Available online www.jocpr.com

Journal of Chemical and Pharmaceutical Research, 2016, 8(7):576-584

Research Article

ISSN : 0975-7384
CODEN(USA) : JCPRC5

Statistical optimization of cultural parameters influencing laccase production by *Pleurotus ostreatus* PKN 04 using response surface methodology

T. V. Preethi[1], A. K. Kathireshan[2*] and G. Narendrakumar[3]

[1]Research Scholar, Department of Microbiology, School of Life Science, Vels University, Chennai – 600 117, Tamilnadu, India
[2]Department of Microbiology, School of Life Science, Vels University, Chennai – 600 117, Tamilnadu, India
[3]Department of Biotechnology, Faculty of Bio and Chemical Engineering, Sathyabama University, Chennai – 600119, Tamilnadu, India

ABSTRACT

Box- Behnken experimental design for cultural optimization engages a specific study on the area of individual factors such as temperature, pH, Agitation, Inoculum size and Incubation time. Production of laccase increased by modification of cultural conditions. In this study, RSM-BB was used for optimizing culture media for maximizing laccase production by using Pleurotus ostreatus in batch fermentation. The optimized cultural conditions increased two fold of laccase production. 46 experiments runs were attained for optimum enzyme production and the coefficient of determination was 99.1% with adjusted R^2 value of 98.3 and predicted with 96.5 for enzyme production from Pleurotus ostreatus PKN04. For the biomass production the R^2 was 98.6%, adjusted and predicted R^2 were 97.5% and 94.65% respectively. This shows the significance of the model and effective production of enzyme from Pleurotus ostreatus.

Keywords: *Pleurotus ostreatus* PKN 04, ABTS, Optimization, RSM-CCD.

INTRODUCTION

Laccase (benzenediol: oxygen oxidoreductase, EC 1.10.3.2) belongs to the group of enzymes called the blue copper oxidases or the blue copper proteins. These proteins are characterized by containing 4 catalytic copper atoms [1 - 5].

Relating to the use in the industrial biotechnology, fungal laccases have widespread applications, ranging from effluent decolouration and detoxification to pulp bleaching, removal of phenolic from wines, organic synthesis, biosensors, synthesis of complex medical compounds and dye transfer blocking functions in detergents and washing powders, many of which have been patented [6-9].

The main purpose of this study is to reduce the production cost of laccase by optimizing the cultural conditions so that the maximum productivity can be achieved [10-16]. In the present investigation, *Pleurotus ostreatus* PKN04 was identified as a potential candidate for the production of laccase and its cultural conditions were optimized.

EXPERIMENTAL SECTION

Organism

Pleurotus ostreatus PKN 04 was isolated from the decomposed wood and leaf litters of Chennai forest and grown in SDA. The organism was screened for the production of laccase by ABTS method, subcultured to obtain pure culture and identified using 18s rRNA sequencing method [17].

Pleurotus ostreatus PKN 04 was grown in Malt extract medium (MEA) and the spores were washed and inoculated in to the fermentation medium [18].

Calculations and statistics
A statistically significant difference between means was determined according to Student's t-test at a probability level of 0.05. The statistical analyses were performed by SPSS Inc., 2014 [19-22].

Optimization of laccase Production

Effect of initial pH on laccase production
Equal volume of the fungal spore was inoculated in minimal media with various initial pH viz., 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, 9, 9.5 and 10. The flasks were incubated at 30°C for 60 h. The laccase production was estimated after incubation. The initial pH at which maximum production of laccase observed was chosen and maintained in the following studies [24-25].

Effect of temperature on laccase production
Fungal isolate was inoculated into minimal media and incubated at different temperature viz., 10, 15, 20, 25, 30, 35, 40, 45, 50, 55 and 60°C for 120 h. The laccase was estimated after incubation by ABTS assay [26-27].

Effect of agitation on laccase production
Pleurotus ostreatus PKN 04 was inoculated into minimal media and incubated at different agitation 0, 50, 100 and 150 rpm for 120 h. The laccase was estimated after incubation [28-29].

Optimization of cultural conditions by Response Surface Methodology (RSM)
RSM is a statistical experimental method used under suitable experimental design to determine multi-variable equations and establish the relationship among the contributing parameters and the responses acquired. In comparison to the conventional mathematical or one factor at a time method, RSM is time saving and economical [30]. The Box-Behnken design was applied under RSM using Design Expert Version 7.0.0 software [31].

Five factors at three different levels were used in duplicate. Three concentration of pH (4-8), temperature (20-40^{o}C), agitation (50-150 rpm), inoculum size (0.5-1.5 g) and incubation time (4-10 days) were selected as the critical variables and nominated as A, B, C, D and E respectively (Table-1, Table - 2). A total number of 46 runs were carried out to estimate the coefficients for the optimization of cultural condition [32]. The data were displayed to Analysis of Variance (ANOVA) and 3 dimensional response surface graphs were constructed Design Expert Version 7.0.0 programs to study the responses (Enzyme activity and Biomass) and interactions between variables (Table - 4 & Table - 6). The quality of the fit of this model was expressed by the coefficient of determination (R^2) [33].

The general quadratic equation

$$Y = \rho\ 0 + \rho\ 1A + \rho\ 2B + \rho\ 3C + \rho\ 4D + \rho 5E + \rho\ 6A^2 + \rho\ 7B^2 + \rho\ 8C^2 + \rho\ 9D^2 + + \rho\ 10E^2 + \rho\ 11AB + \rho 12\ AC + \rho 13AD + \rho 14AE + \rho 15BC + \rho 16BD + \rho 17BE + \rho 18CD + \rho 19CE + \rho 20DE \quad (1)$$

The quadratic equation where Y is the measured response, A, B, C, D and E are the coded independent input variables, ρ0 is the intercept term, ρ1, ρ2, ρ3, ρ4 and ρ5 are the coefficients showing the linear effects, ρ6, ρ7, ρ8, ρ9 and ρ10, are the quadratic coefficients showing the squared effects and ρ11, ρ12, ρ13, ρ14, ρ15, ρ16, ρ17, ρ18, ρ19 and ρ20 are the cross product coefficients showing the interaction effects.

Table – 1 Design summary

Study Type	Response Surface
Initial Design	Box-Behnken
Design Model	Quadratic
No of Runs	46

Table -2 Coded and Actual values of variables used in RSM

Factor	Name	Unit	Low		High		Mean	Std. Dev.
			Coded	Actual	Coded	Actual		
A	pH		-1	4	1	8	6	1.179536
B	Temperature	C	-1	20	1	40	30	5.897678
C	Agitation	Rpm	-1	50	1	150	100	29.48839
D	Inoculum size	Mg	-1	0.5	1	1.5	1	0.294884
E	Incubation period	Days	-1	4	1	10	7	1.769303

Table -3 Response computation

Response	Unit	Analysis	Min	Max	Mean	Std. Dev.	Ratio	Model
Enzyme Activity	IU/ml	Polynomial	24.3	98.1	63.77391	19.735	4.0370	Quadratic
Biomass	G	Polynomial	1.3	5.4	3.569565	1.0858	4.1538	Quadratic

Laccase assay

Laccase activity was defined by the oxidation of ABTS. The non-phenolic dye ABTS (2, 2´-azino-bis- [3 – ethyl benzothiazoline – 6 –sulphonic acid]) was oxidized by laccase produced by *Pleurotus ostreatus* PKN 04 to the more stable condition of the cation radical. The intense blue-green colour formed was correlated to enzyme activity and read at 420nm.

The mixture contained 0.5mM ABTS, 0.1M sodium acetate (pH 4.5), and an appropriate amount of enzyme. Oxidation of ABTS was observed by determining the increase in A420 (ε420, 3.6×10^4 $M^{-1}cm^{-1}$). The reaction mixture contained 0.5mM substrate (ABTS), 2.8 mL of 0.1 M sodium acetate buffer of pH 4.5, and 100 µL of culture supernatant and incubated for 5 min. Absorbance was read at 420 nm in a spectrophotometer against a suitable blank [35].

One unit was expressed as the amount of the laccase that oxidized 1 µmol of ABTS substrate per min [16]. The absorbance was read after 10 min interval using UV/VIS spectrophotometer (Varian Cary® 100 UV-Vis) [36].

Protein estimation was performed using Lowry et al 1951[19].

Biomass production

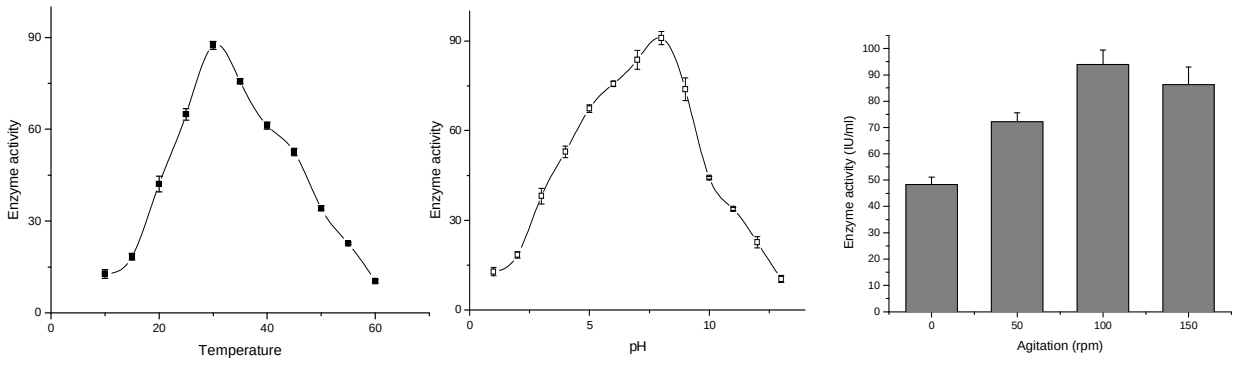
The fungal mycelium was harvested after every 120 hours of growth, cell free filtrate was obtained by filtration through a Whatman No. 1 filter paper. The fungal biomass was repeatedly washed with distilled water and dried at 70°C overnight. The dry weight of the fungus was calculated by using the following formula:

$$\text{Dry weight} = \frac{\text{weight of filter paper} + \text{mycelium}}{\text{weight of filter paper}}$$

Duplicated were used and the average was calculated for minimize the error [37].

RESULTS AND DISCUSSION

The organism isolated was identified as *Pleurotus ostreatus* PKN04 using 18s rRNA analysis and the accession number from NCBI was KX151954. The organism was subjected to different temperature and the enzyme activity was estimated to identify the ideal effect. The optimum temperature was found to be 30ºC and maintaining at the same the pH was optimized. Agitation was adjusted using fixed temperature and pH.



(a) **(b)** **(c)**

Figure-1 Effect of temperature (a) pH (b) and agitation (c) on laccase activity

An aspect of Response surface methodology is the design of experiments, explained by Box and Draper, 1987 [27]. These strategies were originally developed for the model fitting of physical experiments, but can also be applied to numerical experiments. The objective of DoE is the selection of the points where the response should be evaluated.

Optimization using a Box–Behnken design with RSM

In general, the application of a Box–Behnken design with RSM includes following steps. (a) An design of experiment (DOE) is provided based on the chosen conditions. (b) Statistically designed experiments are executed. (c) The coefficients in the mathematical model are estimated and the accuracy of the model is checked. (d) Response analysis is implemented to predict the optimal conditions, and these predictions are confirmed experimentally.

Table -4 BB matrix for laccase production for enzyme activity from *Pleurotus ostreatus* PKN04

Run	pH	Temp	Agitation	Inoculum size	Incubation Period	Enzyme Activity		Residue
						Actual	Predicted	
		C	rpm	mg	Days	IU/ml		
1	4	30	50	1	7	51.2	49.84	1.35
2	6	30	150	1	10	98.1	92.09	6.008
3	6	30	100	1	7	91.1	93.05	-1.95
4	6	30	50	1	10	69.1	70.96	-1.86
5	6	20	100	0.5	7	66.4	66.57	-0.170
6	8	30	150	1	7	51.8	54.06	-2.266
7	6	20	100	1	10	89.2	86.67	2.527
8	6	30	100	1	7	94.1	93.05	1.05
9	6	30	150	1.5	7	72.6	74.30	-1.70
10	6	30	150	1	4	52.1	45.69	6.408
11	6	30	100	0.5	4	43.3	43.71	-0.410
12	4	30	150	1	7	42.1	43.81	-1.71
13	6	20	100	1.5	7	76.2	76.40	-0.208
14	4	30	100	1	10	64.2	65.86	-1.66
15	4	30	100	1	4	24.3	25.46	-1.16
16	6	40	100	1	10	87.9	88.03	-0.13
17	6	30	100	1	7	93.2	93.05	0.15
18	6	30	100	0.5	10	59.6	61.36	-1.76
19	8	30	100	1.5	7	47.5	46.52	0.977
20	8	30	100	0.5	7	39.4	37.98	1.414
21	6	30	50	1.5	7	75.2	74.77	0.422
22	6	20	100	1	4	60.4	60.12	0.277
23	6	40	50	1	7	69.1	68.38	0.71
24	6	20	150	1	7	74.2	77.20	-3.00
25	4	40	100	1	7	45.9	45.23	0.664
26	8	30	50	1	7	37.4	36.59	0.808
27	6	40	150	1	7	72.6	74.56	-1.964
28	8	20	100	1	7	47.6	46.82	0.777
29	6	30	50	0.5	7	48.1	47.73	0.360
30	8	30	100	1	10	53.2	54.96	-1.76
31	6	30	100	1	7	92.4	93.05	-0.65
32	6	40	100	0.5	7	53.4	52.48	0.916
33	6	40	100	1	4	50.2	52.58	-2.38
34	6	30	150	0.5	7	57.9	59.66	-1.76
35	8	40	100	1	7	56.8	55.48	1.314
36	6	20	50	1	7	71.6	71.92	-0.327
37	6	30	50	1	4	53.9	55.36	-1.46
38	4	20	100	1	7	60.2	60.07	0.127
39	4	30	100	1.5	7	61.3	60.32	0.977
40	4	30	100	0.5	7	28.6	27.18	1.414
41	8	30	100	1	4	32.1	33.36	-1.26
42	6	40	100	1.5	7	85.2	84.32	0.87
43	6	30	100	1	7	92.3	93.05	-0.75
44	6	30	100	1	7	95.2	93.05	2.15
45	6	30	100	1.5	10	94.2	95.54	-1.34
46	6	30	100	1.5	4	51.2	51.19	0.002

During the experimental study, the temperature was varied between 20 and 40°C along with pH 4-8, Agitation of 50-150 rpm, inoculum size of 0.5-1.5 g and Incubation time of 4-10 days. Table 4 shows the ANOVA of regression parameters of the predicted response surface quadratic model for enzyme activity.

The experiment was performed based on the experimental design and run is shown in Table 5. The model F-value of 138.44 and a low probability value-(p-value) (Prob >F) less than 0.001 indicate that model terms are significant, while lack of fit is not significant shows the functionality of the model.

Table -5 ANOVA for laccase production for enzyme activity from *Pleurotus ostreatus* PKN04

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	17755.77	20	887.7883	138.5589	< 0.0001 Significant
A-pH	9	1	9	1.404648	0.2471
B-Temperature	38.13063	1	38.13063	5.951122	0.0221
C-Agitation	131.1025	1	131.1025	20.46143	0.0001
D-Inoculum size	1736.806	1	1736.806	271.0667	< 0.0001
E-Incubation period	3844	1	3844	599.9407	< 0.0001
AB	138.0625	1	138.0625	21.54769	< 0.0001
AC	138.0625	1	138.0625	21.54769	< 0.0001
AD	151.29	1	151.29	23.61213	< 0.0001
AE	88.36	1	88.36	13.79052	0.0010
BC	0.2025	1	0.2025	0.031605	0.8603
BD	121	1	121	18.88471	0.0002
BE	19.8025	1	19.8025	3.090615	0.0910
CD	38.44	1	38.44	5.999407	0.0217
CE	237.16	1	237.16	37.01403	< 0.0001
DE	178.2225	1	178.2225	27.81554	< 0.0001
A^2	10114.71	1	10114.71	1578.622	< 0.0001
B^2	440.2	1	440.2	68.70289	< 0.0001
C^2	1458.41	1	1458.41	227.6169	< 0.0001
D^2	2234.764	1	2234.764	348.784	< 0.0001
E^2	1733.531	1	1733.531	270.5557	< 0.0001
Residual	160.1825	25	6.4073		
Lack of Fit	149.6475	20	7.482375	3.551198	0.0823 Not significant
Pure Error	10.535	5	2.107		
Cor Total	17915.95	45			
Source	**Std. Dev.**	**R^2**	**Adjusted R^2**	**Predicted R^2**	**PRESS**
Quadratic	2.531265	0.991059	0.983907	0.965742	613.7604

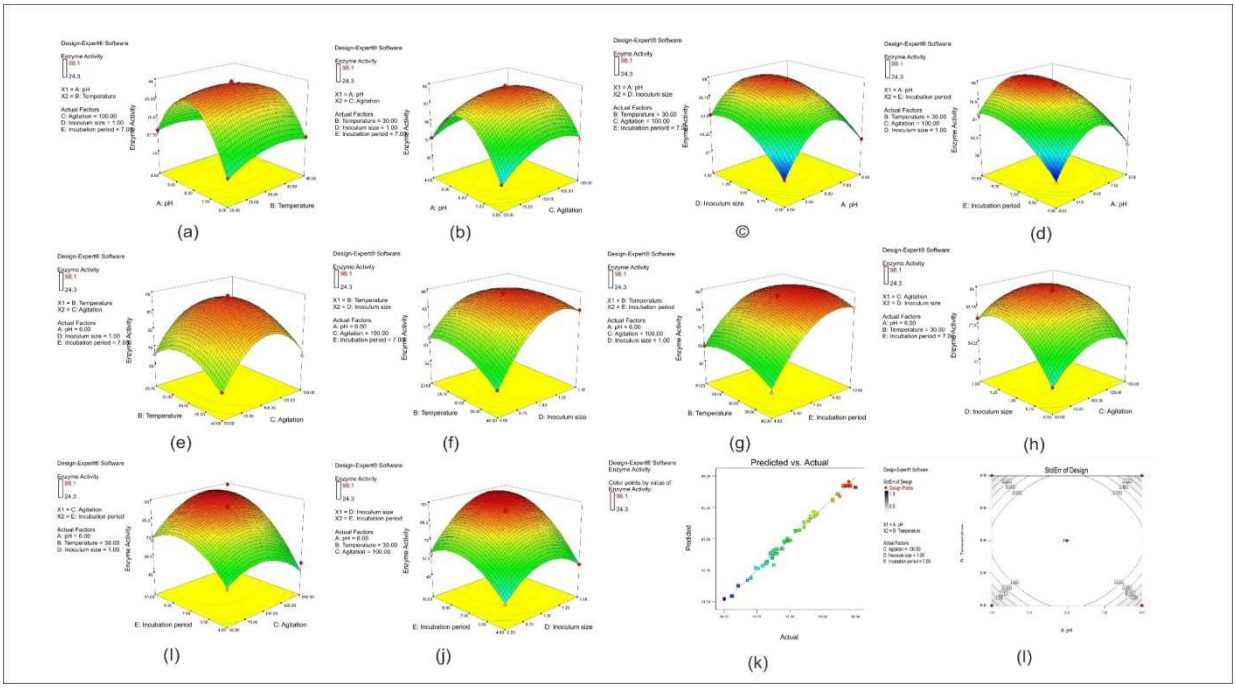


Figure -1 Response surface plots of interaction between process variables in enzyme activity by *Pleurotus ostreatus* PKN04
(a) pH vs temperature (b) pH vs Agitation (c) Inoculum size vs pH (d)Incubation period vs pH (e) Temperature vs Agitation (f) Temperature vs Inoculum size (g) Temperature vs Incubation period (h) Inoculum size vs Agitation (i) Incubation period vs Agitation (j) Incubation period vs Inoculum size (k) Predicted vs Actual (l) Standard error of Design

Model fitting and analysis of variance (ANOVA)

Experiments were performed using the Box–Behnken experimental design. The experimental and predicted R^2 are shown along with the experimental conditions in Table-3. Based on the model analysis in the first part, a quadratic

model was chosen to fit the data. The relationship between the enzyme activity and biomass production and the five chosen factors is shown in Eq. 1 and 2

Based on the BB analysis the final equation in term of coded factor

Enzyme Activity = 93.05 - 0.75 A - 1.54375 B + 2.8625 C + 10.41875 D + 15.5 E+ 5.875 AB + 5.875 AC - 6.15 AD - 4.7 AE + 0.225 BC + 5.5 BD + 2.225 BE - 3.1 CD + 7.7 CE + 6.675 DE - 34.0438A^2 - 7.10208B^2 - 12.9271C^2 - 16.0021 D^2 - 14.0938 E^2 (2)

Table -6 BB matrix for laccase production for biomass production from *Pleurotus ostreatus* PKN04

Run	Factor A	Factor B	Factor C	Factor D	Factor E	Biomass		Residue
						Actual	Predicted	
		C	rpm	Mg	Days	IU/ml		
1	4	30	50	1	7	2.9	2.81	0.09
2	6	30	150	1	10	5.4	5.07	0.33
3	6	30	100	1	7	5.1	5.20	-0.10
4	6	30	50	1	10	3.9	4.06	-0.16
5	6	20	100	0.5	7	3.6	3.68	-0.08
6	8	30	150	1	7	2.9	3.10	-0.20
7	6	20	100	1	10	4.9	4.85	0.05
8	6	30	100	1	7	5.2	5.20	0.00
9	6	30	150	1.5	7	4.1	4.15	-0.05
10	6	30	150	1	4	3.1	2.62	0.48
11	6	30	100	0.5	4	2.3	2.44	-0.14
12	4	30	150	1	7	2.2	2.33	-0.13
13	6	20	100	1.5	7	4.2	4.29	-0.09
14	4	30	100	1	10	3.4	3.54	-0.14
15	4	30	100	1	4	1.3	1.49	-0.19
16	6	40	100	1	10	4.8	4.87	-0.07
17	6	30	100	1	7	5.1	5.20	-0.10
18	6	30	100	0.5	10	3.5	3.54	-0.04
19	8	30	100	1.5	7	2.7	2.66	0.04
20	8	30	100	0.5	7	2.3	2.20	0.10
21	6	30	50	1.5	7	4.2	4.18	0.02
22	6	20	100	1	4	3.4	3.35	0.05
23	6	40	50	1	7	3.8	3.82	-0.02
24	6	20	150	1	7	4.1	4.26	-0.16
25	4	40	100	1	7	2.6	2.45	0.15
26	8	30	50	1	7	2.1	2.09	0.01
27	6	40	150	1	7	3.9	4.14	-0.24
28	8	20	100	1	7	2.7	2.65	0.05
29	6	30	50	0.5	7	2.8	2.77	0.03
30	8	30	100	1	10	3.2	3.21	-0.01
31	6	30	100	1	7	5.3	5.20	0.10
32	6	40	100	0.5	7	3.1	3.01	0.09
33	6	40	100	1	4	2.9	2.97	-0.07
34	6	30	150	0.5	7	3.3	3.33	-0.03
35	8	40	100	1	7	3.2	3.13	0.07
36	6	20	50	1	7	4.1	4.05	0.05
37	6	30	50	1	4	3.1	3.11	-0.01
38	4	20	100	1	7	3.4	3.28	0.12
39	4	30	100	1.5	7	3.3	3.29	0.01
40	4	30	100	0.5	7	1.6	1.52	0.08
41	8	30	100	1	4	1.8	1.86	-0.06
42	6	40	100	1.5	7	4.7	4.62	0.08
43	6	30	100	1	7	5.2	5.20	0.00
44	6	30	100	1	7	5.3	5.20	0.10
45	6	30	100	1.5	10	5.3	5.26	0.04
46	6	30	100	1.5	4	2.9	2.96	-0.06

During the experimental study, the temperature was varied between 20 and 40°C along with pH, Agitation, inoculum size and Incubation time. Table 7 shows the ANOVA of regression parameters of the predicted response surface quadratic model for biomass production.

The experiment was performed based on the experimental design and run is shown in Table 7. The model F-value of 89.095 and a low probability value-(p-value) (Prob >F) less than 0.001 indicate that model terms are significant, while lack of fit is not significant shows the functionality of the model.

Table -7 ANOVA for laccase production for *Pleurotus ostreatus* PKN04

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	53.48697	20	2.674349	89.09546	< 0.0001 Significant
A-pH	0.0025	1	0.0025	0.083287	0.7753
B-Temperature	0.1225	1	0.1225	4.081066	0.0542
C-Agitation	0.275625	1	0.275625	9.182399	0.0056
D-Inoculum size	4.950625	1	4.950625	164.9292	< 0.0001
E-Incubation period	11.56	1	11.56	385.1194	< 0.0001
AB	0.4225	1	0.4225	14.07551	0.0009
AC	0.5625	1	0.5625	18.73959	0.0002
AD	0.4225	1	0.4225	14.07551	0.0009
AE	0.1225	1	0.1225	4.081066	0.0542
BC	0.0025	1	0.0025	0.083287	0.7753
BD	0.25	1	0.25	8.328706	0.0079
BE	0.04	1	0.04	1.332593	0.2593
CD	0.09	1	0.09	2.998334	0.0957
CE	0.5625	1	0.5625	18.73959	0.0002
DE	0.36	1	0.36	11.99334	0.0019
A^2	31.64379	1	31.64379	1054.207	< 0.0001
B^2	1.545606	1	1.545606	51.4916	< 0.0001
C^2	4.430455	1	4.430455	147.5998	< 0.0001
D^2	6.745606	1	6.745606	224.7287	< 0.0001
E^2	5.185606	1	5.185606	172.7576	< 0.0001
Residual	0.750417	25	0.030017		
Lack of Fit	0.710417	20	0.035521	4.440104	0.0528 Not significant
Pure Error	0.04	5	0.008		
Cor Total	54.23739	45			
Source	**Std. Dev.**	**R^2**	**Adjusted R^2**	**Predicted R^2**	**PRESS**
Quadratic	0.17	0.9862	0.9751	0.9465	2.90

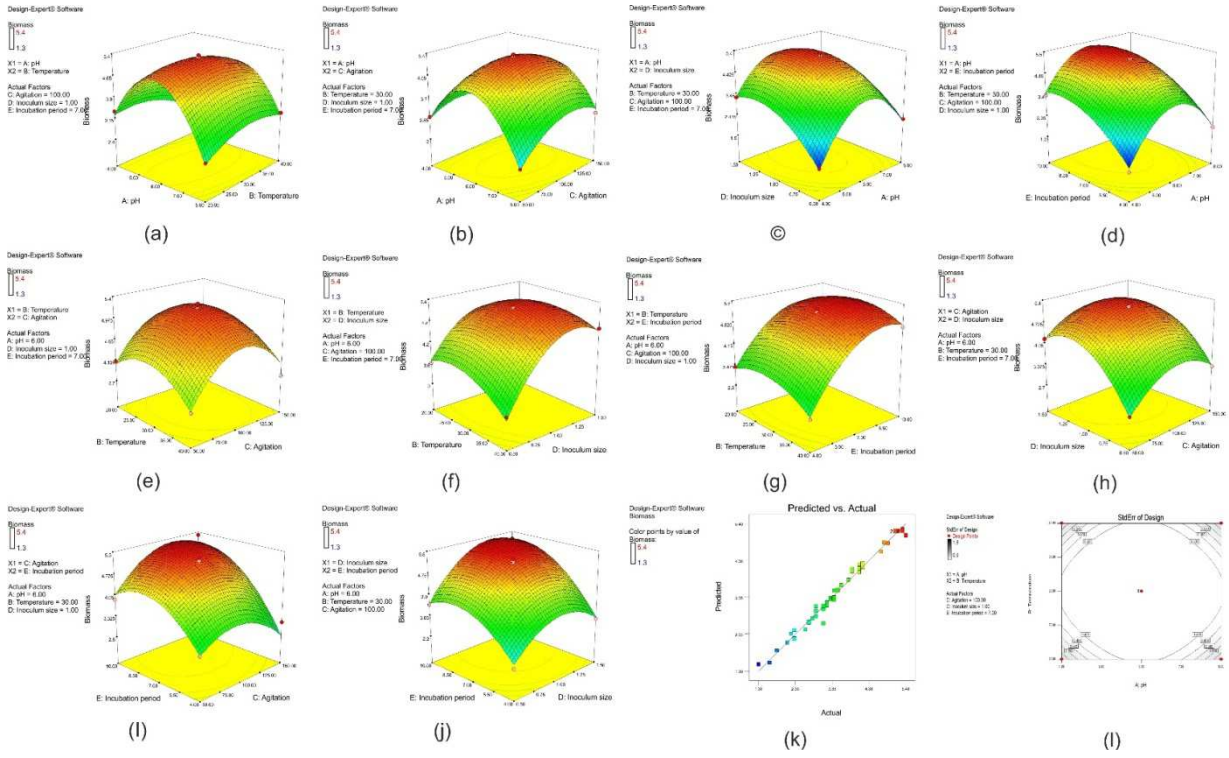


Figure -2 Response surface plots of interaction between process variables in biomass production by *Pleurotus ostreatus* PKN04
(a) pH vs temperature (b) pH vs Agitation (c) Inoculum size vs pH (d)Incubation period vs pH (e) Temperature vs Agitation (f) Temperature vs Inoculum size (g) Temperature vs Incubation period (h) Inoculum size vs Agitation (i) Incubation period vs Agitation (j) Incubation period vs Inoculum size (k) Predicted vs Actual (l) Standard error of Design

CONCLUSION

Pleurotus ostreatus PKN04 was identified and physical parameters such as temperature, pH, agitation and inoculums size were characterized. On identifying the range of variables, RSM design of experiments was formulated and executed. The interactive effects of the parameters were analyzed for biomass production and enzyme activity.

REFERENCES

[1] MC Machczynski, E Vijgenboom, B Samyn, GW Canters, *Protein Sci.*, **2004**, 13,2388–2397.
[2] G Aggelis, D Iconomou, M Christou, D Bokas, S Kotzailias, G Christou, V Tsagou, S Papanikolaou, *Water Res*, **2003**, 37, 3897-3904.
[3] G Hublik, F Schinner, *Enz Microb Technol*, **2000**, 27, 330-336.
[4] G Singh, N Ahuja, M Batish, N Capalash, P Sharma, *Bioresour Technol*, **2008**, 99, 7472-7479.
[5] E Aracri, JF Colom, T Vidal, *Bioresour. Technol*, **2009**, 100, 5911-5916.
[6] P Simphiwe, O Buthelezi Ademola, Balakrishna, *Molecules*, **1987**, 17, 14260-14274.
[7] M Asgher, Q Yasmeen, HMN Iqbal. *Ecol. Eng*, **2014**, 63, 1-11.
[8] DH Van Campen, R Nagtegaal, AJG Schoofs. Multi-criteria Design Optimization, Springer-Verlag, Berlin, Heidelberg, **2009,** 205-228.
[9] Guo-Qing Zhang, Tian Tian, Yue-Ping Liu, He-Xiang Wang, Qing-Jun Chen, *Process Biochemistry*, **2011**, 46, 2336–2340.
[10] DS Arora, RK Sharma, *Applied Biochemistry and Biotechnology*, **2010**,160(6),1760-1788.
[11] RS Couto and JL Toca-Herrera, Biotechnol. *Mol. Bio. Rev*., **2006**, 1, 115-120.
[12] I Muhannad. Massadeh, Abeer Fraij and Khalid Fandi, *Jordan Journal of Biological Sciences*, **2010**, 3 (2), 51-54.
[13] DG Pitcher, NA Saunders and RJ Owen. *Lett. Applied Microbiol*., **1989**,8, 151-156.
[14] Baladhandayutham Suresh, Thangavelu Viruthagiri and Ezhumalai Sasikumar, *As. J. Food Ag-Ind*, **2009**, 2(03), 302-314.
[15] P Giardina, G Palmieri, A Scaloni, B Fontanella, V Faraco, G Cennamo, G Sannia, *Biochem J*, **1996**, 341, 655-663.
[16] A Majcherczyk, C Johannes, A Hüttermann, *Enzyme and Microbial Technology*, **1998**, 22(5), 335–341.
[17] R Bourbonnais, MG Paice. *Appl. Microbiol. Biotechnol.* **1990**,36, 823–827.
[18] OH Lowry, AL Rosebrough AL, Farr, and RJ Randall, *J. Biol. Chem.* **1951**,193,265-275.
[19] RL Plackett, JP Burman, *Biometrika* **1946**, 34, 255–272.
[20] C Kotzamanidis, D Chatzopoulos, C Michailidis, G Papaiakovou and D Patikas, *Journal of Strength and Conditioning Research*, **2005**,19(2), 369-375.
[21] KN Niladevi, RK Sukumaran, P Prema. *J Ind Microbiol Biotechnol*, **2007**, 34 (10), 665-674.
[22] S Trupkin, L Levin, F Forchiassin, A Viale, *J. Ind.Microbiol. Biotechnol*, **2003**, 30, 682- 690.
[23] GS Nyanhongo. J Gomes, GM Giibitz, R Zvauya, J Read, W Steine. *Water Res*, 2002, 36,1449,1456.
[24] G Palmieri, P Giardina, C Bianco, A Scaloni, A Capasso, G Sannia, *J Biol Chem*, **1997**, 272, 31301-31307.
[25] G Mata, P Delpech, JM Savoie, *Revista Iberoamericana de Micología*, **2001,** 18, 118-122.
[26] GEP Box, NR Draper "Empirical Model Building and Response Surfaces", John Wiley & Sons, New York, NY.
[27] Q Yasmeen, M Asgher, MA Sheikh, H Nawa. *Bioresources*, **2013**. 8(1), 944-968.
[28] AFD Vasconcelos, AM Barbosa, RFH Dekker, IS Scarminio, MI Rezende, *Process Biochem*, **2000**, 35, 1131-1138.
[29] T Arunkumar, D Alex Anand, G Narendrakumar, *Journal of Pure and Applied Microbiology*, **2014a**, 8, 727-731
[30] T Arunkumar, D Alex Anand, G Narendrakumar, *International Journal of Pharma and Bio Sciences*, **2014b**, 5, 429-438.
[31] NN Pozdnyakova, J Rodakiewicz-Nowak, OV Turkovskaya. *Journal of Molecular Catalysis B*, **2004**, 30, 1, 19-24.
[32] G Alexandre and I Zhulin , *Trends in biotech*, **2000,** 18, 41– 42.
[33] OV Koroljova - Skorobogat'ko, EV Stepanova, VP Garvrilora, OV Morozova, NV Lumbimova, AN Dzchafarova, AI Jaropolov, A Makowar, *Biotechnology and Applied Biochemistry*, **2003**,28, 47-54.
[34] A Givaudan, A Effosse, D Faure, P Potier, ML Bouillant, R Bally, *FEMS Microbiology Letter*, **1993**,108,205-210.
[35] LO Martins, CM Soares, MM Pereira, M Teixeira, T Costa, GH Jones, AO Henriques, *J. Biol. Chem*, **2002**, 277, 18849-18859.
[36] MM Berrocal, J Rodriguez, AS Ball, MI Perez-Leblic, ME Arias, *Appl. Microbiol. Biotechnol*, **1997**, 48, 379–384.

[37]T Suzuki, K Endo, M Ito Tsujibo , M Miyamoto, YA Inamori, *Biosci Biotechnol Biochem*, **2003**, 67, 2167–2175.