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Enhanced Production of Glutathione from *Candida utilis* using Palm Jaggery

C.S Rathnasabapathy*, Syed Mahin Basha, R.Dhanasekar
Department of Technology, Annamalai University, Annamalai Nagar-608002, Tamil Nadu, India

Corresponding author:cs_saba@yahoo.com

ABSTRACT: The sequential Media Optimization for the production of Glutathione using *Candida utilis* is investigated. In the first step, Plackett- Burman design was used to optimize various media compositions of carbon and nitrogen sources along with other nutrients. The two medium constituents, namely copper sulfate and zinc sulfate were found to be effective trace elements in the production of glutathione. These two identified variables along with another variable, the inoculum size were optimized for enhancing the production of glutathione. Box- Behnken response surface methodology was used to optimize these variables. The optimal values of the medium components were found to be : Copper Sulphate, 0.011g/; zinc sulfate 0.009g/L; inoculum size, 5.3%. An improved Glutathione yield of 76 mg/ml was obtained with optimized medium composition,which is 20% higher production than previously obtained results.
Keywords: Glutathione, *Candida utilis*, statistical optimization,

INTRODUCTION

Glutathione (γ-Glutamylcysteinylglycine; GSH) is a low molecular mass thiol with proposed functions in many cellular processes. In 1921, Hopkins isolate GSH from yeast, animal liver and muscle. The functions of GSH include protection of cells against xenobiotics, carcinogens, radiation and reactive oxygen species. It is used as a toxin scavenger and as a medicine for liver. GSH also elicits more interests in food and additive industry, therapeutics and sports nutrition. GSH can be produced by chemical methods [1], enzymatic reaction [2] and microorganism fermentation [3-5]. In practicality, efficient production of GSH production is by yeast fermentation. Since the production is intracellular in yeast, GSH production can be enhanced in two ways: increasing the cell biomass or by increasing the GSH content of the yeast. Earlier is easier than the latter. GSH is synthesized in two ATP dependent steps. γ-Glutamylcysteine synthase (γ-GCS) catalyses the formation of γ- glutamylcysteine which is the first step. The second step involves glutathione synthase (GS) expediting the formation of glutathione. The first reaction is the limited step. γ-GCS activity was shown to be feedback inhibited by GSH to prevent over accumulation of tripeptide[6,7]. γ-GCS is found to be a highly regulated enzyme and GSH synthetase is found to be a constitutive unregulated enzyme[8]. Being a tripeptide, GSH biosynthesis has close relationship with intracellular amino acids especially those containing sulfur [9]. *Saccharomyces cerevisiae* and *Candida utilis* are currently used to produce Glutathione on an industrial scale[10].

There are a number of reports on optimization of medium components using classical method by changing one independent variable by fixing all the other variables at constant level. This proves to be extremely laborious for large number of variables. Conventional practice of single factor optimization by maintaining other factors at an unspecified constant level does not depict the combined effect of all the factors involved. The method requires a large number of experiments to determine optimum levels, which are unreliable. Optimizing all the effecting parameters can eliminate these limitations of a single factor optimization process collectively by statistical experimental design using Plackett–Burman and response surface methodology (RSM). Plackett–Burman design is a well established and widely used statistical design technique for the screening of the medium components in shake flask. In this work Plackett-Burman Design [11], was adopted to optimise various medium components used in the production of GSH fermentation by *Candida utilis* utilizing. The dominant factors are screened and are used as variables along with the inoculum size in Box-Behnken Design

[12-15] to arrive at an optimum composition. The prediction of GSH was made using the developed regression equation.

MATERIALS & METHODS

Strain Maintenance and Growth

The strain *Candida utilis* (NCIM 3401) was used for the production of glutathione. It was procured from National Collection of Industrial Microorganisms (NCIM), National Chemical Laboratory, Pune, India. The strain was maintained in an agar slant using the following medium composition: yeast extract, 0.3% (w/v); peptone, 0.5% (w/v); malt extract, 0.3% (w/v); and glucose, 1% (w/v). The culture was stored at 4^0C and routinely sub cultured at every fortnight time interval.

Media Composition

The production medium contains of the following composition in g/l: KH_2PO_4, 3.0; $MgSO_4.7H_2O$, 0.25; NH_3SO_4, 8.03; Yeast Extract, 3.0; Malt Extract, 3.0**;** Peptone, 5.0 along with jaggery, 28.7. The medium pH was adjusted to 6.4 using 3M H_2SO_4 or 3M NaOH and was sterilized in an autoclave (121^{o}C; 14.5 psi) for 20minutes.

Inoculum Preparation

About 50ml of the growth medium containing the above mentioned composition along with jaggery (equivalent to 1% (w/v) glucose) was used for the preparation of inoculum. pH of the medium was adjusted to 6.4 and was sterilized in an autoclave (121^{o}C ; 14.5 psi) for 20 minutes. The sterilized medium was cooled and cultured with a loop full of strain from the stock culture. The culture was maintained in an incubator shaker for one day(30^{o}C; 250 rpm). 5%(v/v) of the one day grown culture was used as inoculum for all fermentation studies.

Estimation of Cell Mass

Estimation of Biomass was carried out by spectrophotometric method. The optical density of all the cultures were measured using Elico-SLV 164, Double beam UV –VIS spectrophotometer at 420 nm with blanks of the appropriate growth medium. Suspension with an OD above 1.0 was diluted with the appropriate growth medium. Curve relating OD to dry weight was constructed by harvesting cultures at room temperature, washing with distilled water, and resuspending the cells in distilled waster to about 10 mg of dry weight per ml. Portion (5 ml) was dried at 100^{O}C and weighed. The dry weight of the cells was determined. The strain Candida utilis produces an extra cellular slime and in turn produces turbid solutions. In such cases, the optical density is read against a culture supernatant blank, diluting the blank in the same ration as the culture.

Estimation of Glutathione

Glutathione concentration was determined according to the method described by George L. Ellman[16]. The wet cells were extracted with 40% (v/v) ethanol at 30^{O}C for two hours, centrifuged at 5000 x g for 20 minutes and the supernatant was used for the assay of glutathione. The supernatant solution (3ml) was added with 2ml of phosphate buffer and 5 ml of water. 3ml of this mixture was placed in a photometer cell and 0.02 ml of DTNB reagent was added to it. The reagent was prepared by dissolving 39.6 mg of 5-5' – di thio bis (2-nitrobenzoic acid) (DTNB) in 10ml of phosphate buffer (pH 7). Colour was developed within 2 minutes and the absorbance was read at 412 nm using Elico-SLV 164, Double beam UV-VIS spectrophotometer.

Batch Studies on Biosynthesis of GSH

50 ml of the medium containing the required composition(as mentioned in Section 3.1) was taken in a 250ml Erlen Meyer flask. pH of the medium was adjusted to 6.4 by the addition of 3M NaOH or 3M H_2SO_4. After adjusting the pH, the medium was sterilized in an autoclave (121^{o}C and 1.4 kg/cm^2) for 20 minutes. The medium was cooled and was inoculated with 24 h pre grown culture[5%(v/v)]. The samples at predetermined time intervals were taken and analyzed for cell mass, glutathione and residual sugar as described in the earlier sections. Media Optimization was done using the statistical Design of Experiments. Minitab software was used for the Design and Analysis.

RESULTS AND DISCUSSION

Media Optimization using Plackett Burman Experimental Design

The medium constituents namely, carbon, nitrogen and mineral sources used in GSH fermentation were optimized. Fifteen components under the above said sources were investigated for their dominance in the process of enhancing the yield of GSH. The medium constituents are: $CuSO_4$, $MgSO_4$, $FeSO_4$, $ZnSO_47H_2O$, $CaCl_2$, KH_2PO_4**,** Malt extract, NH_4SO_4, Peptone, $MnSO_4.7H_2O$, $(NH_4)_2HPO_4$, NH_4SO_4, Urea, Yeast Extract, K_2HPO_4, and Palm Jaggery. In order to study the combined effect of these factors, the experiments were performed for different combinations using the statistical design of experiments. A factorial design, Plackett Burman design was used to study the dominance among the fifteen constituents of the medium. Twenty runs are generated for fifteen variables. Two level design (-1indicates the lower level and +1 indicates the higher level) with a set of twenty runs was generated (shown in Table 1).

The experiments were performed for all the twenty runs using the procedure mentioned in the previous sections. The cell mass and glutathione were analyzed for all the runs and were subjected to factorial analysis. From the results of the analysis (Table.2), the effects of the variables and their significance on the production were found using their P values ($P< 0.05$).

A Pareto chart showing the dominance of the individual variables is shown in Figure.1.

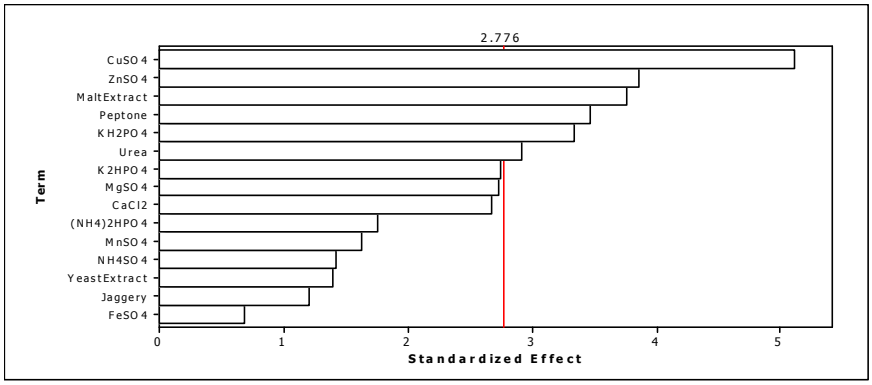

Figure.1 Pareto Chart of Standardized Effects

Among the variables tested, the variables which were found to be dominant on the production of GSH in their order are: $CuSO_4$; $ZnSO_4$; Malt Extract; Peptone; KH_2PO_4; Urea.

To confirm the positive influence of these two salts, further experimentation was conducted without the $CuSO_4$ and $ZnSO_4$. It was evident from the results that $CuSO_4$ and $ZnSO_4$ have strong effect on the production of glutathione. The amount of GSH produced using the normal medium (with $CuSO_4$ and $ZnSO_4$) is 65 mg/L which was approximately 25% higher production than the medium lacking in $CuSO_4$ and $ZnSO_4$(Table 3).

Optimization of dominant factors using Response Surface Methodology

The Plackett- Burman design does the screening of the variables based on their influence on the process. The levels of the significant parameters and their interaction effects on the glutathione production were analyzed and investigated by RSM methodology. A two level (-1 and +1) Box-Behnken Design was adopted for further optimization of the dominant variables (Table.4). The two variables which were identified by the Plackett-Burman design along with the third variable, the inoculum size were considered for the optimization study. The experiments were performed for a set of fifteen runs (given in Table.4) and the readings were recorded. The data was subjected to multiple regression analysis and the results were tabulated (Table.5).

Using the estimated regression coefficients, the following regression equation was formulated

$$Y = 62 - 0.25 * A + 4.5 * B + 3.0 * C - 3.375 * A^2 - 0.375 * B^2 - 3.8750 * C^2 - 4.25 * A* B - 2.25 * A* C + 4.25 * B* C.$$

where, Y the glutathione concentration, A - $CuSO_4$, B - $ZnSO_47H_2O$ and C the inoculum size.

To validate the model, the data was subjected to ANOVA. The value of R^2 (0.9589) indicates that a good correlation exists between the experimental data and predicted values. The ANOVA of the regression analysis demonstrated that the model is highly significant as evident from the calculated F value (12.96) with a very low probability value ($P = 0.006$) (Table. 6). It was also observed that the coefficient for linear and interaction effects were highly significant ($P = 0.004$ and 0.008) when compared with the squared effect. The predicted R^2 value is 0.7191 and the adjusted R^2 value is 0.8849.

The P values are used as a tool to check the significance of each of the coefficients, which, in turn, may indicate the patterns of the interaction among the variables. Larger the magnitude of T and smaller the value of P indicate that the corresponding coefficient is more significant. Values of the probability less than 0.05 indicates that the model terms are significant. In this case B, C, A^2, C^2, AB and AC were the significant model terms. Values greater than 0.10 indicate that the model terms are not significant. This implies that the linear effects of $ZnSO_47H_2O$ ($P < 0.002$) and inoculum size ($P = 0.009$) were more significant. Table 5 also indicate that the other individual, squared and the interactive effects of $CuSO_4$, $ZnSO_47H_2O$ and inoculum size.

The effect of interaction of parameters on the production of glutathione was depicted using contour plots. The interaction effects of $ZnSO_4$-inoculum size, $CuSO_4$-inoculums size and $CuSO_4$-$ZnSO_4$ on the production of GSH were demonstrated (Figures 3, 4 and 5). It was evident from the results that different trends of GSH production profiles were observed in each of the cases. For the case of $ZnSO_4$- inoculum size, the production increased with increase in both the variables. On the contrary, in the case of $CuSO_4$- inoculums size and $CuSO_4$-$ZnSO_4$, there was a fall in the production at higher levels of these variables. From all these three results, the $CuSO_4$ has shown some kind of negative effect on the production of GSH at its higher levels.

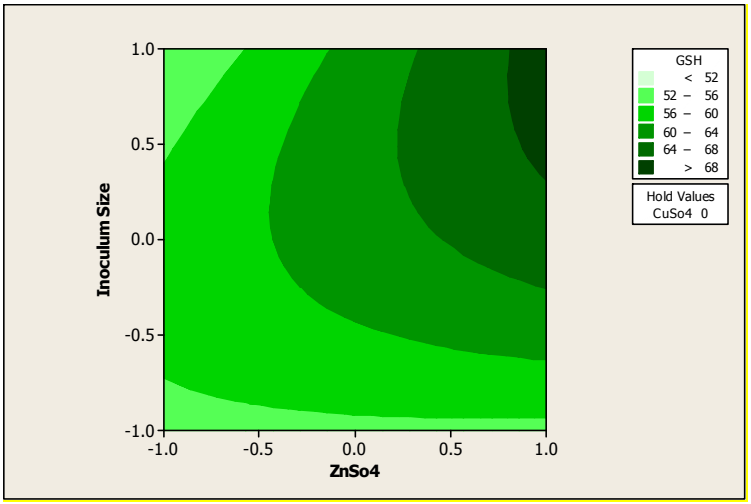

Figure 3.Contour plot showing the effect of Inoculum Size and Zinc Sulphate on the production of Glutathione

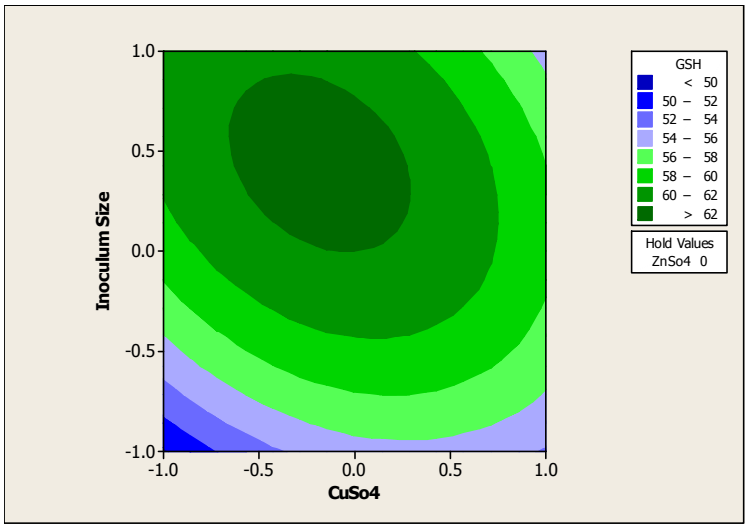

Figure 4.Contour plot showing the effect of Inoculum Size and Copper Sulphate on the production of Glutathione

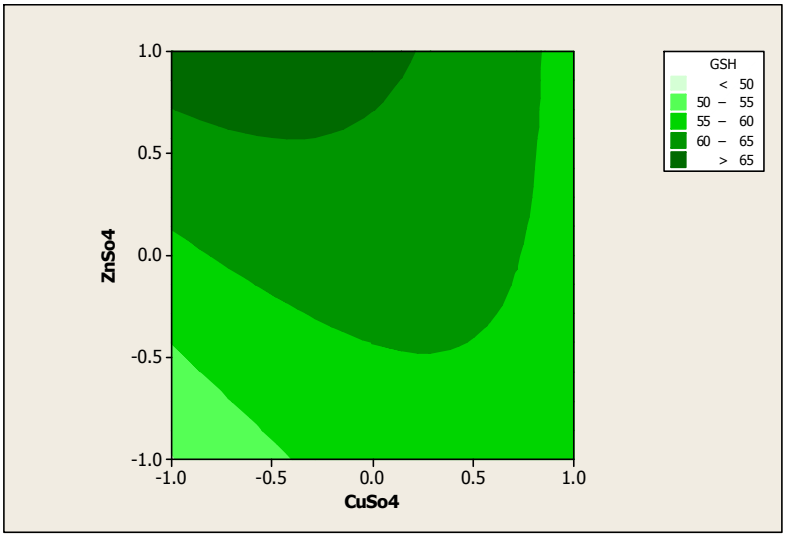

Figure 5.Contour plot showing the effect of Zinc Sulphate and Copper Sulphate on the production of Glutathione

The regression equation obtained from the RSM was used to find the optimum values of $CuSO_4$, $ZnSO_4$ and the inoculum size. The sequential quadratic programming of MATLAB 7 was used to solve the second-degree polynomial regression equation The optimum values thus resulted were: $CuSO_4$, 0.011 mg/l, $ZnSO_4$, 0.009 mg/l and inoculum size, 5.3%(v/v). A run carried out with these optimized values of the variables yielded 76mg/l of glutathione, which is 18% higher than the yield obtained from previous optimization.

CONCLUSION

Batch Glutathione synthesis from palm jaggery using *Candida utilis* was investigated. An improved yield of around eighteen percent was obtained by means of employing a sequential optimization technique for the medium used. Palm Jaggery, a cheaply available sugar source could be effectively utilized for the GSH fermentation. Yield obtained from palm jaggery is comparable to the yields obtained from different synthetic carbon sources. The two minerals, $CuSO_4$ and $ZnSO_4$ were found to have a strong influence on the production of GSH. The enhanced production of GSH from palm jaggery favours the commercial feasibility of the GSH fermentation.

Table 1 Plackett-Burman Two Level Design

S.No	A	B	C	D	E	F	G	H	J	K	L	M	N	O	P	Wet cell mass (g/L)	GSH (mg/L)
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	33.6	65
2	-1	1	-1	-1	1	1	1	1	-1	1	1	-1	-1	-1	-1	29.4	51
3	-1	-1	1	-1	-1	1	1	1	1	-1	-1	1	-1	-1	-1	27.4	43
4	1	-1	-1	1	-1	-1	1	1	1	1	1	-1	1	-1	-1	32.8	61
5	1	1	-1	-1	1	-1	-1	1	1	1	-1	1	-1	1	-1	30.1	53
6	-1	1	1	-1	1	1	-1	-1	1	1	1	-1	1	-1	1	29.4	51
7	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	-1	1	-1	29.4	51
8	-1	-1	-1	1	1	-1	-1	1	-1	-1	1	1	1	-1	1	27	44
9	-1	-1	-1	-1	1	1	-1	-1	1	-1	1	1	1	1	-1	31.4	60
10	1	-1	-1	-1	-1	1	1	-1	-1	1	-1	1	1	1	1	31.6	62
11	-1	1	-1	-1	-1	-1	1	1	-1	-1	-1	-1	1	1	1	29.2	51
12	1	-1	1	-1	-1	-1	-1	1	1	-1	1	-1	-1	1	1	32.6	59
13	-1	1	-1	1	-1	-1	-1	-1	1	1	-1	1	-1	-1	1	27.8	43
14	1	-1	1	-1	1	-1	-1	-1	-1	1	-1	-1	1	-1	-1	28	50
15	1	1	-1	1	-1	1	-1	-1	-1	-1	1	-1	-1	1	-1	30	51
16	1	1	1	-1	1	-1	1	-1	-1	-1	1	1	-1	-1	1	31.6	59
17	-1	1	1	1	1	1	1	1	1	1	1	1	1	-1	1	31.6	60
18	1	1	1	1	-1	1	1	1	1	1	1	-1	-1	1	-1	30.6	55
19	-1	-1	-1	-1	-1	-1	-1	-1	1	1	-1	-1	1	-1	-1	28	46
20	1	1	1	1	-1	-1	-1	-1	1	1	1	1	1	1	1	27.8	40

A, Jaggery (2.583-3.15 g/l); **B**, $MgSO_4$(0.022-0.027g/l); **C**, K_2HPO_4(0.09-0.11g/l); **D**, $MnSO_4$(0.009-0.011 g/l); **E**, $ZnSO_47H_2O$(0.009-0.011 g/l); **F**, FeSO4(0.011-0.013 g/l); **G**,$CuSO_4$(0.011-0.013 g/l); **H**, $CaCl_2$(0.009-0.011 g/l) ; **J**, KH_2PO_4(0.27-0.33 g/l); **K**, $(NH_4)_2HPO_4$(0.27-0.33 g/l); **L**, NH_4SO_4(0.72-0.88 g/l); **M**, Peptone(4.5-5.5 g/l); **N**, Yeast Extract(2.7-3.3 g/l); **O**, Malt Extract(2.7-3.3 g/l); **P**, Urea(1.17-1.43 g/l)

Table2. Estimated Effects and Coefficients for Glutathione

Term	Effect	Coef	SE Coef	T	P
Constant		56.149	1.611	34.85	0.000
Jaggery	2.753	1.376	1.136	1.21	0.292
$MgSO_4$	-8.869	-4.434	1.626	--2.73	0.053
K_2HPO_4	-6.722	-3.361	1.224	-2.75	0.052
$MnSO_4$	6.293	3.147	1.942	1.62	0.180
$ZnSO_47H_2O$	18.454	9.227	2.386	3.87	0.018
$FeSO_4$	-1.624	-0.812	1.193	-0.68	0.534
$CuSO_4$	18.829	9.415	1.843	5.11	0.007
$CaCl_2$	-9.058	-4.529	1.698	-2.67	0.056
KH_2PO_4	11.227	5.613	1.681	3.34	0.029
$(NH_4)_2HPO_4$	-4.987	-2.493	1.416	-1.76	0.153
NH_4SO_4	-5.229	-2.615	1.843	-1.42	0.229
Peptone	-13.421	-6.711	1.932	-3.47	0.025
Yeast Extract	-4.381	-2.191	1.565	-1.40	0.234
Malt Extract	10.683	5.342	1.422	3.76	0.020
Urea	9.033	4.516	1.547	2.92	0.043

Table 3. Validation of Plackett-Burman Design

S.No	Medium	Wet cell mass(g/L)	GSH(mg/L)
1	Medium Without $CuSO_4$	32.81	50
2	Medium Without $ZnSO_4$	52.76	52
3	Normal medium	54.28	62

Table 4. Box-Behnken Design and Response.

RunOrder	$CuSO_4$	$ZnSO_4$	Inoculum Size	GSH(mg/l)
1	1	0	1	55
2	-1	0	-1	50
3	1	1	0	58
4	0	0	0	60
5	1	0	-1	55
6	-1	0	1	59
7	0	-1	-1	54
8	0	1	-1	54
9	0	-1	1	53
10	1	-1	0	57
11	0	0	0	65
12	-1	1	0	68
13	0	1	1	70
14	0	0	0	61
15	-1	-1	0	50

Table 5.Estimated Regression Coefficients for GSH

Term	Coef	SE Coef	T	P
Constant	62.0000	1.1832	52.400	0.000
CuSO4	-0.2500	0.7246	-0.345	0.744
ZnSO4	4.5000	0.7246	6.211	0.002
Inoculum Size	3.0000	0.7246	4.140	0.009
CuSO4*CuSO4	-3.3750	1.0665	-3.164	0.025
ZnSO4*ZnSO4	-0.3750	1.0665	-0.352	0.739
Inoculum Size*Inoculum Size	-3.8750	1.0665	-3.633	0.015
CuSO4*ZnSO4	-4.2500	1.0247	-4.148	0.009
CuSO4*Inoculum Size	-2.2500	1.0247	-2.196	0.080
ZnSO4*Inoculum Size	4.2500	1.0247	4.148	0.009

S = 2.04939 PRESS = 143.5; R-Sq = 95.89% R-Sq(pred) = 71.91% R-Sq(adj) = 88.49%

Table 6. Analysis of Variance for GSH

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	489.933	489.933	54.437	12.96	0.006
Linear	3	234.500	234.500	78.167	18.61	0.004
Square	3	90.683	90.683	30.228	7.20	0.029
Interaction	3	164.750	164.750	54.917	13.08	0.008
Residual Error	5	21.000	21.000	4.200		
Lack-of-Fit	3	7.000	7.000	2.333	0.33	0.808
Pure Error	2	14.000	14.000	7.000		
Total	14	510.933				

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