubation Period 10
Veratryl alcohol 100
H2O2 0.1
FeSO4 0.055
MgSO4 0.055
Peptone 2
CuSO4
Temperature

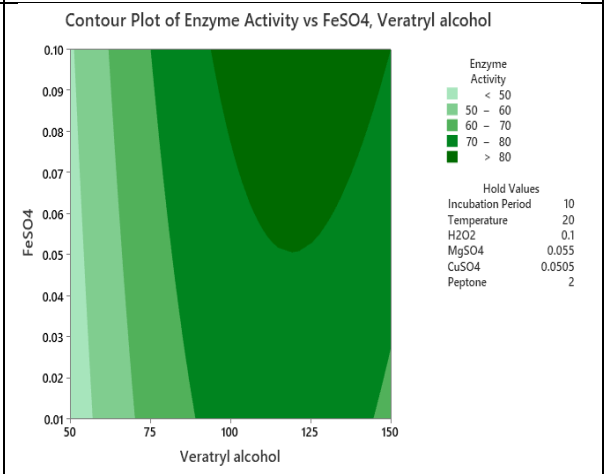

Surface Plot of Enzyme Activity vs Peptone, Temperature
Hold Values
Incubation Period 10
Veratryl alcohol 100
H2O2 0.1
FeSO4 0.055
MgSO4 0.055
CuSO4 0.0505
Enzyme Activity
Temperature
Peptone

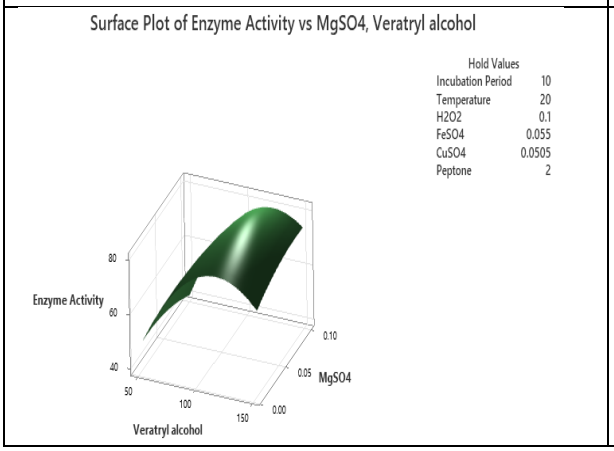

Contour Plot of Enzyme Activity vs Peptone, Temperature
Enzyme Activity
< 40
40 – 50
50 – 60
60 – 70
> 70
Hold Values
Incubation Period 10
Veratryl alcohol 100
H2O2 0.1
FeSO4 0.055
MgSO4 0.055
CuSO4 0.0505
Peptone
Temperature

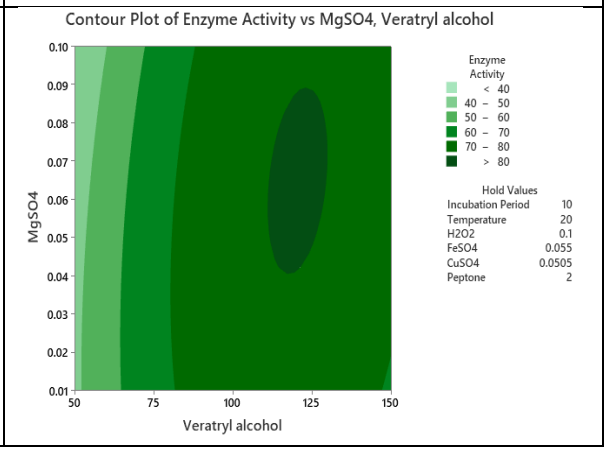

Surface Plot of Enzyme Activity vs H2O2, Veratryl alcohol
Hold Values
Incubation Period 10
Temperature 20
FeSO4 0.055
MgSO4 0.055
CuSO4 0.0505
Peptone 2
Enzyme Activity
Veratryl alcohol
H2O2


Contour Plot of Enzyme Activity vs H2O2, Veratryl alcohol
Enzyme Activity
< 40
40 – 50
50 – 60
60 – 70
70 – 80
> 80
Hold Values
Incubation Period 10
Temperature 20
FeSO4 0.055
MgSO4 0.055
CuSO4 0.0505
Peptone 2
H2O2
Veratryl alcohol


Surface Plot of Enzyme Activity vs FeSO4, Veratryl alcohol
Hold Values
Incubation Period 10
Temperature 20
H2O2 0.1
MgSO4 0.055
CuSO4 0.0505
Peptone 2
Enzyme Activity
Veratryl alcohol
FeSO4

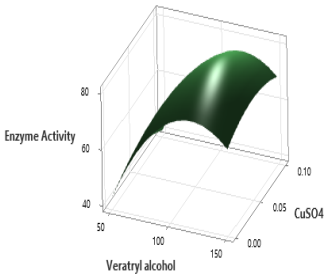

Contour Plot of Enzyme Activity vs FeSO4, Veratryl alcohol
Enzyme Activity
< 50
50 – 60
60 – 70
70 – 80
> 80
Hold Values
Incubation Period 10
Temperature 20
H2O2 0.1
MgSO4 0.055
CuSO4 0.0505
Peptone 2
FeSO4
Veratryl alcohol


Surface Plot of Enzyme Activity vs MgSO4, Veratryl alcohol
Hold Values
Incubation Period 10
Temperature 20
H2O2 0.1
FeSO4 0.055
CuSO4 0.0505
Peptone 2
Enzyme Activity
Veratryl alcohol
MgSO4

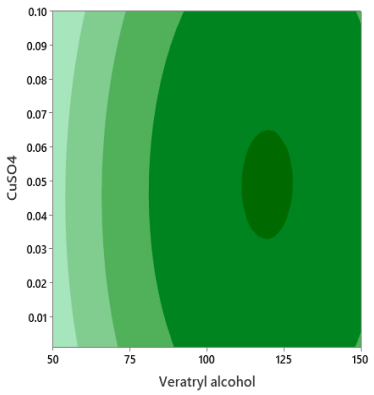

Contour Plot of Enzyme Activity vs MgSO4, Veratryl alcohol
Enzyme Activity
< 40
40 – 50
50 – 60
60 – 70
70 – 80
> 80
Hold Values
Incubation Period 10
Temperature 20
H2O2 0.1
FeSO4 0.055
CuSO4 0.0505
Peptone 2
MgSO4
Veratryl alcohol

Surface Plot of Enzyme Activity vs CuSO4, Veratryl alcohol

Hold Values	
Incubation Period	10
Temperature	20
H2O2	0.1
FeSO4	0.055
MgSO4	0.055
Peptone	2



Contour Plot of Enzyme Activity vs CuSO4, Veratryl alcohol

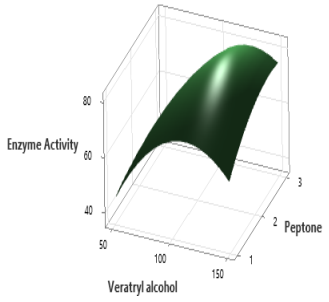


Enzyme Activity
< 50
50 – 60
60 – 70
70 – 80
> 80

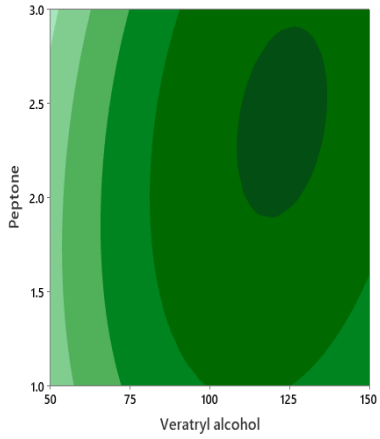
Hold Values	
Incubation Period	10
Temperature	20
H2O2	0.1
FeSO4	0.055
MgSO4	0.055
Peptone	2

Surface Plot of Enzyme Activity vs Peptone, Veratryl alcohol

Hold Values	
Incubation Period	10
Temperature	20
H2O2	0.1
FeSO4	0.055
MgSO4	0.055
CuSO4	0.0505



Contour Plot of Enzyme Activity vs Peptone, Veratryl alcohol

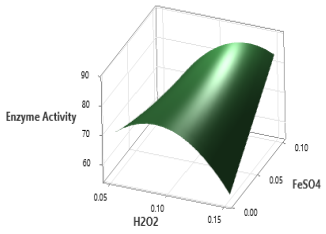


Enzyme Activity
< 40
40 – 50
50 – 60
60 – 70
70 – 80
> 80

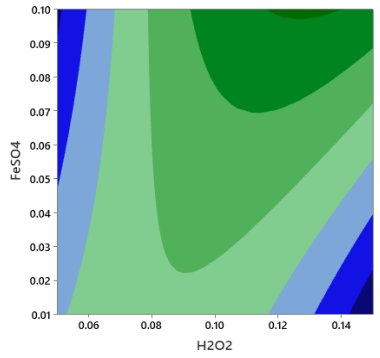
Hold Values	
Incubation Period	10
Temperature	20
H2O2	0.1
FeSO4	0.055
MgSO4	0.055
CuSO4	0.0505

Surface Plot of Enzyme Activity vs FeSO4, H2O2

Hold Values	
Incubation Period	10
Temperature	20
Veratryl alcohol	100
MgSO4	0.055
CuSO4	0.0505
Peptone	2



Contour Plot of Enzyme Activity vs FeSO4, H2O2

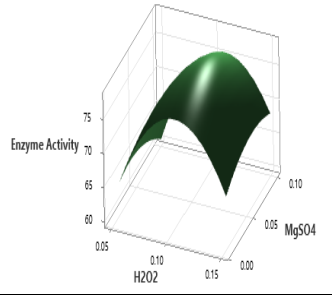


Enzyme Activity
< 60
60 – 65
65 – 70
70 – 75
75 – 80
80 – 85
> 85

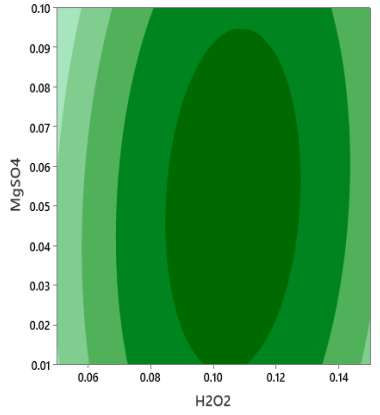
Hold Values	
Incubation Period	10
Temperature	20
Veratryl alcohol	100
MgSO4	0.055
CuSO4	0.0505
Peptone	2

Surface Plot of Enzyme Activity vs MgSO4, H2O2

Hold Values	
Incubation Period	10
Temperature	20
Veratryl alcohol	100
FeSO4	0.055
CuSO4	0.0505
Peptone	2



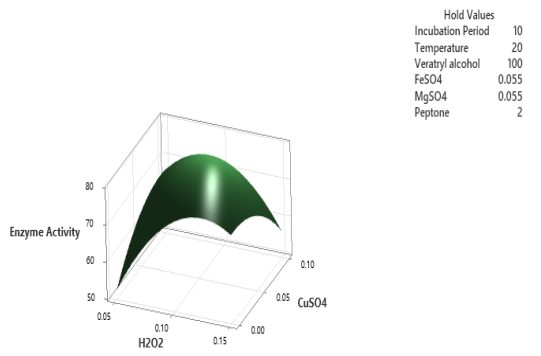
Contour Plot of Enzyme Activity vs MgSO4, H2O2



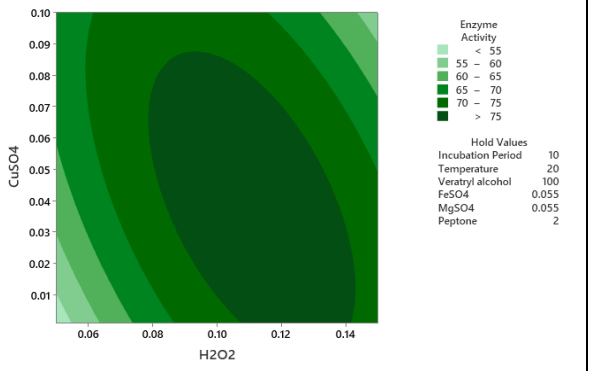
Enzyme Activity
< 64
64 – 68
68 – 72
72 – 76
> 76

Hold Values	
Incubation Period	10
Temperature	20
Veratryl alcohol	100
FeSO4	0.055
CuSO4	0.0505
Peptone	2

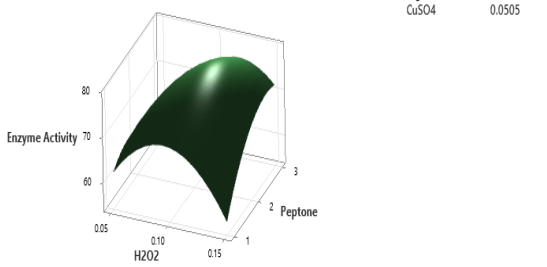
Surface Plot of Enzyme Activity vs CuSO4, H2O2



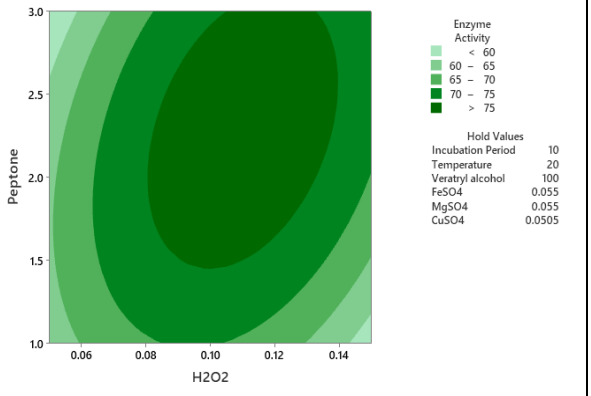
Contour Plot of Enzyme Activity vs CuSO4, H2O2



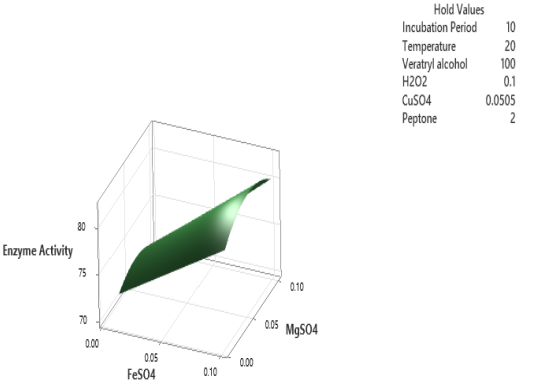
Surface Plot of Enzyme Activity vs Peptone, H2O2



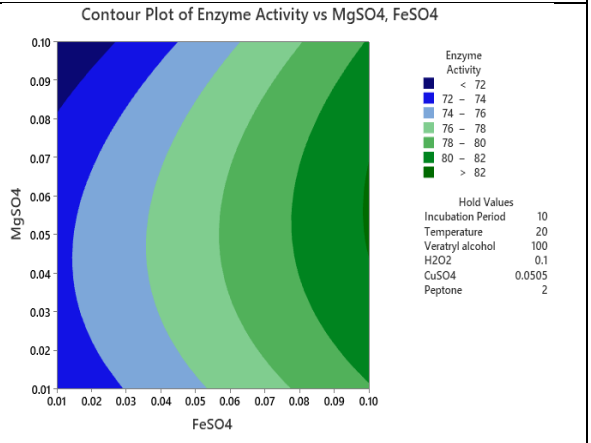
Contour Plot of Enzyme Activity vs Peptone, H2O2



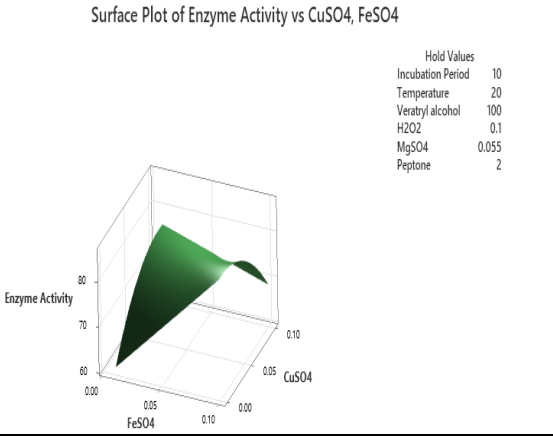
Surface Plot of Enzyme Activity vs MgSO4, FeSO4



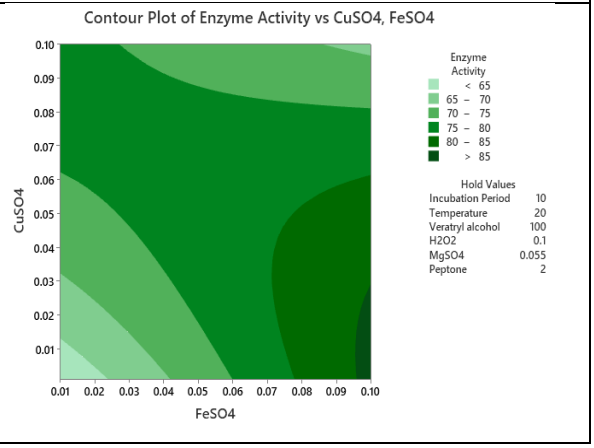
Contour Plot of Enzyme Activity vs MgSO4, FeSO4



Surface Plot of Enzyme Activity vs CuSO4, FeSO4



Contour Plot of Enzyme Activity vs CuSO4, FeSO4



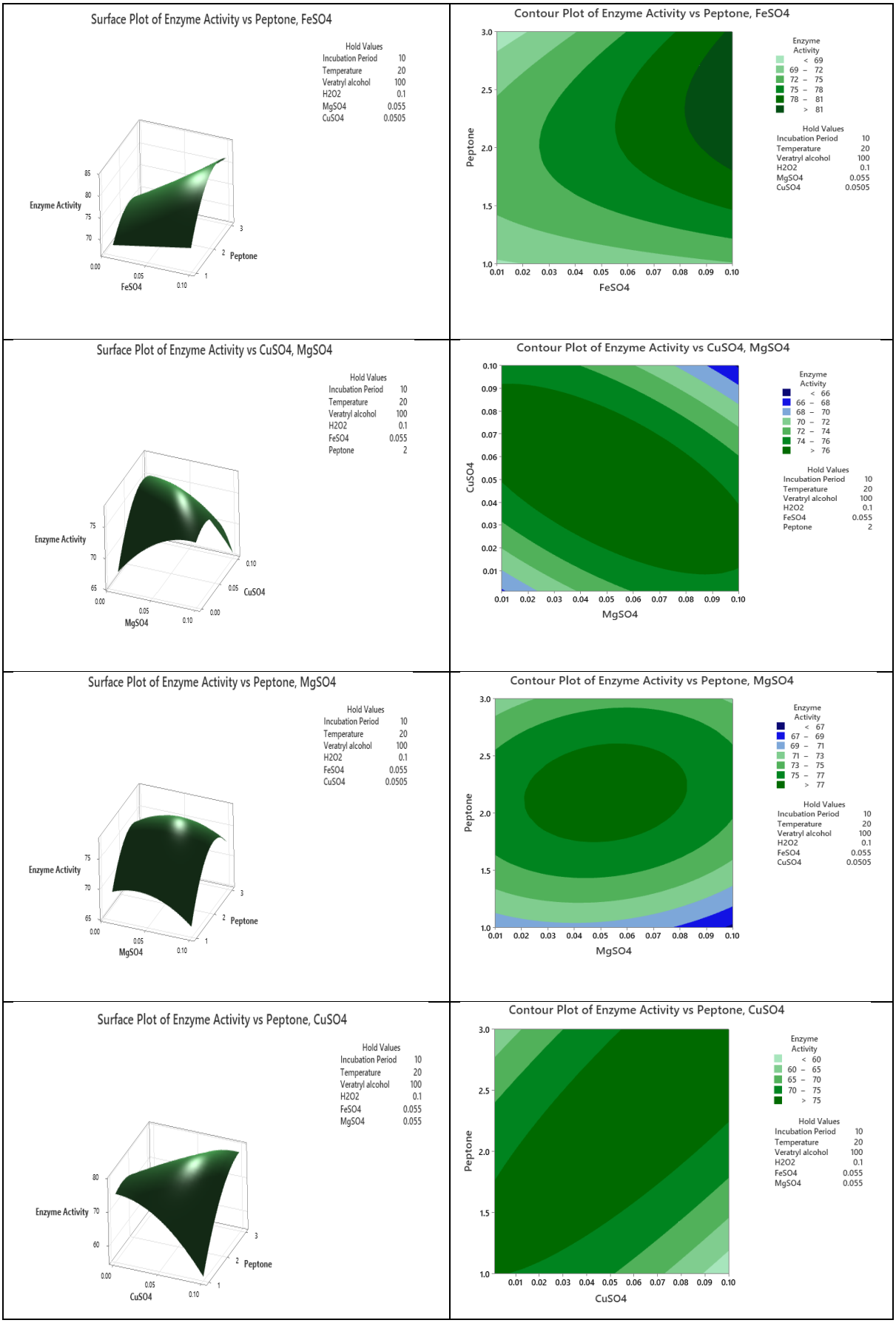


Figure 2: Response surface plot of enzyme activity *versus* Incubation Period, Temperature, Veratryl alcohol, H_2O_2, $FeSO_4$, $MgSO_4$, $CuSO_4$ and Peptone

Response Optimization:
The optimal conditions for each factor to maximize enzyme activity were determined using the desirability function approach. Following optimization, the stationary point was identified at an 11-day incubation period, a temperature of 18°C, 150mM veratryl alcohol, 0.148mM H_2O_2, 0.1 g/L $FeSO_4$, 0.1 g/L $MgSO_4$, 0.001 g/L $CuSO_4$, and 3.00 g/L peptone. Under these conditions, the maximum enzyme activity achieved was 115.8, with a desirability value of 1, as shown in Figure 3.

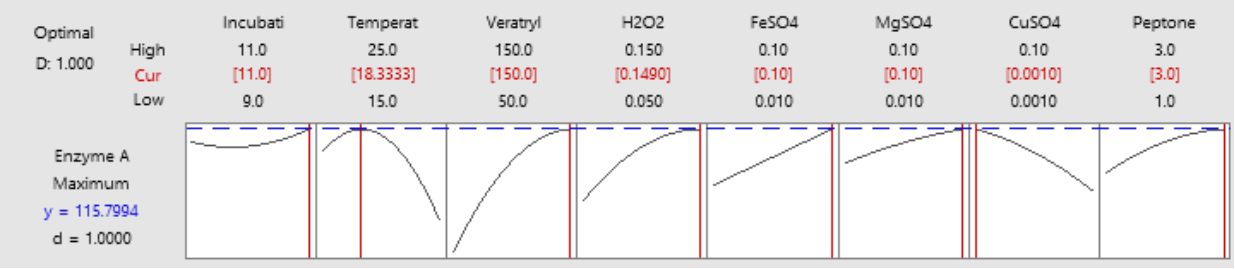


Figure 3: Optimization of parameters using the desirability function approach

CONCLUSION

The soils collected from the Nashik district in Maharashtra, India, are a rich source of lignocellulolytic fungi with high lignin peroxidase activity, according to the current study, which also showed that the strain LP8i has the potential to produce a lot of lignin peroxidase enzyme. *Purpureocillium lilacinum* (LP8i) is isolated from soil and identified using ITS region sequencing. Under unoptimized conditions, *Purpureocillium lilacinum* shows 80U/ml enzyme activity. In optimized media conditions using RSM *Purpureocillium lilacinum* shows lignin peroxidase activity 105.947U/ml. The enzyme production enhances over the un-optimized condition. The enzyme from this isolated fungus can be used for broad-spectrum applications such as bioethanol production, dying industries, toxicity reduction, wastewater treatment, and the cosmetic industry. However, additional work related to protein characterizations and pilot-scale production of the LiP enzyme from this fungus needs to be studied.

Acknowledgment:
The authors thank the Principal, K.T.H.M. College, Nashik as well as M.V. P's K.S.K.W.A.S.C College CIDCO, Nashik for providing all the facilities for conduct of the work.

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