

EVALUATION OF C19 STEROID INTERMEDIATES DURING MICROBIAL TRANSFORMATION OF PHYTOSTEROL

ABSTRACT

Aim: To assay for C19 steroid intermediates during biotransformation of phytosterol.

Study of Design: Pretreatment and processing of wheat bran and rice bran, Sulphate-phosphate-ferric technique, phytosterol screening, fermentation process, enzymatic assay and steroid intermediate assay.

Place and Duration of Study: Microbiology Unit, Department of Biological Sciences, College of Natural and Applied Sciences, Fountain University Osogbo, Osun State, Nigeria between April, 2015 and March, 2016.

Methodology: Preliminary screening of agricultural residues (wheat bran and rice bran) for presence of phytosterols was determined using Sulphate-phosphate-ferric technique. *Saccharomyces cerevisiae* strain was screened for its ability to transform phytosterol to steroid intermediates after fermentation process using gas chromatography-flame ionization detector (GCFID) and enzymes such as 3-ketosteroid-9-hydroxylase (Ksh) and 3-ketosteroid-1-dehydrogenase (KstD) required for transformation were also assayed through Ion exchange and gel permeation chromatography respectively.

Results: Steroid intermediates such as Boldenone, Testosterone, 4-androstene-3, 17-dione (AD), Androsta-1,4-diene-3,17-dione (ADD), 9-OH-Testosterone and 9-OH-AD were obtained from crude extract after fermentation using GCFID. The result shows the ability of *Saccharomyces*

cerevisiae to transform rice bran and wheat bran phytosterols to precursors for synthesis of steroid hormones.

Conclusion: Sterol uptake takes place via the direct contact between the cells and the sterol particles, thus *Saccharomyces cerevisiae* ability to transform phytosterols to steroids shows its adaptive mechanism which allows microorganisms to use minimally water-soluble hydrocarbons as carbon sources.

Keywords: *Saccharomyces cerevisiae*, phytosterol, boldenone, androsta-1,4-diene-3,17-dione, 3-ketosteroid-9-hydroxylase,

INTRODUCTION

Phytosterols are plant derived compounds structurally related to mammalian cell- delivered cholesterol that functions as essential constituents of plant cell membranes [1]. Phytosterols also referred to as plant sterols are common plant constituents present in human diet which are structurally related to cholesterol, but differ in their side chain structure. The most abundant sterols in plants and human diets are sitosterols and campesterols which are essential to maintaining normal function in plant cell membranes. Phytosterols consist of a steroid skeleton with a hydroxyl group attached to the C-3 atom of the A-ring and an aliphatic side chain attached to the C-17 atom of the D-ring [2]. Consumption of 1.5–3.0 g of phytosterols lead to a reduction of 8–15% in the blood levels of LDL-cholesterol [3] thereby reducing serum levels of low-density lipoproteins and the risk of atherosclerosis [4]. The major sources of phytosterols in human diet are vegetable oils, vegetable oil-based food products, cereal grains, cereal-based foods especially rye, nuts and margarine.

Steroid is an organic compound containing twenty carbon atoms bonded together to form four fused rings i.e. three cyclohexane rings and one cyclopentane ring (the D ring); e.g. dietary fat

cholesterol, sex hormones testosterone and inflammatory drug dexamethasone [5]
Biotransformation of phytosterols is triggered by cholesterol oxidases (Cho) through oxidation of
3-hydroxyl-5-ene moiety of sterols to 4-ene-3-one moiety [6]. Researchers also reported the
microbial transformation of Soybean phytosterols (usually β -sitosterol, stigmasterol,
campesterol, and brassicasterol) to steroid hormones intermediates (C19-steroids and C22-
steroids) [7].

The C19-steroids are the products of complete side chain cleavage which are used as precursors
for all kinds of steroid hormones such as sex hormones, anabolic steroids, and adrenocortical
hormones based on their pharmacological activity [8]. Traditional mutagenesis screening
techniques have long been employed as a practical means of producing C19- steroid-producing
strains which eventually leads to significant deficiencies between the strains selected from
random mutations; slight differences in structure between C19 steroids making it difficult to
separate in industrial applications [9].

The aim of this research is to transform phytosterols present in agricultural residues into C19
steroid intermediates using local strain of *Saccharomyces cerevisiae* in order to prevent increased
production costs and limits deficiencies of mutant strains which include residual decomposition
of the steroid nucleus and reduced transformation efficiency.

METHODOLOGY

Chemicals and Steroid standards

Chemical reagents, C19-steroid intermediate standards [androsta-4-ene-3,17-dione (AD),
androsta-1,4-dien-3,17-dione (ADD), 9 β -hydroxyandrosta-4-ene-3,17-dione (9-OH-AD),

testosterone, 9-hydroxy-testosterone (9-OH-testosterone), Boldenone and Estrone] and Enzyme standards (99% purity) were procured from Aldrich Sigma.

Extraction of Phytosterol from Agricultural Residue

Phytosterol was extracted from agricultural residue (wheat bran and rice bran) using Sulphate-Phosphate-Ferric (SPF) method [10]. Wheat bran and rice bran (10 g) dissolved in 100 ml of ethanol separately were kept on the orbital shaker at 100 rpm for 20 minutes. The crude extract was filtered and stored in the refrigerator for further use. Ten milliliters of chromogenic reagent (2.5 g of Ferric chloride in 25 ml of Phosphoric acid) was transferred into 100 mL of sulphuric acid to obtain the Sulphate- Phosphate- Ferric (SPF) required for the extraction.

Preliminary Screening of Phytosterol

Crude extract (20 mL) of each sample was added to 2 mL of the chromogenic reagent to test for the presence of phytosterol in the samples. The presence of phytosterol in the substrate was confirmed by mixture colour change from colourless to dark brown colour after addition of chromogenic reagent. Phytosterol present in the crude extract was quantified using spectrophotometric method at 500nm [10].

Biotransformation of Crude Extract to Steroid Intermediate

a. Sterol Biotransformation Medium

The biotransformation medium comprised of 0.075g of KH_2PO_4 , NaCl (0.0125g), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (1g), CaCl_2 (0.01375g), $(\text{NH}_4)_2\text{SO}_4$ (0.025g), FeSO_4 (0.0025g), agricultural residue (10g) was inoculated with 24 hours old *Saccharomyces cerevisiae* BY15 and incubated at 30°C on an orbital shaker at 200 rpm for 72 hours. The medium was centrifuged after incubation period and crude extract (supernant) was analyzed for steroid intermediate formation by adding 1 mL of chloroform to 1 mL of crude extract at 24 hours interval [11].

b. Qualitative Test for Steroid Present in the Medium

Presence of steroid intermediate in the biotransformation medium was determined using Liberman- Burchad test by adding 2 mL of chloroform, acetic anhydride (10 drops) and sulfuric acid (2drops) to the crude extract [10]. Color change from bluish red to cherry red color is indicated presence of steroid intermediate.

c. Quantification of Steroid Intermediate

Extraction and analysis of steroids were carried out according to the modified methodology [12]. The crude extract (supernatant) was separated with petroleum ether (extracting agent) and evaporated to dryness using nitrogen stream. Extract obtained was saponified at 95°C for 30 minutes using 3 mL of 10% KOH in ethanol to which 0.20 mL of benzene was added to ensure miscibility. De-ionised water (3 mL) and 2 mL of hexane was added to the mixture to extract the non-saponifiable materials. Extraction was done thrice with 2 mL of hexane for 1 hour, 30 minutes and 39 minutes respectively to achieve complete extraction of the steroids. The hexane was concentrated to 2 mL in agilent vial for gas chromatography analysis.

The steroid intermediates obtained were quantified using Gas chromatography- flame ionization detector HP 6890 Powered with HP Chemstation Rev.A 09.01 (1206) software. The chromatography was done in Ultra-1 capillary column (25 m× 0.20 mm× 0.33 µm) with a split ratio of 20:1, inlet temperature (280 °C), detector temperature (325°C), hydrogen and compressed pressure (22 and 35 psi) using flame ionization detector detector signal and helium as the mobile phase.

d. Enzyme Purification and Quantification

Saccharomyces cerevisiae ability to produce enzymes required for biotransformation of phytosterol to steroid intermediate was assayed by subjecting the crude extract to enzyme

dialysis, ion exchange chromatography (DEAE- cellulose chromatography) and gel permeation chromatography using Superdex- 75 column respectively.

e. Determination of Enzymes Activity

Enzyme activity was quantified using 3, 5- dinitrosalicylic acid reagent thus the colour complex formed was subjected to spectrophotometric assay at 540 nm. Enzyme activity is defined as the amount of enzyme that catalyzes the conversion of 1 μ mol of phytosterol per minute [4]. Enzyme activity was calculated as follows;

Standard factor = (Concentration (μ mol/ml) of standard solution) $\div$ (Absorbance at 540 nm)

Enzyme activity = (Standard Factor $\times$ Absorbance $\times$ Dilution) $\div$ (Time of incubation)

Molecular Weight Determination

Discontinuous reducing technique was employed using 15 % and 5 % w/v acryl amide resolving and stacking gels respectively [13]. Samples were treated with reducing agent buffer and boiled for 5 minutes prior to the application of the gel. High and low molecular weight standards were co- resolved on each gel for subunit molecular weight determination enzyme.

RESULTS AND DISCUSSION

Phytosterols are steroid alcohols that occur naturally in plants that regulate the fluidity of plant cell membranes and feature in cellular differentiation and proliferation [14]. Microbial transformation of phytosterol to steroid intermediates requires side chain cleavage [15]; thus C19-steroids namely androsta-4-ene-3,17-dione (AD), androsta-1,4-dien-3,17-dione (ADD), 9^l-hydroxyandrosta-4-ene-3,17-dione (9-OH-AD), testosterone, 9-hydroxy-testosterone (9-OH-testosterone), Boldenone and Estrone (Figures I and II) obtained in this research shows the ability of *Saccharomyces cerevisiae* to transform rice bran and wheat bran phytosterols to

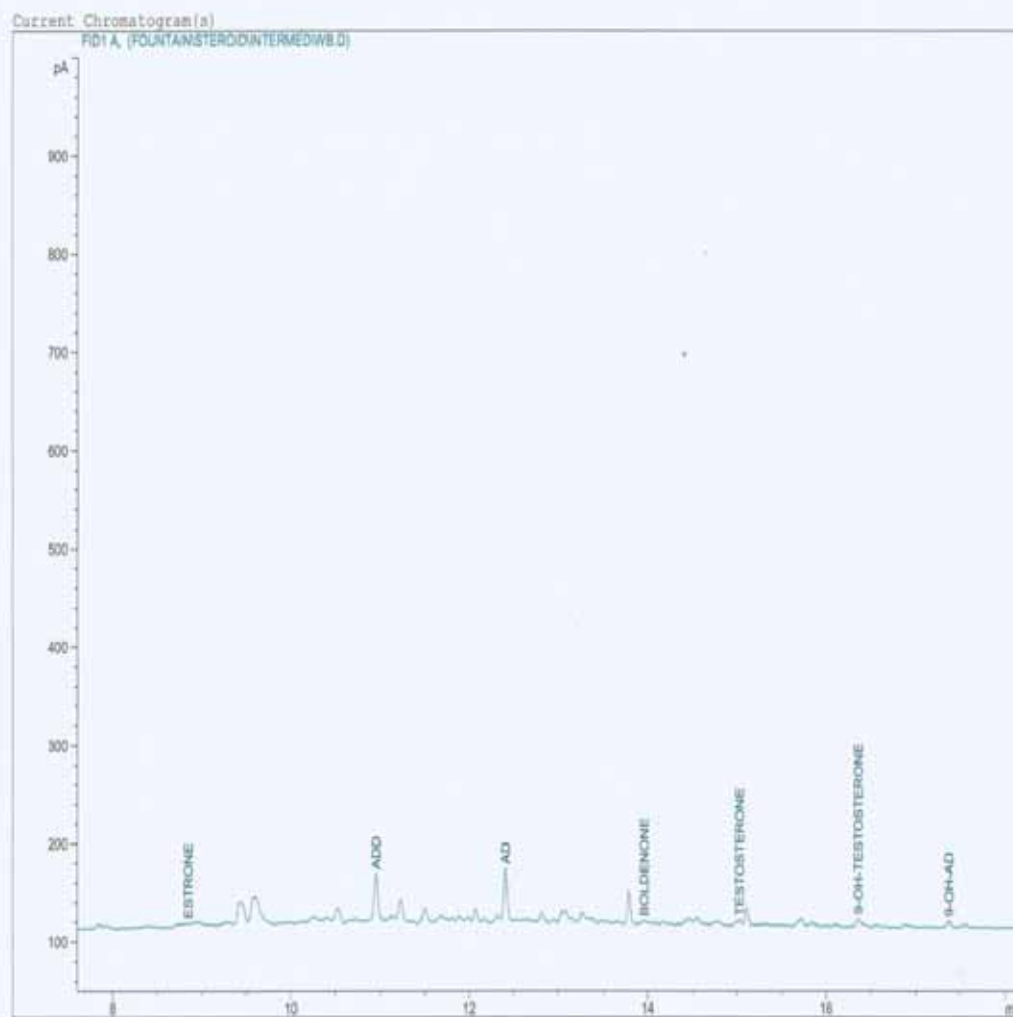
precursors for synthesis of steroid hormones such as sex hormones, anabolic steroids and adrenocortical hormones through complete side chain cleavage [7].

Microbial transformation of rice bran and wheat bran phytosterols to different steroid intermediates by *Saccharomyces cerevisiae* also indicated its ability to initiate oxidation of 3-hydroxyl-5-ene moiety of sterols to 4-ene-3-one moiety through expression of cholesterol oxidases (Cho) enzyme [6]. Low steroid intermediate concentration (4.61 mg/100g) obtained from rice bran transformation medium compared to 7.12 mg/100g of wheat bran (Tables I and II) shows the cellular toxicity of the steroid intermediates (AD and ADD) to *Saccharomyces cerevisiae* microbial cells which inhibit cell growth, respiration and also suppress enzyme activity in the sterol transformation pathway causing low product yield [16] which is a major factor limiting the productivity of conversion of phytosterol to steroid.

Table I: Steroid Intermediates obtained from Wheat bran biotransformation medium

RetTime	Area	Amt/Area	Amount	Steroid intermediates
[min]	[pA*s]		[mg/100g]	
8.849	21.04	1.65563e-5	3.48e-4	Estrone
10.955	261.98	7.56467e-3	1.98	Androsta-1,4-dien-3,17-dione (ADD)
12.408	196.09	1.75565e-2	3.44	Androsta-4-ene-3,17-dione (AD)
13.791	124.91	1.45488e-4	1.82e-2	Boldenone
15.029	43.64	3.12500e-6	1.36e-4	Testosterone
16.364	60.16	1.56250e-6	9.40e-5	9-hydroxy-testosterone (9-OH-testosterone)
17.382	28.98	5.77548e-2	1.67	9 ^β -hydroxyandrosta-4-ene-3,17-dione (9-OH-AD)
Total	736.80		7.12	

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147
148 **Figure I: Chromatogram of Steroid Intermediates obtained from Wheat bran**
149 **biotransformation medium**

150 **Table II: Steroid Intermediates obtained from Rice bran biotransformation medium**

RetTime	Area	Amt/Area	Amount	Steroid intermediates
[min]	[pA*s]		[mg/100g]	
8.764	3.82	1.65563e-5	6.33e-5	Estrone
11.370	100.41	1.22168e-2	1.23	Androsta-1,4-dien-3,17-dione (ADD)
12.833	39.24	6.53573e-2	2.56	Androsta-4-ene-3,17-dione (AD)
13.826	49.45	3.42883e-4	1.70e-2	Boldenone
15.029	20.04	3.12500e-6	6.26e-5	Testosterone
16.250	34.02	1.56250e-6	5.32e-5	9-hydroxy-testosterone (9-OH-testosterone)
17.381	10.99	7.26643e-2	7.99e-1	9 ^β -hydroxyandrosta-4-ene-3,17-dione (9-OH-AD)
Total	257.97		4.61	

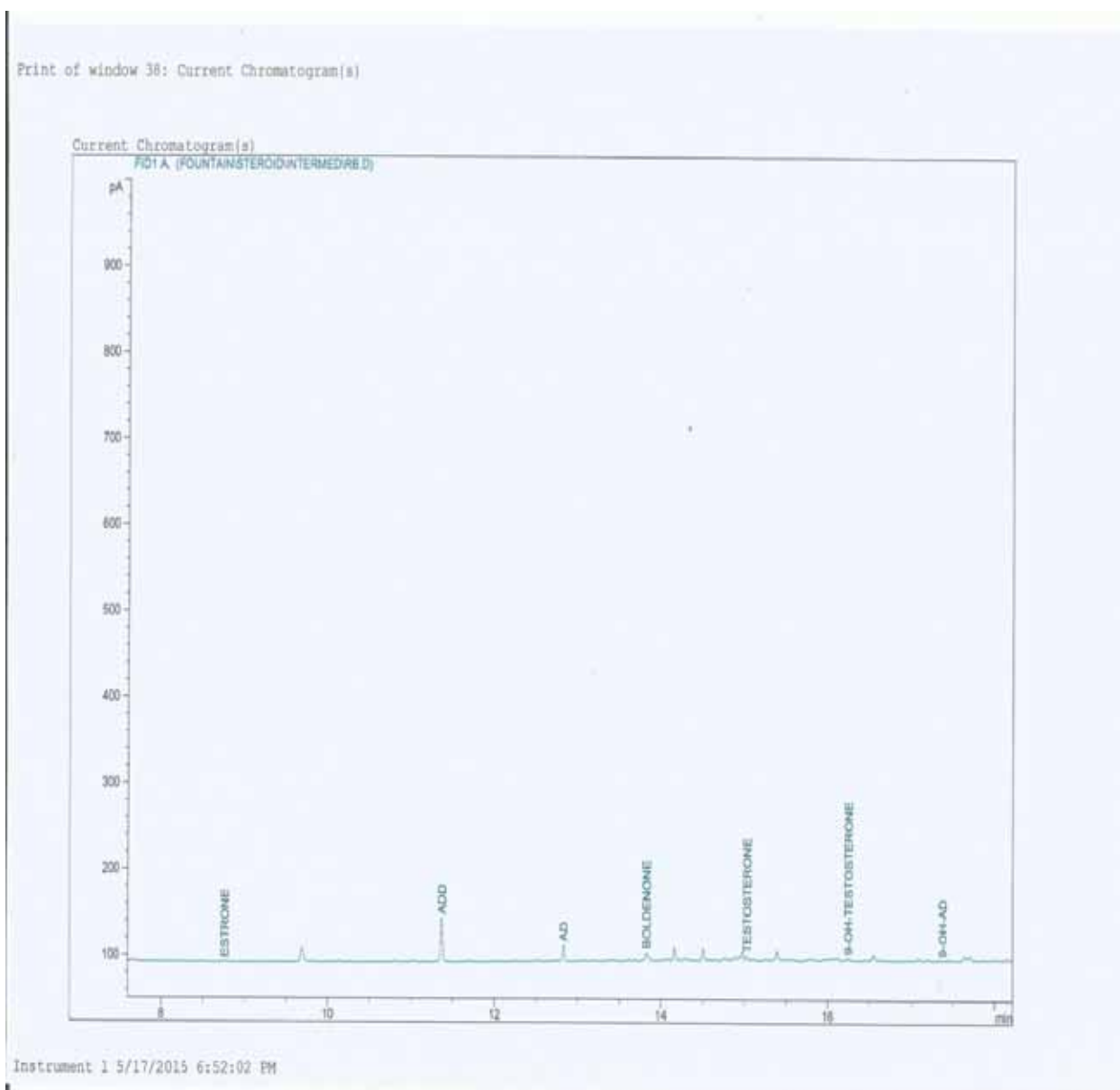


Figure 11: Chromatogram of Steroid Intermediates obtained from Rice bran biotransformation medium

Maintaining an intact steroid nucleus is required to producing valuable steroids from the catabolism of phytosterols thus microbial degradation of steroid intermediate produced by *Saccharomyces cerevisiae* to unstable chemicals was determined through enzymatic activity assay for 3-ketosteroid-9-hydroxylase (Ksh) and 3-ketosteroid-1-dehydrogenase (KstD) enzymes

respectively (Table III). Thus, low Ksh and KstD enzyme activities (85.84 and 0.92 U/mg) obtained using wheat bran transformation medium compared to 105.34 and 1.12 U/mg enzyme activities obtained from rice bran transformation medium implies that steroid hormones intermediates obtained from wheat bran had intact steroid nucleus compared to rice bran steroid intermediates which eventually leads to variation in concentrations (4.61mg/100g of rice bran and 7.12mg/100g of wheat bran) as a result of decomposition of steroid nucleus, thus forming 1,4-dien-9-hydroxy-steroids which is structurally unstable chemicals [17].

Phytosterols are more hydrophobic and its poor solubility retards their bioavailability because of inadequate mass transfer. Many efforts such as organic solvent and surfactant-facilitated emulsification have been used as means to enhancing the bioavailability of phytosterols by improving the dispersity and dissolubility of phytosterols in reaction media which raises the complexity of the process, production costs and also leads to environmental pressures. C19-steroids obtained from wheat and rice brans are highly important precursors of steroid hormones and can be used to synthesize almost all the steroid hormones used medically [8]. The C19-steroids can also be used as precursors for the synthesis of C21 corticosteroids [18]. Inactivation of *Saccharomyces cerevisiae* Kshs, KstDs, or both to block the decomposition of the steroid nucleus can result to increase in concentration or accumulation of C19 steroids such as AD, ADD, and 9-OHAD using rice bran [19].

Table III: Quantification of Enzymes Required for Phytosterol Biotransformation

Enzyme	Molecular Weight (Da)	Activity (U/mg)	
		Wheat bran	Rice bran
3-ketosteroid-9-alpha-hydroxylase	38,247.18	85.84	105.34

180	3-ketosteroid-1-dehydrogenase	55,100.12	0.92	1.12
181	3-Hydroxymethyl-3-glutamyl coenzyme A	204,452.50	0.74	1.15

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183 CONCLUSION

184 Microbial phytosterol transformation by *Saccharomyces cerevisiae* using wheat bran is hereby
 185 recommended due to its mechanism of sterol uptake through microbial cells adherence to the
 186 surfaces of sterol particles, forming stable agglomerates. Sterol uptake takes place via the direct
 187 contact between the cells and the sterol particles [4], thus *Saccharomyces cerevisiae* ability to
 188 transform phytosterols to steroids shows its adaptive mechanism which allows microorganisms
 189 to use minimally water-soluble hydrocarbons as carbon sources.

190 REFERENCES

- 191 1. Lichtenstein AH. Plant Sterols and Blood Lipid Levels. *Journal of Current opinion in*
 192 *Clinical Nutrition and Metabolic Care*, 2002; 5:147-152. [http://journals.lww.com/co-](http://journals.lww.com/co-clinicalnutrition/toc/2002/03000)
 193 [clinicalnutrition/toc/2002/03000](http://journals.lww.com/co-clinicalnutrition/toc/2002/03000).
- 194 2. Moreau RA, Whitaker BD, Hicks KB. Phytosterols, Phytostanols, and Their Conjugates
 195 in Foods: Structural Diversity, Quantitative Analysis, and Health-Promoting Uses.
 196 *Journal of Progress in Lipid Research*, 2002 41(6):457-500.
 197 [https://doi.org/10.1016/S0163-7827\(02\)00006-1](https://doi.org/10.1016/S0163-7827(02)00006-1)
- 198 3. Quilez J, Garcia- Lorda P, Salas-Salvado J. Potential Uses and Benefits of Phytosterols In
 199 Diet: Present Situation and Future Directions. *Journal of Clinical Nutrition.*, 2003; 22:
 200 342-351. [http://dx.doi.org/10.1016/S0261-5614\(03\)00060-8](http://dx.doi.org/10.1016/S0261-5614(03)00060-8)

4. Fernandes P, Cruz A, Angelova B, Pinheiro HM, Cabral JMS. Microbial Conversion of Steroid Compounds: Recent Developments. *Journal of Enzyme and Microbial Technology*, 2003; 32(6):688-705. [https://doi.org/10.1016/S0141-0229\(03\)00029-2](https://doi.org/10.1016/S0141-0229(03)00029-2).
5. Zollner G, Marschall HU, Wagner M, Trauner M. Role of Nuclear Receptors in the Adaptive Response to Bile acids and Cholestasis: Pathogenetic and Therapeutic Considerations. *Journal of Molecular Pharmacology*, 2006; 3(3):231-251. DOI:10.1021/mp060010s
6. Doukyu N. Characteristics and Biotechnological Applications of Microbial Cholesterol oxidases. *Appl Microbiol Biotechnol*; 2009, 83:825. doi:10.1007/s00253-009-2059-8.
7. Feng-Qing W, Kang Y, Dong-Zhi W. From Soybean Phytosterols to Steroid Hormones, Soybean and Health, Prof. Hany El-Shemy (Ed.), ISBN: 978-953-307-535-8, InTech, Available from: <http://www.intechopen.com/books/soybean-and-health/from-soybean-phytosterols-to-steroid-hormones>; 2011; Pp 231-253
8. Malaviya A, Gomes J. Androstenedione Production by Biotransformation of Phytosterols. *Bioresource Technology*, 2008; 99(15):6725–6737. <https://doi.org/10.1016/j.biortech.2008.01.039>
9. Gulla V, Banerjee T, Patil S. Bioconversion of Soy Sterols to Androstenedione by *Mycobacterium fortuitum* Subsp. *fortuitum* NCIM 5239, a Mutant Derived from Total Sterol Degradation Strain. *Journal of Chemical Technology and Biotechnology*, 2010; 85: 1135-1141. doi 10.1002/jctb.2410
10. Yanliang L, Xin S, Juan F, Jianqiang L, Yinbo Q. Microbial transformation of phytosterol in corn flour and soybean flour to 4-androstene-3,17-dione by *Fusarium*

- moniliforme Sheld. *Bioresource Technology*; 2009; 100: 1864–1867.
doi:10.1016/j.biortech.2008.09.040
11. Wadhwa L, Smith KE. Progesterone side-chain cleavage by *Bacillus Sphaericus*. *FEMS Microbiology Letters*. 2000; 192: 179–183. DOI: 10.1111/j.1574-6968.2000.tb09379.x
12. Ju-Yeon M., Hee-June S, Hyun-Hwa S, Jeongae Lee, Uhee J, Sung-Kee J, Hyun SK, Kyung-Hoon K, Kyu Hwan P, Bong CC, Man HC. Metabolic changes in serum steroids induced by total-body irradiation of female C57B/6 mice. *The Journal of Steroid Biochemistry and Molecular Biology*; 2014; 141:52-59.
<https://doi.org/10.1016/j.jsbmb.2014.01.004>
13. Laemmli UK. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. *Nature*, 1970; 227, 680 – 685. doi:10.1038/227680a0
14. Piironen V, Lindsay DG, Miettinen TA, Toivo J, Lampi AM. Review Plant sterols: Biosynthesis, biological function and their importance to human nutrition. *Journal of the Science of Food and Agriculture*, 2000; 80: 939-966. DOI: 10.1002/(SICI)1097-0010(20000515)80:7<939::AID-JSFA644>3.0.CO;2-C
15. Szentirmai A. Microbial physiology of sidechain degradation of sterols. *Journal of Industrial Microbiology*, 1990; 6(2): 101-115.doi:10.1007/BF1576429
16. Donova, M.V. (2007) Transformation of steroids by actinobacteria: A review. *Appl Biochem Microbiol* ; 43(1): 1-14. doi:10.1134/S0003683807010012
17. Van der GR, Yam K, Heuser T, Wilbrink MH, Hara H, Anderton MC, Sim E, Dijkhuizen L, Davies JE, Mohn WW, Eltis LD. A Gene Cluster Encoding Cholesterol Catabolism in a Soil Actinomycete Provides Insight into *Mycobacterium tuberculosis* Survival in Macrophages. *Journal of Proceedings of the National Academy of Sciences of the United*

246 *States of America*, 2007; 104: 1947-1952.
247 www.pnas.org/cgi/doi/10.1073/pnas060572810
248 18. Andrews DR, Giusto RA, Sudhakar AR. A Corticoid Synthesis from 9 α -
249 Hydroxyandrost-4-ene-3,17-dione via a Steroidal Allene. *Tetrahedron Letters* 1996; 37:
250 3417-3420. [https://doi.org/10.1016/0040-4039\(96\)00583-7](https://doi.org/10.1016/0040-4039(96)00583-7)
251 19 Petrusma M, Dijkhuizen L, Van Der GR. *Rhodococcus rhodochrous* DSM 43269 3-
252 ketosteroid 9 α -hydroxylase, a two-component iron-sulfur-containing monooxygenase
253 with subtle steroid substrate specificity. *Appl Environ Microbiol.*, 2009; 75: 5300–
254 5307.[doi:10.1128/AEM.00066-09](https://doi.org/10.1128/AEM.00066-09)