Chapter 7

Microbial Biotransformation for the Production of Steroid Medicament

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Abstract

Androstenedione (AD) is a steroid intermediate valuable for the production of steroid medicaments. Microbial biotransformation of phytosterol to produce AD is a well-researched area. However, low substrate solubility of phytosterol in aqueous media and nucleus degradation of AD to androstadienedione (ADD) or 9-hydroxy-AD are the major obstacles for AD production leading to detailed research for optimization of biotransformation process. In this review, microbial transformation of AD with respect to the existing methods of chemical or biochemical synthesis of AD are extensively discussed. This review examines the microbial biotransformation process and limitations for enhanced AD production. Factors affecting the effective biotransformation process to obtain AD are discussed and limitations are highlighted. The main content of this review focuses on the recent and futuristic biotechnological advances and strategies in techniques to enhance AD bioprocess.

Keywords: androstenedione, microbial biotransformation, phytosterol, steroids, nucleus degradation

1. Introduction

Steroids are terpenoid lipids of specific structure that contain the nucleus of four cycloalkane rings. Androstenedione (AD) is a natural steroid which belongs to the 17-ketosteroid family. It is produced by the adrenal cortex and gonads. In the body, cholesterol leads to the formation of steroidal hormones. AD is a compound specifically used as a precursor for the majority of pharmaceutically active steroids such as testosterone, estradiol, ethinylestradiol, testolactone,

progesterone, cortisone, cortisol, prednisone and prednisolone [1]. The steroid pharmaceuticals are of great importance for their role in the management of human fertility, osteoporosis, menopause and blood pressure regulation [2, 3]. Commercially, steroid production represents one of the largest sectors of medical products manufactured by the pharmaceutical industry [4]. Production of AD and androstadienedione (ADD) exceeds 1000 tons per year in the world. Therefore, production of AD on a large scale with cost effective process becomes an area of demanding research in biotechnology. Apart from the broadly used natural compounds, phytosterols gained an increasing importance as raw materials for the synthesis of steroidal drugs such as pregnenolone, boldenone, androstenedione and androstadienedione. The utilization of cholesterol and phytosterol as the sole carbon source by *Mycobacterium* sp. for growth and proliferation led to a serious development in microbial biotransformation processes for production of large number of steroidal compounds [1].

Phytosterols are thoroughly widespread in plants and are similar to cholesterol in terms of physiological functions and structure. Phytosterols differ from cholesterol by having a methyl or ethyl group at C-24 (**Figure 1**). Phytosterols participate in essential cellular processes since they modulate permeability and fluidity of membranes. In addition, they are precursors for the synthesis of steroid hormones and are involved in plant defense mechanisms. β-Sitosterol, campesterol, and stigmasterol are the main phytosterols found in plants (**Figure 1**). The most important phytosterols, β-sitosterol (C-29 carbon skeleton), campesterol (C-28), and stigmasterol (C-29), contribute up to 98% of all the phytosterols found in plants [5]. As cholesterol acts as a starting material for steroid production by adrenal cortex and gonads, phytosterol can act as a starting material for AD production by many microorganisms which are able to utilize phytosterols as carbon and energy source.

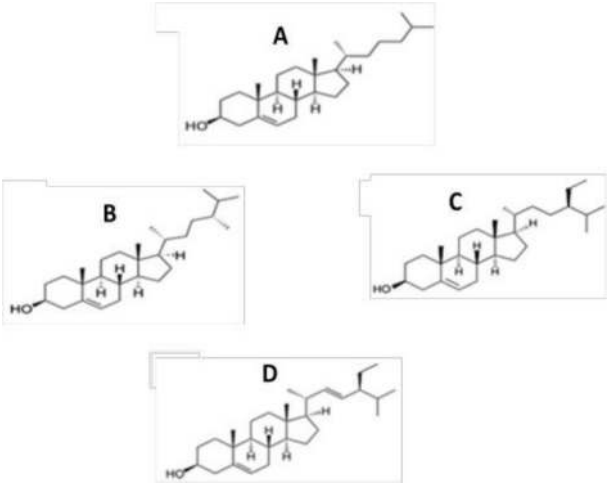

Figure 1. (A) Structure of cholesterol, (B) structure of campesterol, (C) structure of β-sitosterol, (D) structure of stigmasterol.

This property is a key element in the steroid precursor production and, therefore can be exploited to make AD production a commercially viable process.

Further, phytosterols being a polar substance, has very low solubility in aqueous media. Because of low substrate solubility of phytosterols, the process of production of AD gets hampered. Current review focuses on the mechanisms used for enhanced AD production, and includes current trends & future perspectives.

2. Synthesis of androstenedione from phytosterols

AD is synthesized in the body by adrenal cortex and gonads stimulating formation of testosterone or estradiol. Adrenocorticotropic hormone (ACTH) stimulates the steroid synthesis pathway from cholesterol leading to formation of AD. Phytosterol being similar to cholesterol is able to stimulate the formation of AD by its side-chain cleavage. (Elaboration of the pathway in **Figure 2**).

As shown in the **Figure 2**, CYP11A1 is an enzyme that is responsible for side chain cleavage of the sterol resulting in AD formation.

2.1. Chemical synthesis

Marsh demonstrated a chemical synthesis of AD (aromatase inhibitors) which involves multiple steps [6]. AD can be produced chemically by addition of thiol reagents to appropriate dienone intermediates. C19 steroids should retain the steroidal nucleus for their effective functions. However, the steroidal nucleus is sensitive to a temperature leading to its degradation [7]. Steroidogenic enzymes were also sensitive to endocrine-disrupting chemicals (EDCs) [8]. Apart from the above mentioned limitations, chemical synthesis leads to added costs and lower yield of AD with lengthy and sensitive procedures. Additionally, chemical synthesis of AD also requires use of toxic/harmful chemicals which affects the environment. Hence, there is a need to develop a process which is environment friendly and cost effective. Microbial biotransformation of phytosterol fulfills the need.

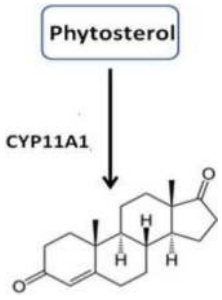

Figure 2. Side chain cleavage of sterol leading to AD formation.

2.2. Biotransformation and substrates used

Sterol rich substrates are used to obtain steroidal intermediates by biotransformation. There are a number of organisms known to transform phytosterols to AD such as *Aspergillus, Arthrobacter, Bacillus, Brevibacterium, Chryseobacterium, Fusarium, Gordonia, Nocardia, Pseudomonas, Rhodococcus, Streptomyces* and *Mycobacterium* sp. [2, 3].

3. Factors affecting androstenedione production

3.1. Substrate solubility

Several researchers have reported their work in developing a microbial biotransformation process with high yield of AD with wide industrial applications [9–11]. Therefore, it was important to understand the factors affecting the actual biotransformation process. The basic and the most reported research is on low solubility of phytosterols in the aqueous media. This leads to low mass transfer rate and low substrate availability for conversion of phytosterol to AD. Oxygen transfer rate was also reported to be another critical parameter in the biotransformation of phytosterol [12]. Along with oxygen transfer rate (OTR) the effect of by-products such as 1,4-HBC formed during the bioprocess was found to affect the amount of the products produced leading to a loss in yield by around 16.3 percent [12].

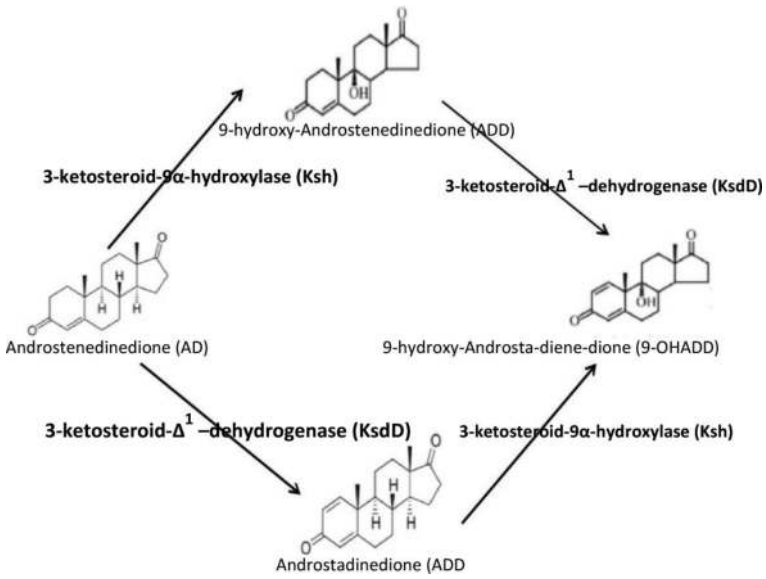

Figure 3. Enzymes involved in nucleus degradation of AD.

3.2. Nucleus degradation

The change in the steroidal nucleus of AD is one of the major obstacles reported in the microbial biotransformation of phytosterols. After formation of AD during microbial biotransformation, further degradation of AD takes place producing either ADD or 9-hydroxy androstenedione (9-OHAD) [7]. In a phenomenon which was coined as nucleus degradation by the researchers, two major enzymes have been implicated so far: 3-ketosteroid-1,2-dehydrogenase (KsdD) was found to be responsible for AD to ADD conversion and hence lead to low yield of AD. 3-ketosteroid-9-hydroxylase (Ksh), was observed to be responsible for AD to 9-OHAD conversion, leading to nucleus degradation and low yield of AD (**Figure 3**).

Xu et al. demonstrated that the enzymes involved in nucleus degradation of AD are temperature sensitive [7]. 3-ketosteroid-1,2-dehydrogenase (KsdD) and 3-ketosteroid-9-hydroxylase (Ksh) are enzymes sensitive at 37°. These enzymes break the B ring of steroidal nucleus which leads to degradation of the steroid. Hence it can be concluded that the microbial biotransformation of phytosterol is a temperature sensitive process.

4. Techniques to enhance production of androstenedione

As described earlier, to develop a process which is industrially applicable for high yields of AD, different techniques need to be incorporated. In the following section, we discuss the effective techniques to enhance AD production; thus making the process more economical.

4.1. Screening of micro-organisms

In the process of microbial biotransformation, selection of the microorganisms is a very crucial step. Microorganisms which are able to utilize sterols as their sole energy and carbon source are reported to have the ability to produce AD as an intermediate in the sterol degradation pathway [13]. Such microorganisms e.g., *Mycobacterium* sp., *Mycobacterium smegmatis*, *Rhodococcus* sp., *Deinococcus radiodurans*, *Pseudomonas* sp. have been documented to have an ability to produce AD or ADD. **Table 1** illustrates the organisms which are able to utilize sterols as their sole carbon and energy source to produce steroidal intermediates, AD, ADD, 9-hydroxy AD or 9-hydroxy ADD and also the various strategies implemented for enhancing the growth of the microorganisms.

4.2. Genome modifications

As mentioned in the earlier sections, researchers have demonstrated the pathways followed by microorganisms for biotransformation of phytosterol, the enzymes responsible for AD production and its nucleus degradation. Various studies have been performed on genome sequencing and proteomics to determine the sequence of the genes involved in the side chain cleavage of phytosterol. CYP11A1 in *Mycobacterium neoaurum* was reported to be involved in side chain cleavage of phytosterol leading to formation of AD [14]. Further, as described in Section 3.2, 3-ketosteroid-1,2-dehydrogenase (KsdD) and 3-ketosteroid-9-hydroxylase (Ksh) are the

Organism	Product	Strategy	Reference
Mycobacterium neoaurum	AD, ADD	Three-stage fermentation	[13]
Pseudomonas aeruginosa	AD,ADD, B	Selective media containing 8- hydroxyquinoline	[10]
Nocardioides simplex	AD, ADD, T	Selective media containing Hp-β- CD	[15]
Mycobacterium smegmatis	AD, ADD	Selective media containing Hp-β- CD	[16]
Mycobacterium tuberculosis	AD, ADD	Selective media containing Hp-β- CD	[17]
Moraxella ovis	AD, ADD	Natural medium containing rice bran oil	[11]
Deinococcus radiodurans,	AD, ADD	Natural medium containing rice bran oil	[11]
Corynebacterium equi	AD, ADD	Natural medium containing rice bran oil	[11]
Corynebacterium urealyticum	AD, ADD	Natural medium containing rice bran oil	[11]
Mycobacterium smegmatis	AD, ADD	Selective media	[18]

AD: Androstenedione, ADD: androstadienedione, T: Testosterone, B: Boldenone.

Table 1. Microorganisms reported to have the ability to transform sterols to steroidal intermediates.

enzymes responsible for nucleus degradation of AD. To avoid the nucleus degradation genome modification or mutation studies can be performed. Wei et al. has demonstrated a study to increase the yield of AD or ADD by mutations in Ksh or KsdD genes [14]. Ksh enzyme has two subunits namely A and B known as monooxygenase and reductase respectively. As described by Wei et al., the whole genome sequencing of *Mycobacterium neoaurum* explains six distantly placed gene clusters encoding KsdD enzyme and two gene clusters encoding KshA and KshB each. It was found that NwIB-02 *Mycobacterium neoaurum* with mutation in KsdD gene accumulated AD as the main product. Besides that, double mutated *mycobacterium neoaurum* i.e. mutations in both KsdD and Ksh genes accumulated AD as the main product of microbial biotransformation of phytosterol. Cloning of any one of the genes encoding these enzymes into a more economically cultivable strain may lead to enhanced production of desired products.

4.3. Liquid polymer-based systems

As described earlier, low substrate solubility is one of the major obstacles in aqueous bioconversion systems involving hydrophobic compounds. Researchers derived an alternative of using organic solvents in the aqueous media to increase the substrate solubility and mass transfer. On the other hand, organic solvents are not environment friendly. An alternative to organic solvents are the use of supercritical fluids, liquid polymers, ionic liquids and natural oils [19, 20]. Such strategies also negate the drawbacks caused by organic solvents i.e. damaging effects on microbial cells and its hazardous nature. Carvalho et al. demonstrated the use of poly (methylphenylsiloxane) oil (Silicone B oil) for AD extraction. It provides a suitable media for sitosterol side chain cleavage. AD yields close to 10 mM were obtained in almost 4–5 days of incubation, for an initial substrate concentration of 12 mM (referred to the polymer/organic solvent phase), with a biocatalyst concentration of 5 mg dry cell weight/ml. The researchers concluded the use of silicone B oil as non-volatile and non-toxic for providing a sustainable environment for the microbial side chain cleavage of sitosterol, both in single liquid phase system and in oil: aqueous two liquid phase systems.

To enhance the production of AD from phytosterol ionic liquid-aqueous biphasic systems were established by Yuan et al. [19]. Ionic liquids (ILs) with different cations and anions provided distinct but favorable substrate solubilization and product distribution for two phase conversion. The results of this study showed that AD production reached 2.23 g L^{-1} after 5 days of biotransformation with substrate concentration of 5 g L^{-1}. Further, ionic liquids which are easy to recycle produce negligible vapor pressure which indicates the industrial application of ILs in biphasic transformation process. Hence this strategy can be built up further for a more scaled up production.

4.4. Three-stage fermentation system

A recent strategy employed for enhanced AD production is the use of three-stage fermentation system. The well-known two-stage fermentation system uses a complex sugar (fructose in this case) as an initial carbon source followed by additional supplementation of simple sugar (glucose) which leads to elimination of the lag phase of the microorganism as well as an increase in biomass [21]. Shao et al. showed the effect of different carbon and energy sources on *Mycobacterium neoaurum* for enhancement of the desired steroid intermediate (AD/ADD) [13]. The researchers found that fructose acted as an optimal initial carbon source. Subsequent feeding by glucose maintained the metabolism of *mycobacterium neoaurum*. The researchers proposed three-stage fermentation by addition of phytosterol as third source and found enhanced ADD production (18.6 g/ l) which is reported to be the highest using *mycobacterium neoaurum*.

4.5. Use of PHB granules

Gerber et al. developed a whole–cell system based on recombinant *Bacillus megaterium* which encodes CYP11A1, the enzyme responsible for side chain cleavage of sterols [4]. The microorganism's PHB granules, aggregates of bioplastic coated with a protein/phospholipid monolayer act as substrate storage entities. As described earlier, substrate solubility of sterols in microbial biotransformation process is one of the major obstacles; PHB granules increase the conversion rate by serving as substrate storage entity. This phenomenon leads to increase in the mass transfer thereby increasing the desired product. Microorganisms which code for PHB production or the organisms having the ability to produce PHB granules can be screened for the biotransformation of sterols. These organisms may help in substrate availability thereby enhancing the efficiency of biotransformation process.

5. Conclusions

As per the available literature, there are number of strategies to increase the product yield. However, this area of research is still a hot topic as new strategies need to be developed which can lead to high yields of AD from cheaper substrates. Techniques involving industrially appropriate strains or addition of bioavailable catalysts to enhance the conversion or analysis of molecular pathway for generation of by-products. This may lead to development of a commercially cost effective process with high yields of the desired product. AD being one of the most important steroids which has high demand in pharmaceuticals as it is the precursor for

widely marketed drug category known as steroids. Therefore, the industrial production of this compound will greatly benefit the biomedical sector.

6. Future perspectives

The industrial production of androstenedione is challenging and economically important.

Therefore upcoming research in this field will be focused on:

a. Bioprospecting for finding organisms with enhanced production of androstenedione

b. Process optimization for economically viable production

c. Developing industrially applicable techniques to enhance the yield of AD.

Although there are a number of strains reported to have the biotransformation ability, yet the efficacy of these strains does not match the industrial applicability. Hence the need of the hour is to look for organisms having the capacity of transforming phytosterols to AD with high efficiency. Strategies can be applied for screening microorganisms having the ability to produce natural compounds like biosurfactants which increase the availability of complex substrates. Such organisms might play a role in substrate solubility of phytosterol thus minimizing the need of additional compounds.

Process optimization involves various parameters such as substrate inhibition, product inhibition, activities of biocatalysts, etc. To date, to the best of our knowledge, these parameters have not been addressed in the available literature. These parameters may result in the low yield of AD. Once addressed, these parameters might open a vast area of research. The mechanism of phytosterol biotransformation by various organisms has been reported but the enlisted parameters have not been paid attention to. All the aspects of the process need to be considered so as to develop an industrially applicable process for biotransformation of phytosterols.

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Conflict of interest

None declared.

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References

[1] Malaviya A, Gomes J. Androstenedione production by biotransformation of phytosterols. Bioresource Technology. 2008;**99**:6725-6737

[2] Liu M, Zhu ZT, Tao XY, Wang FQ, Wei DZ. Single nucleotide polymorphism analysis for the production of valuable steroid intermediates in Mycobacterium neoaurum. Biotechnology Letters. 2016;**38**:1881-1892

[3] Liu M, Zhu ZT, Tao XY, Wang FQ, Wei DZ. RNA-Seq analysis uncovers non-coding small RNA system of Mycobacterium neoaurum in the metabolism of sterols to accumulate steroid intermediates. Microbial Cell Factories. 2016;**15**:1-17. DOI: 10.1186/s12934-016-0462-2

[4] Gerber A, Kleser M, Biedendieck R, Bernhardt R, Hannemann F. Functionalized PHB granules provide the basis for the efficient side-chain cleavage of cholesterol and analogs in recombinant *Bacillus megaterium*. Microbial Cell Factories. 2015;**14**:107. DOI: 10.1186/s12934-015-0300-y

[5] Miras-Moreno B, Sabater-Jara AB, Almagro L, Pedreño MA. Bioactivity of phytosterols and their production in plant in vitro cultures. Journal of Agricultural and Food Chemistry. 2016;**64**:7049-7058

[6] Marsh DA, Brodie HJ, Garrett W, Tsai-Morris CH, Brodie AM. Aromatase inhibitors. Synthesis and biological activity of androstenedione derivatives. Journal of Medicinal Chemistry. 1985;**28**:788-795

[7] Xu XW, Gao XQ, Feng JX, Wang XD, Wei DZ. Influence of temperature on nucleus degradation of 4-androstene-3, 17-dione in phytosterol biotransformation by *Mycobacterium* sp. Letters in Applied Microbiology. 2015;**61**:63-68

[8] Lacey M, Bohday J, Fonseka SM, Ullah AL, Whitehead SA. Dose–response effects of phytoestrogens on the activity and expression of 3β-hydroxysteroid dehydrogenase and aromatase in human granulosa-luteal cells. The Journal of Steroid Biochemistry and Molecular Biology. 2005;**96**:279-286

[9] Donova MV, Egorova OV. Microbial steroid transformations: Current state and prospects. Applied Microbiology and Biotechnology. 2012;**94**:1423-1447

[10] Eisa M, El-Refai H, Amin M. Single step biotransformation of corn oil phytosterols to boldenone by a newly isolated Pseudomonas aeruginosa. Biotechnology Reports. 2016:36-43

[11] Sallam LAR, Osman ME, Hamdy AA, Zaghlol GM. Microbial transformation of phytosterols mixture from rice bran oil unsaponifiable matter by selected bacteria. World Journal of Microbiology and Biotechnology. 2008;**24**:1643-1656

[12] Gao X, Feng J, Hua Q, Wei D, Wang X. Investigation of factors affecting biotransformation of phytosterols to 9-hydroxyandrost-4-ene-3,-17-dione based on the HP-β-CD-resting cells reaction system. Biocatalysis and Biotransformation. 2014;**32**:343-347

[13] Shao M, Zhang X, Rao Z, Xu M, Yang T, Li H, Xu Z. Enhanced production of androst-1, 4-diene-3, 17-dione by Mycobacterium neoaurum JC-12 using three-stage fermentation strategy. PLoS One. 2015;**10**:e0137658

[14] Wei W, Wang FQ, Fan SY, Wei DZ. Inactivation and augmentation of the primary 3-ketosteroid-Δ1-dehydrogenase in *Mycobacterium neoaurum* NwIB-01: Biotransformation of soybean phytosterols to 4-androstene-3, 17-dione or 1, 4-androstadiene-3, 17-dione. Applied and Environmental Microbiology. 2010;**76**:4578-4583

[15] Shtratnikova VY, Schelkunov MI, Fokina VV, Pekov YA, Ivashina T, Donova MV. Genome-wide bioinformatics analysis of steroid metabolism-associated genes in Nocardioides simplex. Current Genetics. 2016;**62**:643-656

[16] Galán B, Uhía I, García-Fernández E, Martínez I, Bahíllo E, Barredo JL, García JL. *Mycobacterium smegmatis* is a suitable cell factory for the production of steroidic synthons. Microbial Biotechnology. 2017;**10**:138-150

[17] Capyk JK, D'Angelo I, Strynadka NC, Eltis LD. Characterization of 3-ketosteroid 9α-hydroxylase, a Rieske oxygenase in the cholesterol degradation pathway of *Mycobacterium tuberculosis*. Journal of Biological Chemistry. 2009;**284**:9937-9946

[18] Jenkins RL, Wilson EM, Angus RA, Howell WM, Kirk M, Moore R, Nance M, Beown A. Production of androgens by microbial transformation of progesterone in vitro: A model for androgen production in rivers receiving paper mill effluent. Environmental Health Perspectives. 2004;**112**:1508-1511

[19] Yuan JJ, Guan YX, Wang YT, Wang HQ, Yao SJ. Side-chain cleavage of phytosterols by *Mycobacterium* sp. MB 3683 in a biphasic ionic liquid/aqueous system. Journal of Chemical Technology and Biotechnology. 2016;**91**:2631-2637

[20] Carvalho F, Marques MP, de Carvalho CC, Cabral JM, Fernandes P. Sitosterol in liquid polymer based systems. Bioresource Bioconversion with Resting Cells Technology . 2009;**100**:4050-4053

[21] Du J, Qian Y, Qu H, Xi Y, Huang H, Jin H, Chang Z. Comparative analysis of single- stage and two-stage fermentation systems under various process conditions. BioResources. 2016;**12**:326-343