International Journal of Engineering Science Invention
ISSN (Online): 2319 – 6734, ISSN (Print): 2319 – 6726
www.ijesi.org Volume 2 Issue 11‖ November 2013 ‖ PP.29-37

Biotransformation Of Benzaldehyde To L- Phenylacetylcarbinol By Free Cells Of Yeast (*Saccharomyces Cerevisae*), Effects Of B-Cyclodextrin And Its Optimization

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ABSTRACT: *In this study, an Artificial Neural Network (ANNs) was engaged to optimize L-Phenylacetylcarbinol production form biotransformation of benzaldehyde by free cell of yeast and the effect ofβ-Cyclodetrin.In developing ANN model, performance of ANN is heavily influenced by its network structure,five-level-five-factors design was applied, which generate 50 experimental runs. The inputs for the ANNs arecell weight (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-level (%): X_5. The learning algorithms used was QP with MNFF, the transfer function was Tanh. Meanwhile, RMSE was determined to be 7.245. The coefficient of determination R^2 and the adj. R^2 were found to be 0.9985 and 0.997, respectively.It was observed that 1000 (mg/100 ml) benzaldehyde with 900 (µg/100 ml) acetaldehyde in the presence of 1.8% β-cyclodextrin gave the highest yield (664 mg/100 ml) of L-Phenylacetylcarbinol. Hence, the organism can tolerate higher levels of acetaldehyde and benzaldehyde.*

KEYWORDS:*Artificial neural network (ANNs), Biotransformation, Yeast, L-Phenylacetylcarbinol, Optimization*

I. INTRODUCTION

One of the key intermediate for the synthesis of most pharmaceutical products (norephedrine,nor-pseudoephedrine, L-ephedrine, pseudoephedrine etc.) is L-Phenylacetylcarbinol. Although, chemical synthesis from cyanohydrins can be used in the production, but the biotransformation pathusing benzaldehyde for it industrially production is preferable, Brusse*et al.*(1988). Neuberg and Lieberman (1921) first experimented the biotransformation of benzaldehyde to optically L-PAC, since then the demand for industrial application of this process came about when the chemical synthesis of ephedrine using 1-acetyl phenylcarbinol 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 β-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. Adepoju *et al*. (2013) carried out research on an innovative approach to biotransformation of benzaldehyde to L-PAC via free cells of saccharomyces cerevisae in the presence of β-cyclodextrin, using response surface methodology and the process was optimized using CCRD. The results obtained were good. In this work, an Artificial Neural Network (ANNs) was engaged to optimize L-

Phenylacetylcarbinol production form biotransformation of benzaldehyde by free cell of yeast (*Saccharomyces cerevisae*) and the effect of β-Cyclodetrin was evaluated.

II. MATERIAL AND METHODS

2.1 MATERIALS

All chemicals used such as; diethyl ether, anhydrous sodium sulphate, benzaldehyde, acetaldehyde, β-cyclodextrin were of analytical grade and need no further purification.

2.2 METHODS

2.2.1 MICROORGANISMS

Yeast 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; Adepoju *et al*., 2013).

2.2.2 THE GROWTH MEDIUM

The growth medium for *Saccharomyces cerevisae* (Long *et al.,* 1989; Adepoju *et al.,* 2013) 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^{o}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 ^{o}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 ^{o}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 ^{o}C and 240 rpm.

2.2.5 EFFECT OF β-CYCLODEXTRIN ADDITION ON BIOTRANSFORMATION OF BENZALDEHYDE

Effect of various levels of β-cyclodextrin 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^{o}C and 240 rpm. To study the effect of β-CD level, concentration of β-CD was optimized in the range of 0.4 to 3.2%. Semi-continuous feeding of different levels of benzaldehyde and acetaldehyde was also carried out according to design software (Table 1).

2.3ANALYSIS 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 diethyl ether. 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 preparedfor gas chromatography (GC) analysis.

2.4GAS 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, Adepoju *et al.,* 2013). The experiment was replicated in triplicate until it was found to be reproducible within ± 3 percent limits.

2.5EXPERIMENTAL DESIGN

In developing ANN model, performance of ANN is heavily influenced by its network structure; therefore, the learning algorithms used was QuickProp (QP), multilayer connection type used was multilayer normal feed forward (MNFF), three total layer numbers was used and the node number of input layer was five.

For the output layer, Node Number was 1, the transfer function was Tanh and the slope of transfer function and the hidden Layer was 1, the node number was 12, transfer function was also Tanh and slope of transfer function was also 1 (Fig. 1). Meanwhile, the optimum ANN structure was determined first using mean square error (MSE) approach. The higher coefficient R^2 was determined; the variable analysis also was conducted to study the effects of variables towards the L-Phenylacetylcarbinol production using 3D curvature's surface plots. A hybrid ANN model was used in conducting process optimization.

Table 1 show the independent factors 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, and their five levels for ANNs design.Table 2 depicts the L-Phenylacetylcarbinol yields, the observed values and the difference. The effects of unexplained variability in the L-Phenylacetylcarbinol yield response due to extraneous factors were minimized by randomizing the order of experiments.

Table 1: Factors and their Levels for ANNs Design

Variable	Symbol	Actual factor levels				
Cell weight (wet. wt)	X_1	2	3	4	5	6
Incubation time (min)	X_2	40	50	60	70	80
Acetaldehyde conc. (µg/100 ml)	X_3	400	700	1000	1300	1600
Benzaldehyde conc. (mg/100 ml)	X_4	500	700	900	1100	1300
ß-CD level (%)	X_5	0.4	0.8	1.2	1.6	3.2

Table 2: ANNs Design for Biotransformation of Benzaldehyde to L-PAC and the by products with Five Independent Variables using actual values

Std. run	X_1	X_2	X_3	X_4	X_5	L-PAC (mg/100 ml)	Observed value (mg/100 ml)	Difference
1	2.00	40.00	400.00	500.00	0.40	212	212	0.00029631
2	6.00	40.00	400.00	500.00	0.40	220	220	6.9505E-5
3	2.00	80.00	400.00	500.00	0.40	211	211	0.00033853
4	6.00	80.00	400.00	500.00	0.40	210	210	0.0008403
5	2.00	40.00	1600.00	500.00	0.40	209	209	0.0012446
6	6.00	40.00	1600.00	500.00	0.40	213	213	0.0011119
7	2.00	80.00	1600.00	500.00	0.40	211	211	0.00067939
8	6.00	80.00	1600.00	500.00	0.40	206	206	0.00055253
9	2.00	40.00	400.00	1300.00	0.40	205	205	0.00040242
10	6.00	40.00	400.00	1300.00	0.40	206	206	0.00017716
11	2.00	80.00	400.00	1300.00	0.40	205	205	0.00030121
12	6.00	80.00	400.00	1300.00	0.40	197	197	0.00058316
13	2.00	40.00	1600.00	1300.00	0.40	201	201	0.0013218
14	6.00	40.00	1600.00	1300.00	0.40	196	196	0.00118
15	2.00	80.00	1600.00	1300.00	0.40	204	204	0.00085186
16	6.00	80.00	1600.00	1300.00	0.40	191	191	0.00071778
17	2.00	40.00	400.00	500.00	3.20	332	332	9.2085E-5
18	6.00	40.00	400.00	500.00	3.20	364	364	0.00020485
19	2.00	80.00	400.00	500.00	3.20	368	368	0.00032902
20	6.00	80.00	400.00	500.00	3.20	391	391	0.00066427
21	2.00	40.00	1600.00	500.00	3.20	392	392	0.0010404
22	6.00	40.00	1600.00	500.00	3.20	419	419	0.00086966
23	2.00	80.00	1600.00	500.00	3.20	430	430	0.00055557
24	6.00	80.00	1600.00	500.00	3.20	449	449	0.00039127
25	2.00	40.00	400.00	1300.00	3.20	477	477	0.00021363
26	6.00	40.00	400.00	1300.00	3.20	500	500	0.00013199
27	2.00	80.00	400.00	1300.00	3.20	514	514	0.000386
28	6.00	80.00	400.00	1300.00	3.20	529	529	0.00072191
29	2.00	40.00	1600.00	1300.00	3.20	534	534	0.001586
30	6.00	40.00	1600.00	1300.00	3.20	554	554	0.0015233
31	2.00	80.00	1600.00	1300.00	3.20	574	574	0.0011736
32	6.00	80.00	1600.00	1300.00	3.20	585	585	0.00099812
33	2.00	60.00	1000.00	900.00	1.80	345	345	0.00016085
34	6.00	60.00	1000.00	900.00	1.80	369	369	8.843E-5
35	4.00	40.00	1000.00	900.00	1.80	305	323	18

36	4.00	80.00	1000.00	900.00	1.80	341	323	18
37	4.00	60.00	400.00	900.00	1.80	277	308	31
38	4.00	60.00	1600.00	900.00	1.80	339	308	31
39	4.00	60.00	1000.00	500.00	1.80	216	216	0.000197
40	4.00	60.00	1000.00	1300.00	1.80	370	370	3.2114E-5
41	4.00	60.00	1000.00	900.00	0.40	52	52	0.00010651
42	4.00	60.00	1000.00	900.00	1.80	664	664	0.00035117
43	4.00	60.00	1000.00	900.00	1.80	386	386.5	0.50002
44	4.00	60.00	1000.00	900.00	1.80	387	386.5	0.49998
45	4.00	60.00	1000.00	900.00	1.80	386	386.5	0.50002
46	4.00	60.00	1000.00	900.00	1.80	387	386.5	0.49998
47	4.00	60.00	1000.00	900.00	1.80	386	386.5	0.50002
48	4.00	60.00	1000.00	900.00	1.80	387	386.5	0.49998
49	4.00	60.00	1000.00	900.00	1.80	386	386.5	0.50002
50	4.00	60.00	1000.00	900.00	1.80	387	386.5	0.49998

2.5.1 STATISTICAL DATA ANALYSIS

ANNs structure was used for modelling the L-PAC production. The optimum ANN structure was determined using mean square error (MSE) approach. The higher coefficient R^2 was determined; the variable analysis also was conducted to study the effects of variables towards the L-Phenylacetylcarbinol yield using relative importance and 3D curvature's surface plots. A hybrid ANN model was used in conducting process optimization. The difference between the experimental and observed values was also used to proof the validity of ANNs for the optimization of L-Phenylacetylcarbinol production.

III. RESULTS AND DISCUSSION

Table 2 depicts the actual factors considered in this study with experimental L-PAC yields, the observed yields as well as the difference obtained by ANNs software. The effects of unexplained variability in the L-PAC yield response due to extraneous factors were minimized by randomizing the order of experiments. Table 3 shows the paameters of the best network that described the results of of the normal data type for ANNs. Considering the large QP-values (the number of repetition) and low corresponding RMSE-values (root mean squared error) which was used to compare the predicted values of L-PAC yield obtained from the model with experimental data, shows that all the model terms are significant and have very strong effects on the L-PAC yield. 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 0.9985 indicated that the sample variation of 99.85% 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) 0.997 was also evaluated to be 0.997.

Generally, the three-dimensional (3D) curvature 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 4.2c, f, h, j, suggested mutual reciprocal interaction of cell weight with benzaldehyde conc., incubation time with benzaldehyde conc., acetaldehyde conc. with benzaldehyde conc., and benzaldehyde conc. with β-CD level, respectively. On the other hand, the nature of curvatures' of 3D surfaces in Figure 4.2a, b, d, e, g, i, indicated moderate interactions of cell weight with incubation time, cell weight with acetaldehyde conc., cell weight with β-CD level, incubation time with acetaldehyde conc., incubation time with β-CD level, and acetaldehyde conc. with β-CD level, respectively.

Table 3: Parameters of the Best Network for L-PAC Describing the Results of the Normal Data Type For ANNs

Data	**Values**
Iteration (QP)	309200
RMSE	7.245
Average R	0.9985
Average DC	0.997

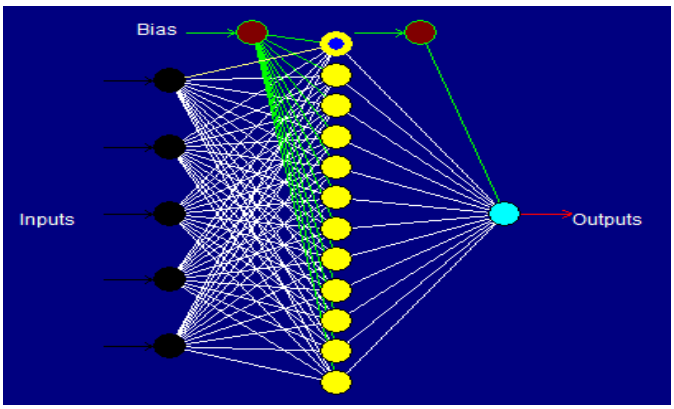
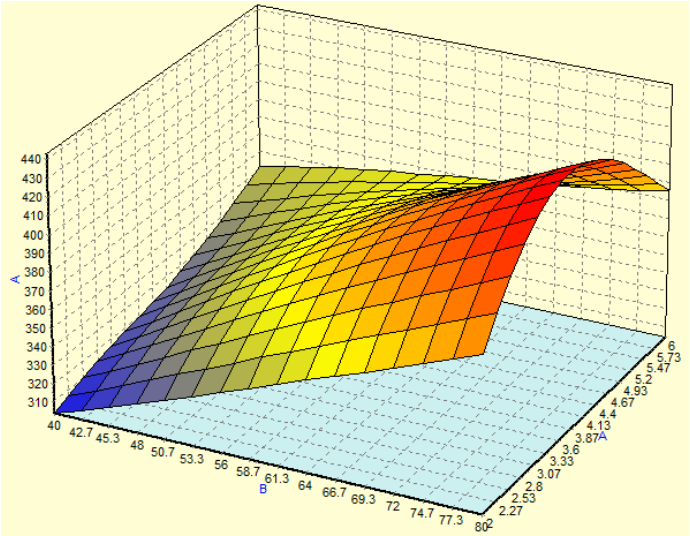
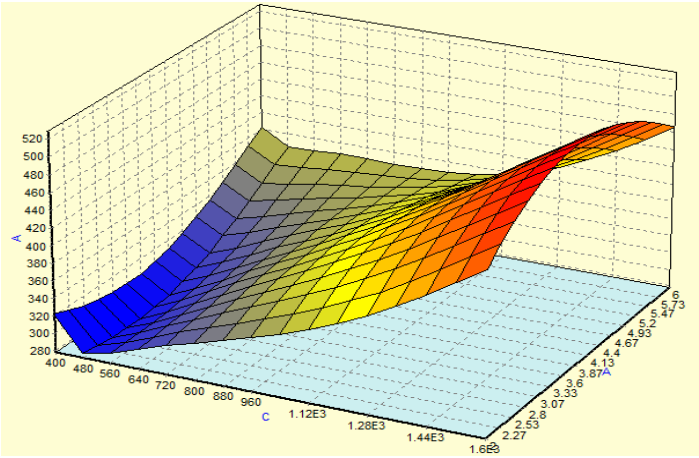


Figure 1: Network Structure with Twelve Transfer Functions



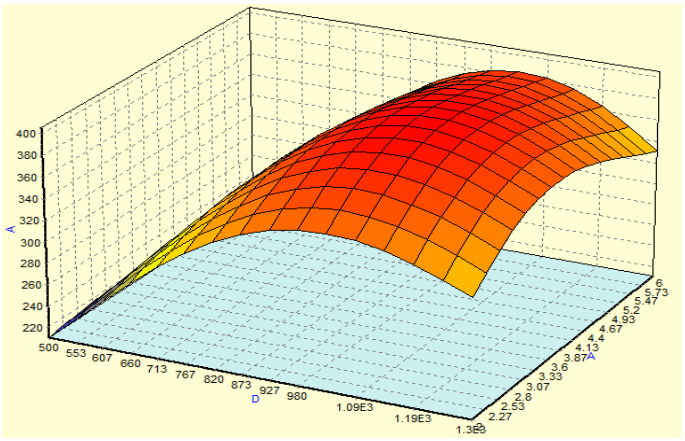
(a)

A(vertical) = L-PAC yield (mg/100 ml), A(horizontal) = Cell weight g(wet.wt), B(horizontal) = Incubation time (min)



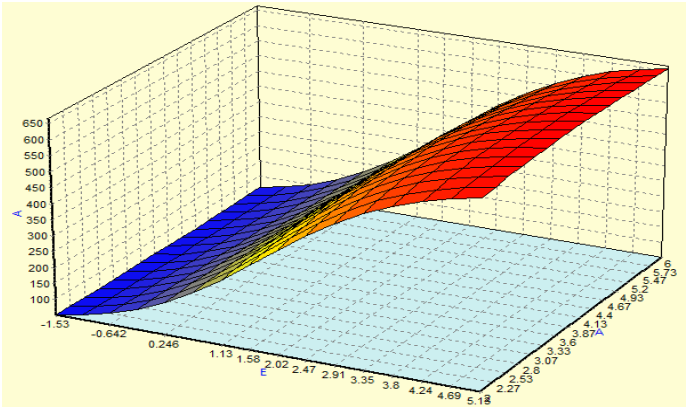
(b)

A(vertical) = L-PAC yield (mg/100 ml), A(horizontal) = Cell weight g(wet.wt), C(horizontal) = Acetaldehyde conc. (µg/100 ml)



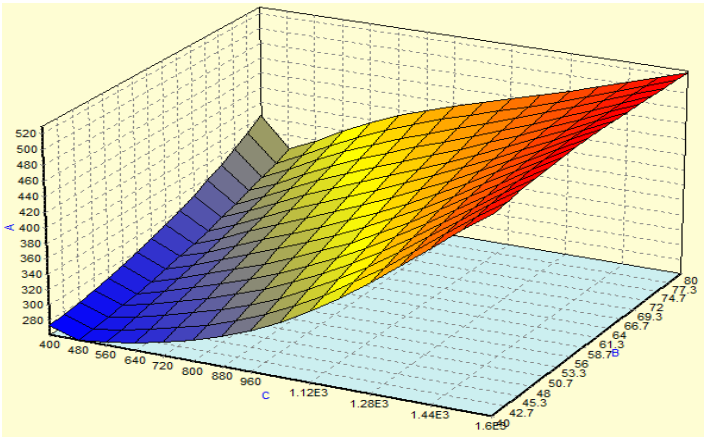
(c)

A(vertical) = L-PAC yield (mg/100 ml), A(horizontal) = Cell weight g(wet.wt), D(horizontal) = Benzaldehyde conc. (mg/100 ml)



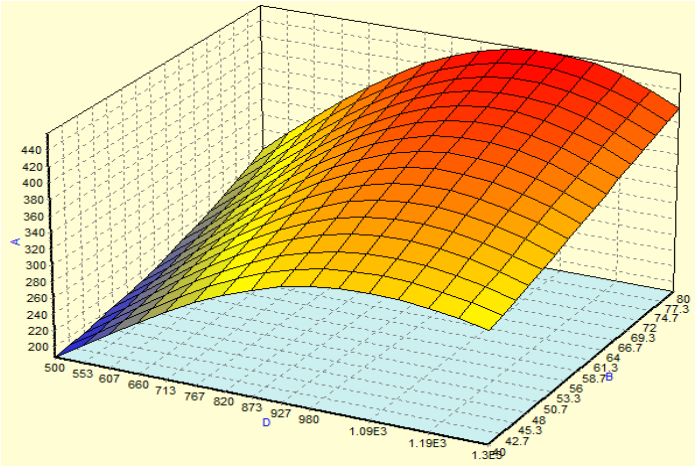
(d)

A(vertical) = BA yield (mg/100 ml), A(horizontal) = Cell weight g(wet.wt), C(horizontal) = ß-CD level (%)



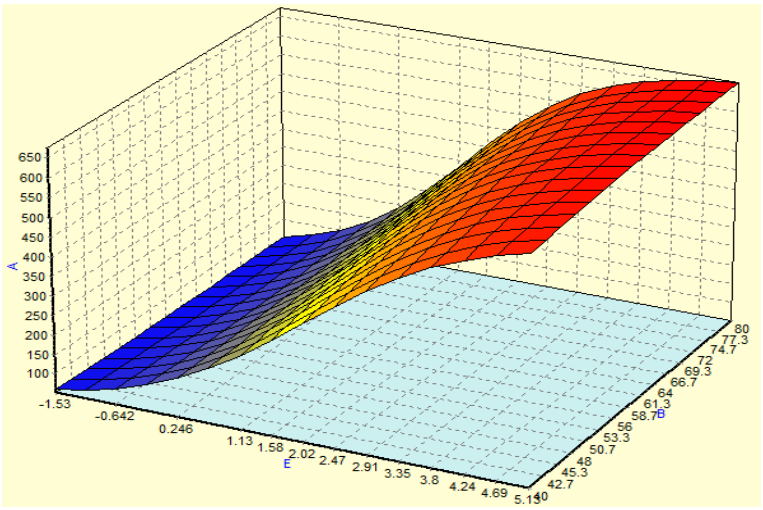
(e)

A(vertical) = L-PAC yield (mg/100 ml), B(horizontal) = Incubation time (min), C(horizontal) = Acetaldehyde conc. (µg/100 ml)



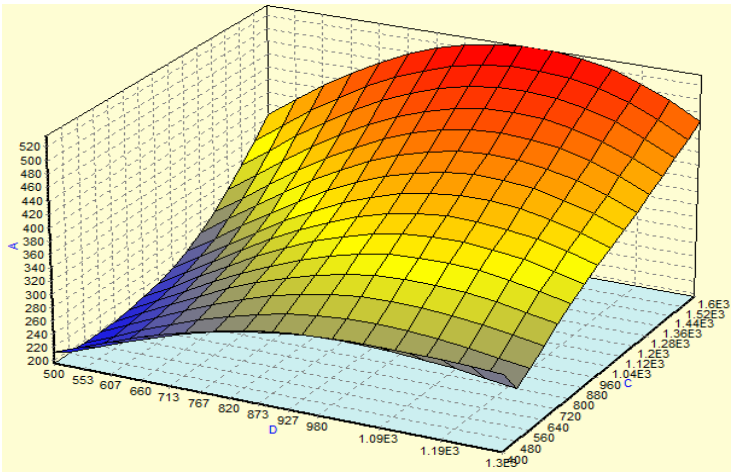
(f)

A(vertical) = L-PAC yield (mg/100 ml), B(horizontal) = Incubation time (min), D(horizontal) = Benzaldehyde conc. (mg/100 ml)



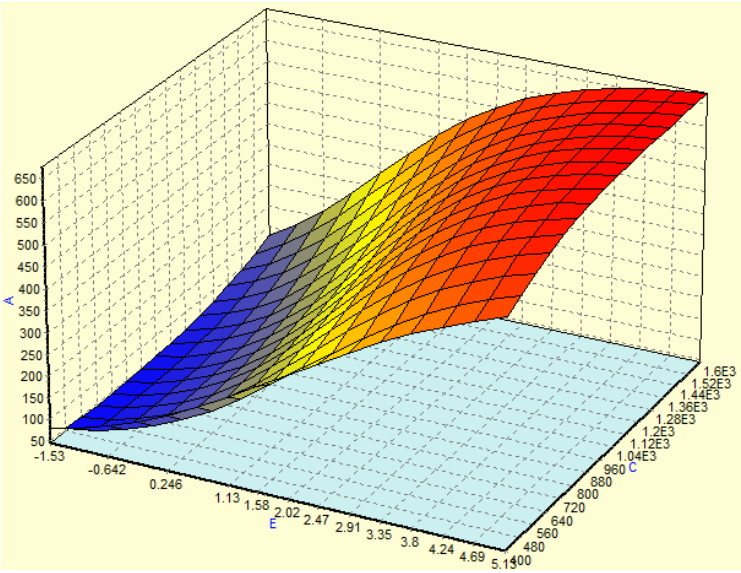
(g)

A(vertical) = L-PAC yield (mg/100 ml), B(horizontal) = Incubation time (min), E(horizontal) = ß-CD level (%)



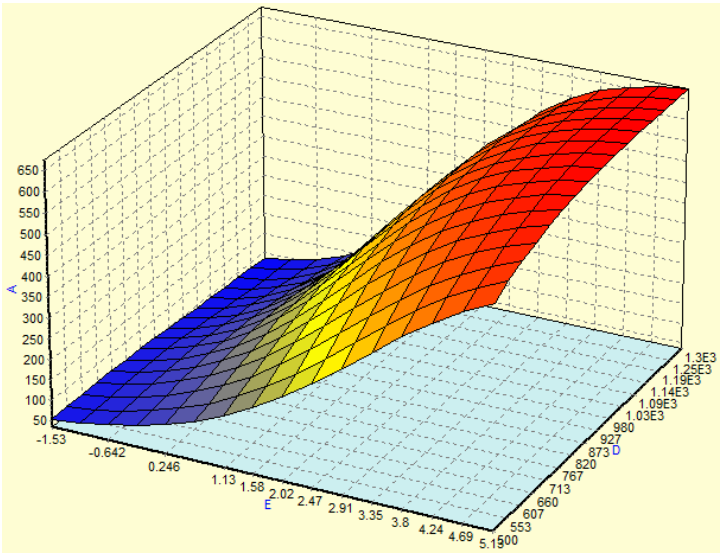
(h)

A(vertical) = L-PAC yield (mg/100 ml), C(horizontal) = Acetaldehyde conc. (µg/100 ml), D(horizontal) = Benzaldehyde conc. (mg/100 ml)



(i)

A(vertical) = L-PAC yield (mg/100 ml), C(horizontal) = Acetaldehyde conc. (µg/100 ml), E(horizontal) = ß-CD level (%)



(j)

A(vertical) = L-PAC yield (mg/100 ml), D(horizontal) = Benzaldehyde conc. (mg/100 ml), E(horizontal) = ß-CD level (%)

Figure 2: (a-j): 3-D curvatures' plots

IV. CONCLUSIONS

The results obtained in this study using Artificial neural network 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-Phenylacetylcarbinolby free cells yeastand effects of β-Cyclodextrin, indicate that ANNs is a good optimization tools for L- Phenylacetylcarbinol production. The Root Mean Square Error (RMSE) obtained was 3.0739. The coefficient of determination R^2 and the adj. R^2 were

found to be 0.99206 and 0.98419, respectively. It was observed that 1000 (mg/100 ml) benzaldehyde with 900 (µg/100 ml) acetaldehyde in the presence of 1.8% β-cyclodextrin gave the highest yield of L-Phenylacetylcarbinol. Hence, theorganism can tolerate higher levels of acetaldehyde and benzaldehyde. The lower values of difference obtained between experimental and observed values showed the power of ANNs for L-Phenylacetylcarbinol.

V. ACKNOWLEDGEMENT

The Authors acknowledge the effort of the technical staff of biology and chemistry laboratories of Landmark University.

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