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## TWEEN 80 ALTERS THE PRODUCTION RATIO OF PHARMACEUTICALLY IMPORTANT STEROID INTERMEDIATES, 4-AD AND ADD DURING BIOTRANSFORMATION OF SOY STEROL BY *MYCOBACTERIUM SP. NRRL B-3805*

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**ABSTRACT:** 4-androstene-3, 17-dione (4-AD) and androsta-1,4-diene-3,17-dione (ADD) are pharmaceutically important steroid intermediates produced by steroid biotransformation. Non-pathogenic mutants of saprophytic *Mycobacterium* have been used for production of 4-AD and ADD. Being an intracellular process, the substrate entry is the rate limiting step for 4-AD and ADD production. This biotransformation is also limited by hydrophobic nature of sterol substrates. Uniform dispersion of sterol substrates in aqueous biotransformation medium is desired for efficient 4-AD and ADD production. Tween 80, a non ionic detergent is a promising agent to improve dispersion of hydrophobic substrates. In this study, besides the biotransformation, effects of tween 80 on growth, aggregation, cell wall fluidity and mycolic acid contents of *Mycobacterium sp. NRRL B-3805* were estimated. Presence of 20g/L tween 80 led to 380 % increase in biomass. No toxic effect on biomass accumulation was evident at any of the tested concentrations (0.31 to 20g/l). In presence of 1.24 g/L tween 80, biotransformation was shifted towards ADD, resulting in 121 % more ADD production. The ratio of 4 - AD and ADD was changed from 12:1 (control) to 5:1. In presence of 5 g/L tween 80, ~50% reduction in mycolic acid was recorded. Presence of tween 80, significantly enhanced the biomass accumulation and altered the 4 - AD and ADD accumulation ratio by *Mycobacterium sp. NRRL B-3805*. Decrease in cell wall mycolic acid content could be correlated with increased ADD production.

**INTRODUCTION:** Steroid biotransformation is an important microbial process for production of pharmaceutically important intermediates<sup>1-3</sup>. Steroid biotransformation is placed amongst the important and highly valuable fermentations along with antibiotics and Vitamins. Production of 4-androstene-3, 17-dione (4-AD) and androsta-1,4-diene-3,17-dione (ADD) is dependent on biotransformation of easily available steroid precursors, mainly plant sterols<sup>4-7</sup>.

4-AD and ADD can be converted to almost all types of known steroid molecules of medical importance, including but not limited to anti-inflammatory, anti-allergic, cardio-tonic, geriatric, progestational, anabolic, immunosuppressive and contraceptive agents<sup>1, 2, 7-10</sup>.

Production of 4-AD and ADD is catalyzed by fast growing mutant strains of *Mycobacterium*<sup>11</sup>. Microbial sterol catabolism is a multistep degradation pathway aided by several intracellular enzymes<sup>12</sup>. Due to involvement of several cytochrome dependent reactions, cascade of intracellular enzymes and energy pool, the steroid biotransformation needs viable cells of *Mycobacterium* in order to produce 4-AD and ADD. Steroid biotransformation faces two major challenges.

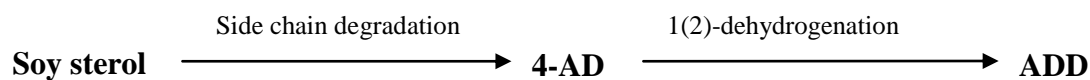
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Mycobacterium cell wall is a complex meshwork of peptidoglycan and arabinogalactan on which huge molecules of mycolic acids are attached. This structural complexity and hydrophobicity is thought to be a great barrier for entry of substrates inside the *Mycobacterium* cell. Moreover, the hydrophobic nature of sterols opposes their mixing in aqueous biotransformation medium and induces aggregate formation. In order to break the sterol aggregates and to improve substrate availability, detergents have been studied by several workers<sup>5, 13-16</sup>. Detergents are the molecules which improve the water interaction of hydrophobic substances. Detergents were theorized as part of sterol uptake system, named as flexible multicomponent mesophase<sup>15</sup>. Low toxicity of tween 80 for *Mycobacterium* can be expected due to its ability to degrade tween<sup>17</sup>.

Detergents from triton series (Triton X-100 and Triton X-114) and Tween 80 had been used to prepare  $\beta$ -sitosterol stock for biotransformation by *Mycobacterium* sp. NRRL B-3805<sup>18</sup>.

Detergents, including tween 80, have been used to prepare microemulsion of sterols, which were found suitable for production of 4-AD and ADD from  $\beta$ -sitosterol<sup>13, 19, 20</sup>. Bacteriostatic and inhibitory effect of Tween 80 (0.05 to 0.5 g/l) on growth of *Mycobacterium avium*, *Mycobacterium intracellulare* complex has been reported<sup>21, 22</sup>.

In this study *Mycobacterium* sp. NRRL B-3805 was used as model organism. This mutant strain is considered as major producer of 4-AD from natural sterol, while ADD is produced in minor quantities by 1(2)-dehydrogenation of 4-AD (**Fig.1**).



**FIG. 1: SOY STEROL DEGRADATION PATHWAY OF *MYCOBACTERIUM* SP. NRRL B-3805**

This study is aimed to assessing effects of tween 80 on steroid biotransformation and effects on physiology of *Mycobacterium* sp. NRRL B-3805, when added prior to substrate addition.

## MATERIALS AND METHODS:

**Chemicals and Culture Media:** Nutrient broth medium (purchased from HiMedia Laboratories Pvt. Ltd. India) was used as growth and bio-transformation medium. Tween 80 was purchased from Merck, India Pvt. Ltd. TLC plates (Merck, Silica gel 60 F<sub>254</sub>) were purchased from Merck, Germany. Molybdophosphoric acid for development of mycolic acid TLC plates was purchased from HiMedia Laboratories Pvt. Ltd. India. Soy sterol (95%) was obtained from Xi'an Healthful Biotechnology co. Ltd., China.

**Microbial Strain:** *Mycobacterium* sp. NRRL B-3805 was procured from Northern Regional Research Laboratory, Illinois (USA). Nutrient agar slants were used for cultivation and maintenance of the strain. After 5 days incubation (32 °C) the well grown slants were preserved at 4 °C in a refrigerator.

**Effect on Growth and Aggregation:** To study the effect of tween 80 on biomass accumulation of

*Mycobacterium* sp. NRRL B-3805, nutrient broth medium was supplemented with different concentrations of tween 80. The final volume of the medium was kept to 45 ml in 250 ml capacity Erlenmeyer flask. After sterilization, the flasks were inoculated by 10 % (5 ml) overnight grown cells of *Mycobacterium* sp. NRRL B-3805 in medium of same composition. Inoculated flasks were incubated at 32 ± 1 °C in gyratory shaker at 200 rpm.

The growth was estimated by measuring the optical density at 600 nm (OD<sub>600</sub>) at 1:9 dilutions in phosphate buffer saline (pH 7.2). To determine dry cell weight the whole medium was subjected to centrifugation (4000 rpm for 10 minutes at 25 °C), supernatant was discarded and cell pellets were washed twice with phosphate buffer saline. The cell pellet was then dried in a lyophilizer and weighed using a precision balance.

Determination of biomass in presence of soy sterol (during bio-transformation), needed an additional step to remove residual sterol. To remove sterol, cell pellet was washed twice with 10 ml, methanol-chloroform (1:1, v/v) mixture, followed by centrifugation. The cell pellet was then completely dehydrated by lyophilisation and weighed.

**Steroid Biotransformation:** To study effect of tween 80 on AD and ADD production by *Mycobacterium* sp. NRRL B - 3805, the growing cells were first exposed to different concentrations of tween 80 supplemented in nutrient broth medium for 8 hours in a gyratory shaker (200 rpm) at 32 °C. 0.1 gram sterilized soy sterol was then added aseptically in the form of micro suspension in distilled water. The soy sterol micro suspension was prepared by milling the sterol in water overnight in a conical flask using glass beads (300 rpm at room temperature).

For estimation of biotransformation products, 1 ml aliquot was taken from the flask and extracted by 500 µL chloroform twice. After a brief centrifugation, the chloroform layer was collected in a fresh microcentrifuge tube. The pooled chloroform extracts were allowed to evaporate at room temperature. After evaporating the chloroform completely, the extract was re-dissolved into 250 µL chloroform and used for quantitative TLC analysis<sup>23</sup>. Bioconversion per unit biomass was determined by dividing the quantity (mg) of bioconversion product (4-AD or ADD) by biomass (g) accumulated during biotransformation.

Biotransformation per gram biomass =

$$\frac{\text{Bioconversion product (mg)}}{\text{Biomass (g)}}$$

**Estimation of Cell Wall Mycolic Acids:** Mycolic acids were estimated in the form of methyl esters<sup>24</sup> using a slightly modified method. Briefly, 100 mg lyophilized cells were digested by 5 ml esterification reagent (methanol: sulphuric acid; 4:1, v/v) in Teflon capped glass tubes. The samples were refluxed for 8 hours at 85°C in a water bath. After cooling to room temperature, the samples were extracted three times by petroleum ether. The extracts were evaporated in nitrogen stream at 40°C. For TLC, the evaporated samples were reconstituted by 200 µl petroleum ether. 20 µl of sample was spotted on TLC plate.

The plates were developed in hexane-ethyl acetate solvent system (94:6 v/v) and sprayed with 10% molybdo-phosphoric acid (w/v in ethanol), followed by heating for 10 minutes at 180°C. The

relative quantification of spots was done by image densitometric analysis. Briefly; TLC plates were scanned on Samsung flatbed scanner at 400 dpi. Image analysis was carried out using Image Quant TL software (GE Life Sciences, USA).

**Estimation of Cell Wall Fluidity:** Cell wall fluidity of *Mycobacterium* sp. NRRL B-3805 cells was estimated by measuring thermal transition temperature by Differential Scanning Calorimeter (DSC) (Mettler Toledo STAR<sup>o</sup> System). Previously described method<sup>25</sup> was adopted for DSC measurements. Lyophilized, whole cells were subjected to -50 to 140 °C melt/upward scan and the heat flux was recorded. All the experiments were carried out in triplicates. Data represented in graphs as average of three independent experiments with ± SD. Student's t-test was applied to test significance of the results. \* denotes significance of data at 95 % confidence level (p<0.05).

## RESULTS AND DISCUSSION:

**Growth and Aggregation of *Mycobacterium* sp. NRRL B-3805 in Presence of Tween 80:** All the tested concentrations of tween 80 led to improved biomass accumulation by *Mycobacterium* sp. NRRL B-3805 (**Fig. 2**). Maximum ~447 % enhancement in biomass accumulation was recorded at 20 g/L tween 80. 28 %, 54 %, 85 %, 142 %, 225 % and 380 % enhancement in biomass accumulation was recorded at 0.31, 0.63, 1.25, 2.5, 5, and 10 g/l tween 80.

Enhancement in biomass accumulation was significantly correlated with tween 80 dosage (r = 0.94). No inhibitory effect was found up to 20 g/L (maximum tested concentration). Hydrophobic nature of the *Mycobacterium* cell envelope induces clumping, which is a frequent phenomenon in the cultures of *Mycobacterium*.

The OD<sub>600</sub>: biomass ratio represents the aggregation of cells. Up to 5g/L tween 80, the ratio of dry cell weight: OD<sub>600</sub> remained more or less constant, indicating no significant effect on clumping. With further increase in the tween 80 concentration (10 and 20 g/L), ratio of cell weight: OD<sub>600</sub> increased, indicating greater cell clumping (**Table 1**). Increase in cell density could be the possible reason of increase in clumping of cells at higher concentrations of tween 80.

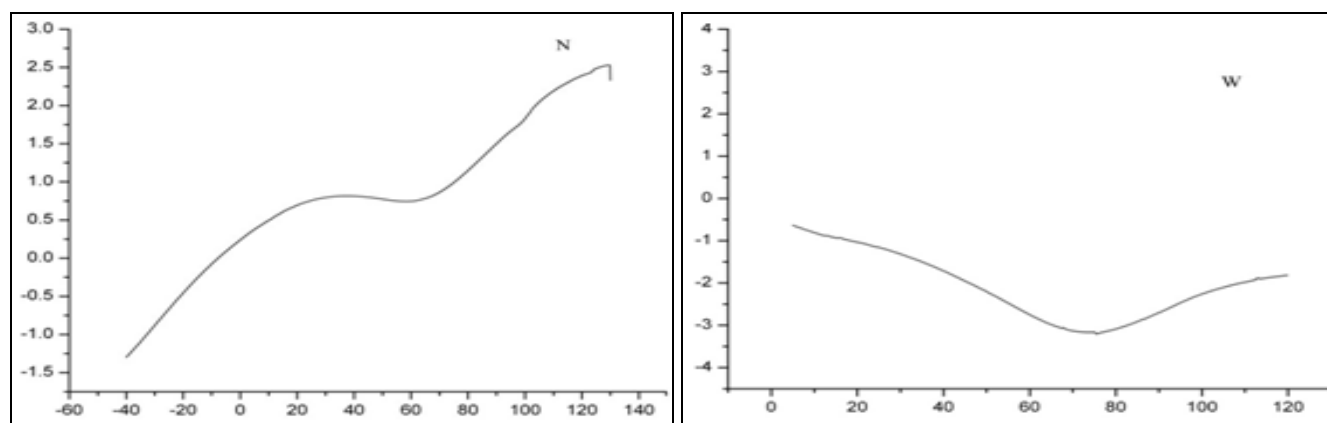
**TABLE 1: AGGREGATION OF MYCOBACTERIUM SP. NRRL B-3805 CELLS IN PRESENCE OF TWEEN 80**

Tween 80 (g/L)	0	0.31	0.62	1.25	2.5	5.0	10.0	20.0
Ratio of Dry cell weight/OD <sub>600</sub>	0.033	0.033	0.035	0.036	0.035	0.035	0.043	0.048

Enhancement in growth of *Mycobacterium* in presence of tween 80 can possibly be attributed to enhanced nutrient uptake due to removal of outer layer and reduced cellular aggregation. It has been reported that brief exposure (1 - 4 hours) of tween 80 leads to removal of outer layer and prevents cell clumping of *Mycobacterium*<sup>26</sup>. However, it can be assumed that high cell density could nullify the anti clumping effect of tween 80. Other mechanisms like, improved oxygen transfer and utilization of oleic acid generated by tween 80 break down has been reported for *Mycobacterium smegmatis*<sup>27</sup>.

**Effect of Tween 80 on Cell Wall Fluidity and Mycolic Acid Content of *Mycobacterium* sp. NRRL B-3805:** Cells grown at 0.62 g/L tween 80 were used for determination of thermal transition temperature and mycolic acid content, as at this

concentration the biomass was increased by two fold, in comparison with control. Thermal transition temperature was measured by DSC which determines the heat flux at various applied temperatures. In this study, presence of tween 80 in growth medium, led to increase in thermal transition temperature by ~ 15 °C. The thermal transition temperature of control and tween 80 grown cells was 59.46 °C and 74.16 °C respectively. Thermal transition temperature is a measure of the change in fluidity of the cell wall of *Mycobacterium* (25), greater the thermal transition temperature less is the fluidity of the cell wall. The increase in thermal transition temperature along with alteration in thermal transition curve indicates reduction in cell wall fluidity (Fig. 3).



**FIG. 3: THERMAL TRANSITION TEMPERATURE OF CONTROL (N) AND TWEEN 80 (W) TREATED CELLS. THE INCREASE IN THERMAL TRANSITION TEMPERATURE INDICATES REDUCTION IN FLUIDITY OF TWEEN 80 GROWN CELLS**

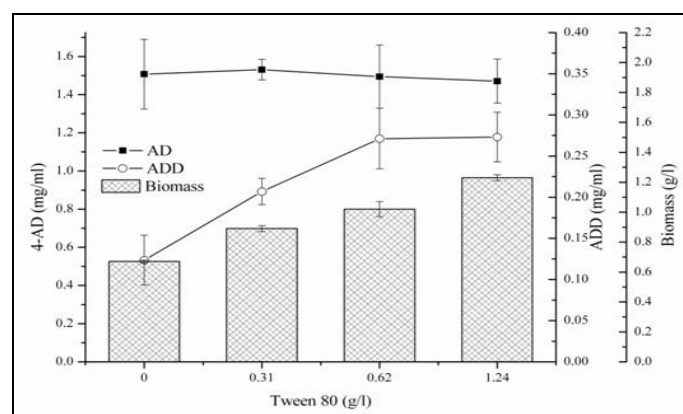
The mycolic acids from *Mycobacterium* cell wall are divided into three major classes;  $\alpha$ , methoxy and keto-mycolates. Relative content of these mycolic acids was determined in *Mycobacterium* sp. NRRL B-3805, grown with or without tween 80. Relative concentrations of all the three mycolates were found to be lesser in tween 80 grown cells. Approximately 38.5, 67 and 87 % decrease in  $\alpha$ -mycolic, methoxy-mycolic and keto-mycolic acids, respectively. The reduction in methoxy and keto-mycolic acids was significantly higher compared with reduction in  $\alpha$ -mycolic acids. The proportional ratio of mycolates in the cell wall of *Mycobacterium* is ~70% for  $\alpha$ -mycolic acid

while 15 % each for methoxy and keto-mycolates<sup>28</sup>. Considering this ratio, the overall depletion in mycolic acid content was estimated to be reduced by ~50 %. The reduction in hydrophobic components of cell wall (outer layer and mycolic acids) can be responsible of efficient nutrient uptake as reflected by enhanced growth.

The reduction in mycolic acid content explains the increase in thermal transition temperature as well as the broad peak obtained with tween 80 cells. The possible exposure of lipoarabinomannan and peptidoglycan layer due to loss of mycolic acids can be attributed to the enhanced thermal transition

temperature. Cell components other than mycolic acids are required to maintain the structure of the cell, thus are less fluid.

**Bioconversion of Soy Sterol to 4-AD and ADD by *Mycobacterium* sp. NRRL B-3805 in Presence of Tween 80:** Fig. 4 shows the 4-AD, ADD and biomass accumulation during bioconversion at different concentrations of tween 80.



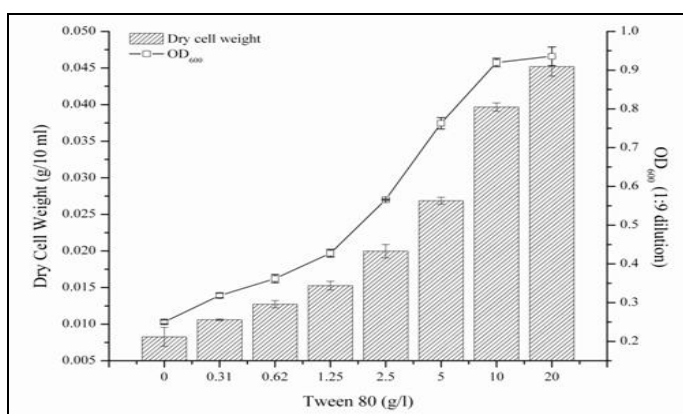
**FIG. 4: BIOCONVERSION OF SOYA STEROL TO 4-AD AND ADD BY *MYCOBACTERIUM* SP. NRRL B-3805 IN PRESENCE OF TWEEN 80, BRAS REPRESENT THE BIOMASS ACCUMULATION DURING BIO-TRANSFORMATION**

These results indicate that C-1(2)-dehydrogenation of 4-AD leading to ADD production was favoured by presence of tween 80 in incubation medium. There was a positive correlation between tween 80 and biomass during soy sterol bioconversion ( $r = 0.98$ ). 32 %, 53 % and 83 % increase in dry cell weight was recorded at 0.31, 0.62 and 1.24 g/L tween 80 respectively. Presence of sterol not affected the extent of increase in biomass accumulation. Analysis of production of steroid intermediates (mg) per unit biomass (g) with increase in concentration of tween 80 concentration revealed constant decrease in efficiency towards 4-AD production. Production of 4-AD per unit biomass reduced by 23 %, 35 % and 47 % at 0.31, 0.62 and 1.24 g/l tween 80 respectively. Per gram productivity of biomass for ADD accumulation increased in presence of Tween 80 **Table 2** with highest specific production recorded at 0.62 g/L of tween 80.

The important outcome of this study was the increase in ADD production, with increasing concentration of tween 80. The loss of mycolic acid and consequent enhanced permeability of the cell can be attributed to increase in ADD accumulation.

Despite considerable enhancement in biomass, 4-AD accumulation was not significantly altered.

However the ADD production increased significantly with increasing concentration of tween 80. An increase of 67 %, 120 % and 121 % in ADD production was recorded at 0.31, 0.62 and 1.24 g/L tween 80 respectively.



**FIG. 2: EFFECT OF TWEEN 80 ON BIOMASS ACCUMULATION AND OD<sub>600</sub> OF *MYCOBACTERIUM* SP. NRRL B-3805**

The improved removal of ADD from the *Mycobacterium* cell reduced the cytotoxic effects of ADD.

**TABLE 2: 4-AD AND ADD ACCUMULATION PER UNIT BIOMASS (MG/G) OF *MYCOBACTERIUM* SP. NRRL B-3805 IN PRESENCE OF TWEEN 80**

Tween 80 (g/L)	4-AD	ADD
0	2245.28	184.02
0.31	1722.19	232.69
0.62	1459.24	264.89
1.24	1197.87	222.29

The evidence from mycolic acid analysis and DSC data support this observation. Increased cell wall permeability, led to enhanced out flux of ADD turning more 4-AD into ADD. It was reported that ADD has toxic effects on respiration of *Mycobacterium* due to inhibition of cytochrome <sup>2, 29</sup>, which results in suppression of sterol transformation and reduced cellular growth. In present study, ADD accumulation was enhanced with increasing concentration of Tween 80. The growth (biomass accumulation) was not suppressed even at presence of 121% more ADD, as compared with control. Despite high ADD concentration the biomass was 83% high as compared to control.

Ratio of 4-AD and ADD had been reported to be 10:1 when *Mycobacterium* sp. MB 3683 is used for biotransformation<sup>30</sup>. In present study, the conversion ratio in control was ~12:1, which corresponds with the previous report. The 4-AD:ADD ratio was altered in presence of Tween 80 and declined with increase in Tween 80 concentration **Table 3**.

**TABLE 3: 4-AD AND ADD RATIO AT DIFFERENT CONCENTRATION OF TWEEN 80**

Tween 80 (g/L)	4-AD: ADD ratio	Total ketosteroid (4-AD+ADD)
0	12.20:1	1.63
0.31	07.40:1	1.74
0.62	05.51:1	1.77
1.24	05.39:1	1.76

However, the total production of 17-ketosteroid (4-AD +ADD) was increased marginally (6-8 % increase). The increase in ADD accumulation was 67 %, 120 % and 121 % at 0.31, 0.62 and 1.24 g/L tween 80 respectively.

**CONSLUSION:** Present study concludes that presence of tween 80 in growth and bio-transformation medium increases biomass of *Mycobacterium* sp. NRRL B-3805. The increase in biomass cannot be translated to higher 4-AD and total ketosteroid production, while ADD production enhanced significantly. The increase in biomass and ADD production can be related to reduction in mycolic acid content. Decrease in mycolic acid content led to enhanced permeability for ADD and nutrients. Enhanced out-trafficking of ADD, shifted the bioconversion flux. However reduction in cell wall mycolate content didn't enhanced entry of sterols.

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**CONFLICT OF INTEREST:** No conflict of interests to disclose.

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