International Journal of Engineering Research and General Science Volume 3, Issue 6, November-December, 2015
ISSN 2091-2730

Physio-Morphological Characterization of *Mycobacterium spp.* followed by substrate selection (Phytosterol) for 9-OH-AD Production

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Abstract— *Mycobacterium spp.* was used for the biotransformation of phytosterol to 9*α*-hydroxyandrost-4-ene-3,17-dione (9-OH-AD). Fast growing *Mycobacterium spp.* rapidly and completely degrades sterols by simultaneously attacking the steroid ring nucleus and 17-alkyl side chains. The 3-ketosteroid 9α-hydrolyase has been reported as a key enzyme in production of 9-OH-AD. The present study signifies the importance of culture characterization and selection of phytosterol. Physio-morphological Characterization of *Mycobacterium* revealed that the microorganism is Gram-positive, non motile and acid-fast rod; colonies varies in size 5-15mm on different agar media and appear as non-pigmented, rough with opaque aspect. Statistical design was used to screen the most suitable source of phytosterol for 9-OH-AD production by *Mycobacterium spp*. Quality of phytosterol plays a crucial role in the biotransformation process. Six different types of phytosterols were studied by Fraction factorial design (FFD). Maximum yield of 9-OH-AD (5.58 mg/g) was obtained with the use of substrate- vendor (A) Micronized phytosterol - 10 g/l. The FFD was very effective in screening of substrate for 9-OH-AD production.

Keywords— Phytosterol, Bioconversion, Characterizaton, *Mycobacterium spp.*, 9*α*-hydroxyandrost-4-ene-3,17-dione, Statistical design and Fraction factorial design.

INTRODUCTION

The 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.The steroid drug production industry demands more than 2000 tons of natural sterols annually and there is an increasing need for cheap and available sterol containing raw materials [1]. A majority of steroid drugs as anti-inflammatory, anti-allergic, cardiotonic, geriatric, progestational, anabolic, immunosuppressive and contraceptive agents have been successfully introduced in the allopathic system of medicine. The commercial production of these pharmaceutically active steroidal drugs depends upon three C-17-ketosteroid precursors namely, androst-4-ene-3,17-dione (AD), androsta-1,4-diene-3,17-dione (ADD) and 9α-hydroxyandrost-4-ene-3,17- dione (9-OH-AD) [2,3]. 9-OH-AD is the key precursor in the synthesis of various glucocorticoids.

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. Selective side chain degradation of sterols to 17-ketosteroids is one of the most widely used bio transformation 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 [4].

Phytosterol is a collective term for plant sterols and stanols. The most common plant sterols are sitosterol, campesterol, and stigmasterol, and the most common plant stanols are sitostanol, campestanol, and stigmastanol [5]. Phytosterols, which are structurally and physiologically similar to cholesterol, are a large group of steroidal triterpenes. The pharmaceutical industry has a long history of converting phytosterols to therapeutic steroid hormones by microbial transformation. However, some functions of phytosterols are often limited because of their poor solubility in aqueous media.The bioavailability of phytosterols can be improved by micronizing the particles.

Micronization means transfer of the coarse drug powder to an ultrafine powder with a mean particle size being typically in the range of 2-5 μm, size distributions normally ranges from approximately 0.1 to 25 μm by very simple technique i.e. wet milling or jet milling.The basic principle of micronization is to increase the dissolution velocity by increasing the surface area. After micronization, the particles have greater specific surfaces which lead to better solubility. Reducing the particle size by the rapid expansion of supercritical solutions (RESS) can enhance the solubility and so improve the bioavailability of phytosterols [6].

Media optimization and strain improvement are two important methods for the enhancement of the yield by biotransformation. Usually media optimization is done 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 [7].

The present study describes the bioconversion using *Mycobacterium species* of phytosterols to 9α-hydroxyandrost-4-ene-3,17-dione (9-OH-AD), a key intermediate for the production of steroidal drugs and hormones, by designing a suitable production medium using statistical optimization. The gene cluster of *Mycobacterium species* involved in sterol catabolism reported as a new starting point for the biotransformation of phytosterols [8]. *Mycobacterium spp.* rapidly and completely degrades sterols such as sitosterol and cholesterol by simultaneously attacking the steroid ring nucleus and 17-alkyl side chains [9, 10].

MATERIALS AND METHODS

Microorganism and Cultivation

Mycobacterium spp. was used for the biotransformation 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, 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-2-5.0 g/l and Antifoam-1.0 ml with pH - 7.00 and agar-25.0 g/l. The slants were incubated at 30ºC for 8 days. Grown culture was perserved with 20% glycerol for further use.

Morphophysiological characterization

Mycobacterium spp. is a non-tuberculous species of the phylum actinobacteria, belonging to the genus mycobacterium. It is a chemoheterotroph and obligate anaerobe. It is also a saprophyte whose natural habitat includes soil, water and dust. Its optimal growing temperature is 30-37°C, making it a thermophile. It was grown on different media for characterization (Table 1) and incubated for 10 days at 30°C. The colony morphology of isolates was observed with the use of a stereomicroscope (10×). Gram and acid-fast stains were used for studies of microscopic morphology and acid-fast stain respectively [11]. *Mycobacterium spp.* morphology was studied further by Scanning electron microscopy. *Mycobacterium spp.* was one of the first bacterial species to be examined using the technique of electron microscopy (EM) [12]. Culture was fixed in a solution of 2.5% glutaraldehyde in 0.1M phosphate buffer, pH 7.4, overnight at 4.0 °C. The samples were rinsed once in the same buffer and dehydrated by increasing concentrations of ethanol (30, 50, 70, 90 and 100%). The samples were dried in a fume hood and fixed on to stubs with conductive self-adhesive carbon tapes, coated with gold film sputtering and used for analysis with scanning electron microscopy (SEM).

Sr. No.	Agar Media	Composition
1	I	L-Asparagine-5 g/l, Potassium dihydrogen phosphate-5.9 g/l, Potassium dihydrogen sulphate-5 g/l, Citric Acid-1.5 g/l, Magnesium Carbonate-0.6 g/l, Glycerol-20 ml, Tween 80-2 ml and agar - 25 g/l, pH - 7.2.
2	II	Peptone-10g/l, Sodium Chloride-5 g/l, Beef Extract-5g/l,Tween 80-2 ml and agar - 25 g/l, pH - 7.4.
3	III	Yeast extract-11.50 g/l, Diammonium Hydrogen Phosphate-1.73 g/l, Potassium Dihydrogen Phosphate-0.58 g/l, Dipotassium Hydrogen Phosphate-0.64g/l, Ferrous sulphate-0.0058g/l, Zinc sulphate-7-hydrate-0.0023g/l, Magnesium Sulphate-0.23 g/l, Glycerol-11.67g/l, Tween 80 -3.50 g/l and Antifoam-1 ml and agar - 25 g/l with pH - 7.00.
4	LA	Dextrose-10 g/l, Malt Extract-20g/l, Yeast Extract-3 g/l, Bacteriological Peptone-10 g/l and agar - 25 g/l with pH - 7.00 .
5	SA	Yeast Extract-5g/l, Ammonium sulphate-1g/l, Potassium Dihydrogen Phosphate-1g/l, Soyaflour-5 g/l, Soya Oil-5g/l, Tween 80-10 g/l and agar - 25 g/l, pH - 7.00 .
6	TRSA (Tryptic Soya Agar)	Casein Enzymic Hydrolysate-15.00 g/l, Papaic digest of Soyabean meal-5.00 g/l, Sodium chloride-10.00g/l, Agar 15.00g/l with Final pH (at 25°C) 7.3±0.2.
7	NA (Nutrient Agar)	Peptic digest of animal tissue-5.00 g/l, Sodium Chloride-5.00 g/l, Beef extract-1.50g/l, Yeast extract-1.50g/l, Agar-15.00g/l with final pH (at 25°C) 7.4±0.2.
8	SFMA (Sautons Fluid Medium Agar)	Ferric Ammonium Citrate-0.0167 g/l, L-Asparagine-1.33 g/l, Citric Acid-0.66 g/l, Magenium Sulphate 7 hydrate-0.166 g/l, Dipotassium hydrogen Phosphate-0.177g/l, Sodium Dihydrogrn Phosohate-0.056 g/l, Sodium Chloride-0.035 g/l, Polysorbite 80-0.833g/l and Agar-20 g.l, pH 7.2.
9	BHIA (Brain Heart Infusion Agar)	Calf brain, infusion from-200.00g/l, Beef heart, infusion from -250.00 g/l, Proteose Peptone-10.00 g/l, Dextrose-2.00g/l, Sodium chloride-5.00g/l, Disodium phosphate-2.50g/l, Agar-15.00 g/l with Final pH (at 25°C) 7.4±0.2.
10	LJ (Lowenstein Jensen Agar)	L-Asparagine-6.0g/l, Monopotassium Phosphate -4.0g/l, Magnesium Sulphate-0.4g/l, Magnesium Citrate-1.0g/l, Potato starch, soluble-50.0g/l, Malachite green-0.6g/l.
11	AV3 Agar	Dextrose-22 g/l, Soyaflour-10g/l, Yeast Extract-2.5 g/l, Citric Acid-2.2 g/l, Urea-0.5 g/l, Diammonium Sulphate-1g/l, Potassium Dihydrogen Phosphate-0.5 g/l, Magnesium Sulphate-0.5 g/l, Ferrous Sulphate-0.05 g/l, Calcium Carbonate-2 g/l, Polypropylene Glycol-0.1 ml and agar - 25 g/l, pH - 6.2.
12	AA2	Dextrose-10 g/l, Soyaflour-3 g/l, Citric Acid-2.2 g/l, Potassium dihydrogen Phosphate-0.5 g/l, Urea-0.5 g/l, Magensium Sulphate-0.5 g/l, Ferrous sulphate-0.05 g/l, Calcium Carbonate-1.5 g/l, Ammonium Chloride-1 g/l and agar - 20 g/l, pH - 6.9
13	Middlebrook 7H9 Agar Base	Ammonium Sulphate-0.50 g/l, Sodium glutamate-0.50g/l, Sodium citrate-0.10g/l, Pyridoxine-0.001g/l, Biotin-0.0005g/l, Disodium phosphate-2.500g/l, Monopotassium phosphate -1.000g/l, Ferric ammonium citrate-0.040g/l, Magnesium sulphate-0.050g/l, Calcium chloride-0.0005g/l, Zinc sulphate -0.001g/l, Copper sulphate-0.001g/l, Malachite green-0.001g/l, Agar-15.00g/l with Final pH (at 25°C) 6.6±0.2.
14	Middlebrook 7H10 Agar Base	Ammonium sulphate-0.500g/l, L-Glutamic acid-0.500g/l, Monopotassium phosphate-1.500g/l, Disodium phosphate -1.500g/l, Sodium Citrate-0.400g/l, Ferric Ammonium Citrate -0.040g/l, Magnesium Sulphate-0.025g/l, Calcium chloride -0.0005g/l, Zinc sulphate-0.001g/l, Copper sulphate-0.001g/l, Pyridoxine hydrochloride-0.001g/l, Biotin-0.0005g/l, Malachite green-0.00025g/l, Agar-15.000g/l with Final pH (at 25°C) 6.6±0.2.

Table 1: Media and their composition

Biotransformation process

Mycobacterium spp. was used for the biotransformation of phytosterol to 9-hydroxyandrostenedione. The grown slant was harvested with normal saline and was used to inoculate the seed medium. This medium is composed of Yeast Extract-12.00 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 on shaking incubator for 24±4 hrs.

Transformation medium (production medium) comprises of the basal components like Phytosterol-10 g/l, Tween 80-3.50 g/l,

Ammonium acetate-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. 10% of the grown seed medium was transferred to production medium in 250 ml conical flasks containing 30 ml of medium. Flasks were incubated at 30ºC and 240 rpm for 120 hrs. The process parameters such as pH, PCV were checked and yield was assessed through HPLC at an interval of 24 hrs.

9-OH-AD estimation by HPLC

9*α*-hydroxyandrost-4-ene-3,17- dione (9-OH-AD) 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 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 9-OH-AD at 238nm. The mobile phase was composed of Water: Acetonitrile, the flow rate was 0.8 ml/min and column temperature at 30°C. Concentration of 9-OH-AD was calculated by comparison of peak areas with the standard 9-OH-AD and subsequently 9-OH-AD activity was calculated.

Substrate (Phytosterol) Selection for 9-OH-AD production

Phytosterols (PSs) are suitable raw material for microbial biotransformation. The effect of different phytosterols on the biotransformation process for production of 9-OH-AD was studied. Six different types of phytosterols from different vendors were used. The applied phytosterol constitute of 2-sitosterol, campesterol, brassicasterol, stigmasterol , sitostanol, campestanol, and stigmastanol. Depending on the vendor, this composition of phytosterol may vary. A list of different Phytosterols is given in Table 2.

Sr.No.	Different Types of Phytosterol	Code
1	(A)- Micronized	A
2	(B)- Micronized	B
3	(C)- Non micronized	C
4	(D)- Non micronized	D
5	(E)- Non micronized	E
6	(F)- Non micronized	F

Table 2: Variation of Phytosterol

Experimental design

In order to optimize the conditions of biotransformation for 9-OH-AD yield and phytosterol conversion, a set of statistically designed experiments were conducted. All the statistical analysis was done using the Design expert software (Stat-Ease Inc., Version 8.0.7.1). First, fractional factorial design (FFD) was used to identify the significant factors affecting 9-OH-AD yield and phytosterol conversion rate by *Mycobacterium spp*. The coded values of variables are given in Table 2. A 2^{5-1} FFD which included 16 sets of trials with four replicates at center point was carried out in duplicate to screen the critical parameters influencing 9-OH-AD yield and phytosterol conversion rate [12].

The 9-OH-AD yield and PS conversion rate are considered as responses (R1) 9-OH-AD. After the analysis of FFD, a first-order model was gained [13].

RESULTS AND DISCUSSION

Isolation and morphological characterization

The colony morphology of *Mycobacterium spp.* on different media (Figure 1) were observed regularly upto 10 days with the use of a stereomicroscope-10× (Figure 2 & Table 3). Morphological studies of culture showed that size of *Mycobacterium spp.* varies from 5-15mm.

Colony Characteristics	Different Plate Media						
	I	**II**	**III**	**LA**	**SA**	**TRSA**	**NA**
Size	5mm	9mm	9mm	10mm	10mm	7mm	15mm
Shape	Round	Round	Round	Round	Round	Round	Round
Color	Off-white	Cream	Off-white	Off-white	Off-white	Off-white	Off-white
Margin	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular
Elevation	Convex	Convex	Convex	Flat	Convex	Convex	Flat
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Morphology	Granulated	Grooved	Rough Furrow	Centrally Furrow	Rough granulated	Shiny granulated	Shiny Furrow

Colony Characteristics	Different Plate Media						
	SFMA	**BHIA**	**LJ**	**AV3 Agar**	**AA2**	**7H9 Agar**	**7H10 Agar**
Size	6mm	7mm	6mm	9mm	9mm	9mm	10mm
Shape	Round	Round	Round	Round	Round	Round	Round
Color	Pale Yellow	Off-white	Buff brown	Off-white	Cream	White	White
Margin	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular	Irregular
Elevation	Convex	Flat	Convex	Flat	Convex	Convex	Flat
Opacity	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque	Opaque
Morphology	Rosette cluster	Rough Furrow	Lobular	Shiny center	Rough Furrow	Glossy appearance	Shiny granulated

Table 3: Morphological Characterization

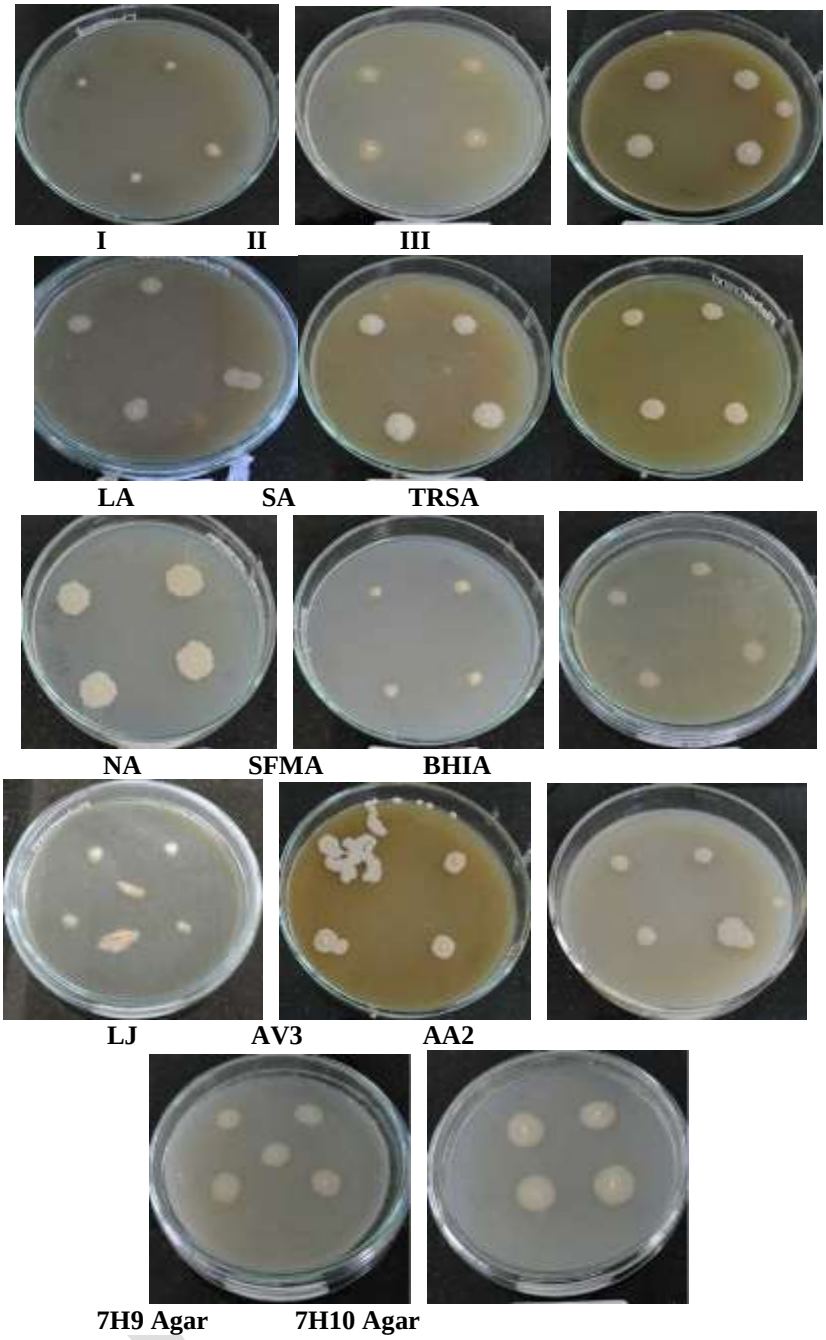

Figure 1: Morphology of *Mycobacterium spp.*on different Agar media

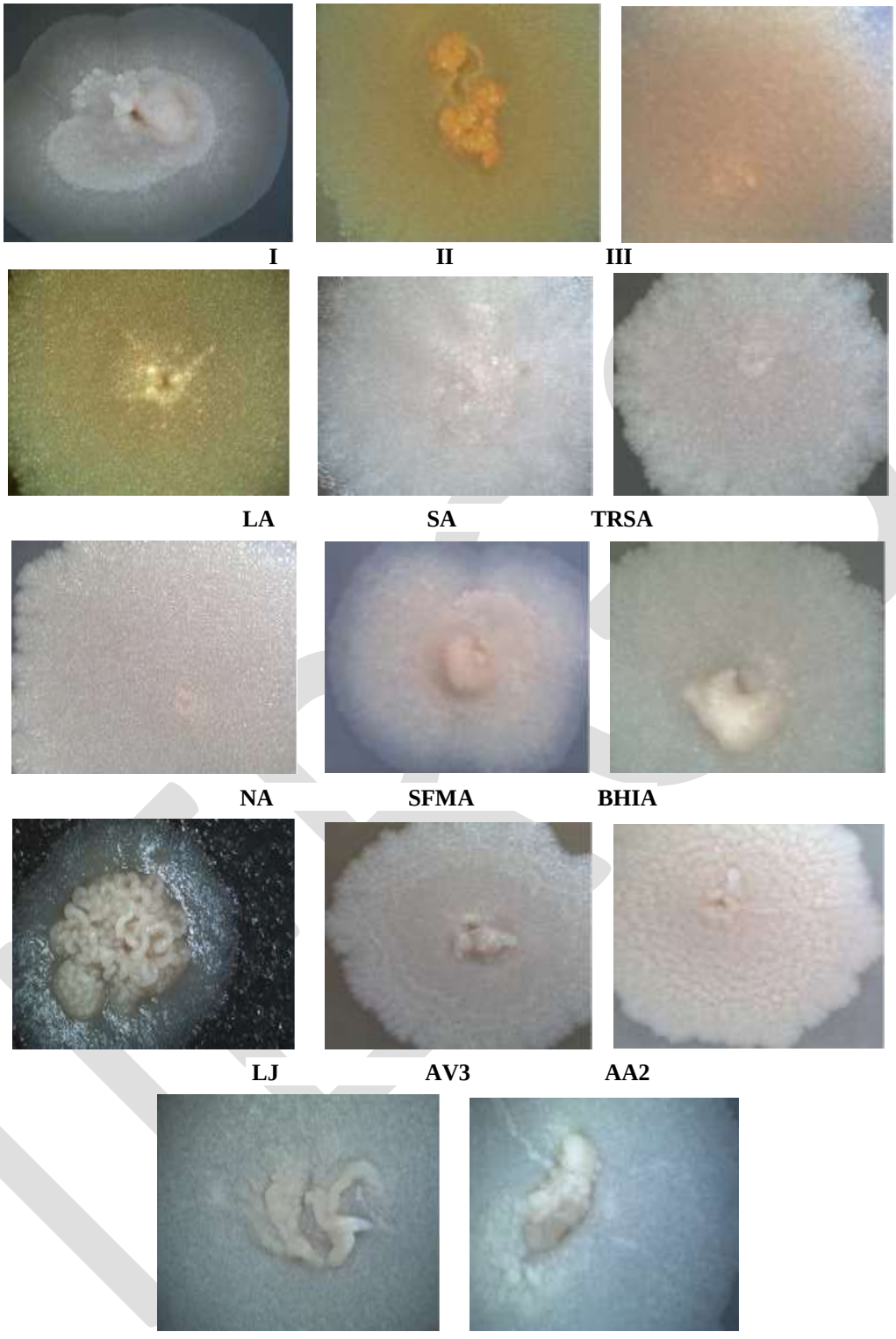

Figure 2: Stereomicroscopic images of colonies on different Agar media

Microscopic Observation During Bioconversion Process

Microscopic studies of culture during bioconversion process showed that *Mycobacterium spp.* is a Gram-positive bacilli (in Gram Staining). In live slide, it was observed that it is non motile rod (1-3 µm x 0.2-0.4 µm). Acid fast staining revealed that it is acid fast organism, sometimes long rods with occasional beaded or swollen cells having non-acid-fast ovoid bodies at one end (Figure 3). Scanning electron microscopy of *Mycobacterium spp.* revealed some of the ultrastructural morphologic details exhibited by a number of "rod-shaped", *Mycobacterium spp.* bacteria (Figure 4).Long filamentous structures are often observed, but spores and capsules are absent.

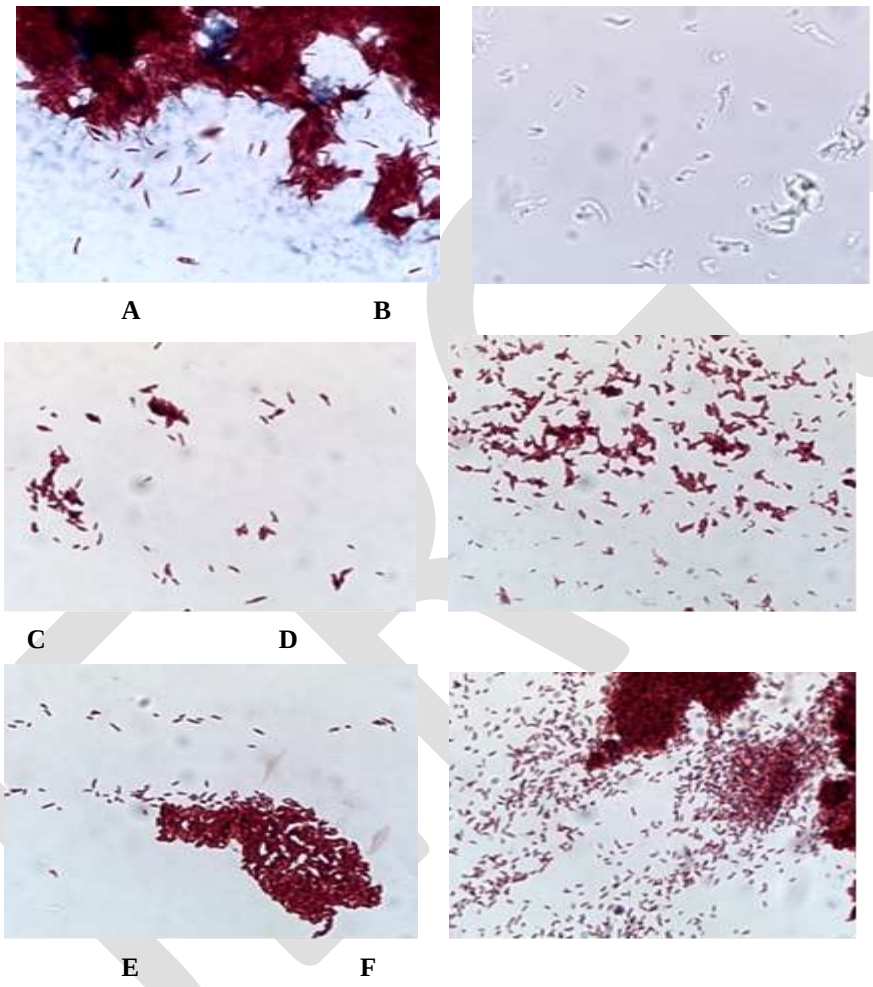

Figure 3: A-Stain image of seed culture, B-Live image of seed culture, C-F: Culture image in Production media at different Hrs:- C-24 Hrs, D-24 Hrs, E-24 Hrs and F-24 Hrs

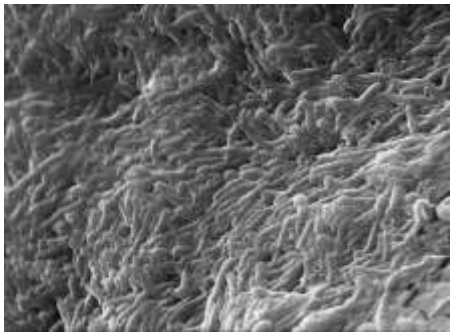

Figure 4: Scanning Electron Micrograph of *Mycobacterium spp.*

Bioconversion Process

It was established that *Mycobacterium spp.* degrades specifically only the side hydrocarbon chain in phytosterols molecules and uses it as a carbon source. In bioconversion medium inoculated with *Mycobacterium spp.* (Figure 5), phytosterol was transformed into product 9*α*-hydroxyandrost-4-ene-3,17- dione (9-OH-AD). The maximum yield of 9-OH-AD was obtained at 96-120 hrs.

A **B**

Figure 5: A- Grown Seed broth , B- Grown Production Broth

FFD analysis for process variables affecting 9-OH-AD yield and PS conversion

A screening design was performed to estimate the effects of six phytosterol. The design and results of FFD are indicated in Table 4. Effects of the six variables were analyzed by multiple regression analysis method and are illustrated in Table 5. The results indicated factor A and factor E are significant but comparatively (A)- Micronized Phytosterol (factor E) was most significant factor (P<0.0001). Thus, use of vendor: (A)- Micronized phytosterol would lead to the increase of 9-OH-AD yield (5.58 mg/g). The analysis of variance (ANOVA) of FFD was carried out and the results are shown in Table 5. P values for 9-OH-AD yield using vendor (A)- Micronized phytosterol was 0.0057. The higher values of determination coefficient (R2=0.7510 for 9-OH-AD yield) further confirmed the effectiveness of the models.

Std	Factor 1 A.A	Factor 2 B.B	Factor 3 C.C	Factor 4 D.D	Factor 5 E.E	Factor 6 F.F	Response 1 R1
1	-1	-1	-1	-1	-1	-1	2.962
2	1	-1	-1	-1	1	-1	5.581
3	-1	1	-1	-1	1	1	2.066
4	1	1	-1	-1	-1	1	3.211
5	-1	-1	1	-1	1	1	4.881
6	1	-1	1	-1	-1	1	2.596
7	-1	1	1	-1	-1	-1	2.348
8	1	1	1	-1	1	-1	5.561
9	-1	-1	-1	1	-1	1	2.248
10	1	-1	-1	1	1	1	5.121
11	-1	1	-1	1	1	-1	4.986
12	1	1	-1	1	-1	-1	5.248
13	-1	-1	1	1	1	-1	3.164
14	1	-1	1	1	-1	-1	5.516
15	-1	1	1	1	-1	1	2.005
16	1	1	1	1	1	1	5.216

Table 4: Design and results of FFD

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 4.83 implies the model is significant. There is only a 1.79% 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.

Source	Sum of Squares	df	Mean Square	F Value	P-value Prob>F	
Model	23.33	6	3.89	4.52	0.0218	Significant
A-A	11.21	1	11.21	13.04	0.0057	
B-B	0.13	1	0.13	0.15	0.7091	
C-C	1.156E-003	1	1.156E-003	1.345E-003	0.9715	
D-D	1.15	1	1.15	1.34	0.2763	
E-E	6.81	1	6.81	7.93	0.0202	
F-F	4.02	1	4.02	4.68	0.0588	
Residual	7.74	9	0.86			
Cor Total	31.06	15				

Table 5: ANOVA analysis for FFD model

Abbreviations: df: degree of freedom

R-squared 0.7510 Pred R-Squared 0.2129

Adeq Precision 5.936 Adj R-squared 0.5859

In this case A, E are significant model term. Values greater than 0.1000 indicate the model terms are not significant. Here model terms B, C and D are not significant. Adeq Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 5.936 indicates an adequate signal and thus model can be used to navigate the design space.

CONCLUSION

Present study reveals characterization of *Mycobacterium spp.* on different plate agar media and selection of phytosterol source by using fraction factorial design (FFD). This work has demonstrated the importance of statistical approach to maximize the bioconversion to 9-OH-AD from phytosterol by using *Mycobacterium spp*. FFD analysis showed that vendor: (A)- Micronized phytosterol is the most suitable source of phytosterol for 9-OH-AD production. Validation results by ANOVA indicates that vendor (A)- Micronized phytosterol is the most appropriate among all studied phytosterols. Data obtained in this study, overall depicts that statistical technique can be used to select the most suitable source for phytosterol.

REFERENCES:

[1] Szentirmai, A. (1990). Microbial Physiology of Sidechain Degradation of Sterols. Journal of Industrial Microbiology and Biotechnology, 6: 101-115.
[2] Fernandes, P., Cruz, A., Angelova, B., Pinheiro, H. M. And Cabral, J. M. S. (2003). Microbial conversion of steroid compounds: recent developments. Enzyme Microb. Technol., 32(6): 688-705.
[3] Malaviya, A. and Gomes, J. (2008). Androstenedione production by biotransformation of phytosterols. Biores. Technol., 99(15): 6725-6737.
[4] Fernandes, P., Cabral, J. M. S. (2007). Phytosterols: applications and recovery methods. Biores. Technol., 98(12): 2335-2350.
[5] Dutta PC, Appelqvist L. (1996). Saturated sterols (sta-nols) in unhydrogenated and hydrogenated edible vegetable oils and in cereal lipids. J Sci Food Ag-ric 71: 383-391.
[6] Tom, J.W. and Debenedetti, P.G. (1991). Particle formation with supercritical fluids, Journal of Aerosol Science, 22:555–584, .
[7] Khan, S., Misra, A. K., Tripathi, C. K. M., Misra, B. N. And Bihari, V. (2006). Response surface optimization of effective medium constituents for the production of alkaline protease from a newly isolated strain of Pseudomonas aeruginosa. Ind. J. Exp. Biol., 44: 151-156.
[8] Van der Geize, R.; Yam, K.; Heuse,r T.; Wilbrink, M.H.; Hara, H.; Anderton, M.C.; Sim, E.;Dijkhuizen, L.; Davies, J.E.; Mohn, W.W. & Eltis, L.D. (2007). A Gene Cluster Encoding Cholesterol Catabolism in a Soil Actinomycete Provides Insight into Mycobacterium tuberculosis Survival in Macrophages. Proceedings of the National Academy of Sciences of the United States of America, 104: 1947-1952.
[9] Da Costa Cruz, J. 1938. Mycobacterium fortuitum urn novo bacilo acido-resistente patogenico para o homen. Acta Med. (Rio de Janeiro) 1:298-301.
[10] Marina, A., Waldburger, C.D., and Hendrickson, W.A. (2005). Structure of the entire cytoplasmic portion of a sensor histidine-kinase protein. EMBO J 24, 4247-4259.
[11] Berd, D. (1973). Laboratory identification of clinically important aerobic actinomycetes. Appl Microbiol 25, 665–681.
[12] Werner, G.H. (1951) Electron-microscopic studies of the cellular morphology of tubercle bacilli. Bibl. Tuberc. 5, 53–90.
[13] Chen, Q.H., He, G.Q., Mokhtar, A.M.A., (2002). Optimization of medium composition for the production of elastase by Bacillus sp. EL31410 with response surface methodology. Enzyme Microb. Technol., 30(5):667-672.
[14] Chen, Q.H., Fu, M.L., Liu, J., Zhang, H.F., He, G.Q., Ruan, H., (2008). Optimization of ultrasonic-assisted extraction (UAE) of betulin from white irch bark using response surface methodology. Ultrason. Sonochem., 16(5):599-604.