An innovative approach to biotransformation of benzaldehyde to L-PAC via free cells of *Saccharomyces cerevisae* in the presence of β-Cyclodextrin

T. F. Adepoju[1*], S.K. Layokun[2], Ojediran, J. O[3], Charles, O[4]

[1]Chemical Engineering Department, Landmark University, Omu-aran, P.M.B. 1001, Kwara State, Nigeria

[2]Chemical Engineering Department, Obafemi Awolowo University, Ile-Ife, Osun State, P.M.B, 2200055, Nigeria

[3]Agric&Biosystem Engineering Department, Landmark University, Omu-aran, P.M.B.1001, Kwara State, Nigeria

[4]Biological Sciences Department, Landmark University, Omu-aran, P.M.B. 1001, Kwara State, Nigeria

*Address of Corresponding Author: Chemical Engineering Department, Landmark University, Omu-aran, P.M.B. 1001, Kwara State, Nigeria.

Email: avogadros2002@yahoo.com, Tel: +234-803-9404673, Fax: +234 (36) 232401

Abstract- Response surface methodology (RSM) was employed to optimize L-Phenylacetylcarbinol (L-PAC) production form biotransformation of benzaldehyde via free cell of *Saccharomyces cerevisae* presence Beta-Cyclodextrin in this work. Specifically, response surface methodology was applied, and the effect of five variables, viz. cell weight, incubation time, acetaldehyde conc., benzaldehyde conc. and β-CD level and their reciprocal were determined. Central composite rotatable design was used to generate 50 individual experiments, which was designed to study the effects of these factors during biotransformation of benzaldehyde to L-PAC. A statistical model predicted the highest biotransformation yield of L-PAC to be 586.938 (mg/100 ml) at the following optimized variables conditions: cell weight of 5.17 g (wet. wt.), incubation time of 74.82 min, acetaldehyde conc. of 1594.05 (µg/100 ml), benzaldehyde conc. of 1300 (mg/100 ml) and β-CD level of 3.20 %. Using these variables under experimental condition in three independent replicates, an actual L-PAC yield of 587.00 (mg/100 ml) was obtained. The physical properties of the produced L-PAC suggested that its could serve as a key intermediate for the synthesis of L-ephedrine, pseudoephedrine, norephedrine, nor-pseudoephedrine as well as adrenaline, amphetamine, methamphetamine, phenylpropanolamine and phenylamine.

Keywords: Biotransformation, *Saccharomyces cerevisae,* optimization, Response surface methodology, L-PAC.

1.0 Introduction

1-hydroxy-1-phenyl-2-propanone or 1-hydroxy-1-phenylacetone or α-hyroxybenzyl

methyl ketone also known as L-Phenylacetylcarbinol (L-PAC) has a molecular formula of $C_9H_{10}O_2$. It performance as a key intermediate for the synthesis of L-ephedrine, pseudoephedrine,norephedrine,norpseudoephedr-ne as well as adrenaline, amphetamine, methamphetamine, phenylpropanolamine and phenylamine (Ellaiah and Krishna, 1988; Shukla and Kulkarni, 2002).

- Adepoju T.F is currently pursuing master degree program in Chemical Engineering at Obafemi Awolowo University, Nigeria. He is a Lecturer in the department of Chemical Engineering, Landmark University, Email: avogadros2002@yah.com
- S. K. Layokun is a professor in the Department of Chemical Engineering, Obafemi Awolowo University, Nigeria.
- Ojediran O.J is a senior Lecturer from the Agric & Biosystem Engineering Department, Landmark University
- C. Okolie is Lecturer in biological Sciences Department, Landmark University, Nigeria

L-PAC can be produced by chemical synthesis from cyanohydrins, but the biotransformation route for its production from benzaldehyde is preferred industrially (Brusse et al., 1988). Biotransformation of benzaldehyde to optically L-PAC was first experimented by Neuberg and Lieberman (1921) and the demand for industrial application of this process came about when the chemical synthesis of ephedrine using 1-acetyl phenyl carbinol was patented.

Meanwhile, almost all the literature concerning the synthesis of L-PAC and benzyl alcohol by fermenting yeast deals with yield optimization by free cells (Agrawal et al., 1986; Cardillo et al., 1991; Zeeman et al., 1992**).** Studies revealed that the formation of L-PAC from benzaldehyde under normal fermentative conditions using yeast, shows that the quantitative conversion of benzaldehyde into L-PAC has never been achieved because of formation of by-products like benzyl alcohol, PAC-diol (Smith and Hendlin, 1953; Gupta *et al.,* 1979; Netraval and Vojtisek, 1982; Agrawal and Basu, 1989). The yeast cannot be used for multiple batches because of the toxic and inhibitory effects of substrate and products (Long et al., 1989; Coughlin *et al.,* 1991).

The use of cyclodextrin always decreased the toxicity of benzaldehyde for bioconversion using immobilized cells has been reported

(Coughlin et al., 1991; Mahmoud *et al.,* 1990). In view of these, Vilas *et al.,* 2002, worked on the effect of addition of b -cyclodextrin on biotransformation of benzaldehyde to L-PAC by the cells of *Torulaspora delbrueckii* in order to increase the optimum yield of L-PAC. Agrwal *et al.,* 1986, worked on the production of L-Acetyl Phenyl Carbinol by yeast employing benzaldehyde as precursor and the results was reported to be acceptable except that the experiment was not optimized.

Production of phenyl acetyl carbinol by yeast was carried out by Gupta *et al.,* 1978, but no report was available showing high yields of L-PAC production by this mechanism. Biotransformation of benzaldehyde to L-phenylacetylcarbinol (L-PAC) by *Torulaspora delbrueckii* and conversion to ephedrine by microwave radiation was reported by Vilas *et al*. (2002). The results obtained were good, except the process condition was not optimized. Shukla and Kulkarni (2001) worked on the process parameters and reusability of the free cell mass of *Torulaspora delbrueckii* for the production of L-PAC without optimization using statistical approach. In the same vein, Shukla and Kulkarni (2000) worked on L-PAC: biosynthesis and industrial application. Vrsalovic *et al*. (2006) carried out research on modeling of transformation processes using numerical method and the process was optimized using the Nelder-Mead algorithm. The numerical values of the parameters were evaluated by fitting the model to the experimental data with the "Scientist" software. The model differential equations were solved numerically by the fourth order Runge-Kutta algorithm, which is also offered in the same software. The results obtained were good except the complexity in the optimization steps. In this work, biotransformation of benzaldehyde to L-PAC was carried out via the use of free cells of *Saccharomyces cerevisiae*. To optimize the biotransformation conditions for the production on L-PAC, RSM was applied to determine the effects of five -level-five factors and their reciprocal interactions on the yield of L-PAC.

2.0 Material and Methods

2.1 Materials

All the chemicals (diethyl ether, anhydrous sodium sulphate, benzaldehyde, acetyladehyde, b-

cyclodextrin ((b- CD) etc.) used were of analytical grade and need no further purification.

2.2 Methods

2.2.1 Microorganisms

Saccharomyces cerevisae used in this study was isolated locally. The culture was consistently maintained on a medium containing 0.4% dextrose, 1% yeast extract, 1% malt extract, and 2% agar at pH 7.2 (Agarwal *et al*., 1986).

2.2.2 The growth medium

The growth medium for *Saccharomyces cerevisae* (Long et al., 1989) contained glucose 2%, peptone 2%, yeast extract 1% and had pH 5.5.

2.2.3 Culture growth

1 ml suspension of cells of the isolate *Saccharomyces cerevisae* containing 10^6 cells was inoculated into 9 ml of growth medium and incubated on a rotary shaker at 30 ± 2°C at 240 rpm for 24 h. The obtained culture was inoculated into 100 ml of the same medium and allowed to grow for 24 h. Under the same conditions, cells were harvested by centrifuging at 10, 000 rpm for 15 min at 15 °C. The biomass obtained was washed with water, centrifuged and was used for biotransformation studies.

2.2.4 Biotransformation of benzaldehyde to L-PAC

100 ml of biotransformation medium containing 5% glucose, 0.6% peptone and had pH 4.5 was inoculated with a known weight of cell mass (biomass) obtained. The reactor was incubated on a shaker at 30 °C and 240 rpm at different time range for adaptation of cells to the medium. Benzaldehyde and acetaldehyde was added and flasks were incubated again for the biotransformation on a shaker at 30 °C and 240 rpm.

2.2.5 Effect of b-cyclodextrin addition on biotransformation of benzaldehyde

Effect of 0.4 – 1.6% β-cyclodextrin (b-CD) was studied at benzaldehyde and acetaldehyde levels ranging from 500 mg to 1600 mg/100 ml and 400 µl to 1300 µl/100 ml, respectively. The reaction was allowed to take place for 3 h at 30 ± 2°C and 240 rpm. Semi-continuous feeding of different levels of benzaldehyde and acetaldehyde was also carried out according to design software (Table 1) at different intervals in presence of β -CD.

2.3 Analysis of biotransformation products

Once biotransformation, the medium was centrifuged at 10,000 rpm for 15 min. The

supernatant were extracted three times with equal volumes of diethylether. The combined extract was dried over anhydrous sodium sulphate and concentrated over a temperature controlled water bath. The residue obtained was dissolved in methanol and subjected to gas chromatography (GC) analysis.

2.4 Gas Chromatography Analysis

The conditions used for GC analysis were as follows- GC model used was Chemito-8510 with Oracle -1 computing integrator. A 4 meter long column of 5% OV-17 was used. The injector temperature and detector temperature (FID) was maintained at 250 ^{o}C. Column programming was as follows: 75 ^{o}C for 3 min, then 10 ^{o}C/ 1 min up to 250 ^{o}C and holding time was for 5 min. Retention times of L-PAC was 17 min. The concentration of the compound was determined using peak area method (Shukla and Kulkarni, 1999). The experiment was replicated in triplicate until it was found to be reproducible within ± 3 percent limits.

2.5 Experimental design

Central Composite Rotatable Design (CCRD) experimental design was employed to optimize the biotransformation of benzaldehyde to L-PAC. Five-level-five-factors design was applied, which generate 50 experimental runs. This included 32 factorial points, 10 axial points, and 8 central points to provide information regarding the interior of the experimental region, making it possible to evaluate the curvature effect. Selected factors for biotransformation of benzaldehyde to L-PAC were; cell weight g (wet. wt): X_1, incubation time (min): X_2, Acetaldehyde conc. (mg/100 ml): X_3, benzaldehyde conc. (mg/100 ml): X_4 and β-CD level (%): X_5. Table 1 show the independent factors and their five levels for Central Composite design, and the combinations of five independent factors in a Central Composite experimental design.

Depicted in Table 2 also are the L-PAC yields, the predicted yields and the residual values. The effects of unexplained variability in the L-PAC yield response due to extraneous factors were minimized by randomizing the order of experiments.

Table 1: Factors and their Levels for Composite Central Design

Variable	Symbol	Coded factor levels				
		-2	-1	0	1	2
CW	X_1	2	3	4	5	6
IT	X_2	40	50	60	70	80
AC	X_3	400	700	1000	1300	1600

BC	X_4	500	700	900	1100	1300
ß-CD level (%)	X_5	0.4	0.8	1.2	1.6	3.2

CW= Cell weight g (wet. wt), IT= Incubation time (min), AC= Acetaldehyde conc. (µg/100 ml), BC= Benzaldehyde conc. (mg/100 ml)

Table 2: Experimental design matrix by central composite rotatable design (CCRD) for five-level-five-factors response surface study

SR	X_1	X_2	X_3	X_4	X_5	L-PAC	Pv	Res.
1	-1	-1	-1	-1	-1	212.00	211.89	0.11
2	1	-1	-1	-1	-1	220.00	220.12	-0.12
3	-1	1	-1	-1	-1	211.00	210.72	0.28
4	1	1	-1	-1	-1	210.00	210.33	-0.33
5	-1	-1	1	-1	-1	209.00	209.04	-0.038
6	1	-1	1	-1	-1	213.00	212.90	0.10
7	-1	1	1	-1	-1	211.00	210.74	0.26
8	1	1	1	-1	-1	206.00	205.98	0.022
9	-1	-1	-1	1	-1	205.00	205.22	-0.22
10	1	-1	-1	1	-1	206.00	205.57	0.43
11	-1	1	-1	1	-1	205.00	205.42	-0.42
12	1	1	-1	1	-1	197.00	197.16	-0.16
13	-1	-1	1	1	-1	201.00	200.49	0.51
14	1	-1	1	1	-1	196.00	196.47	-0.47
15	-1	1	1	1	-1	204.00	203.57	0.43
16	1	1	1	1	-1	191.00	190.93	0.068
17	-1	-1	-1	-1	1	332.00	332.23	-0.23
18	1	-1	-1	-1	1	364.00	364.09	-0.091
19	-1	1	-1	-1	1	368.00	367.94	0.060
20	1	1	-1	-1	1	391.00	391.17	-0.17
21	-1	-1	1	-1	1	392.00	391.51	0.49
22	1	-1	1	-1	1	419.00	418.99	0.01
23	-1	1	1	-1	1	430.00	430.09	-0.091
24	1	1	1	-1	1	449.00	448.95	0.051
25	-1	-1	-1	1	1	477.00	476.69	0.31
26	1	-1	-1	1	1	500.00	500.67	-0.67
27	-1	1	-1	1	1	514.00	513.77	0.23
28	1	1	-1	1	1	529.00	529.13	-0.13
29	-1	-1	1	1	1	534.00	534.09	-0.087
30	1	-1	1	1	1	554.00	553.70	0.30
31	-1	1	1	1	1	574.00	574.04	-0.044
32	1	1	1	1	1	585.00	585.03	-0.027
33	-2	0	0	0	0	345.00	345.61	-0.61
34	2	0	0	0	0	369.00	368.46	0.54
35	0	-2	0	0	0	305.00	305.10	-0.097
36	0	2	0	0	0	341.00	340.97	0.033
37	0	0	-2	0	0	277.00	276.49	0.51
38	0	0	2	0	0	339.00	339.58	-0.58
39	0	0	0	-2	0	216.00	216.09	-0.086
40	0	0	0	2	0	370.00	369.98	0.022
41	0	0	0	0	-2	52.00	52.14	-0.14
42	0	0	0	0	2	664.00	663.92	0.079
43	0	0	0	0	0	386.00	386.51	-0.51
44	0	0	0	0	0	387.00	386.51	0.49
45	0	0	0	0	0	386.00	386.51	-0.51
46	0	0	0	0	0	387.00	386.51	0.49
47	0	0	0	0	0	386.00	386.51	-0.51
48	0	0	0	0	0	387.00	386.51	0.49
49	0	0	0	0	0	386.00	386.51	-0.51
50	0	0	0	0	0	387.00	386.51	0.49

PV=predicted value (mg/100 ml), Res. = Residual, SR= Standard Runs

2.5.1 Statistical Data Analysis

The data obtained from biotransformation of benzaldehyde to L-PAC was analysed statistically using response surface methodology (CCRD), so as to fit the quadratic polynomial equation generated by the Design-Expert software version 8.0.3.1 (Stat-Ease Inc., Minneapolis, USA). To correlate the response variable to the independent variables, multiple regressions was used to fit the coefficient of the polynomial model of the response. The quality of the fit of the model

was evaluated using test of significance and analysis of variance (ANOVA). The fitted quadratic response model is described by Eqn 1:

$$Y = b_0 + \sum_{i=1}^{k} b_i X_i + \sum_{i=1}^{k} b_{ii} X_i^2 + \sum_{i<j}^{k} b_{ij} X_i X_j + e \quad (1)$$

Where: Y is L-PAC yield (response factor), b_o is the intercept value, b_i (i= 1, 2,…… k) is the first order model coefficient, b_{ij} is the interaction effect, and b_{ii} represents the quadratic coefficients of X_i, and e is the random error.

3.0 Results and Discussion

Table 2 shows the coded factors considered in this study with L-PAC yield, predicted value as well as the residual values obtained. Design Expert 8.0.3.1 software was employed to evaluate and determine the coefficients of the full regression model equation and their statistical significance. Table 3 described the results of test of significance for every regression coefficient. Considering the large F-values (the test for comparing the variance associated with all terms with the residual variance) and low corresponding p-values (the probability value that is associated with the F -value for all terms), all the model terms are remarkably significant and have very strong effects on the L-PAC yield witt $p< 0.05$ (Table 3).

Nevertheless, the linear term X_5 with F-value of 3.61×10^6 and p-value of <0.0001, was the most significant model term. In order to minimize error, all the coefficients were considered in the design. The results of the second-order response surface model fitting in the form of ANOVA are presented in Table 4. The model F-value (terms used to estimate effects) of 2.156×10^5 with low p-value (<0.0001) implied a high significance for the regression model (Yuan *et al.,* 2008). The goodness of fit of the model was checked by the coefficient of determination (R^2). R^2 should be at least 0.80 for the good fit of a model (Guan and Yao, 2008). In this case, the R^2 value of 1.00 indicated that the sample variation of 100% for the L-PAC production is attributed to the independent factors (cell weight, incubation time, acetaldehyde conc., benzaldehyde conc. and β-CD level). The value of the adjusted determination coefficient (Adj. R^2 of 1.00) was also identical, supporting a high significance of the model (Akhnazarova and Kefarov, 1982; Khuri and Cornell, 1987) and all p-values were less than 0.05, implying that the model proved suitable for the

adequate representation of the actual relationship among the selected factors. The lack-of-fit term of 0.8317 was not significant relative to the pure error. In this case, a non-significant lack of fit is good. Hence, the model could be used in theoretical prediction of the L-PAC production. The developed regression model equation describing the relationship between the L-PAC yield (Y) and the coded values of independent factors of cell weight (X_1), incubation time (X_2), acetaldehyde conc. (X_3), benzaldehyde (X_4) and β-CD level (X_5) and their respective interactions is described in Eq. (2).

$$\begin{aligned} Y(mg/100\,ml) = {} & 386.51 + 4.80x_1 + 7.54x_2 \\ & + 13.26x_3 + 32.35x_4 + 128.61x_5 \\ & - 2.16x_1x_2 - 1.09x_1x_3 - 1.97x_1x_4 \\ & + 5.91x_1x_5 + 0.72x_2x_3 + 0.34x_2x_4 \\ & + 9.22x_2x_5 - 0.47x_3x_4 \\ & + 15.53x_3x_5 + 37.78x_4x_5 - 5.21x_1^2 \\ & - 11.22x_2^2 - 13.87x_3^2 - 16.52x_4^2 \\ & - 5.03x_5^2 \qquad (2) \end{aligned}$$

Where Y= $L - PAC\ yield\ (mg/100\,ml)$

All negative and positive values in the equation shows that the variables have negative and positive effect on the yield of L-PAC production, respectively. The model coefficients and probability values i.e. coded value are shown in Table 5. The low values of standard error observed in the intercept and all the model terms showed that the regression model fits the data well, and the prediction is good (Table 5). The variance inflation factor (VIF) obtained in this study showed that the 8-centre points are orthogonal to all other factors in the model. The model also proved suitable for the adequate representation of the real relationship among the selected independent factors.

Usually, the three-dimensional (3D) response surface plots are graphical representations of the regression equation for the optimization of the reaction variables, and they are represented in Figure 2. The curvatures' nature of 3D surfaces in Figure 2a, b, e, f, and h suggested reciprocal interaction of cell weight with incubation time, cell weight with acetaldehyde conc., incubation time with acetaldehyde conc., incubation time with benzaldehyde conc. and acetaldehyde conc. with benzaldehyde conc., respectively. On the other hand, the nature of curvatures' of 3D surfaces in Figure 2c, d, g, i, j indicated moderate interactions of cell weight with benzaldehyde conc., cell weight with β-CD

level, incubation time with β-CD level, acetaldehyde conc. with β-CD level, and benzaldehyde conc. with β-CD level, respectively.

The optimal values of the independent factors selected for the biotransformation of benzaldehyde to L-PAC were obtained by solving the regression equation (Equation. 2) using the Design-Expert software package. The optimal conditions for this process were statistically predicted as X_1 = 5.17 g (wet. wt.), X_2 = 74.82 (min), X_3 = 1594.05 (µl/100 ml), X_4 = 1300 (ml/100 ml) and X_5 = 3.20 %. The predicted L-PAC yield under the above set conditions was 586.938 (mg/100 ml). In order to verify the prediction of the model, the optimal conditions were applied to three independent replicates, and the average L-PAC yield obtained was 587.00 (mg/100 ml), which is well within the predicted value for the model equation.

Qualities of L-PAC

In order to ascertain the quality of the L-PAC produced, the physical appearance was found to be in powder form, the density was determined to be 1.115 g/cm^3, the melting and boiling points were found to be 170 ± 2 and 251± 2 ^{0}C, respectively.

Table 3: Test of significance for all regression coefficient terms

Source	SS	df	MS	F-value	p-value
X_1	999.64	1	999.64	5031.47	<0.0001
X_2	2463.02	1	2463.02	12397.09	<0.0001
X_3	7618.98	1	7618.98	38348.51	<0.0001
X_4	45333.77	1	45333.77	2.282x 10^5	<0.0001
X_5	7.164x 10^5	1	7.164x 10^5	3.61x 10^6	<0.0001
X_1X_2	148.78	1	148.78	748.86	<0.0001
X_1X_3	38.28	1	38.28	192.68	<0.0001
X_1X_4	124.03	1	124.03	624.28	<0.0001
X_1X_5	1116.28	1	1116.28	5618.56	<0.0001
X_2X_3	16.53	1	16.53	83.21	<0.0001
X_2X_4	3.78	1	3.78	19.03	<0.0001
X_2X_5	2719.53	1	2719.53	13688.18	<0.0001
X_3X_4	7.03	1	7.03	35.39	<0.0001
X_3X_5	7719.03	1	7719.03	38852.10	<0.0001
X_4X_5	45677.53	1	45677.53	2.299x 10^5	<0.0001
X_1^2	1508.47	1	1508.47	7592.59	<0.0001
X_2^2	6996.22	1	6996.22	35213.98	<0.0001
X_3^2	10693.63	1	10693.63	53824.12	<0.0001
X_4^2	15172.48	1	15172.48	76367.46	<0.0001
X_5^2	1407.85	1	1407.85	7086.11	<0.0001

SS= Sum of Square, MS= mean square

Table 4: Analysis of variance (ANOVA) of regression equation

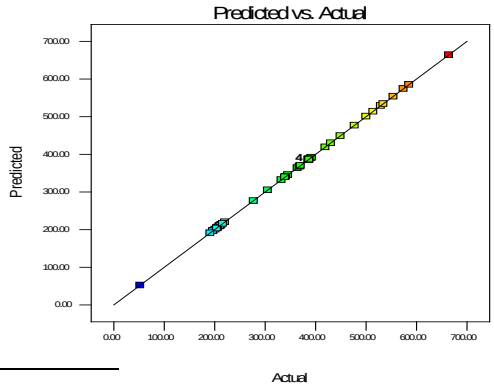
Source	Sum of Squares	df	Mean Square	F-value	p-value

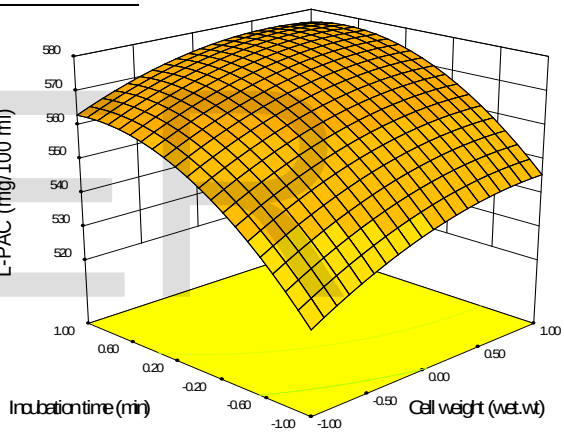
Model	5.568 x 10^5	20	42838.92	2.156 x 10^5	<0.0001
Residual	5.76	29	0.20		
Lack of fit	3.76	22	0.17	0.60	0.8317
Pure error	2.00	7	0.29		
Cor total	8.568 x 10^5	49			

R^2 = 100% R^2 (adj.) = 100% Std. Dev. = 0.45 Mean = 341.58 C.V. % = 0.13

Table 5: Regression coefficients and significance of response surface quadratic

Fact.	CE	df	Standard error	95% CI Low	95% CI high	VIF
Interce	386.51	1	0.16	386.19	386.83	-
X_1	4.80	1	0.068	4.67	4.94	1.00
X_2	7.54	1	0.068	7.40	7.68	1.00
X_3	13.26	1	0.068	13.12	13.12	1.00
X_4	32.35	1	0.068	32.21	32.21	1.00
X_5	128.61	1	0.068	128.47	128.75	1.00
X_1X_2	-2.16	1	0.079	-2.32	-2.00	1.00
X_1X_3	-1.09	1	0.079	-1.25	-0.93	1.00
X_1X_4	-1.97	1	0.079	-2.13	-1.81	1.00
X_1X_5	5.91	1	0.079	5.75	6.07	1.00
X_2X_3	0.72	1	0.079	0.56	0.88	1.00
X_2X_4	0.34	1	0.079	0.18	0.50	1.00
X_2X_5	9.22	1	0.079	9.06	9.38	1.00
X_3X_4	-0.47	1	0.079	-0.63	-0.31	1.00
X_3X_5	15.53	1	0.079	15.37	15.69	1.00
X_4X_5	37.78	1	0.079	37.62	37.94	1.00
X_1^2	-5.21	1	0.060	-5.33	-5.09	1.05
X_2^2	-11.22	1	0.060	-11.34	-11.10	1.05
X_3^2	-13.87	1	0.060	-13.99	-13.75	1.05
X_4^2	-16.52	1	0.060	-16.65	-16.40	1.05
X_5^2	-5.03	1	0.060	-5.16	-4.91	1.05





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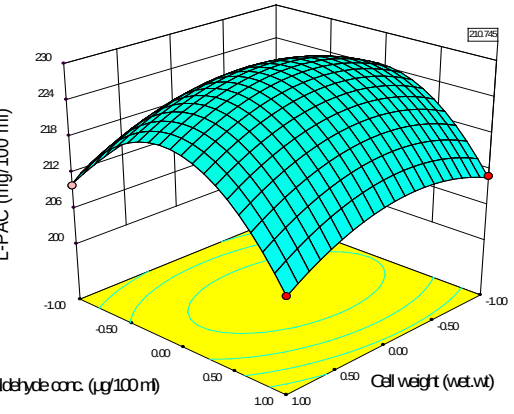

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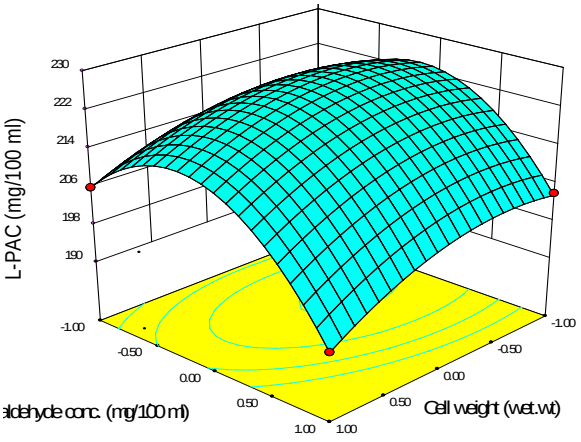
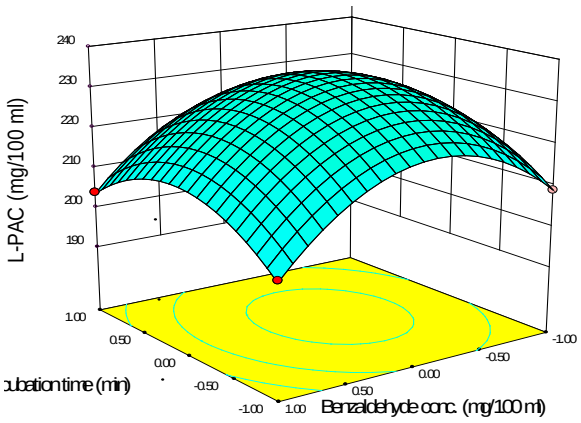
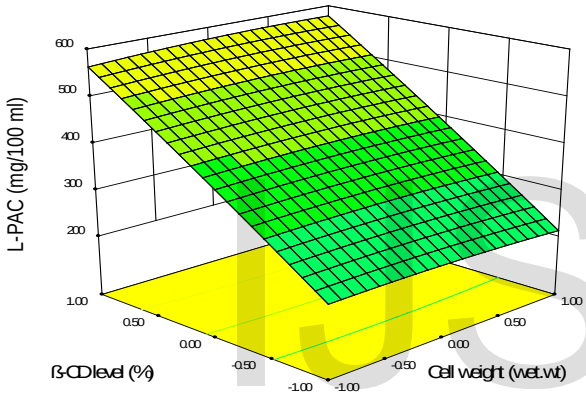


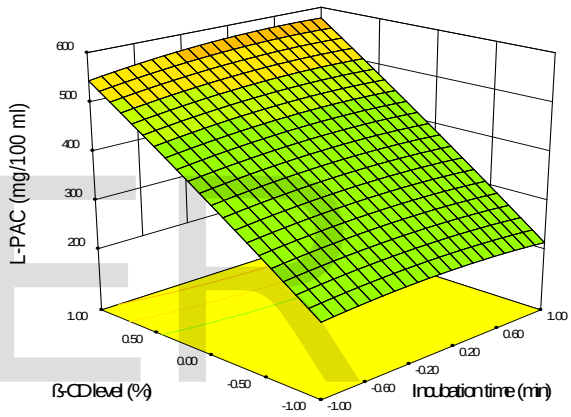
(c)



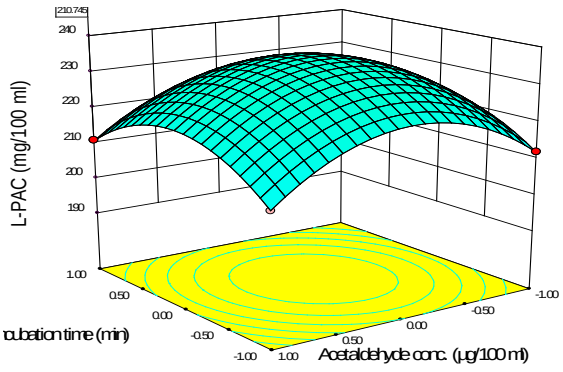
(f)



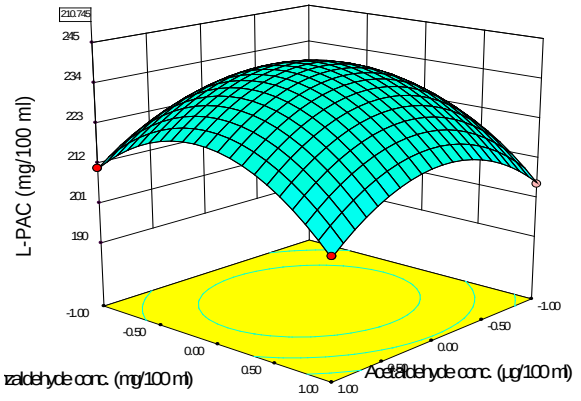
(d)



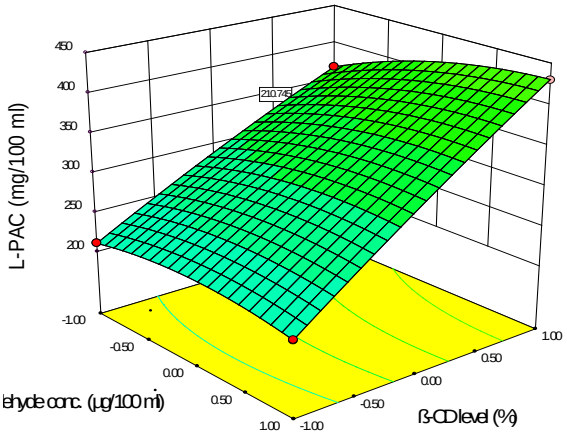
(g)



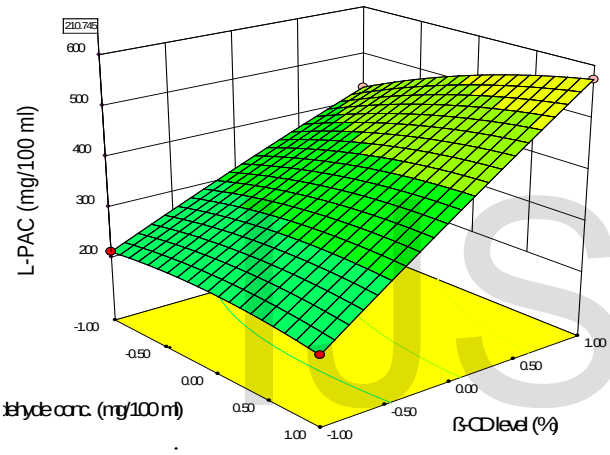
(e)



(h)



(i)



(j)

Figure 2: The curvatures' nature of 3D surfaces

Conclusions

The results obtained in this study using response surface methodology to determine the effects of five reaction variables, namely, cell weight, incubation time, acetaldehyde conc., benzaldehyde conc. and β-CD level on biotransformation of benzaldehyde to L-PAC yield via free cells *Saccharomyces cerevisae* presence of Beta- Cyclodetrin, indicate that RSM is a good optimization tools for L-PAC production. The statistical model predicted that the optimal conditions for the selected biotransformation variables as cell weight of 5.17 g (wet. wt), incubation time of 74.82 min, acetaldehyde conc. of 1594.05 (µl/100 ml), benzaldehyde conc. of 1300 (ml/100 ml) and β-CD level of 3.20 % with an actual L-PAC yield of 587.00 (mg/100 ml). Hence, this work established the usefulness of RSM for the optimum biotransformation of benzaldehyde to L-PAC and also the quality of L-PAC produced suggested that it could be used effectively as precursor for the production of L-ephedrine and D-pseudoephedrine.

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