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Media Optimization for 9α-hydroxyandrost-4-ene-3,17- dione Production by Mycobacterium spp. using Statistical Designs

Dr. Umesh Luthra
Sr. General Manager
Ipca Laboratories Ltd.,
Biotech R&D, Mumbai.

Dr. Nishtha K. Singh
Assistant Manager
Ipca Laboratories Ltd.,
Biotech R&D, Mumbai.

Archana Tripathi
Sr. Research Associate
Ipca Laboratories Ltd.,
Biotech R&D, Mumbai.

Vrushali Bhosle
Jr. Research Associate
Ipca Laboratories Ltd.,
Biotech R&D, Mumbai.

Khushboo Mota
Jr. Research Associate
Ipca Laboratories Ltd.,
Biotech R&D, Mumbai.

Abstract

9α-hydroxyandrost-4-ene-3,17- dione is a steroidal derivative and the key precursor in the synthesis of various glucocorticoids. In the present study, the effect of medium components on the biotransformation of phytosterol to 9α-hydroxyandrost-4-ene-3,17- dione using Mycobacterium spp. was studied. This work demonstrates the use of statistical approach to obtain optimum yield of 9α-hydroxyandrost-4-ene-3,17- dione. Plackett-Burman statistical experimental design was applied to evaluate the fermentation medium components. The concentrations of Phytosterol, Yeast Extract and Glycerol were found to have more significance on the 9α-hydroxyandrost-4-ene-3,17- dione conversion. The Pareto chart was employed to highlight the most important among a (typically large) set of factors and to determine the order of significance of the factors affecting bioconversion. A Central Composite Design of "response surface methodology" was conducted to optimize the selected factors to maximize the yield. The optimized model include Phytosterol - 25.0 g/l, Yeast Extract - 7.5 g/l and Glycerol - 20.0 g/l for maximum conversion to 9α-hydroxyandrost-4-ene-3,17- dione (9.10 mg/gm). The statistical design of experiment offers efficient methodology to identify the significant variables and to optimize the factors with minimum number of experiments for biotransformation of phytosterol to 9α-hydroxyandrost-4-ene-3,17- dione by Mycobacterium spp.

Keywords: *9α-hydroxyandrost-4-ene-3,17- dione, Mycobacterium spp., Placket-Burman Design, Central Composite Design*

1. Introduction

Steroids are a large group of organic compounds with the perhydro-1,2 cyclopentano- phenanthrene nucleus, which consists of four fused rings. Steroid biotransformation is a multimillion dollar industry and pharmaceutical uses of steroids are numerous. Steroid medications are widely used in clinical applications ranging from anti-inflammatory, immunosuppressive, progestational, diuretic, and anabolic to contraceptive agents [1]. Among the steroidal derivatives, 4-androstene-3,17-dione (AD), 1,4-androstadiene-3,17-dione (ADD) and 9α-hydroxyandrost-4-ene-3,17- dione (9-OH-AD) are major products [2, 3]. 9α-hydroxyandrost-4-ene-3,17- dione is the key precursor in the synthesis of various glucocorticoids.

Specific microbial transformation steps have been incorporated for synthesis of new steroids and their evaluation as drugs and hormones. Biotransformations have provided adequate tools for the large scale production of natural or modified steroid analogues [4]. Selective side chain degradation of sterols to17-ketosteroids is one of the most widely used biotransformation reactions of steroids. It is well-known that phytosterols (PSs) are suitable raw materials for microbial degradation to 17-ketosteroids because of low cost and easy availability [5]. In Industrial level, 9α-

hydroxyandrost-4-ene-3,17- dione is mainly produced by *Mycobacterium* spp. through biotransformation. *Mycobacterium* spp is a nontuberculous species of the phylum actinobacteria, belonging to the genus mycobacterium [6].

The study of factors influencing the biotransformation of steriod is very much essential in any bioprocess development. Generally a higher productivity has been achieved by culture medium optimization. Medium optimization by single dimensional search is laborious and time consuming, especially for a large number of variables and it does not ensure desirable conditions. Plackett-Burman design is widely used in screening experiment as the number of experiment run required are very few, leading to saving of time, chemicals and man power [7].

Response Surface Methodology (RSM) is an effective tool for optimizing the process condition that uses quantitative data from an appropriate experimental design to determine and simultaneously solve multivariate equations [8]. It usually involves an experimental design such as Central Composite Design (CCD) to fit a second-order polynomial by a least squares technique. An equation is used to describe the test variables, and describe the combined effect of all the test variables in the response.

A statistical approach has been employed in the present study for which a Plackett-Burman design is used for identifying significant variables influencing 9α-hydroxyandrost-4-ene-3,17-dione (9α-hydroxyandrost-4-ene-3,17- dione) production through biotransformation using *Mycobacterium* spp. The levels of the significant variables were further optimized using response surface methodology and central composite design.

2. Materials And Methods:

2.1 Microorganism

In this study, *Mycobacterium* spp. was used for the bioconversion of phytosterol to 9α-hydroxyandrost-4-ene-3,17-dione. The strain was cultivated in culture media comprising of Yeast Extract-12.00 g/l, Ammonium Sulphate-1.73 g/l, Potassium dihydrogen phosphate-0.50 g/l, Dipotassium hydrogen phosphate-0.70 g/l, Ferrous sulphate-0.0050 g/l, Zinc sulphate-7-hydrate-0.0020 g/l, Magnesium sulphate-0.25 g/l , Glycerol-12.50 g/l, Polypropylene Glycol-2-5 g/l and Antifoam-1.0 ml with pH - 7.0 and agar-25.0 g/l. The slants were incubated at 30ºC for 8 days. Grown culture was preserved with 20% glycerol for further use.

2.2 Biotransformation process

Mycobacterium spp. was used for the biotransformation of phytosterol to 9α-hydroxyandrost-4-ene-3,17- dione. The grown slant was harvested with normal saline and was used to inoculate the seed medium. Medium consist of Yeast Extract-12.00 g/l, Ammonium Sulphate-1.70 g/l, Potassium dihydrogen phosphate-0.50 g/l, Dipotassium hydrogen phosphate-0.70 g/l, Ferrous sulphate-0.0050 g/l, Zinc sulphate-7-hydrate-0.0020 g/l, Magnesium sulphate-0.25 g/l, Glycerol-12.50 g/l, Polypropylene Glycol-2-5 g/l and Antifoam-1.0 ml with pH - 7.0. Inoculum flasks were incubated at 30ºC at 240 rpm in shaking incubator for 24±4 hrs.

10% of the grown seed culture was transferred to production medium in 250 ml Erlenmeyer flasks containing 30 ml of medium. Transformation medium (production medium) comprises of the basal components like Phytosterol-25 g/l, Tween 80-3.50 g/l, Ammonium sulphate-2.50 g/l, Potassium dihydrogen phosphate-0.75 g/l, Dipotassium hydrogen Phosphate-4.00 g/l, Yeast Extract-2.50 g/l, Ferrous Sulphate-0.002 g/l, Zinc Sulphate-0.0008 g/l, Glycerol-12.50 g/l and Magnesium sulphate-0.50 g/l with pH 7.0. Flasks were incubated at 30ºC at 240 rpm for 120 hrs. The process parameters such as pH, PCV and microscopy were checked to understand the morphology of the culture during growth and biotransformation and 9α-hydroxyandrost-4-ene-3,17- dione yield was assessed through HPLC at an interval of 24 hrs.

2.3 Quantification of 9α-hydroxyandrost-4-ene-3,17- dione production by HPLC analysis

Quantification of 9α-hydroxyandrost-4-ene-3,17-dione produced in the culture broth was done by HPLC analysis. The culture broth of 2.5 gm was taken in 25 ml volumetric flask with 10 ml methanol and sonicated for 20 minutes.The extract was filtered and diluted with methanol and injected in the system. The HPLC (Waters 2496) having C-18 column (Betasil) was used for the estimation of 9α-hydroxyandrost-4-ene-3,17- dione at 238nm. The mobile phase was composed of Water: Acetonitrile, the flow rate was 0.8 ml/min and column temperature set at 30°C. Yield of 9α-hydroxyandrost-4-ene-3,17- dione was calculated by comparison of peak areas with the standard area. Subsequently 9α-hydroxyandrost-4-ene-3,17- dione activity was calculated in all experimental runs of statistical analysis.

2.4 Experimental Design and Data Analysis

Plackett-Burman (PB) design was used to determine the likely effects of medium components on 9α-hydroxyandrost-4-ene-3,17- dione production. Plackett-

Burman design is an efficient screening design where main effects are considered. This is a very economical design, with the run number a multiple of four and comprises of two Level screening designs. Eight assigned variables were screened in Plackett-Burman design with 3 dummy variables in 12 experimental runs. Eight factors consisting of major medium components prepared at two levels –1 for low level and +1 for high level [9]. The factors (g/l) such as Phytosterol, Dipotassium Hydrogen Phosphate, Potassium Dihydrogen Phosphate, Glycerol, Ammonium Sulphate, Yeast extract, Urea and Magnesium Sulphate at the same level were studied. The actual values of the variables at low level (−1) and high level (+1) are given in (Table 1).

All the statistical analysis was done using the Design expert software (Stat-Ease Inc., Version 8.0.7.1). All experiments were performed in duplicates and the average of it was taken as the response.

2.4.1 Screening of medium components by using Plackett Burman

A set of eight medium components were screened by Plackett Burman design. Plackett-Burman Design was introduced in this study as a first optimization step to identify the factors that have significant effects on the 9α-hydroxyandrost-4-ene-3,17- dione production. Selection of medium components plays a key role in biotransformation to 9α-hydroxyandrost-4-ene-3,17-dione. This design was used to screen and evaluate the important factor(s) that influence the response of eight assigned factors and three dummy variable to estimate experimental error in 12 experimental designs. The low level (– 1) and high level (+ 1) of each factor are listed in Table 1. Plackett-Burman design matrix for 8 variables is given in Table 2.

Table 1: Two levels of the factors used in Plackett-Burman Design

Code	Factor	Low Level (-)	High Level (+)
A	Phytosterol	5.00	15.00
B	Dipotassium Hydrogen Phosphate	3.50	7.50
C	Potassium Dihydrogen Phosphate	0.50	2.00
D	Glycerol	5.00	15.00
E	Ammonium Sulphate	2.50	5.00
F	Yeast extract	1.50	5.00
G	Urea	0.15	0.50
H	Magnesium Sulphate	0.15	0.50

Table 2: Twelve run Plackett-Burman design matrix for 8 variables with coded values along with the yield.

Run	A	B	C	D	E	F	G	H	Yield (mg/gm)
1	+	+	-	+	+	+	-	-	6.549
2	-	+	+	-	+	+	+	-	2.962
3	+	-	+	+	-	+	+	+	6.291
4	-	+	-	+	+	-	+	+	3.768
5	-	-	+	-	+	+	-	+	4.526
6	-	-	-	+	-	+	+	-	4.515
7	+	-	-	-	+	-	+	+	3.298
8	+	+	-	-	-	+	-	+	6.566
9	+	+	+	-	-	-	+	-	3.375
10	-	+	+	+	-	-	-	+	4.329
11	+	-	+	+	+	-	-	-	5.021
12	-	-	-	-	-	-	-	-	2.501

2.4.2 Optimization of the independent variables

(a) Response Surface Methodology (RSM)

A Central Composite Design (CCD) was employed to optimize the three most significant factors screened by Plackett-Burman design. The CCD is one of the most commonly used response surface design for fitting second-order models. A central composite design consists of F factorial points, 2k axial points (±α), and nc center points. According to CCD for the three variables, 17 experimental runs were executed (Table 4).

Table 3: Coded values for each factor of the central composite design.

Code	Factors	-α	-1	0	+1	+α
A	Phytosterol	11.59	15.00	20.00	25.00	28.41
B	Yeast Extract	1.47	3.00	5.25	7.50	9.03
C	Glycerol	14.64	16.00	18.00	20.00	21.36

Each parameter was studied at different levels (-α, −1, 0, +1, +α) [Table 3]. The minimum and maximum ranges of parameters were investigated and the full experimental plan with respect to their values. A matrix of seventeen experiments with three factors was generated using the software package STAT EASE Version 8.0 USA. 9α-hydroxyandrost-4-ene-3,17- dione activity was taken as the dependent variable or response (Y). The following second-order polynomial equation was adopted to study the effects of variables to the response.

$$Y=\beta_o + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (1)$$

Where Y is the response (9α-hydroxyandrost-4-ene-3,17- dione yield mg/gm), A, B and C are independent variables (Phytosterol, Yeast Extract and Glycerol); βo is the regression coefficient at center point; β1, β2 and β3 are linear coefficients; β11, β22 and β33 are the quadratic coefficients; and β12, β13 and β23 are the second order interaction coefficients

Table 4: Experimental code and levels of factors in CCD

Run	A	B	C	Yield (mg/gm)
1	-1.00	-1.00	1.00	5.23
2	α	0.00	0.00	6.15
3	0.00	0.00	0.00	6.23
4	0.00	α	0.00	5.65
5	0.00	0.00	α	7.23
6	-1.00	1.00	1.00	5.68
7	-α	0.00	0.00	4.95
8	-1.00	-1.00	-1.00	7.86
9	0.00	0.00	0.00	5.82
10	1.00	-1.00	-1.00	7.98
11	0.00	0.00	0.00	5.96
12	0.00	-α	0.00	6.56
13	1.00	1.00	1.00	9.10
14	-1.00	1.00	-1.00	6.21
15	0.00	0.00	-α	7.53
16	1.00	1.00	-1.00	5.52
17	1.00	-1.00	1.00	5.96

(b) Verification of the CCD model:

In order to verify the adequacy of the developed CCD (RSM) model, analysis of variance (ANOVA) was used.

3. Result And Discussion

3.1 Screening of parameters using Plackett-Burman design

Plackett-Burman design was used as a screening method to determine which of the components in the fermentation medium significantly effect the production. The experimental results with the Plackett-Burman design are shown in Table 2. These are tested simultaneously by shifting factors from a low value (−1) to a high value (+1). The significant variables screened via Plackett Burman design were shown in Pareto chart (Fig.1). The experimental results were interpreted based on the partition of the overall effect of all the factors to the response into individual factor effect, this partition has been made statistically. When the value of the concentration effect of the tested variable is positive, the conclusion is that the influence of the concerning variable is greater at a high concentration tested, and when negative, this indicates that the influence of the given variable is greater at a low concentration [10]. Hence to optimize 9α-hydroxyandrost-4-ene-3,17- dione production, it is necessary to optimize the concentrations of Phytosterol, Yeast extract and Glycerol using different concentrations whereas other components of the medium can be kept constant.

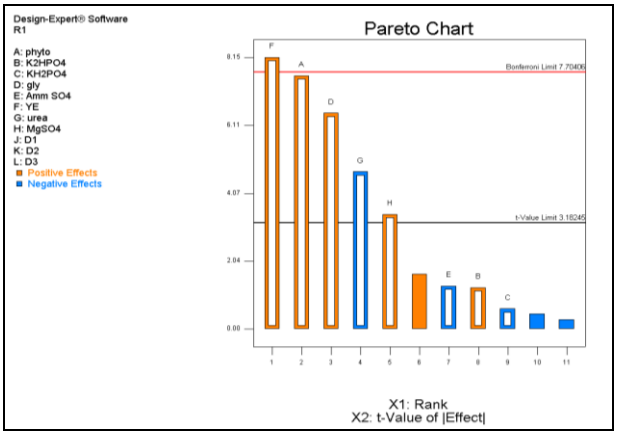


Figure 1: Pareto Chart of Main Effects for Plackett-Burman Design variable

The Pareto chart as shown in Fig.1 offers a convenient way to view the response obtained by Plackett Burman design matrix and the order of significance of the variable affecting biotransformation of phytosterol to 9α-hydroxyandrost-4-ene-3,17- dione. Screened variables of Plackett Burman was further proceeded with CCD to optimize the actual required concentration.

3.2 Optimization of the independent variables by RSM via CCD

Phytosterol (A), Yeast Extract (B) and Glycerol (C) was screened *via* Plackett-Burman design were further proceeded with (RSM) a Central Composite Design, consisting of a set of 17 runs of experiment with three replicates at central point was conducted. Table 3 shows variables and their levels for central composite design (CCD). The CCD matrix of the independent variables in coded units (experimental design) and experimental values of response is given in Table 4. All the experiments were performed in 250 ml Erlenmeyer flask containing 30 ml of media.

Multiple regression analysis was used to analyze the data and polynomial equation derived from regression analysis for 9α-hydroxyandrost-4-ene-3,17- dione production was shown in equation(2).

$$Y = 5.98 + 0.41A - 0.15B - 0.15C + 0.23AB + 0.59AC + 0.96BC - 0.088A^2 - 0.11B^2 + 0.56C^2 \quad \text{.....(2)}$$

Where, Y is response of 9α-hydroxyandrost-4-ene-3,17- dione production, A is Phytosterol , B is Yeast Extract and C is Glycerol.

3.2.1 ANOVA for Response Surface Quadratic Model

Analysis of variance (ANOVA) was used to check the adequacy of the model (Table 5).

Table 5: ANOVA for Central composite design

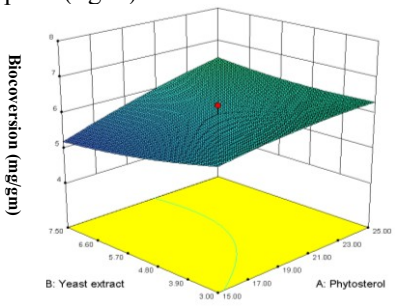
Source	Sum of Squares	df	Mean Square	F-Value	*p-value* Prob>F
Model	17.88	9	1.99	6.42	0.0114
A	2.29	1	2.29	7.41	0.0296
B	0.31	1	0.31	0.99	0.3518
C	0.32	1	0.32	1.05	0.3401
AB	0.44	1	0.44	1.43	0.2711
AC	2.78	1	2.78	9.00	0.0200
BC	7.41	1	7.41	4.68	0.0018
A^2	0.087	1	0.087	23.95	0.6128
B^2	0.13	1	0.13	0.28	0.5335
C^2	3.53	1	3.53	0.43	0.0118
Residual	2.17	7	0.86	11.39	
Cor Total	20.05	16			

Abbreviations: df- degree of freedom

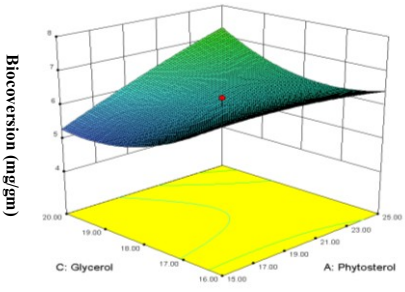
R-squared = 89.19% Pred R-Squared = 8.25%
Adeq Precision = 8.495 Adj R-squared = 75.30%

The model F-value 6.42 represents that the model was significant. There is only a 1.17 % chance that a "Model F-Value" this large could occur due to noise.

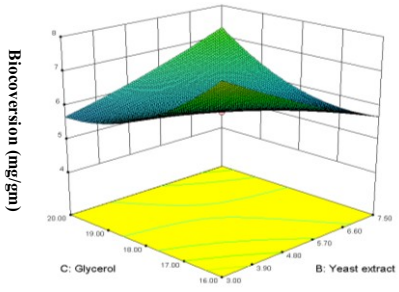
Values of "Prob > F" less than 0.0500 indicates model terms are significant. In this case A, AC, BC and C^2 are significant model terms.Values greater than 0.1000 indicate the model terms are not significant.The "Lack of Fit F-value" of 9.58 implies the Lack of Fit is not significant relative to the pure error. There is a 9.72 % chance that a "Lack of Fit F-value" this large could occur due to noise. The R-Squared value is 89.19% and "Adj R-Squared" value is 75.30%. Adequate precision measures the signal to noise ratio. This model can be used to navigate the design space. The optimum level of variables and interaction effects were found out by 3D surface plots (fig. 2).



(A)



(B)



(C)

Figure 2: 3D surface plot for 9-OH-AD production showing interaction between (A) Phytosterol and Yeast Extract (B) Phytosterol and Glycerol (C) Yeast Extract and Glycerol.

Each graph of figure represents the effect of two factors on 9α-hydroxyandrost-4-ene-3,17- dione production while the third factor was held at zero level. The interaction between Phytosterol (A), Yeast Extract (B) and Glycerol (C) was significant for 9α-hydroxyandrost-4-ene-3,17- dione production. Synergetic effect of Phytosterol (A), Yeast Extract (B) and Glycerol (C) showed enhancement in the bioconversion to 9α-hydroxyandrost-4-ene-3,17- dione. The optimization of the analyzed responses *via* CCD (RSM) demonstrated that the maximum 9α-hydroxyandrost-4-ene-3,17- dione conversion (9.10 mg/gm) was obtained with Phytosterol - 25.0 g/l, Yeast Extract - 7.5 g/l and Glycerol - 20.0 g/l

3.3 Validation of Model

To validate the obtained CCD model for biotransformation of phytosterol to 9α-hydroxyandrost-4-ene-3,17- dione by *Mycobacterium* spp., three sets of experiments were performed.

4. Conclusion

This work has demonstrated the use of statistical approach to obtain optimum yield of 9α-hydroxyandrost-4-ene-3,17- dione from *Mycobacterium* spp. This methodology could therefore be successfully employed for process development where an analysis of effects and interactions of many experimental factors are required. Central composite experimental design maximizes the amount of information that can be obtained, while limiting the numbers of individual experiments required. Response curves are very helpful in visualizing the main effects and interaction of factors. Thus, less time consuming experimental designs could generally suffice for the optimization of many processes. From the above Pareto chart of standardized effects, it can be seen that Phytosterol (A), Yeast Extract (B) and Glycerol (C) have significant effect on biotransformation to 9α-hydroxyandrost-4-ene-3,17- dione. Therefore, to increase the productivity, it is necessary to optimize the concentrations of Phytosterol, Yeast Extract and Glycerol with CCD whereas other components of the medium can be kept constant. The optimization of the analyzed responses demonstrated that the maximum 9α-hydroxyandrost-4-ene-3,17- dione conversion (9.10 mg/gm) was obtained with Phytosterol - 25.0 g/l, Yeast Extract - 7.5 g/l and Glycerol - 20.0 g/l. All points were located near the central point of the design. The significant interactions between three variables were also observed. Thus, the response surface methodology was found to be a favourable strategy to optimize the bioconversion of phytosterol to 9α-hydroxyandrost-4-ene-3,17- dione. Being convenient and effective, this method might be useful in optimization of the overproduction of other metabolites as well.

Hence statistical experimental designs are powerful tools for the rapid search of key factors from a multi-variable system and minimizing the error in determining the effect of parameters and the results are achieved in an economical manner.

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