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Optimization of Downstream Process Parameters for Isolation of Androstenedione Using Statistical Approach

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Abstract **- 4-Androstenedione (AD), a 19-carbon steroid hormone is an oxidation product of phytosterol fermentation by *Mycobacterium sp.* The isolation of the product from the fermentation broth is a challenging task. Downstream processing is an important aspect of all biotechnological processes and has significant implications on quality and yield of the final product. In the present study, the statistical approach was employed to optimize the various parameters for the downstream processing of AD. The quantity of Hyflow (filtration aid) was optimized as 5%, initially using the one variable at a time method. Further the factors: rpm, volume, solvent polarity and extraction temperature were studied using full factorial design. Analysis of the experiment showed that the polarity and the extraction temperature had maximum impact on the AD extraction. Selection of solvent for extraction procedure was carried out using one variable at a time (OVAT) technique. Ethyl acetate showed maximum product recovery. A Central Composite Design of "response surface methodology" was conducted to optimize the most significant factors of downstream process, to maximize the product extraction. The optimized model shows rpm-3500, solvent volume-5V and temperature-30°C gives maximum extraction of androstenedione.**

Index Terms **- Androstenedione, downstream processing, Hyflow, solvent extraction, full factorial design, response surface methodology.**

I. INTRODUCTION

4-Androstenedione (AD), a 19-carbon steroid hormone is an oxidation product of phytosterol fermentation. Since phytosterol is easily available from plant source, this route is selected for manufacturing of AD. Pharmaceutical steroid precursor: androst-4-ene-3,17-dione (AD) play a key role in the management of human fertility, menopause, osteoporosis, and blood pressure regulation because of its therapeutic (glucocorticoids) and contraceptive properties (e.g. progesterone and estrogen)[1]. AD is a direct precursor of testosterone and estrone. Fig. 1: represents the structure for androstenedione[2].

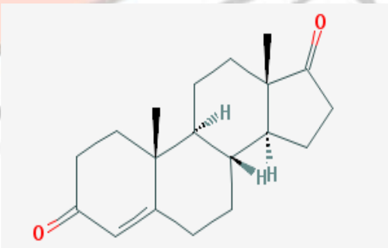

Figure 1: Chemical structure of androstenedione

Androstenedione is produced through biotransformation of phytosterol by *Mycobacterium sp. Mycobacterium sp.* are actinobacteria which are non pathogenic, aerobic, non motile. The harvested fermentation broth is further processed by downstream processing to produce crude product of AD, which can then be utilized to synthesize other steriods.

Downstream processing is an important aspect of all biotechnological processes and has significant implications on quality and yield of the final product. The downstream process involves recovery, isolation, and purification of the microbial products from harvested fermentation broth (cell debris, processing medium and contaminating biomolecules). Fig. 2 shows the various steps in the downstream processing of a product.

Although, strategies of purification depend on desired use of the final product and in most cases several different processes like cell separation, solvent extraction, decolorization, chromatographic purification etc. are involved in purification of fermentation products. All these processes have significant effect on the cost of the final product and may even account for more than 60% of total cost for production[3]. Generally the extraction processes consumes a lot of time and bulk amount of solvents. Downstream process design has the greatest impact on overall biomanufacturing cost. Therefore, development of a simplified and cost effective downstream process for microbial fermentation products is a major challenge for their commercialization.

Normally, in the downstream processes, the optimum conditions are determined by keeping one variable parameter and others at constant level, which is a very time consuming process [4]. Statistical experimental design techniques are very useful tools when more than one factor is studied at a time. These statistical models help in understanding the interactions among the different variables at various levels and in calculating the optimal level of each variable[5]. The statistical approach enables the evaluation of various components at a time thus making it cost effective and time saving process.

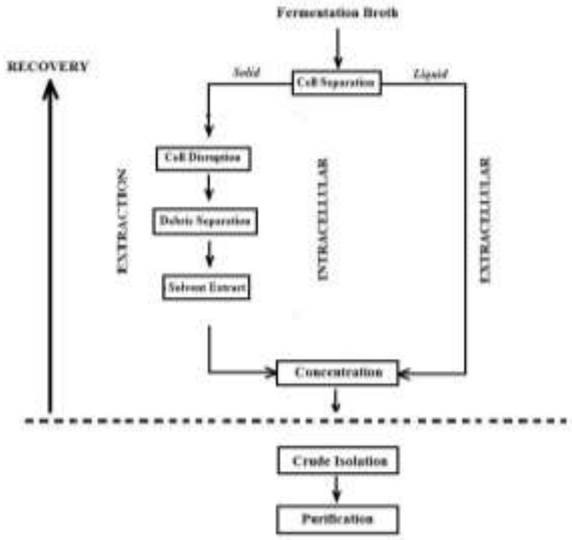

Figure 2: shows the various steps in the downstream processing of a product.

The present research aims to develop a suitable and efficient downstream process for the maximum recovery of AD. The current study deals with the optimization of the various process parameters involved in the purification of Androstenedione at the various stages of the downstream processing using the statistical approach.

II. MATERIAL AND METHODS

Biotransformation of phytosterol to androstenedione (AD) was carried out using *Mycobacterium sp.*

Procedure

1. Fermentation

Fermentative production of Androstenedione is the commonly used method. AD is produced through fermentation of phytosterol by *Mycobacterium sp.* The harvested fermentation broth is further processed by downstream processing to isolate crude product of AD.

2. Downstream Processing

The harvested broth was proceeded for downstream processing, the pH was monitored. 10% sulfuric acid was added dropwise to adjust the pH to get the desired value i.e. 2.5±0.1. After the pH adjustment, the acidified broth was extracted with ethyl acetate. Extraction procedure was done in two stages. In the first stage, 1V of ethyl acetate was used while in the second stage 0.5V was used. The two layers involving ethyl acetate and aqueous layer were separated. The aqueous layer was recovered again using ethyl acetate. Charcoal was added to the obtained ethyl acetate layer and was stirred for 1 hr, later on charcoal was filtered.

With the filtrate, distillation process was carried out with reduced pressure at < 80°C. The concentrated mass was obtained in form of viscous liquid (consistency like oil) or sticky solid.Then 4V of hexane was added to the viscous liquid followed by heating and stirring at 60-65°C for 1 hr . The reaction mixture was then cooled to 25°C. The cake was filtered and washed with 1V of hexane. It was then dried at 80°C of temperature and the crude AD product was obtained. The assay purity was checked by HPLC.

3. Quantification of product by HPLC

Androstenedione produced in the fermentation broth was determined by HPLC. The harvested 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 was maintained at 30°C. Concentration of AD was calculated by comparison of peak areas with those of standard AD and subsequently AD activity was calculated.

Optimization of Downstream Process Parameters

1. Quantity of Hyflow

Androstenedione is an intracellular product and the harvested fermentation broth was slimy in nature. Separation of cell mass from broth by using ordinary filtration method was slow. To overcome this problem, separation of cell mass is done by using filtration aid i.e. Hyflow. In the current study, the quantity of Hyflow was first optimized using the classical method of One variable at a time. A popular way to deal with optimization is the One Variable at a Time (OVAT) approach.

The quantity of Hyflow added for the study were 1%, 2% and 3% in fermentation broth. Once the quantity of Hyflow was finalized with OVAT then other process parameters were optimized.

2. Full Factorial

The full factorial design was employed in the selection of most significant variable for AD isolation. Full factorial design determines the effects of multiple variables on product extraction. In addition to evaluating the impact of each variable, it also determines the impact of interaction among the variables.The two level factorial design is considered to be a multivariable sequential search technique in which the effects of two or more factors are studied simultaneously and the responses are analyzed statistically to arrive at a decision.[6, 7]. A two level four factorial design was carried out for the optimization of AD downstream process parameters.

The parameters taken into consideration were

– rpm
– Volume
– Polarity
– Extraction process temperature

The low level (– 1) and high level (+ 1) of each factor are listed in Table 1. Experiment was designed using Design expert software (Stat-Ease Inc., Version 8.0.7.1).

Table 1: Experimental code and levels of factors in the Full factorial design

Code	Factor	Low Level (-)	High Level (+)
A	rpm	2000	3000
B	Volume	1V	3V
C	Polarity	Non Polar	Polar
D	Temperature	25°C	30°C

3. Selection of Solvent

Solvents play an important role in the product extraction and isolation step of the downstream processing. It is involved in the removal of the impurities that vary markedly from the product. In the study, solvent used for recovery of AD was optimized using OVAT technique. The solvents used for the study were ethyl acetate, acetone and methanol since AD showed good solubility in these solvents. All the solvents were utilized for the extraction of AD. Once the solvent was finalized with OVAT, it was further used to optimize other process parameters.

4. Optimization of process parameters using RSM

The three most significant parameters (A: rpm, B: solvent volume, and C: temperature) in downstream processing, affecting the isolation of AD, were further optimized by using CCD. The CCD is one of the most commonly used response surface design for fitting second-order models. To produce and examine the experimental design, the statistical software "Design Expert 8.0" was employed. A set of 20 different runs were carried out and the maximum recovery of AD was used as response on accomplishment of the experimental design.

To obtain an empirical model, linking the response measured with the independent variables, multiple regression of the data was also carried out. Using the "Design Expert" software, 3D graphs were formed to define the optimum levels of the variables for maximum yield of AD. Table 2 shows the experimental range for each variable using the classical optimization approach where one parameter was changed while others were kept constant.

Table 2: Ranges of various independent process factors used in RSM.

Code	Factors	Low Level	High Level
A	rpm	3000	4000
B	Solvent Volume	3V	5V
C	Temperature	30°C	40°C

III. RESULT AND DISCUSSION

The optimization of downstream process parameters for isolation of Androstenedione was done using statistical approach

Quantity of Hyflow

The quantity of Hyflow was optimized using the classical method of One variable at a time experiment. Hyflow plays an important role as an filtration aid for separation of cell mass during downstream process. The results of experiment is given in table 3.

It was observed that as the quantity of Hyflow was increased, the filtration time decreases. They are inversely proportional to each other. Even after increasing the concentration of Hyflow from 5% to 7%, the time of separation was same. Thus for economic feasibility, the optimized quantity of Hyflow using OVAT was 5% for fast separation of cell mass.

Table 3: Different Quantity of Hyflow

Quantity of Hyflow (%)	Filtration Time (min)
1	22
2	19
3	14
4	9
5	5
7	5

Full Factorial

Full factorial design was employed for selection of variables effecting extraction of AD from fermentation broth. The factors studied were rpm, volume, polarity of solvent and temperature. The experimental run along with the yield is represented in table 4.

Table 4: Experimental design for full factorial experiment for 4 variables with yield.

Run	rpm	Volume	Polarity	Temperature	Yield (mg/gm)
1	-	-	-	-	2.8
2	+	-	-	-	2.1
3	-	+	-	-	3.7
4	+	+	-	-	2.0
5	-	-	+	-	7.0
6	+	-	+	-	7.8
7	-	+	+	-	8.5
8	+	+	+	-	9.2
9	-	-	-	+	4.6
10	+	-	-	+	5.9
11	-	+	-	+	5.1
12	+	+	-	+	6.4
13	-	-	+	+	8.3
14	+	-	+	+	7.0
15	-	+	+	+	10.2
16	+	+	+	+	16.0

The result was analyzed using the half normal plot and pareto chart is represented in fig. 3 and 4 respectively.

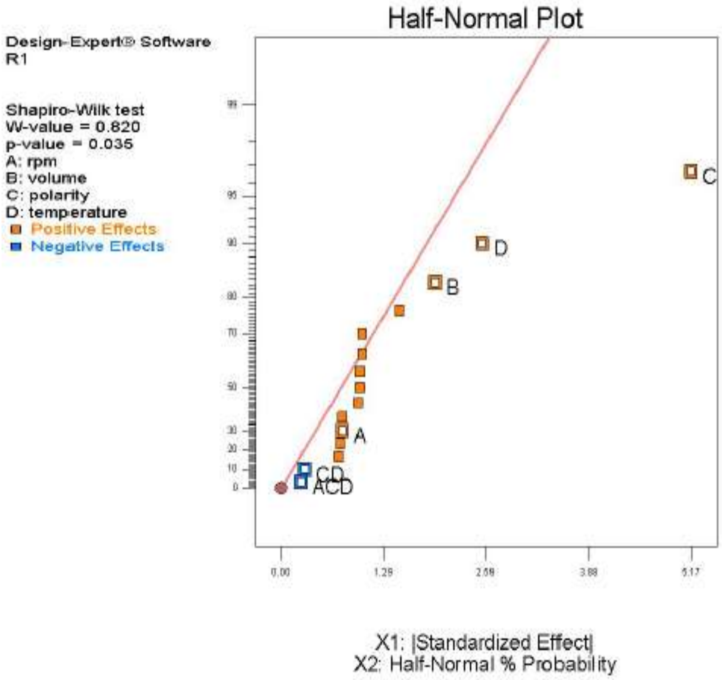

Figure 3: Half normal plot for screening through full factorial design

The plot shows that all the factors A, B, C and D have a positive impact on the extraction of AD. This implies that increasing rpm to 3000, volume to 3V, temperature to 30°C and using polar solvent for recovery will give better extraction in downstream.

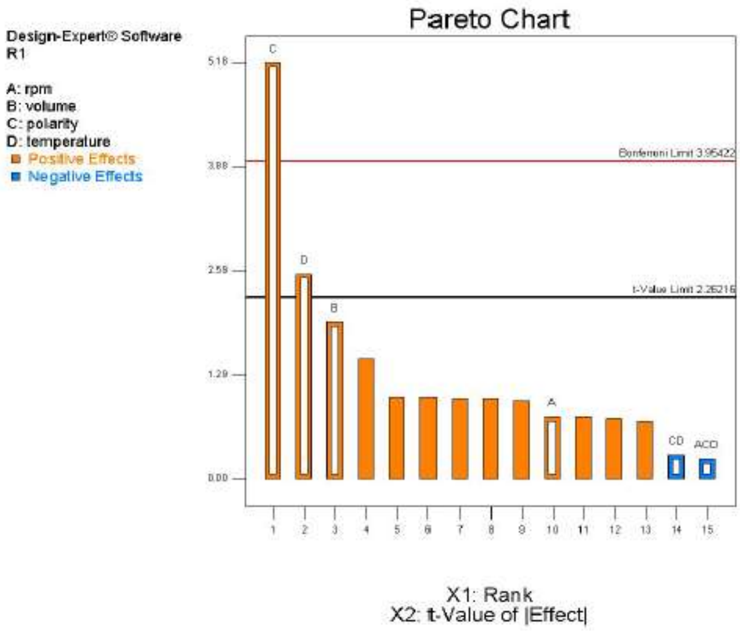

Figure 4: Pareto chart for screening through full factorial design.

In the Pareto chart, the bars are arranged in descending order of height from left to right. This means the categories represented by the tall bars on the left are relatively more significant than those on the right.8 According to the pareto chart components A and D are more significant as compared to the other parameters and interactions. Further use of polar solvent and increasing the extraction temperature would increase the AD yield.

1. Verification of the significant factors

ANOVA was used to verify the significant factors obtained through the above analysis. It is depicted in table 5.

Table 5: ANOVA analysis

Source	**Sum of squares**	**df**	**Mean square**	**F value**	**p value Prob > F**
Model	151.35	6	25.23	6.31	0.0076
A-rpm	2.40	1	2.40	0.60	0.4580
B-Volume	15.21	1	15.21	3.81	0.0828
C-Polarity	107.12	1	107.12	26.81	0.0006
D-Temperature	26.01	1	26.01	6.51	0.0311
CD	0.36	1	0.36	0.090	0.7709
ACD	0.25	1	0.25	0.63	0.8081
Residual	35.96	9	35.96		
Cor Total	187.32	15			

In this analysis, the outstanding effects are incorporated into the "model" and the smaller effects are pooled together to estimate the error called "residual". "Cor total" values are the total sum of squares corrected for the mean. It represents the total system variation using the average response as a baseline [9].

Abbreviations: df: degree of freedom

R-squared 0.8080

Adj R-squared 0.6800

Pred R-Squared 0.3932

Adeq Precision 7.714

The variables which scored a Probability (P) value less than 0.0500 were considered as influential factors affecting the response. Values greater 0.100 indicate that the factors are not significant. The model F value of 6.31 implies that the model is significant. There is only a 0.76% chance that a 'Model F value" this large could occur due to noise."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The obtained ratio of 7.714 indicates an adequate signal. This model can be used to navigate the design space. Analysis of the 4 factors and their interactions indicate that the factor C and D is the most significant factor impacting the extraction of AD in downstream processing.

Selection of Solvent

Solvents are used in bulk quantity during recovery of product. Downstream process has the greatest impact on overall biomanufacturing cost in fermentation process. Therefore, solvent used for recovery of AD was optimized using OVAT technique.

Table 5: Impact of type of solvent on AD yield

Solvents	Yield (mg/gm)
Ethyl acetate	8.1
Acetone	8.0
Methanol	7.8

Among the solvents analyzed it was observed that recovery of Ethyl acetate extraction mass was the highest. The lowest yield of AD is with use of methanol recovery.

Optimization of process parameters using RSM

The three process parameters (A: rpm, B: Solvent volume, and C: temperature) plays an important role in downstream processing, which effect the extraction of AD, were optimized using CCD. A set of 20 different runs were carried out using different combination of three factors. The CCD matrix of the independent variables in coded units (experimental design) and experimental values of response is given in Table 6.

Table 6: Experimental code and levels of factors in CCD

Std	Run	A	B	C	Yield (mg/gm)
20	1	3500.00	4.00	35.00	13.681
19	2	3500.00	4.00	35.00	13.524
13	3	3500.00	4.00	26.59	10.264
14	4	3500.00	4.00	43.41	9.963
11	5	3500.00	2.32	35.00	10.125
10	6	4340.00	4.00	35.00	12.561
15	7	3500.00	4.00	35.00	13.012
16	8	3500.00	4.00	35.00	12.996
9	9	2659.10	4.00	35.00	11.125
1	10	3000.00	3.00	30.00	8.562
5	11	3000.00	3.00	40.00	10.358
18	12	3500.00	4.00	35.00	13.446
8	13	4000.00	5.00	40.00	9.147
12	14	3500.00	5.68	35.00	18.200
3	15	3000.00	5.00	30.00	16.559
17	16	3500.00	4.00	35.00	13,497
7	17	3000.00	5.00	40.00	12.562
6	18	4000.00	3.00	40.00	10.117
2	19	4000.00	3.00	30.00	11.785
4	20	4000.00	5.00	30.00	13.998

Multiple regression analysis was used to analyze the data and polynomial equation derived from regression analysis for androstenedione extraction was shown in equation(1).

$Y = 13.37 - 0.042A + 1.83B - 0.68C - 1.12AB - 0.54AC - 1.12BC - 0.60A^2 + 0.22B^2 - 1.22C^2$ ....(1)

Where, Y is response of androstenedione extraction, A is rpm , B is Solvent Volume and C is Temperature.

1. ANOVA for Response Surface Quadratic Model

   Analysis of variance (ANOVA) was used to check the adequacy of the model (Table 7).

Table 7: ANOVA Analysis for CCD

Source	Sum of Squares	df	Mean Square	F-Value	*p-value* Prob>F
Model	101.37	9	11.26	11.09	0.0004
A	0.025	1	0.025	0.024	0.8796
B	45.85	1	45.85	45.14	0.0001
C	6.23	1	6.23	6.14	0.0327
AB	10.03	1	10.03	9.87	0.0105
AC	2.33	1	2.33	2.29	0.1608
BC	10.07	1	10.07	9.91	0.0104
A^2	5.26	1	5.26	5.18	0.0461
B^2	0.67	1	0.67	0.66	0.4255
C^2	21.30	1	21.30	20.97	0.0010
Residual	10.16	10	1.02		
Cor Total	111.53	19			

Abbreviations: df: degree of freedom
R-squared 0.9089
Adj R-squared 0.8269
Pred R-Squared 0.3188
Adeq Precision 12.898

The model F-value 11.09 represents that the model was significant. There is only a 0.04 % chance that a "Model F-Value" this large could occur due to noise.The model F value of 11.09 with low p value (>0.0001) implied a high significance for the regression model [10].
Values of "Prob > F" less than 0.0500 indicates model terms are significant. In this case B, C, AB, BC, A2 and C2 are significant model terms.Values greater than 0.1000 indicate the model terms are not significant.The "Lack of Fit F-value" of 23.80 implies the Lack of Fit is not significant relative to the pure error. There is a 0.17 % chance that a "Lack of Fit F-value" this large could occur due to noise. The optimum level of variables and interaction effects were found out by 3D surface plots (fig. 5).

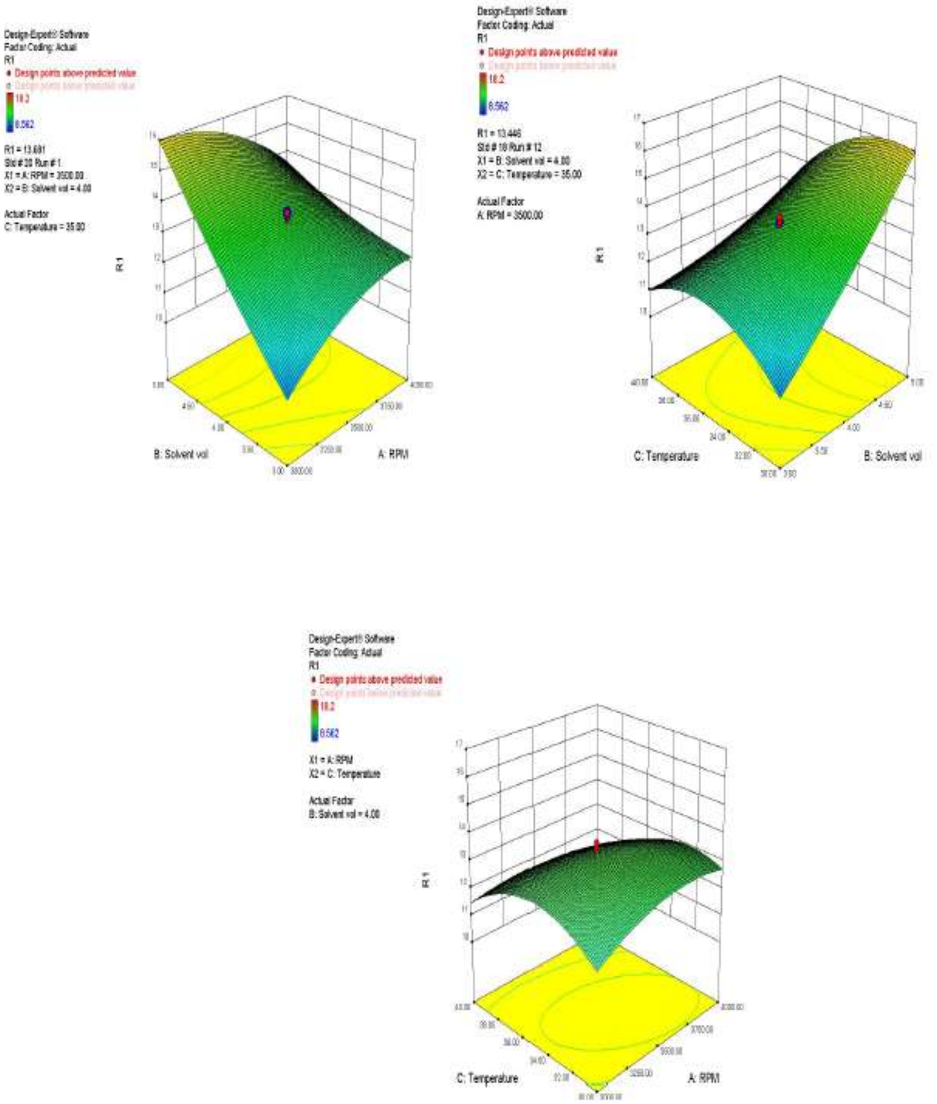

Figure 5: 3D surface plot for AD extraction showing interaction between (A) rpm and Solvent Volume (B) Solvent Volume and Temperature (C) rpm and Temperature.

Each graph of figure represents the effect of two factors on Androstenedione isolation while the third factor was held at zero level. The interaction between rpm (A), Solvent Volume (B) and Temperature (C) was significant for androstenedione isolation. Synergetic effect of rpm (A), Solvent Volume (B) and Temperature (C) showed enhancement in the extraction of Androstenedione. The optimization of the analyzed responses via CCD (RSM) demonstrated that the maximum yield of androstenedione (12.23 mg/gm) was obtained with rpm 3500, solvent volume 5V and temperature 30.

IV. CONCLUSION

This work has demonstrated the use of statistical approach to obtain optimum yield of androstenedione (AD) from *Mycobacterium spp.* by optimizing downstream process (extraction of AD) from harvested fermentation broth. Optimization of Hyflow quantity through OVAT showed that 5% of Hyflow reduces the filtration time to 5 min, which decreases time of extraction. Full factorial run using the four factors namely, extraction temperature, volume, rpm and the solvent polarity for extraction was carried out. The maximum extraction of AD was obtained by full factorial design. The analysis through pareto and half normal plot showed that the polarity and the extraction temperature are the most significant factors having the maximum impact. Thus, use of polar solvents and increasing the temperature can help in maximizing the AD extraction. By assessing selection of polar solvents through OVAT technique showed that use of ethyl acetate was significant as a solvent for extraction. RSM was employed in order to maximize the extraction of AD from fermentation broth. The optimization of the analyzed responses demonstrated that the maximum extraction of androstenedione was obtained with rpm 3500, solvent volume 5V and temperature 30°C.

A statistical approach is very resourceful to develop a suitable and efficient downstream process for the maximum product recovery. It enables the evaluation of various process parameters involved in product extraction in a single experiment thus making it cost effective and time saving process.

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