ANDROSTENEDIONE PRODUCTION BY BIOTRANSFORMATION OF PHYTOSTEROL

by

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Dedicated to My Parents

CERTIFICATE

This is to certify that the thesis entitled "**Androstenedione Production by Biotransformation of Phytosterol**" being submitted by **Mr. Alok Kumar Malaviya** to the Indian Institute of Technology, Delhi, for the award of degree of "Doctor of Philosophy", is a record of the bonafide research carried out by him, which has been prepared under my supervision and guidance in conformity with the rules and regulations of Indian Institute of Technology, Delhi. The results contained in it have not been submitted in part or full to any other university or institute for award of any degree / diploma.

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"So many of our dreams seem impossible, then improbable, then inevitable",
Quotes the famous former film star, Christopher Reeve, bedridden with paralysis, giving hope and a new dimension to people in distress to look up to fulfill their dreams despite the many roadblocks in front of them. When one pursues the dreams relentlessly, the dreams do become inevitable and accessible. I have also taken inspiration from these brave words and feel one can achieve anything if one pursues it with diligence and perseverance and finally the day has came when I am going to submit my doctoral thesis.

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Alok Kumar Malaviya

Abstract

This thesis embodies the work carried out using free *Mycobacterium* sp. cells as whole cell biocatalysts for androstenedione (AD) production from β-sitosterol by selective side chain cleavage. While studying sitosterol to AD biotransformation in aqueous medium, different strategies were tried to improve the production of AD. In the first step, a mutant strain of *Mycobacterium* sp DSMZ 2966 was developed. A two stage strain improvement program was applied for this purpose. Acclimatization to stage wise increasing concentration of sitosterol was applied and a strain (AS-08) capable of growing on 20 gl^{-1} sitosterol was obtained. A total of 2.2 fold increase in AD accumulation ability was exhibited by AS-08. AS-08 was further subjected to chemical mutagenesis resulting in isolation of an improved strain (PL-17). This strain was resistant to 1 gl^{-1} AD and exhibited a 3.3 fold increase in AD accumulation capacity of the parent *Mycobacterium* sp. While studying the effect of operational parameters, maximum AD formation was observed at 28°C, 300 rpm and at 7 pH in aqueous medium. Addition of cyclodextrin (CD) in the fermentation medium at substrate to CD ratio of 1:3 resulted in 2.20 fold increase in AD formation.

Mycobacterial cell wall is rigid and offers a high resistance to the transport of sitosterol into the cytosol. Therefore, the effect of cell wall permeabilizing antibiotics, such as ethambutol, penicillin, polymixin and bacitracin, on biotransformation of sitosterol to androstenedione by modification of cell wall permeability was examined. Drug sensitivity assay results established that bacitracin increased the permeability of the cell wall to hydrophobic compounds. Growth inhibitory study of bacitracin and rifamycin individually as well as in combination showed that these two antibiotics act

synergistically to reduce cell growth. A comparison of transmission electron micrograph results of the bacitracin treated cells with untreated cell, revealed deformities caused in the cell wall structure by bacitracin treatment. These deformities increased the cell wall permeability and transport of sitosterol inside the cell, and thus enhanced androstenedione (AD) production. A maximum of 1.37, 1.44, 1.65 and 1.76 gram AD per gram dry cell weight of mycobacterial cells was produced in the presence of ethambutol, penicillin, polymixin and bacitracin respectively. Below the minimum inhibitory concentration, bacitracin can be used as potent enhancer of permeability of hydrophobic substances across the mycobacterial cell wall.

Surfactants are known to improve the solubility, mobility and availability of the insoluble hydrophobic substrates to the biocatalysts. Therefore, effect of eight different surfactants (ionic and non ionic) on biotransformation of β-sitosterol to androstenedione by *Mycobacterium* sp. was also investigated as a strategy to improve the product formation in aqueous medium. A maximum of 1.9 fold increase in product concentration was achieved by using DMSO and an increase of 1.6 fold by using Triton X-114. Effect of these two surfactants on mycobacterial cell growth was studied. Inoculum was prepared in nutrient broth and was transferred to the medium containing 10 gl^{-1} DMSO and the medium containing 10 gl^{-1} Triton X114 as well as to the medium without any surfactant. DMSO was found to be inhibitory for cell growth when compared with control. After 72 hours of cell growth, DMSO led to 21.2±3.6 % decrease in dry cell weight of mycobacterial cells in comparison to the control culture. The cell growth was comparatively unaffected in presence of 1% Triton X-114. Sitosterol biotransformation

study in optimized conditions exhibited a maximum of 1.09 mM AD production in aqueous medium.

With the aim to further improve the sitosterol to AD biotransformation process, the focus was shifted towards designing a biotransformation system with improved substrate solubility. For this purpose, the application of microemulsion (ME) medium was investigated. Since microemulsions are dispersed micro-heterogeneous systems composed of water, oil and surfactant and are also isotropic, optically clear and thermodynamically stable, microemulsion medium possess extremely large interfacial are for biotransformation. Although, ME has been employed for chemical and biochemical reactions, there are no reports on its application for microbial transformation of sitosterol to AD. During the course of present work, a systematic study on preparation of microemulsions containing nutrient broth and PEG 200 (1:1) as aqueous phase, 40 g/l sitosterol dissolved in chloroform as organic phase, Triton X114 and Tween 80 (1:1) as surfactant phase, was investigated. The phase behavior of this system was studied for 10 different ratios(w/w), 10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 4:6, 3:7, 2:8, 1:9 and 0:10 of the organic phase and surfactant at 30°C. A pseudoternary phase diagram was constructed to demarcate the region giving stable microemulsions. Seventeen different compositions were found to give stable MEs. Finally, it was found that microemulsions can effectively be applied as a reservoir of solubilized hydrophobic substrates. The maximum solubility of sitosterol in microemulsion medium was observed to be 8 gl^{-1}, three orders of magnitude higher than the reported sitosterol solubility of 2 – 4 mgl^{-1} in aqueous medium. During sitosterol biotransformation studies, ME medium was found to exhibit better sitosterol biotransformation results as compared to the aqueous system. Highest

percent molar conversion (88.5%) of solubilised sitosterol was observed in ME medium containing 1: 9: 90 ratio of oil phase, surfactant phase and aqueous phase. However, a maximum concentration of AD (1.6 mM) was produced in ME medium containing 6:14:80 ratio of oil phase, surfactant phase and aqueous phase. Operational parameters were also optimized and highest sitosterol biotransformation was obtained at 250 rpm and at 32°C. Addition of external electron acceptor (menadione) and maintenance of dissolved oxygen concentration at the level of 30% of maximum saturation resulted in enhanced biotransformation of sitosterol to AD. Finally, a maximum of 2.6 mM AD could be produced in ME medium containing 4:16:80 ratio of oil phase, surfactant phase and aqueous phase. Hence, ME based medium offers a better alternative for carrying improved biotransformation of hydrophobic compounds.

Parallel to these studies, on screening and isolation of organisms capable of sitosterol to AD biotransformation was also carried out. This involved screening and isolation of microorganisms from soil samples using the conventional enrichment technique as well as by using a reverse genetics based approach. Random screening and isolation resulted in isolation of a novel bacterial strain, *Stenotrophomonas maltophilia*. Using reverse-genetics approach, *Aspergillus oryzae* was identified as the organism capable of sitosterol to AD biotransformation. Novelty lies in the methodology used for screening and isolation of microorganisms with requisite functional attributes. To the best of our knowledge, both the isolates - *Stenotrophomonas maltophilia* and *Aspergillus oryzae* are also novel in terms of reported sitosterol to AD biotransformation capability of these two organisms.

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