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RESEARCH ARTICLE

OPTIMIZATION OF BIOTRANSFORMATION PROCESS OF PHYTOSTEROL TO ANDROSTENEDIONE BY *MYCOBACTERIUM SPP.* AT SHAKE FLASK LEVEL USING STATISTICAL TECHNIQUE

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ABSTRACT

Mycobacterium spp. was used for biotransformation of phytosterol to androstenedione. The study signifies the importance of media design and that medium constituents play a crucial role in the biotransformation process. Various statistical experiments namely, categorical, numerical factorial design and central composite design were applied to optimize the medium. The carbon and nitrogen sources, emulsifier source and the type of phytosterol were screened through categorical design. The significant components among the four were analyzed through full factorial design. Based on the results obtained, the optimal concentration of the significant components was determined using central composite design. With the tween 80, soya oil and phytosterol concentration to be 15 g/l, 25 g/l and 40 g/l there was a 50% enhancement in the yield of androstenedione. The methods used were very effective in screening the significant components in a limited number of experiments.

INTRODUCTION

Steroids are natural, organic compounds widely distributed in eukaryotic organism. Steroids are terpenoid lipids of specific structure that contain the gonane nucleus of four cycloalkane rings. Steroids and its derivatives are known to play a key role in the management of human fertility, menopause, osteoporosis, and blood pressure regulation because of their therapeutic (glucocorticoids) and contraceptive properties (e.g. progesterone and estrogen). Two important pharmaceutical steroid precursors, androst-4-ene-3,17-dione (AD) and androsta- 1,4-diene-3,17-dione (ADD), which belong to 17-ketosteroid family can be further used to produce a wide range of pharmaceutical steroid derivatives (**Fernandes *et al*., 2003**). Microbial steroid transformation which exploits the metabolic and biocatalytic potential of the microorganisms, is a powerful tool for generation of novel steroidal drugs, as well as their key intermediates. The technique have many advantages like reaction-specific processes; higher stereo-and region-specificity; milder reaction condition and are therefore preferred over chemical transformation. Being an alternative to chemical methods, bioconversion of steroids has been studied extensively (**Malaviya and Gomes, 2008**). Selective side chain degradation of sterols to 17-ketosteroids is one of the most widely used biotransformation reactions of steroids. It is well-known that phytosterols are suitable raw materials for microbial degradation to 17-ketosteroids because of low cost and easy availability (**Fernandes and Cabral,2007; Zhang *et al.*, 2013**). In recent years, microbial selective cleavages of phytosterol to androstenedione using different strains such as *Mycobacterium, Rhodococcus, Bacillus, Nocardia* etc have been reported by many researchers (**Huang *et al*., 2006; Pérez *et al*., 2006; Sripalakit *et al*., 2006; Wang *et al*., 2006; Zhang *et al.*, 2013**).

Media optimization and strain improvement are two important methods for the enhancement of the yield by biotransformation. Usually media optimization is done so as to obtain maximum yield from minimum possible inputs, thus minimizing the amount of non utilized components at the end of fermentation. The conventional method involves varying one parameter at a time while keeping the others at a fixed level. It is very time consuming and expensive. The statistical approach enables evaluation of various components at a time thus making it cost effective and time saving process (**Khan *et al.*, 2006**). The present study is a novel approach, in designing a suitable production medium using statistical optimization, resulting in improved biotransformation of phytosterol to androstenedione using *Mycobacterium species.*

MATERIALS AND METHODS

Microorganism

The bacteria *Mycobacterium spp.* was used for the biotransformation of phytosterol to androstenedione. They are actinobacteria which are non pathogenic, aerobic, non motile.

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The organism was grown on Grown Medium I comprising of soya flour - 7.5 g/l, tween 80 - 11 g/l, soya oil - 6.5 g/l, yeast extract powder - 2.5 g/l, potassium dihydrogen phosphate - 0.5 g/l, ammonium sulphate - 0.5 g/l with pH - 7.00 and agar - 25 g/l. The slants were incubated at 30ºC for 8 days. The grown slant was harvested with normal saline and was used to inoculate medium II (Inoculum medium).

Biotransformation process

Inoculum medium is composed of yeast extract 5 g/l, malt extract 15 g/l, bacteriological peptone 5 g/l and dextrose 20 g/l, pH 7.00. 1 ml of the harvested suspension was inoculated in the inoculum medium in 100 ml conical flasks with 15 ml medium. Inoculum flasks were incubated at 30ºC at 240 rpm on shaking incubator for 48±4 hrs. Growth medium III (Seed medium) comprises of soya flour 5 g/l, tween 80 10 g/l, soya oil 5 g/l, yeast extract powder 5 g/l, potassium dihydrogen phosphate 1 g/l, ammonium sulphate 1g/l with pH 7.00. Three percent of grown inoculum was transferred to seed medium in 250 ml conical flasks containing 35 ml medium. Seed medium flasks were incubated at 30ºC at 240 rpm on shaking incubator for 40 hrs. Transformation medium (production medium) comprises of the basal components like phytosterol 24 g/l, soya oil 18 g/l, tween 80 12 g/l, corn steep liquor 20 g/l soya flour 7.5 g/l, ammonium sulphate 8.5 g/l, calcium carbonate 4 g/l, potassium dihydrogen phosphate 2 g/l, potassium nitrate 2.5 g/l, sodium bicarbonate 1 g/l and magnesium sulphate 0.5 g/l with pH 7.00. Ten percent of the grown seed medium was transferred to production medium in 250 ml conical flasks containing 35 ml medium. Flasks were incubated at 30ºC and 240 rpm. The yield was assessed through HPLC.

AD estimation by HPLC

Androstenedione produced in the culture broth was determined by HPLC. The culture broth of 2.5 gm was taken in 25 ml volumetric flask with 10 ml isopropyl alcohol and sonicated for 20 minutes. Further, the volume was made up with isopropyl alcohol. The extract was filtered and diluted 1:10 with isopropyl alcohol and injected in the system. The HPLC (Waters 2496) having C-18 column (Hypersil ODS, 5u C18 (250 mm X 4.6 mm) was used for the estimation of AD. The mobile phase was composed of methanol and water (80:20, v/v), the flow rate was 1 ml/min and column temperature at 30°C. Concentration of AD was calculated by comparison of peak areas with those standard AD and subsequently AD activity was calculated.

Experimental Design

All the statistical analysis was done using the Design expert software (Stat-Ease Inc., Version 8.0.7.1).

Preliminary Screening: Categorical Full factorial Design

Preliminary screening of the carbon source, nitrogen source, phytosterol type and emulsifier was done through categorical full factorial design. Categorical design helps in selecting the best carbon, nitrogen source, emulsifier and substrate type that supports maximum biotransformation. In the design, soya oil and coconut oil were chosen as the carbon source, corn steep liquor and peptone bacteriological were chosen as the nitrogen sources. Tween 80 and polypropylene glycol (PPG) were selected as the source of emulsifiers and the substrate was phytosterol A from Biogen and phytosterol B from Arboris. Table 1 depicts the code sheet for the categorical experiment.

Table 1. Code sheet for the Categorical Design

Source	Code	Low level (-)	High level (+)
Substrate	A	B	A
Emulsifier	B	PPG	Tween 80
Carbon Source	C	Coconut Oil	Soya Oil
Nitrogen Source	D	Peptone Bacteriological	Corn steep liquor

Secondary screening: Numerical Full factorial Design

Based on the result obtained in the previous experiment and literature review, the numerical full factorial design involving 4 variable namely phytosterol, tween 80, soya oil and corn steep liquor was designed. Full factorial design determines the effects of multiple variables on yield. In addition to evaluating the impact of each variable on the yield it also determines the impact of interaction among the variables. Table 2 shows the code sheet of the full factorial design conducted.

Table 2. Code sheet of full factorial design

Component	Code	Low level (-)	High level (+)
Tween 80	A	10	30
Phytosterol	B	30	50
Soya Oil	C	10	30
Corn steep liquor	D	10	25

Central composite design for Optimization of Significant Parameters

Central composite design (CCD) was employed to get the exact range of each of the significant components. This is a very useful tool to determine the optimal level of medium constituents and their interaction. The significant factors that were screened through factorial design were further optimized using CCD. In this study, CCD involved a 20 experiment design which included 8 cube points, 6 star points and 6 replica of the central point.

RESULTS AND DISCUSSION

To screen the best carbon and nitrogen source, emulsifier and type of substrate which support the growth of *Mycobacterium species* and also enhances the biotransformation process, the 2^4 categorical full factorial designs was employed. The design matrix along with the corresponding response is depicted in Table 3.

The result obtained was analyzed by the half normal plot represented in Figure 1. The half-normal probability plot is a graphical tool that uses the ordered estimated effects to help assess which factors are significant and which are not significant. According to the half normal plot factor B (Emulsifier) is seen to be very far away, factor D (Nitrogen

source) is slightly away from the noise line, whereas factor A (Phytosterol) and factor C (Carbon source) are almost on the line. The more the distance from the noise line, the greater is the impact of the factor on the yield of AD.

Table 3. Categorical design along with the response

Run	A:Phytosterol	B:Emusifier	C:Carbon Source	D: Nitrogen source	Yield g/l
1	-	-	-	-	0.053
2	+	-	-	-	0.12
3	-	+	-	-	4.827
4	+	+	-	-	1.695
5	-	-	+	-	0.068
6	+	-	+	-	0.42
7	-	+	+	-	6.451
8	+	+	+	-	2.281
9	-	-	-	+	0.096
10	+	-	-	+	0.031
11	-	+	-	+	7.537
12	+	+	-	+	6.395
13	-	-	+	+	0.38
14	+	-	+	+	3.819
15	-	+	+	+	6.37
16	+	+	+	+	3.66

The factors with their positive levels were considered for further experiment. According to the analysis, phytosterol type B, tween 80, soya oil and corn steep liquor were chosen as the best substrate, emulsifier, carbon and nitrogen source respectively.

Secondary Screening: Full factorial design

The chosen carbon, nitrogen, substrate and emulsifier sources through the preliminary categorical design were further optimized using numerical 2*4 full factorial design of experiment. Factorial designs help the scientist to perform method optimization experiments in a manner that can obtain maximum information in a small number of experiments. These designs can identify main effects for the factors being studied as well as uncover any existing interactions between factors. The experimental design for screening of medium components is shown in Table 4. Each variable was set at two levels, that is, high level and low level. The high level of each variable was set far enough from the low level to identify which ingredients of the medium have significant influence on the AD production.

The Design Expert software 8.0 was used in the experimental design and data analysis. Pareto was obtained to compare the significance of each effect. The Pareto Chart of the effects (Figure 2) assists to determine the magnitude and the importance of an effect. Pareto chart displays the absolute value of the effects and draws a reference line on the chart at t-value limit, where t is the (1 -α/2) quantile of a t-distribution with degrees of freedom equal to the degrees of freedom for the error term. Any effect that extends within this reference line is statistically insignificant (**Kukreja *et al.*, 2011**).

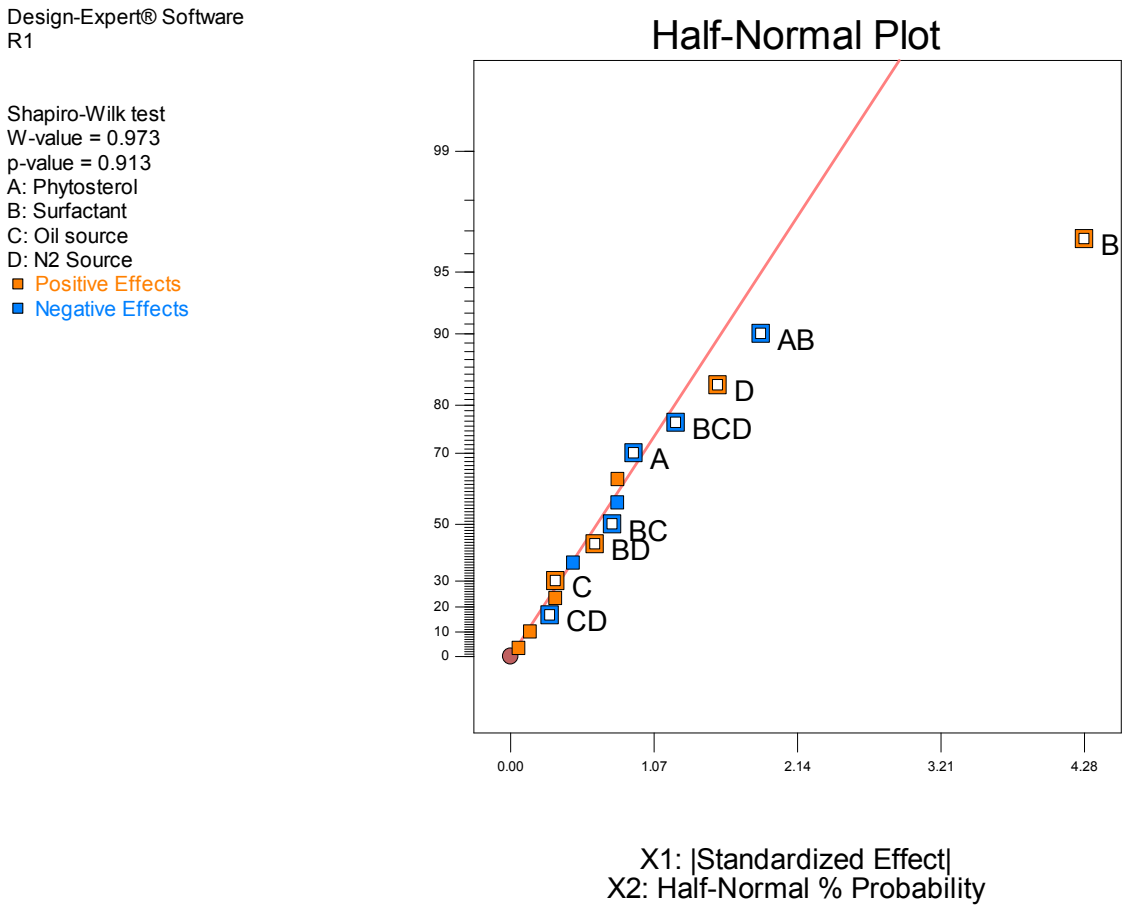

Figure 1. Half normal plot for categorical design

Table 4. The experimental design using full factorial method for screening of medium components

Run	A:Tween 80	B:Phytosterol	C:Soya Oil	D: Corn steep liquor	Yield g/l
1	-	-	-	-	5.868
2	+	+	-	-	7.961
3	-	+	-	-	2.869
4	+	+	-	-	8.256
5	-	-	+	-	2.382
6	+	-	+	-	4.84
7	-	+	+	-	2.458
8	+	+	+	-	2.304
9	-	-	-	+	5.828
10	+	-	-	+	6.5
11	-	+	-	+	4.396
12	+	+	-	+	5.575
13	-	-	+	+	3.322
14	+	-	+	+	2.523
15	-	+	+	+	3.19
16	+	+	+	+	5.799

According to Figure 2, the factors C and A are the most significant factors than the others affecting the yield. Factor C that is soya oil affects the yield on the negative scale whereas factor A that is tween 80 affects the yield on the positive scale.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	38.6	4	9.65	5.06	0.0147
A-Tween 80	11.3	1	11.3	5.92	0.0332
B-Phytosterol	1.2	1	1.2	0.63	0.4449
C-Soya Oil	26.1	1	26.1	13.69	0.0035
D-CSL	2.38E-03	1	2.38E-03	1.25E-03	0.9725
Residual	20.98	11	1.91		
Cor Total	59.57	15			

Abbreviations: df: degree of freedom
R-squared0.6479
Adj R-squared0.5199
Pred R-Squared0.2551
Adeq Precision 6.226

In this case A and C are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. Here model terms B and D are not significant. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable.

The ratio of 6.226 indicates an adequate signal and thus model can be used to navigate the design space.

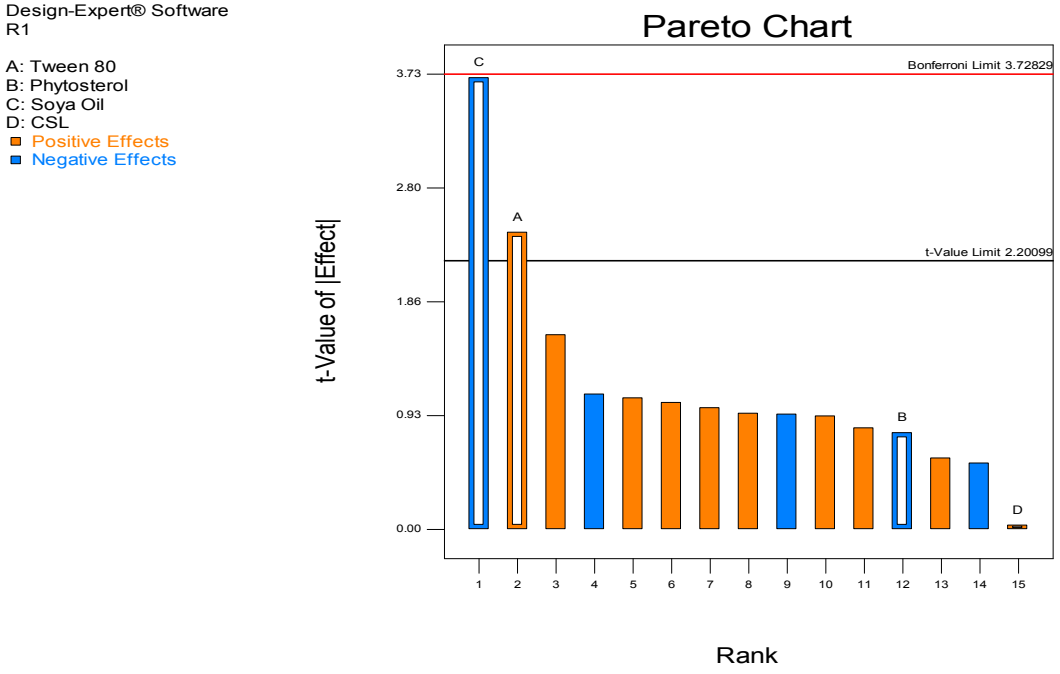

Figure 2. Paretto chart for the full factorial design

Verification of significant factors

The analysis of variance (ANOVA) was applied to evaluate the statistical significance of the design and to verify the above results (Table 5).

The Model F-value of 5.06 implies the model is significant. There is only a 1.47% chance that a "Model F-Value" this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant.

Central Composite Design for Optimization of Significant Parameters

In order to optimize the significant components screened through full factorial a central composite design (CCD) was employed. It was conducted to obtain a quadratic model consisting of factorial trials and star points to estimate quadratic effects and central points to estimate the pure process variability with AD yield as a response (**Kiruthika *et al.*, 2011**). The significant factors, tween 80 and soya oil along

with the substrate (phytosterol) were taken into account for the study. A set of 20 experiments with replicates at center point was designed. Table 6 represents the levels of the factors selected for the CCD design.

Table 6. Factors along with their levels

Variable	Symbol	Coded Factor Level				
		-2	-1	0	1	2
Tween 80	A	9.773	20	35	50	60.226
Soya Oil	B	1.227	6	13	20	24.772
Phytosterol	C	19.773	30	45	60	70.226

Table 7 shows the CCD matrix of the independent in the coded units along with observed and predicted yield.

Table 7. CCD matrix with observed and predicted yield

Run	Tween 80	Soya Oil	Phytosterol	Observed Yield g/l	Predicted Yield
1	-1	-1	-1	2.377	3.392
2	1	-1	-1	2.694	3.034
3	-1	1	-1	8.969	9.662
4	1	1	-1	3.554	3.359
5	-1	-1	1	3.325	3.392
6	1	-1	1	3.613	3.034
7	-1	1	1	10.75	9.662
8	1	1	1	4.537	3.359
9	-2	0	0	9.571	7.663
10	2	0	0	2.805	2.061
11	0	-2	0	2.862	2.089
12	0	2	0	7.061	7.635
13	0	0	-2	3.509	4.862
14	0	0	2	4.133	4.862
15	0	0	0	3.854	4.862
16	0	0	0	5.879	4.862
17	0	0	0	5.032	4.862
18	0	0	0	3.932	4.862
19	0	0	0	4.246	4.862
20	0	0	0	4.19	4.862

Model Fitting

ANOVA was used to evaluate the adequacy of the fitted model. The result of second order response surface model fitting in the form of ANOVA is shown in Table 8.

Table 8. ANOVA analysis of CCD matrix

Source	Sum of Square	df	Mean Square	F value	p-value Prob > F
Model	91.92	6	15.32	18.45	< 0.0001
A-Tween 80	32.39	1	32.39	39	< 0.0001
B-Soya oil	38.27	1	38.27	46.08	< 0.0001
C-Phytosterol	2.36	1	2.36	2.84	0.1155
AB	18.71	1	18.71	22.52	0.0004
AC	0.085	1	0.085	0.1	0.7534
BC	0.1	1	0.1	0.12	0.7334
Residual	10.8	13	0.83		
Lack of Fit	7.72	8	0.96	1.56	0.3228
Pure Error	3.08	5	0.62		
Cor Total	102.72	19			

Abbreviations: df: degree of freedom
R-Squared0.8949
Adj R-Squared0.8464
Pred R-Squared0.7482
Adeq Precision15.191

The model F value of 18.45 with low p value (>0.0001) implied a high significance for the regression model (**Yuan *et al.*, 2008**). The Lack of Fit F-value of 1.56 implies the Lack of Fit is not significant relative to the pure error. There is a 32.28% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good. Hence the model can be used in theoretical prediction of AD yield. The goodness of fit of the model was checked by the coefficient of determination (R^2). R^2 is the proportion of variability in response values explained or accounted for by the model. R^2 should be at least 0.80 for the good fit of model (**Guan and Yao, 2008**). In this case the R^2 value of 0.8949 which is close to 1 indicated that the model is accurate and predicts a better response. Adj R^2 value of the model was 0.8464 which indicates high significance of the model (**Akhnazarova and Kefarov, 1982, Khuri and Cornell, 1987**).

Table 9. Regression coefficients and significance of response surface quadratic

Factor	Coefficient estimate	df	Standard Error	95% CI Low	95% CI High	VIF
Intercept	4.81	1	0.2	4.37	5.25	
A-Tween 80	-1.54	1	0.25	-2.07	-1.01	1
B-Soya oil	1.67	1	0.25	1.14	2.21	1
C-Phytosterol	0.42	1	0.25	-0.12	0.95	1
AB	-1.53	1	0.32	-2.23	-0.83	1
AC	-0.1	1	0.32	-0.8	0.59	1
BC	0.11	1	0.32	-0.58	0.81	1

The model coefficients and coded value are shown in table 9. The low value 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. The variance inflation factor (VIF) obtained in this study showed that the six center points are hexagonal to all other factors in the model. The model also proved suitable for the adequate representation of the real relationship among the selected independent factor (**Adepoju *et al.*, 2013**). Parity plot (Figure 3) showed the distribution of observed and model predicted values where data points are localized close to the diagonal line suggesting that the model is adequate enough to explain AD production.

The 3D response surface plots are graphical representations of the regression equation for the optimization of the reaction variable which are represented in the Figure 4 a, b and c. The nature of curvature of 3D surface in the Figure 4 a, b and c indicated moderate interactions of soya oil and phytosterol, tween 80 and phytosterol and tween 80 and soya oil with AD yield, respectively.

Numerical optimization of factors

Based on Table 7, run number 7, the highest AD yield 10.750 g/l was obtained when the concentration of phytosterol, tween 80 and soya oil was 60 g/l, 20 g/l and 20 g/l respectively. To obtain the maximum optimum yield the factor levels and response were set accordingly. The concentration of tween 80, soya oil and phytosterol was set at 15 g/l, 25 g/l and 40 g/l respectively. From four replications of the experiments under suggested optimal concentrations an average yield of AD was 12 g/l.

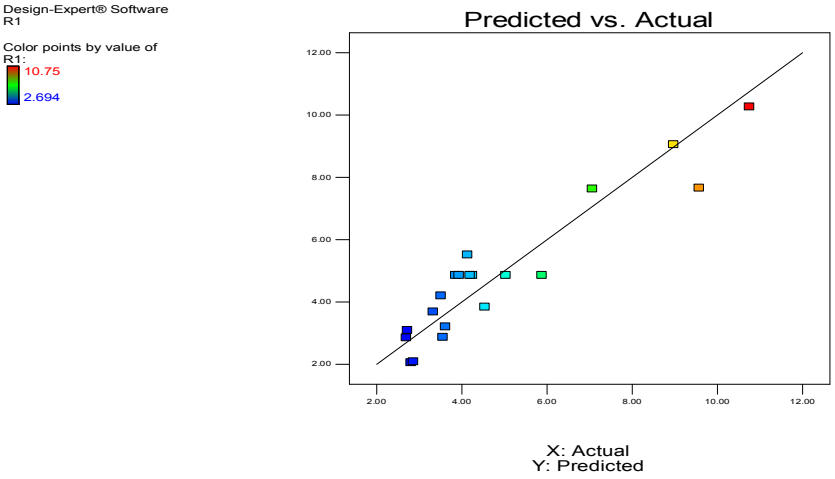

Figure 3. Parity plot showing predicted and observed values

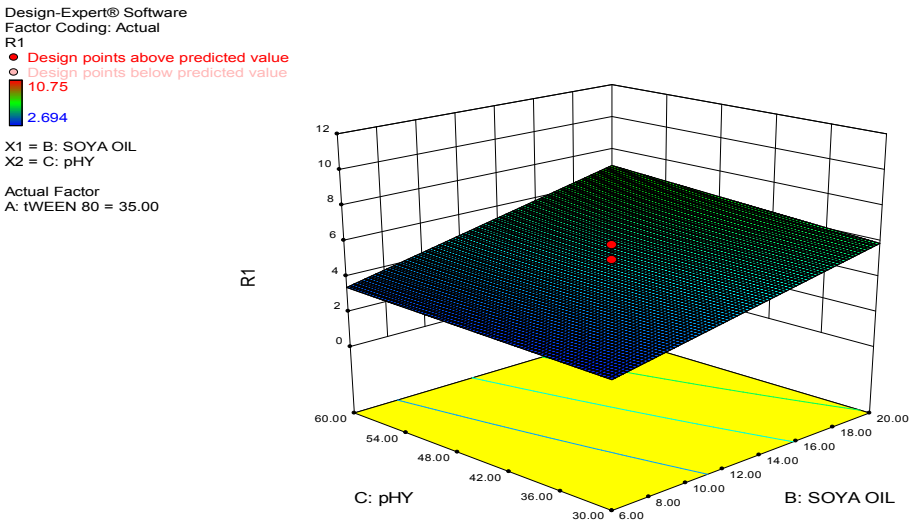

Figure 4a. 3D Curvature of Soya oil versus Phytosterol

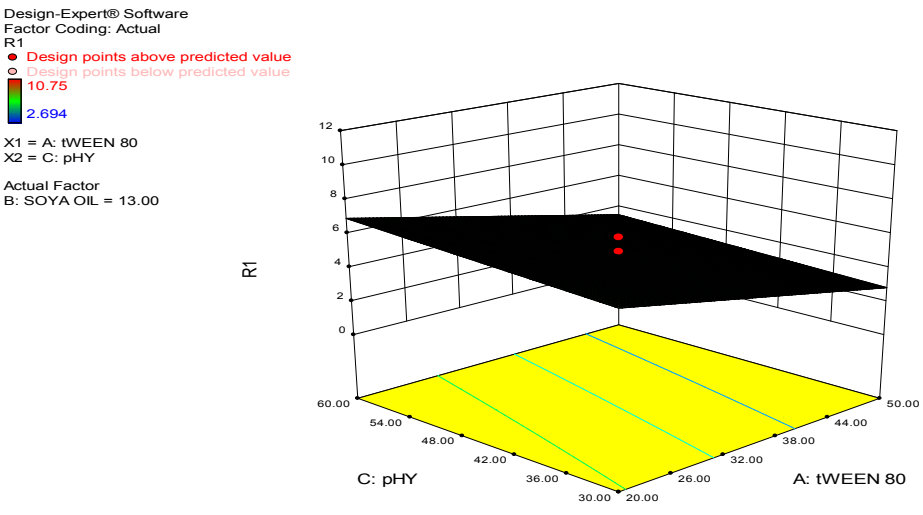

Figure 4b. 3D Curvature of Tween 80 versus Phytosterol

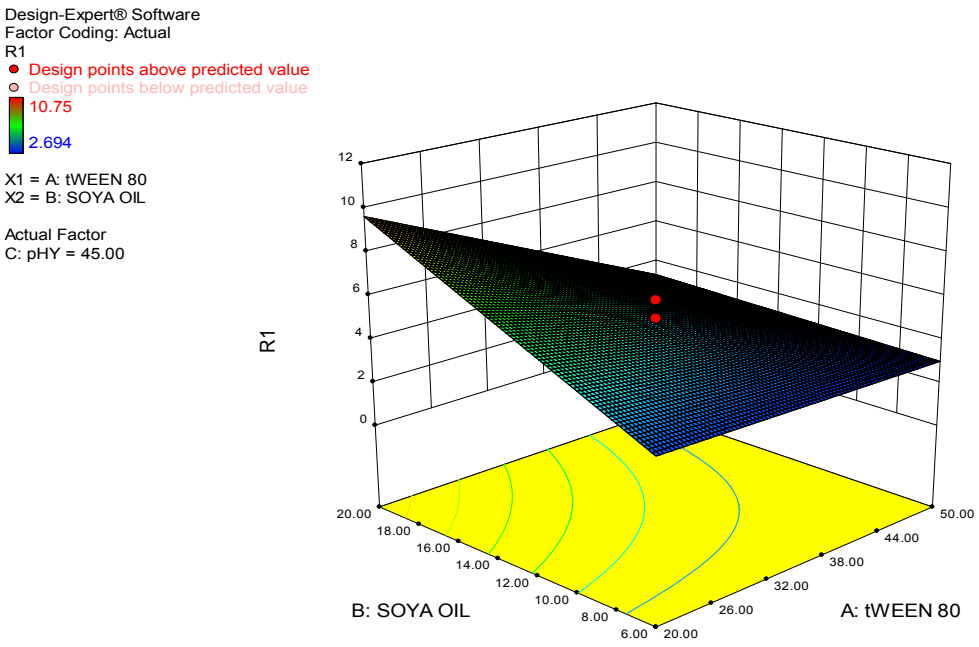

Figure 4c. 3D Curvature of Tween 80 versus Soya oil

Conclusion

An efficient approach that significantly reduced experimental efforts was used to optimize the biotransformation medium for AD production using *Mycobacterium spp.* This included categorical factorial screening, full factorial design and central composite design. Categorical full factorial analysis showed that phytosterol type B, tween 80, soya oil and corn steep liquor as the best substrate, emulsifier, carbon and nitrogen source respectively. The full factorial studies and paretto chart at 2 levels showed that phytosterol and soya oil concentration needs to be lowered for better yield. Tween 80 and corn steep liquor concentration needs to be increased for increasing the yield. Validation of results by ANOVA indicates that tween 80 and soya oil are the most significant component out of the four components analyzed. From further optimization studies, using CCD the optimized values of the variables for AD production were as follows: tween 80 - 15 g/l, soya oil - 25 g/l, phytosterol - 40 g/l. The results show a close concordance between the expected and obtained activity level. Using this statistical approach, there was a 50% increase in the yield of AD. Data obtained in this study overall depicts that statistical technique has successfully increased the AD yield.

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