Optimization of Benzyl Alcohol production via biotransformation of benzaldehyde using free cell of *Saccharomyces cerevisiae* in presence the β-Cyclodextrin

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Abstract: In this work, Response surface methodology (RSM) was employed to optimize the production of benzyl alcohol (BA) via the biotransformation of benzaldehyde using free cell of *Saccharomyces cerevisiae* presence β -Cyclodextrin. 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 (CCRD) was used to generate 50 individual experiments, which were designed to study the effects of these factors during the process. A statistical model predicted the highest biotransformation yield of BA to be 327.259 (mg/100 ml) at the following ooptimized variables conditions: cell weight of 6.00 g (wet. wt), incubation time of 80 min, acetaldehyde conc. of 400.00 (µg/100 ml), benzaldehyde conc. of 500.00 (mg/100 ml) and β-CD level of 3.20 %. Using these variables under experimental condition in three independent replicates, an actual BA yield of 326.00 (mg/100 ml) was obtained. The physical properties of produced BA suggests that its could be used effectively in health care as well as in industries.

Index Terms: Biotransformation, *Saccharomyces cerevisiae,* optimization, Response surface methodology, benzyl alcohol (BA)

1.0 Introduction

Benzyl alcohol (BA) also known as phenyl methanol is an aromatic alcohol with the molecular formula $C_6H_5CH_2OH$. The benzyl group is regularly abbreviated "Bn" not to be confused with "Bz" which is used for benzoyl, hence BA general formula is denoted as BnOH. It's a colorless liquid with a mild pleasant aromatic odour. Meanwhile, it's partially soluble in water and completely miscible in alcohols and diethyl ether. It is a widely used organic solvent due to its polarity, low toxicity, and low vapor pressure.

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BA is used as a general solvent for inks, paints, lacquers, and epoxy resin coatings, Furuta et al. (1995). It is also a precursor to a variety of esters, used in the soap, perfume, and flavor industries. It is often added to intravenous medication solutions as a preservative due to its bacteriostatic and antipruritic properties. It is also used as a photographic developer, used as a dielectric solvent for the dielectrophoretic reconfiguration of nanowires (Wissner-Gross, 2006). It is oxidized rapidly in healthy individuals to benzoic acid, conjugated with glycine in the liver, and excreted as hippuric acid. Although, high concentrations can result in toxic effects including respiratory failure, vasodilation, hypotension, convulsions, and paralysis. Newborns, especially if critically ill, may not metabolize BA as readily as adults.

U.S. FDA approved 5% solution usage of BA in the treatment of head lice in children older than 6 months and in adults (Sciele Pharmaceuticals, Inc., 2009). Benzyl alcohol lotion is used to treat head lice (small insects that attach themselves to the skin) in adults and children 6

months of age and older. But it's not advisable to be used in children less than 6 months of age. BA has nearly the same refraction index of quartz and wool fibre. If a clear quartz object is immersed in BA, it becomes almost invisible. This has been used as a method to non-destructively recognize if an object is made of true quartz or not. Similarly, white wool immersed in BA also becomes almost invisible clearly revealing contaminants such as dark and medullated fibres and vegetable matter.

BA is produced naturally by many plants and is commonly found in fruits and teas. It is also found in a variety of essential oils including jasmine, hyacinth, and ylang-ylang (Merck ECDB, 1989). It is also one of the chemical compounds found in castoreum. This compound is gathered from the beaver plant food (Muller-Schwarze, 2003). It is also a bi-production in biotransformation of benzyladehyde to Phenylacetylcarbinol (L-PAC). 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 BA from benzaldehyde under normal fermentative conditions using yeast, shows that the quantitative conversion has never been achieved because of formation of by-products like L-PAC, 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). Although, 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).

Response Surface Methodology (RSM), a useful optimization tool has been applied in research to study the effect of individual variables and their interactions on response variables. It has been used extensively in the optimization processes. (Mohammed *et al*., 2008; Mitra *et al*., 2009; Njoku *et al*., 2009; Tan *et al*., 2009). The main advantage of RSM is the ability to reduced number of experimental runs needed to provide sufficient information for statistically acceptable results. In

view of these, this work dwells on optimization of production of BA via the biotransformation of benzaldehyde to L-PAC using the free cells of *Saccharomyces cerevisiae*. To optimize the biotransformation conditions, RSM was applied to determine the effects of five -level-five factors and their reciprocal interactions on the yield of BA.

2.0 Material and Methods

2.1 Materials

All the chemicals (diethyl ether, anhydrous sodium sulphate, benzaldehyde, acetyladehyde, β-cyclodextrin ((β- CD) etc.) used were of analytical grade and need no further purification.

2.2 Methods

2.2.1 Microorganisms

Saccharomyces cerevisiae 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 cerevisiae* (Long et al., 1989) contained glucose 2%, peptone 2%, yeast extract 1% and had pH 5.5.

2.2.3 Culture growth

Suspension of cells of the isolate *Saccharomyces cerevisiae* (1.0 ml) 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

Biotransformation medium (100 ml) 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 – 3.2% β-cyclodextrin (β-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

After 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 °C. Column programming was as follows: 75 °C for 3 min, then 10 °C/ 1 min up to 250 °C and holding time was for 5 min. Retention times of BA (benzyl alcohol) was 13 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 process. 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 processes 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 experimentally obtained benzyl alcohol yields, the predicted yields and the residual values. Figure 1 showed the plot of actual against the predicted yield. The effects of unexplained variability in benzyl alcohol 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	BA yield	PY	Res
1	-1	-1	-1	-1	-1	259.00	258.28	0.72
2	1	-1	-1	-1	-1	276.00	276.23	-0.23
3	-1	1	-1	-1	-1	300.00	299.78	0.22
4	1	1	-1	-1	-1	298.00	298.10	-0.10
5	-1	-1	1	-1	-1	309.00	309.00	0.00
6	1	-1	1	-1	-1	311.00	311.32	-0.32
7	-1	1	1	-1	-1	338.00	338.12	-0.12
8	1	1	1	-1	-1	321.00	320.82	0.18
9	-1	-1	-1	1	-1	273.00	273.15	-0.15
10	1	-1	-1	1	-1	294.00	294.23	-0.23
11	-1	1	-1	1	-1	304.00	303.53	0.47
12	1	1	-1	1	-1	305.00	304.98	0.024
13	-1	-1	1	1	-1	313.00	313.25	-0.25
14	1	-1	1	1	-1	319.00	318.70	0.30
15	-1	1	1	1	-1	331.00	331.24	-0.24
16	1	1	1	1	-1	317.00	317.07	-0.069
17	-1	-1	-1	-1	1	305.00	305.43	-0.43
18	1	-1	-1	-1	1	321.00	320.51	0.49
19	-1	1	-1	-1	1	332.00	331.81	0.19
20	1	1	-1	-1	1	327.00	327.26	-0.26
21	-1	-1	1	-1	1	300.00	300.03	-0.028
22	1	-1	1	-1	1	299.00	299.48	-0.48
23	-1	1	1	-1	1	314.00	314.03	-0.026
24	1	1	1	-1	1	294.00	293.85	0.15
25	-1	-1	-1	1	1	282.00	281.93	0.067
26	1	-1	-1	1	1	300.00	300.13	-0.13
27	-1	1	-1	1	1	297.00	297.18	-0.18
28	1	1	-1	1	1	296.00	295.76	0.24
29	-1	-1	1	1	1	266.00	265.90	0.099
30	1	-1	1	1	1	268.00	268.48	-0.48
31	-1	1	1	1	1	269.00	268.77	0.23
32	1	1	1	1	1	251.00	251.73	-0.73
33	-2	0	0	0	0	300.00	300.37	-0.37
34	2	0	0	0	0	302.00	301.44	0.56
35	0	-2	0	0	0	337.00	336.69	0.31
36	0	2	0	0	0	366.00	366.12	-0.12
37	0	0	-2	0	0	297.00	297.43	-0.43
38	0	0	2	0	0	306.00	305.38	0.62
39	0	0	0	-2	0	320.00	320.11	-0.11
40	0	0	0	2	0	288.00	287.70	0.30
41	0	0	0	0	-2	266.00	266.22	-0.22
42	0	0	0	0	2	245.00	244.59	0.41
43	0	0	0	0	0	277.00	277.48	-0.48
44	0	0	0	0	0	278.00	277.48	0.52
45	0	0	0	0	0	277.00	277.48	-0.48
46	0	0	0	0	0	278.00	277.48	0.52

47	0	0	0	0	0	277.00	277.48	-0.48
48	0	0	0	0	0	278.00	277.48	0.52
49	0	0	0	0	0	277.00	277.48	-0.48
50	0	0	0	0	0	278.00	277.48	0.52

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

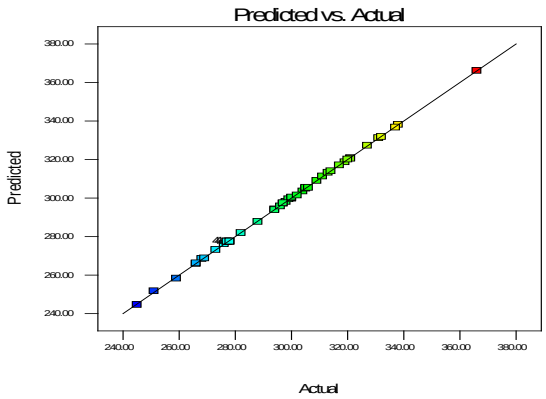


Figure 1: Plot of Actual against predicted

2.5.1 Statistical Data Analysis

The data obtained from biotransformation process to produce BA (benzyl alcohol) 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 Eq. 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 BA 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 BA 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 test for comparing the variance associated with all terms with the residual variance (large F-values) and low corresponding probability value that is associated with the F -value for all terms (p-values), all the model terms are remarkably significant and have very strong effects on the BA yield with $p< 0.05$ (Table 3).

Nevertheless, the linear term X_2^2 with F-value of 42611.15 and p-value <0.0001, is 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 29283.62 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 0.9998 indicated that the sample variation of 99.98% for the BA production is attributed to the independent factors (cell weight, incubation time, acetaldehyde conc., benzaldehyde conc. and β-CD level) only 0.02% of the total variations were not explained by the model. The value of the adjusted determination coefficient (Adj. R^2 of 0.996) was also very high, 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 0.7490 was not significant relative to the pure error. In this case, a non-significant lack of fit is

Table 3: Test of significance for all regression coefficient terms

Sour	SS	df	MS	F-value	p-value
X_1	2.20	1	2.20	9.87	<0.0001
X_2	1657.91	1	1657.91	7444.86	0.0039
X_3	121.04	1	121.04	543.52	<0.0001
X_4	2010.67	1	2010.67	9028.94	<0.0001
X_5	895.51	1	895.51	4021.32	<0.0001
X_1X_2	770.28	1	770.28	3458.96	<0.0001
X_1X_3	488.28	1	488.28	2192.64	<0.0001
X_1X_4	19.53	1	19.53	87.71	<0.0001
X_1X_5	16.53	1	16.53	74.23	<0.0001
X_2X_3	306.28	1	306.28	1375.36	<0.0001
X_2X_4	247.53	1	247.53	1111.54	<0.0001
X_2X_5	457.53	1	457.53	2054.55	<0.0001
X_3X_4	225.78	1	225.78	1013.88	<0.0001
X_3X_5	6300.03	1	6300.03	28290.42	<0.0001
X_4X_5	2945.23	1	2945.23	13225.84	<0.0001
X_1^2	952.62	1	952.62	4277.77	<0.0001
X_2^2	9489.13	1	9489.13	42611.15	<0.0001
X_3^2	993.73	1	993.73	4462.36	<0.0001
X_4^2	1212.29	1	1212.29	5443.80	<0.0001
X_5^2	846.47	1	846.47	3801.08	<0.0001

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

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	29283.62	20	1464.18	6574.93	<0.0001
Residual	6.46	29	0.22		
Lack of fit	4.46	22	0.20	0.71	0.7490
Pure error	2.00	7	0.29		
Cor total	29290.08	49			

R^2 = 99.98%, R^2 (adj.) = 99.96%

SS= Sum of Square, MS= mean square

good. Hence, the model could be used in theoretical prediction of the BA production. The developed regression model equation describing the relationship between the BA 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).

$$Y_2 = 277.48 + 0.23x_1 + 6.19x_2 + 1.67x_3 - 6.81x_4 - 4.55x_5 - 4.91x_1x_2 - 3.91x_1x_3 + 0.78x_1x_4 - 0.72x_1x_5 - 3.09x_2x_3 - 2.78x_2x_4 - 3.78x_2x_5 - 2.66x_3x_4 - 14.03x_3x_5 - 9.59x_4x_5 + 4.14x_1^2 + 13.07x_2^2 + 4.23x_3^2 + 4.67x_4^2 - 3.90x_5^2 \quad (2)$$

Where Y_2 = BA yield (mg/100 ml)

The linear (x_3, x_4, x_5), all the cross products (x_1x_2, x_1x_3, $x_1x_5, x_2x_3, x_2x_4, x_2x_5, x_3x_4$, x_3x_5 *and* x_4x_5) and the quadratic (x_5^2) have negative impact on BA yield, whereas, the rest of the products have positive effect on the yield. 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

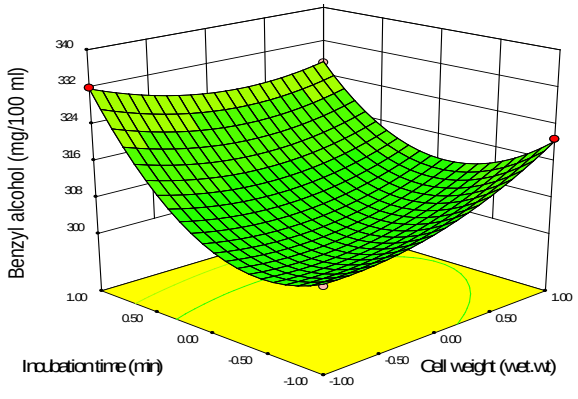
Table 5: Regression coefficients and significance of response surface quadratic

Fact.	CE	df	SE	95% CI Low	95% CI high	VIF
Inter	277.48	1	0.17	277.15	277.82	-
X_1	0.23	1	0.072	0.079	0.37	1.00
X_2	6.19	1	0.072	6.04	6.33	1.00

X_3	1.67	1	0.072	1.53	1.82	1.00
X_4	-6.81	1	0.072	-6.96	-6.67	1.00
X_5	-.4.55	1	0.072	-4.69	-4.40	1.00
X_1X_2	-4.91	1	0.083	-5.08	-4.74	1.00
X_1X_3	-3.91	1	0.083	-4.08	-3.74	1.00
X_1X_4	0.78	1	0.083	0.61	0.95	1.00
X_1X_5	-0.72	1	0.083	-0.89	-0.55	1.00
X_2X_3	-3.09	1	0.083	-3.26	-2.92	1.00
X_2X_4	-2.78	1	0.083	-2.95	-2.61	1.00
X_2X_5	-3.78	1	0.083	-3.95	-3.61	1.00
X_3X_4	-2.66	1	0.083	-2.83	-2.49	1.00
X_3X_5	-14.03	1	0.083	-14.20	-13.86	1.00
X_4X_5	-9.59	1	0.083	-9.76	-9.42	1.00
X_1^2	4.14	1	0.063	4.01	4.27	1.05
X_2^2	13.07	1	0.063	12.94	13.20	1.05
X_3^2	4.23	1	0.063	4.10	4.36	1.05
X_4^2	4.67	1	0.063	4.54	4.80	1.05
X_5^2	-3.90	1	0.063	-4.03	-3.77	1.05

Figure 2. The curvatures' nature of 3D surfaces in Figure 2a, b, e, f, g, and i suggested reciprocal interaction of cell weight with incubation time, cell weight with acetaldehyde conc., incubation time with acetaldehyde conc., incubation time with benzaldehyde conc. incubation time with β-CD level and benzaldehyde conc. with β-CD level, respectively. Meanwhile, the nature of curvatures' of 3D surfaces in Figure 2c, d, h, j showed moderate interactions of cell weight with benzaldehyde conc., cell weight with β-CD level, acetaldehyde conc. with benzaldehyde conc., and acetaldehyde conc. with β-CD level, respectively.

The optimum values of the independent factors selected for the biotransformation of benzaldehyde to BA were obtained by solving the regression equation (Eq. 2) using the Design-Expert software package. The optimum conditions for this process were statistically predicted as X_1 = 6.0 g (wet. wt.), X_2 = 80 (min), X_3 = 400.00 (µg/100 ml), X_4 = 500 (mg/100 ml) and X_5 = 3.20 %. The predicted BA yield under the above set conditions was 327.259 (mg/100 ml). In order to verify the prediction of the model, the optimum conditions were applied to three independent replicates, and the average BA yield obtained was 326.00 (mg/100 ml), which was well within the range predicted by the model equation.



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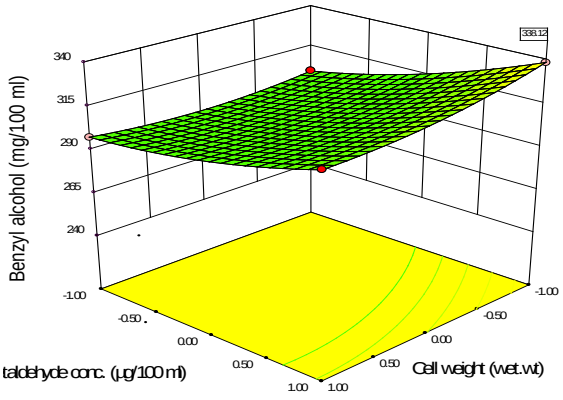

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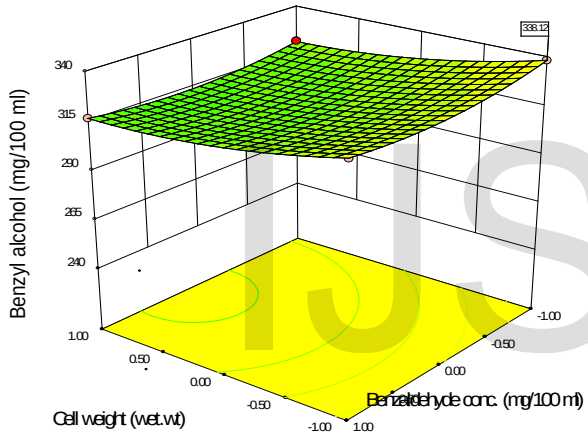

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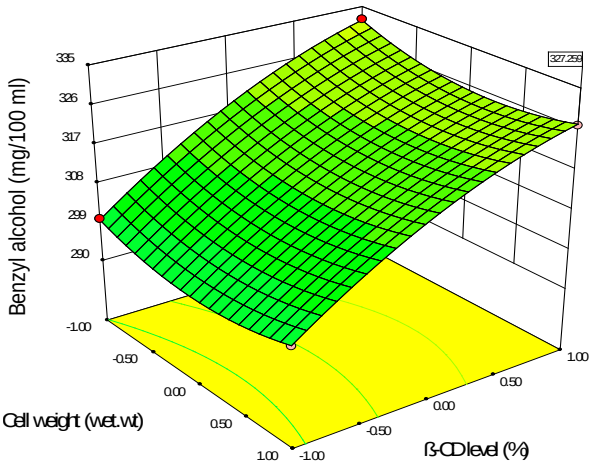
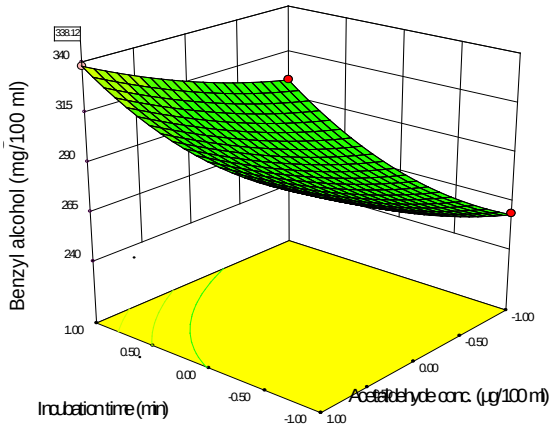
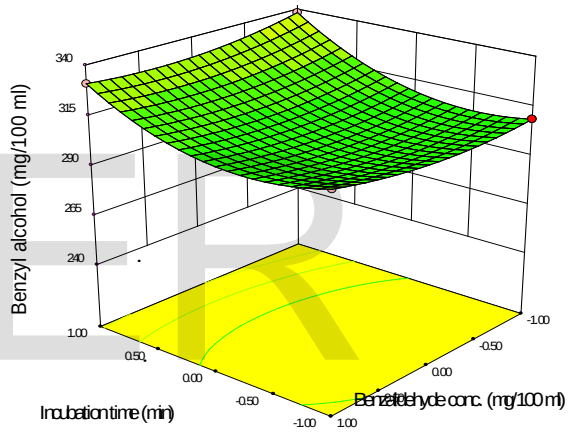


(d)

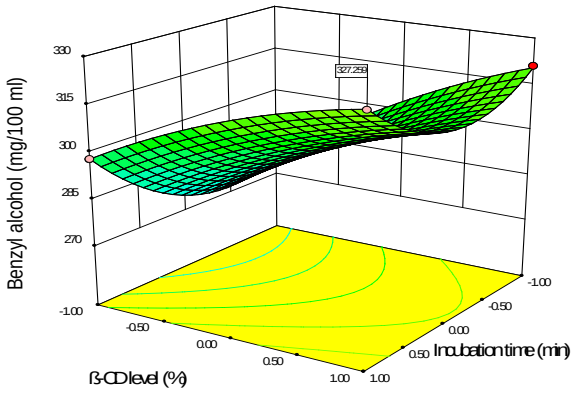


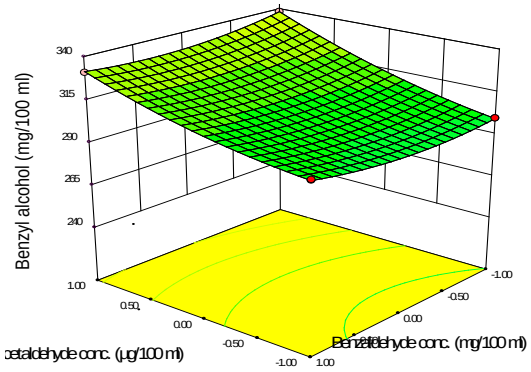
(e)



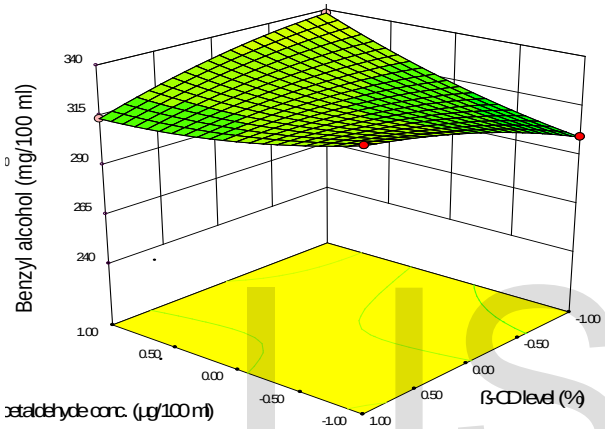
(f)

(g)

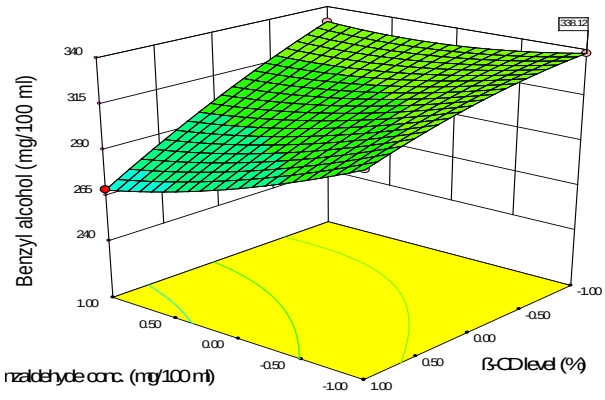




(h)



(i)



(j)

Figure 2: The curvatures' nature of 3D surfaces plots

Physical properties of BA

In other to ascertain the quality of the L-PAC produced, the physical properties was carried out, the appearance was found to be colourless liquid with density 1.03 kg/dm^3, the boiling points was found to be 204 ± 2 ^{0}C and its partially soluble in water.

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 via free cell *Saccharomyces cerevisiae* presence of Beta-Cyclodetrin, indicate that the BA produced was high. The statistical model predicted the optimal conditions for the selected biotransformation variables as cell weight of 6.00 g (wet. wt), incubation time of 80 min, acetaldehyde conc. of 400.00 (µl/100 ml), benzaldehyde conc. of 500.00 (ml/100 ml) and β-CD level of 3.20 % with an actual BA yield of 326.00 (mg/100 ml). Hence, this work established the usefulness of RSM for the

optimum biotransformation of benzaldehyde to BA and also the quality of BA produced advocated that it could be used effectively in health care as well as other industrial applications.

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