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Optimization of media components for the production of laccase from *Pleurotus ostreatus* PKN 04 using Response surface methodology

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ABSTRACT

The influence of media components plays an important role on the enhanced production of products from microorganism. An approach was done to enhance the production of laccase from Pleurotus ostreatus – white rot fungi by manipulating the different concertation of variables using Response surface methodology. The Enzyme production was correlated with the biomass produced during the production process.6 g/L $NaNO_3$, 1.5 g/L K_2HPO_4, 1 g/L Tryptone, 10 g/L Glucose, 0.5 g/L $MgSO_4$ combination showed maximum enzyme activity of 96 IU/ml with an agreeable increase in biomass (5.2 g/L). 50 experiments runs were attained this optimum conditions for enzyme production and the coefficient of determination was 99.5%.

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

INTRODUCTION

Laccases are oxidoreductases produced by white rot fungi. These enzymes present several biotechnological applications including catalyze the oxidation of various phenolic compounds [1-2], delignification of lignocellulosic materials, biobleaching [3-4], effluents decolorization [5], bioremediation [6] and organic synthesis.

The *Pleurotus* genus (higher Basidiomycetes) comprises of many group of comestible fungi that has high nutritional value due to the presence of high protein, fiber, vitamins and minerals [7]. Many species of *Pleurotus* represent valuable protein source, especially in the developing countries [8]. In addition to their nutritional value, these fungi produce important biomolecules that include proteins, enzymes, glycoproteins, polysaccharides and organic acids with a number of biologic activities.

RSM is a group of mathematical and statistical methods for developing experimental model. Design of experiments (DOE) was carefully aimed to optimize a response which is influenced by many independent variables. An experiment has a series of tests called runs, in that the variations are made in the input variables in order to recognize the motives for variations in the output.

RSM was established to model experimental results [9] and then drift into the modelling of numerical trials. The variation is in the kind of error produced by the response. In the conventional experiments, inaccuracy can be due to measurement errors while, *in silico* experiments, numerical noise will result in incomplete union of iterative

processes, round-off errors or the discrete representation of continuous physical phenomena [10]. In RSM, the errors are assumed to be random.

In this communication, media optimization was performed for enhanced growth of *Pleurotus ostreatus*PKN04 using Response surface methodology – Central composite design

MATERIALS AND METHODS

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 [11].

Pleurotus ostreatus PKN 04was grown in Malt extract medium (MEA) and the spores were washed and inoculated in the fermentation medium [12].

Optimization of media components 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 optimization at a time method, RSM is timesaving and economical [13]. The Central composite design was applied under RSM using Design Expert Version 7.0.0 software [14].

Five factors at three different levels were used in duplicate. Three concentration of $NaNO_3$ (3,6 9), K_2HPO_4 (1, 1.5,2), Tryptone (0.5,1,1.5), Glucose (5,10,15) and $MgSO_4$ (0.25, 0.5, 0.75) were selected as the critical variables and nominated as A, B, C, D and E respectively (Table-1, Table - 2). A total number of 50 runs were carried out to estimate the coefficients for the optimization of media [15]. 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 -3). The quality of the fit of this model was expressed by the coefficient of determination (R^2) [16].

The general quadratic equation

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

The quadratic equation where Y is the measured response, A, B, C, D and E are the coded independent input variables, $\vartheta 0$ is the intercept term, $\vartheta 1$, $\vartheta 2$, $\vartheta 3$, $\vartheta 4$ and $\vartheta 5$ are the coefficients showing the linear effects, $\vartheta 6$, $\vartheta 7$, $\vartheta 8$, $\vartheta 9$ and $\vartheta 10$, are the quadratic coefficients showing the squared effects and $\vartheta 11$, $\vartheta 12$, $\vartheta 13$, $\vartheta 14$, $\vartheta 15$, $\vartheta 16$, $\vartheta 17$, $\vartheta 18$, $\vartheta 19$ and $\vartheta 20$ are the cross product coefficients showing the interaction effects.

Table -1 Design summary

Study Type					Response Surface			
Initial Design					Central Composite			
Design Model					Quadratic			
Runs					50			
Factor	Independent Variable	Units	Low Coded	Low Actual	High Coded	High Actual	Mean	Std. Dev.
A	$NaNO_3$	g/L	-1	3	1	9	6	2.792216
B	K_2HPO_4	g/L	-1	1	1	2	1.5	0.465369
C	Tryptone	g/L	-1	0.5	1	1.5	1	0.465369
D	Glucose	g/L	-1	5	1	15	10	4.653693
E	$MgSO_4$	g/L	-1	0.25	1	0.75	0.5	0.232685

Table -2 Response computation

Response	Units	Minimum	Maximum	Mean	Std. Dev.	Ratio	Model
Enzyme activity	U/mL	10	96	60.14	18.87539	9.6	Quadratic
Biomass	Mg	0.8	5.4	3.318	1.043396	6.75	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 420nm in a spectrophotometer against a suitable blank.

One unit was expressed as the amount of the laccase that oxidized 1 µmol of ABTS substrate per min [17]. The absorbance was read after 10 min interval using UV/VIS spectrophotometer (Varian Cary® 100 UV-Vis).

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:

Dry weight = (weight of filter paper + mycelium) - (weight of filter paper).

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

RESULTS AND DISCUSSION

Isolation and identification of organism

The isolated organism was identified using Macroscopic and microscopic method.

Microscopic Features: Spores 8-10.5 x 3-3.5 µ; smooth; cylindrical to narrowly kidney-shaped. These are the measurements given by Petersen & Krisai-Greilhuber for an epitype collection of *Pleurotus ostreatus* [18].

The enzyme activity and the biomass was calculated for every run that was obtained from DOE.

Table -3CCD matrix for laccase production from *Pleurotus ostreatus* PKN04

Run	$NaNO_3$	K_2HPO_4	Tryptone	Glucose	$MgSO_4$	Response (Enzyme activity) U/mL		Response (Biomass) mg	
	g/L	g/L	g/L	g/L	g/L	Actual	Predict	Actual	Predict
1	3	1	1.5	5	0.25	55	57.2	3.4	3.3
2	9	1	1.5	15	0.75	57	56.3	2.7	2.7
3	9	2	0.5	15	0.25	70	68.6	3.7	3.8
4	3	2	0.5	5	0.75	45	45.3	2.4	2.4
5	3	1	0.5	5	0.25	48	48.2	2.8	2.8
6	3	2	1.5	15	0.75	66	65.7	3.7	3.8
7	9	1	0.5	15	0.75	51	50.1	2.6	2.7
8	3	1	0.5	5	0.75	60	59.7	3.4	3.4
9	9	2	1.5	5	0.25	47	48.3	2.8	2.8
10	3	2	1.5	5	0.25	45	44.9	2.4	2.4
11	6	1.5	1	10	-0.0946	68	68.4	3.7	3.8
12	6	1.5	1	10	0.5	95	94.2	5.1	5.2
13	6	1.5	-0.18921	10	0.5	38	37.3	2	2.0
14	9	2	1.5	15	0.75	55	53.5	2.8	2.9
15	9	2	0.5	15	0.75	57	56.6	3.1	3.1
16	9	1	1.5	5	0.75	55	56.8	3.1	3.1
17	3	2	1.5	15	0.25	58	57.4	3.2	3.2
18	9	2	0.5	5	0.25	57	57.3	3.1	3.1
19	6	1.5	1	10	0.5	92	94.2	5.2	5.2
20	3	2	0.5	15	0.75	58	60.0	3.5	3.5
21	6	0.3107	1	10	0.5	56	57.2	3.2	3.2
22	6	1.5	1	10	0.5	94	94.2	5.3	5.2
23	6	1.5	1	-1.89207	0.5	61	59.6	3.3	3.4
24	6	1.5	1	10	0.5	93	94.2	5.4	5.2

25	6	2.6892	1	10	0.5	51	50.4	2.7	2.7
26	3	1	1.5	15	0.75	77	75.9	4.1	4.0
27	13.135	1.5	1	10	0.5	10	10.8	0.8	0.8
28	3	2	0.5	15	0.25	58	58.0	3.1	3.1
29	9	1	0.5	5	0.25	51	52.8	3.3	3.3
30	3	2	0.5	5	0.25	45	45.2	2.2	2.2
31	9	1	0.5	5	0.75	51	50.3	2.9	2.9
32	9	2	1.5	15	0.25	59	59.3	3.4	3.3
33	6	1.5	1	10	0.5	94	94.2	5.1	5.2
34	6	1.5	1	10	0.5	95	94.2	5.2	5.2
35	3	1	1.5	15	0.25	57	56.2	2.7	2.9
36	6	1.5	1	10	0.5	94	94.2	5.2	5.2
37	9	2	1.5	5	0.75	40	40.6	2.1	2.1
38	6	1.5	1	21.89207	0.5	72	74.1	4.1	4.0
39	9	1	1.5	5	0.25	58	53.0	3.3	3.3
40	3	2	1.5	5	0.75	52	51.2	2.8	2.8
41	3	1	0.5	15	0.75	61	61.0	3.4	3.5
42	3	1	0.5	15	0.25	49	47.5	2.7	2.6
43	9	2	0.5	5	0.75	43	43.4	2.3	2.2
44	9	1	0.5	15	0.25	50	50.6	2.9	2.9
45	6	1.5	2.189207	10	0.5	43	44.3	2.3	2.3
46	3	1	1.5	5	0.75	75	74.9	4.1	4.1
47	**6**	**1.5**	**1**	**10**	**0.5**	**96**	**94.2**	**5.2**	**5.2**
48	-1.1352	1.5	1	10	0.5	20	19.8	1.3	1.3
49	9	1	1.5	15	0.25	50	50.5	2.7	2.7
50	6	1.5	1	10	1.0946	75	75.3	4.1	4.0

Table -4 ANOVA for laccase production for *Pleurotus ostreatus* PKN04

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F
Model	17737.15	20	886.8573	334.5615	< 0.0001 Significant
A-$NaNO_3$	154.4233	1	154.4233	58.25524	< 0.0001
B-K_2HPO_4	88.43917	1	88.43917	33.36313	< 0.0001
C-Tryptone	94.24722	1	94.24722	35.55419	< 0.0001
D-Glucose	403.2659	1	403.2659	152.1296	< 0.0001
E-$MgSO_4$	90.6153	1	90.6153	34.18407	< 0.0001
AB	112.5	1	112.5	42.43993	< 0.0001
AC	153.125	1	153.125	57.76547	< 0.0001
AD	4.5	1	4.5	1.697597	0.2029
AE	392	1	392	147.8796	< 0.0001
BC	171.125	1	171.125	64.55586	< 0.0001
BD	364.5	1	364.5	137.5054	< 0.0001
BE	264.5	1	264.5	99.781	< 0.0001
CD	0.125	1	0.125	0.047155	0.8296
CE	78.125	1	78.125	29.47218	< 0.0001
DE	8	1	8	3.017951	0.0930
A^2	10797.79	1	10797.79	4073.398	< 0.0001
B^2	2827.91	1	2827.91	1066.812	< 0.0001
C^2	4943.377	1	4943.377	1864.859	< 0.0001
D^2	1299.388	1	1299.388	490.1863	< 0.0001
E^2	867.7832	1	867.7832	327.3659	< 0.0001
Residual	76.87335	29	2.650805		
Lack of Fit	65.99835	22	2.999925	1.930986	0.1886 Not significant
Pure Error	10.875	7	1.553571		
Cor Total	17814.02	49			
Std. Dev.	1.628129		R^2		0.995685
Mean	60.14		Adj R^2		0.992709
C.V. %	2.707232		Pred R^2		0.985753
PRESS	253.795		Adeq Precision		78.98923

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

R1 =94.17815-1.88818A-1.42893B + 1.4751C + 3.051287D + 1.446399E + 1.875AB-2.1875AC-0.375AD-3.5AE-2.3125BC3.375 BD-2.875BE-0.0625CD + 1.5625CE + 0.5DE-13.9396A^2-7.13373B^2-9.43183C^2-4.83564D^2-3.95175E^2 (2)

To authenticate the statistical results and the model equation, ANOVA was executed as displayed in Table-5, the F value is a measure of difference of the data about the mean, the P value functions as a tool for checking the significance of each and every coefficient. The predicted R^2 value is reasonably agreement with the adjusted R^2 value. The R2 value of enzyme activity is 0.995 that ensures the satisfactory adjustment of the quadratic model to the experimental data.

Optimization of biomass production using RSM.

Biomass production by *Pleurotus ostreatus* was optimized using CCD -RSM by Design Expert, version 7.0.0 software (Stat –Ease Corp). Biomass production of this fungi was optimized by varying the concentrations of the medium components especially$NaNO_3$, K_2HPO_4, Tryptone, Glucose, $MgSO_4$ and the incubation time, temperature and pH were not taken under consideration during cultivation.

The results predicted the maximum biomass production 5.2 g by *Pleurotus ostreatus* PKN 04 in 100 ml of designed medium under the experimental conditions of 6 g/L $NaNO_3$, 1.5 g/L K_2HPO_4, 1 g/L Tryptone, 10 g/L Glucose, 0.5 g/L $MgSO_4$ (Table - 3 Run 50). All the five variables $NaNO_3$, K_2HPO_4, Tryptone, Glucose, $MgSO_4$ and quadratic terms (AB, AC, AE, BC, BD, BE, CD, CE, and squared terms (A^2, B^2,C^2, D^2, E^2) and ABCDE were significant, indicating biomass production depended on all the five factors selected and optimum conditions for maximum biomass production was verified by three dimensional response surface plots.

Table -5 ANOVA for Biomass production by *Pleurotus ostreatus* PKN04

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob> F
Model	54.20622	20	2.710311	345.3756	< 0.0001 Significant
A-$NaNO_3$	0.424745	1	0.424745	54.1254	< 0.0001
B-K_2HPO_4	0.507661	1	0.507661	64.6913	< 0.0001
C-Tryptone	0.157699	1	0.157699	20.09556	0.0001
D-Glucose	0.777391	1	0.777391	99.06312	< 0.0001
E-$MgSO_4$	0.117022	1	0.117022	14.91211	0.0006
AB	0.300313	1	0.300313	38.26889	< 0.0001
AC	0.475313	1	0.475313	60.56918	< 0.0001
AD	0.112813	1	0.112813	14.37572	0.0007
AE	2.257813	1	2.257813	287.7135	< 0.0001
BC	0.165313	1	0.165313	21.06581	< 0.0001
BD	2.475313	1	2.475313	315.4296	< 0.0001
BE	0.427813	1	0.427813	54.51624	< 0.0001
CD	0.052813	1	0.052813	6.729908	0.0147
CE	0.090313	1	0.090313	11.50854	0.0020
DE	0.090313	1	0.090313	11.50854	0.0020
A^2	30.22317	1	30.22317	3851.345	< 0.0001
B^2	8.962777	1	8.962777	1142.129	< 0.0001
C^2	16.38638	1	16.38638	2088.12	< 0.0001
D^2	4.021866	1	4.021866	512.5073	< 0.0001
E^2	3.034231	1	3.034231	386.6527	< 0.0001
Residual	0.227576	29	0.007847		
Lack of Fit	0.158826	22	0.007219	0.73506	0.7295Not significant
Pure Error	0.06875	7	0.009821		
Cor Total	54.4338	49			

Std. Dev.	0.088586	R^2	0.995819
Mean	3.318	Adj R^2	0.992936
C.V. %	2.669853	Pred R^2	0.987784
PRESS	0.664971	Adeq Precision	76.77009

R2 = 5.210662- 0.09903 A - 0.10826 B + 0.060339 C + 0.13397 D + 0.051978 E + 0.096875 AB - 0.12188AC - 0.05938 AD - 0.26563 AE - 0.07188 BC + 0.278125 BD - 0.11563 BE - 0.04063 CD + 0.053125 CE + 0.053125 DE - 0.73749 A^2 - 0.40161 B^2 - 0.54303 C^2 - 0.26903 D^2 - 0.23367 E^2 (3)

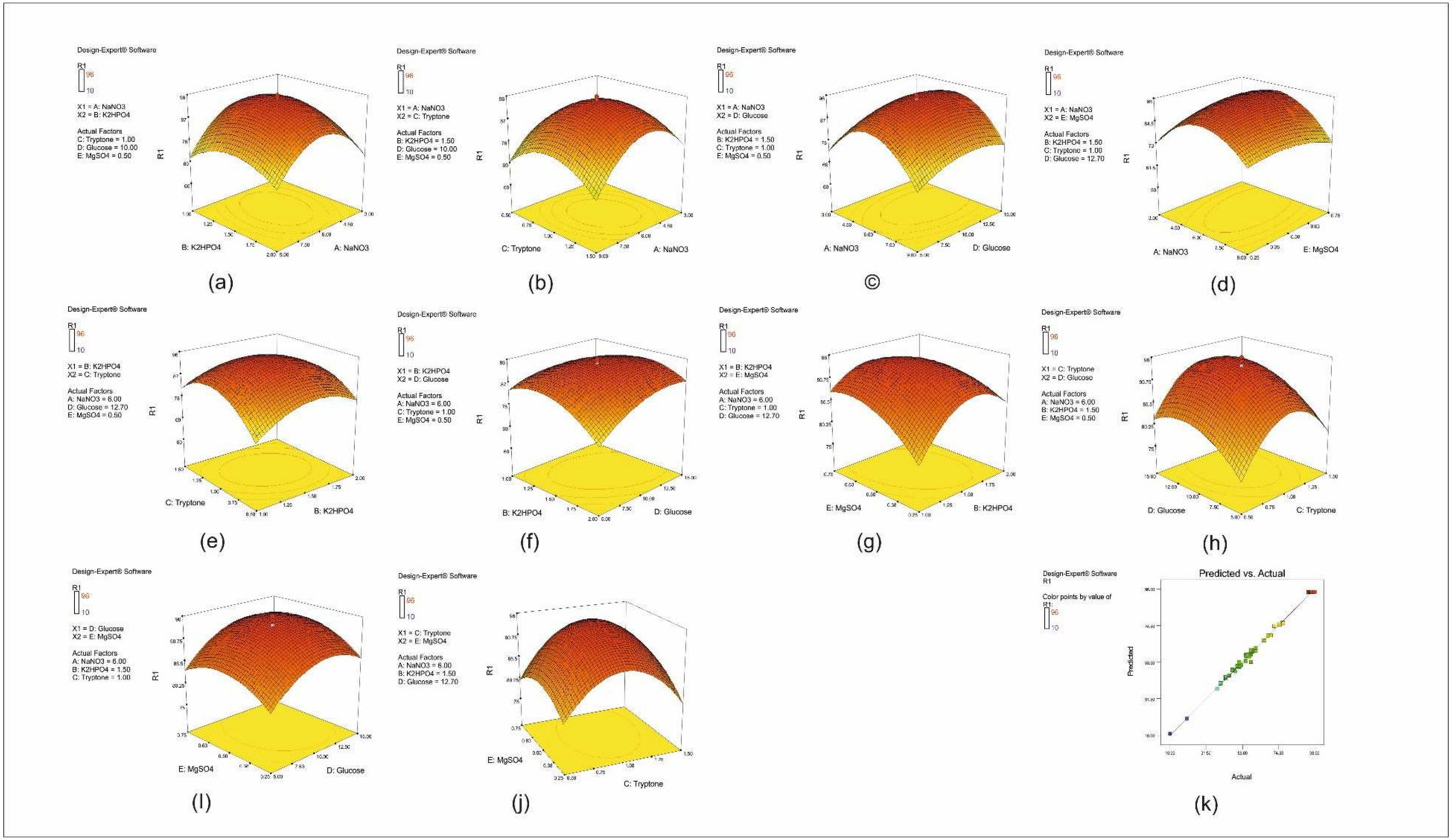

Figure -1 Response surface plots of interaction between process variables in enzyme activity by *Pleurotus ostreatus* PKN04

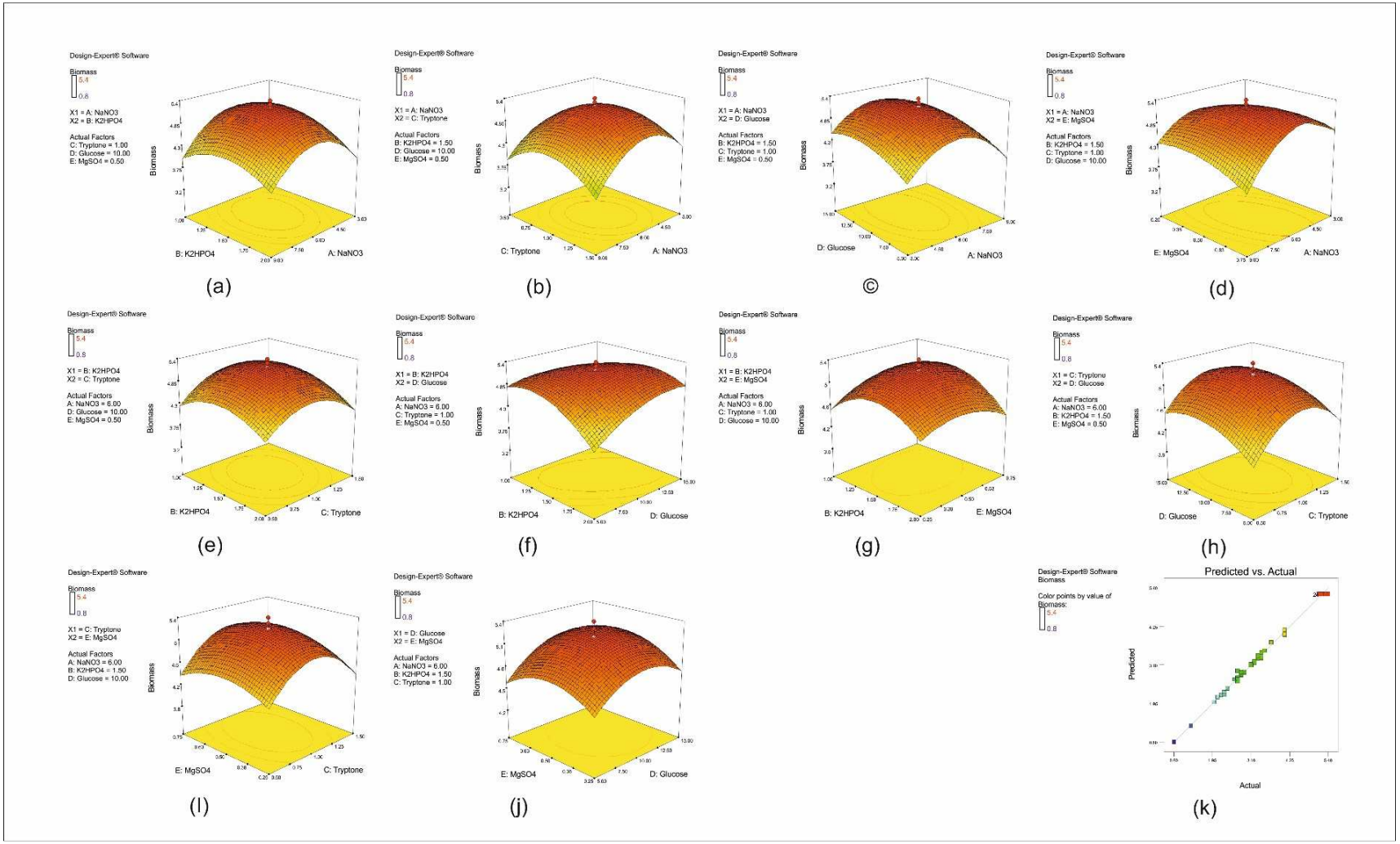

Figure -2 Response surface plots of interaction between process variables in biomass production by *Pleurotus ostreatus* PKN04

CONCLUSION

Central composite experimental design for nutritional optimization engages a specific study on the area of individual factors. Production of laccase increased by modification of media components. In this study, RSM-CCD was used for optimizing culture media for maximizing laccase production by using *Pleurotus ostreatus* in batch fermentation. The optimized medium composition increased two fold of laccase production.

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