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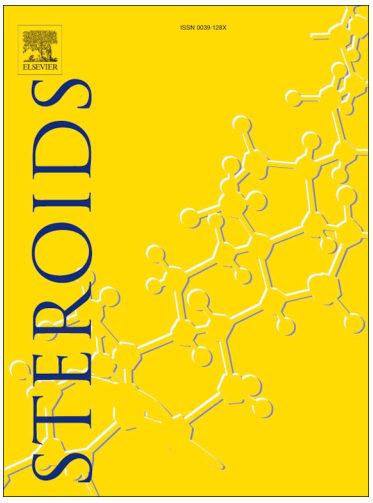

Production aspects of testosterone by microbial biotransformation and future prospects

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Production aspects of testosterone by microbial biotransformation and future prospects

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Abstract

In human males, TS plays a key role in maintaining health and sexual functioning. Cholesterol acts as a precursor molecule for its biosynthesis. The microbial biotransformation of cholesterol by numerous microbes like bacteria, fungi, yeasts, etc. has led to the synthesis of TS out of human body making it a great example for industrial steroid production due to its therapeutic properties. Bioransformation through microbes is more advantageous over chemical synthesis as it gives higher conversion rates, higher specificity; reaction goes under mild conditions like temperature and neutral pH, thus being an effective alternate to chemical route. Current review focuses on production aspects of TS by microbial biotransformation and its future prospects with recent advancement.

Table of Content

Keywords: Testosterone, Production, Microbial biotransformation.

Abbreviations: TS, Testosterone; AD, 4- androstene-3, 17-dione; ADD, androsta-l,4-diene-3,17-dione; 17β-HSD, 17β- hydroxysteroiddehydrogenase; LC, Leydig cells; C, Carbon; NADP, nicotinamide adenine dinucleotidephosphate ; EC, Enzyme Commission; sp., species;

SCP2, Sterol Carrier Protein2; ER, Endoplasmic Reticulum; LH, Luteinizing Hormone; FSH, Follicle Stimulating Hormone; DHT, Dihydrotestosterone; HTS, High-throughput screening.

1. Introduction

At industrial scale, the production of steroids and drugs serves to be one of the best examples to showcase successful application of microbial technology. To produce highly functionalized steroidal compounds with both, commercial and therapeutic values, specific reactions are required. Steroids are derived from cyclopentanoperhydrophenantrene (Sterane) structurally (Fig.1) and their compounds show pharmaceutical importance [1]. Cholesterol, androsterone, TS, progesterone, estrone/oestrone, cholic acid, estradiol, AD and ADD are some of the steroidal compounds found in vertebrates [2].

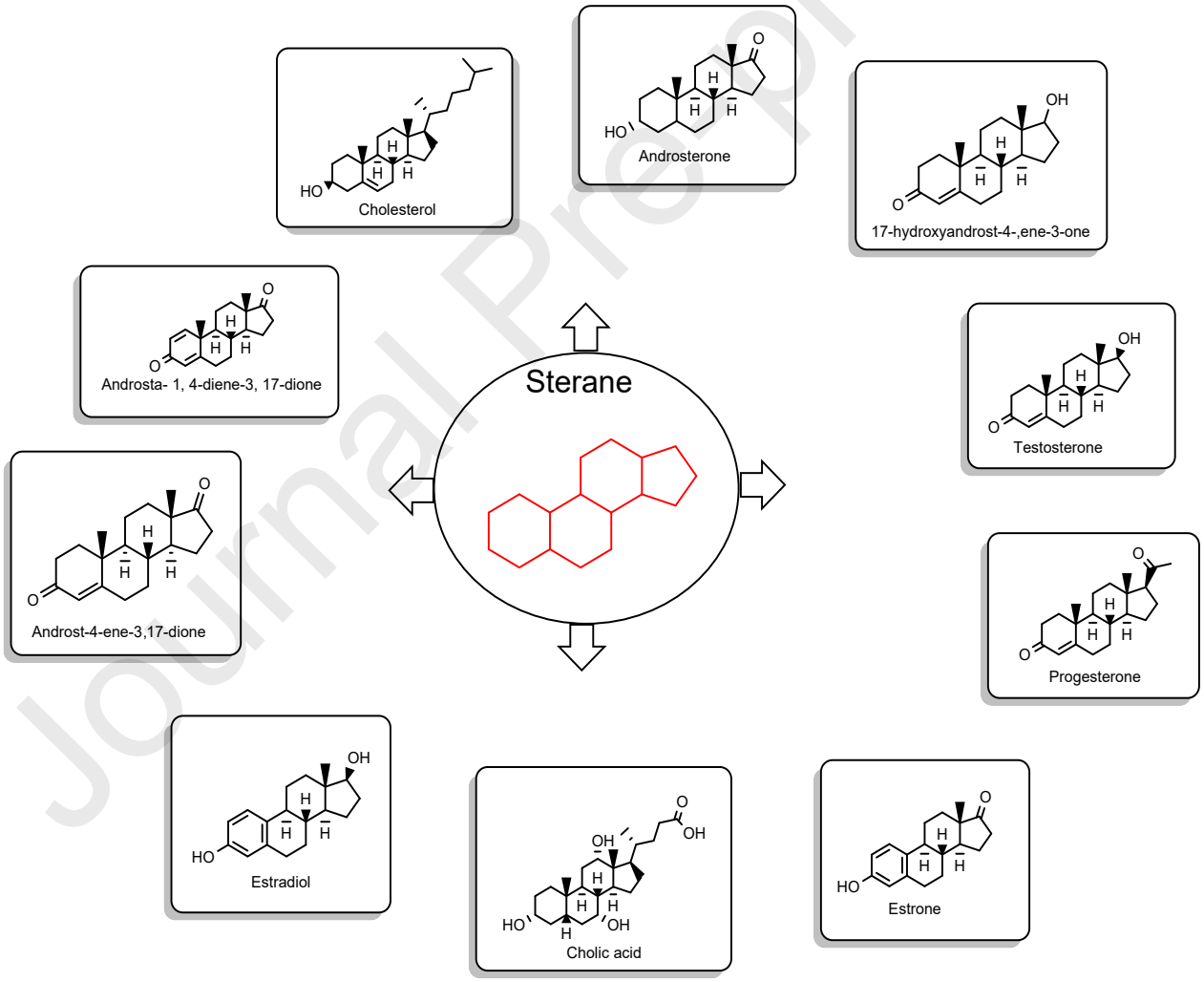

Fig. 1. Origin of steroidal compounds from Sterane, the parent ring structure of steroid compounds in vertebrates.

TS (17-hydroxyandrost-4-,ene-3-one) is a principal male sex androgen produced by LCs and released by testis throughout the life with a well-known role in maintaining human health and sexual functioning in men [3, 4]. It is an important pharmaceutical steroid used in medicine from a long time with safe record and great efficacy for hormone replacement therapy in androgen deficient men since it is responsible for the development of sexual characteristics in males [3, 5, 6]. Androgens are significant for developing male reproductive tissues like epididymis, seminal vesicle, prostate, testis, penis, etc. and maintaining properties like hair growth, increased muscle strength, etc. [7, 8]. Cholesterol is precursor molecule for synthesizing steroid hormones, bile acids and Vitamin D [9]. As compared to its biosynthetic precursor, the C-17 aliphatic side chain is absent from TS, belonging to C-19 androgen group, making it more water soluble than cholesterol [10]. The reduction of AD to TS is the final step in its biosynthesis [11] catalysed by microsomal 17-ketosteroid reductase enzyme (17β-HSD; 17β-hydroxysteroid: NADP 17-oxidoreductase, EC: 1.1.1.64) [3, 5, 12]. In history, Ernst Laqueur and co-workers extracted TS from 100kg of bull testis and isolate 10mg of a purified androgen, 17β-hydroxy-4-androstene-3one and named it 'testosterone' which was known to be more active than androsterone as observed in biological tests performed in 1935 [13, 14]. After that synthetic route was also tried for the synthesis of TS but due to complex structure and asymmetric centres, chemical synthesis was avoided. Since TS has significant importance as a steroid hormone, its microbial synthesis is of utmost interest [15]. Different microorganisms have been reported for production of TS and its derivatives that are not readily accessible by chemical synthesis [16] such as filamentous fungi [17], yeasts [18], bacteria [19] and plants [20]. They have also shown synthesis of TS from enzymatic reduction of AD by 17β-HSD [5, 21]. Especially, fungi have a great ability to carry out diverse chemical reactions like oxidation, reduction, hydroxylation, hydrolysis, degradation, etc., on steroidal compounds [16, 22]. Several *Mycobacterium* sp. mutants have been reported to exhibit a single step process of microbial transformation for the production of TS from sterols [4, 5]. The maintenance and functioning of normal prostate glandular structure depend on presence of constant TS and modifications or changes in it can link to prostate cancer [23, 24]. TS exhibit anabolic properties promoting muscle growth and maintenance. It is also used for male birth control and treatment of male menopause [2, 25]. Since the steroidal biological activities are dependent on their proper functioning, transformations through microbes can serve as a great tool for producing pharmaceuticals with high value from available steroid raw materials [16, 26].

2. Biosynthesis of Testosterone

In human males, the end product of steroid biosynthesis is TS with much importance as a hormone [16] since it links directly to the development of masculine sexual properties. The isolation and synthesis of the actual molecule was done back in 1935 [10]. Cholesterol acts as a precursor molecule for the synthesis of all steroid hormones including TS secreted by ovaries of females and testis of males but mammals lack the ability to degrade these steroid hormones. TS is highly water soluble than cholesterol, its biosynthetic precursor, due to the absence of aliphatic side chain on C-17 [10]. Out of total testicular volume, approximately 500 million LCs are required for the metabolic conversion and circulation of cholesterol into androgens with contributions from adrenal cortex as well. Brain cells also produce a minute amount of TS [27] but their contribution is very small towards the circulating hormone levels. *De novo* synthesis of the substrate cholesterol can be done from acetate or it can be taken up from lipoproteins of plasma membrane with LCs possessing its storage capacity. The intracellular transport of cholesterol towards mitochondria occurs via a vesicle-mediated transport system having an endosomal/lysosomal network with SCP2 acting as transfer proteins for supplying cholesterol to the outer membrane of mitochondria [28]. This coupled intracellular transport mechanism results in the production of pregnenolone (C_{21}) from cholesterol (C_{27}), beginning the steroidogenic cascade where pregnenolone is converted to a variety of C_{19}-steroids by enzymes in the ER. These oxidative enzymes belong to cytochromes P450, a group of heme-containing proteins. In humans, the capacity of the pregnenolone-converting enzyme system is not capable of converting all available pregnenolone into TS under normal circumstances, resulting in leakage of many intermediates as progesterone derivatives out of the LCs. This indicates that the rate-limiting step for TS production is localized at ER levels while the rate-determining step for steroidogenesis, regulated by LH is at the level of cholesterol side chain cleavage activity in mitochondria [29]. The testicular functions are developed and maintained by LH & FSH with LH being the most significant hormone to control functions and population size of LC via controlling proliferation and differentiation [8, 30]. The final step of TS biosynthesis is the reduction of AD to TS (fig. 2) catalysed by microsomal 17-ketosteroid reductase enzyme (17β-hydroxysteroid: NADP 17-oxidoreductase) [6, 31].

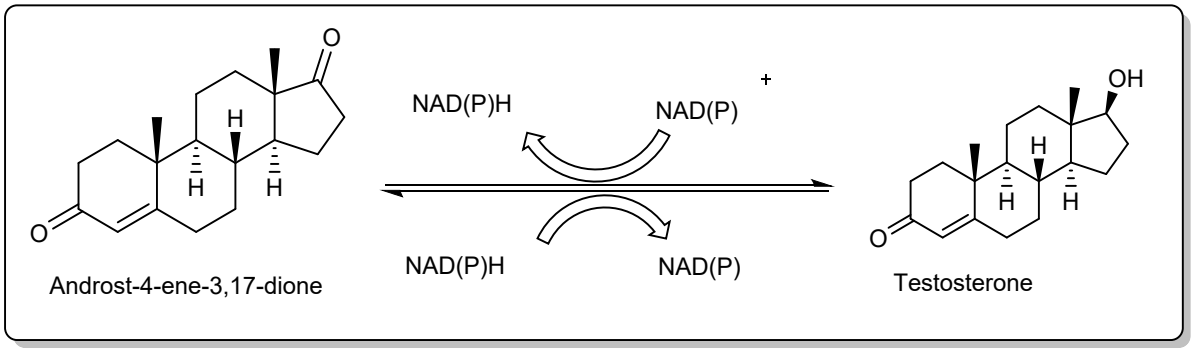

Fig. 2. Scheme representing transformation of AD into TS by 17β-HSD

Approximately, 6-7 mg of TS is released per day by the testis [32]. The production of androgens is balanced by metabolic clearance and excretion rates to maintain the appropriate levels of androgen concentration [8]. Due to the easy availability and low cost, natural sterols like cholesterol and 3-sitosterol are used as raw materials for TS production semi-synthetically (Fig. 3) [33]. To obtain TS from sterols, multistep chemical synthesis [34] can also be performed or production of intermediates AD or ADD can be done using combination of different methods [4, 21]. The process of *in vitro* TS production from AD has been developed using the recombinant murine 17β-HSD type V (aldo-keto-reductase instead of short-chain dehydrogenase: reductase superfamily) with glucose dehydrogenase as cofactor recycling enzyme [5, 35]. For the total synthesis of TS, hydrochrysene approach was applied by Johnson et al. which was a multi-step reaction with 55% yield at final stage [36]. This was the first synthetic route reported for the synthesis of TS but synthetic methods have several drawbacks like multistep reaction, non-specific, creation of hazardous non-degradable compounds and fewer yields making it non-preferable for pharmaceutical industries. TS molecule has several asymmetric centres making its synthetic synthesis very difficult, therefore microbial biotransformation is preferred.

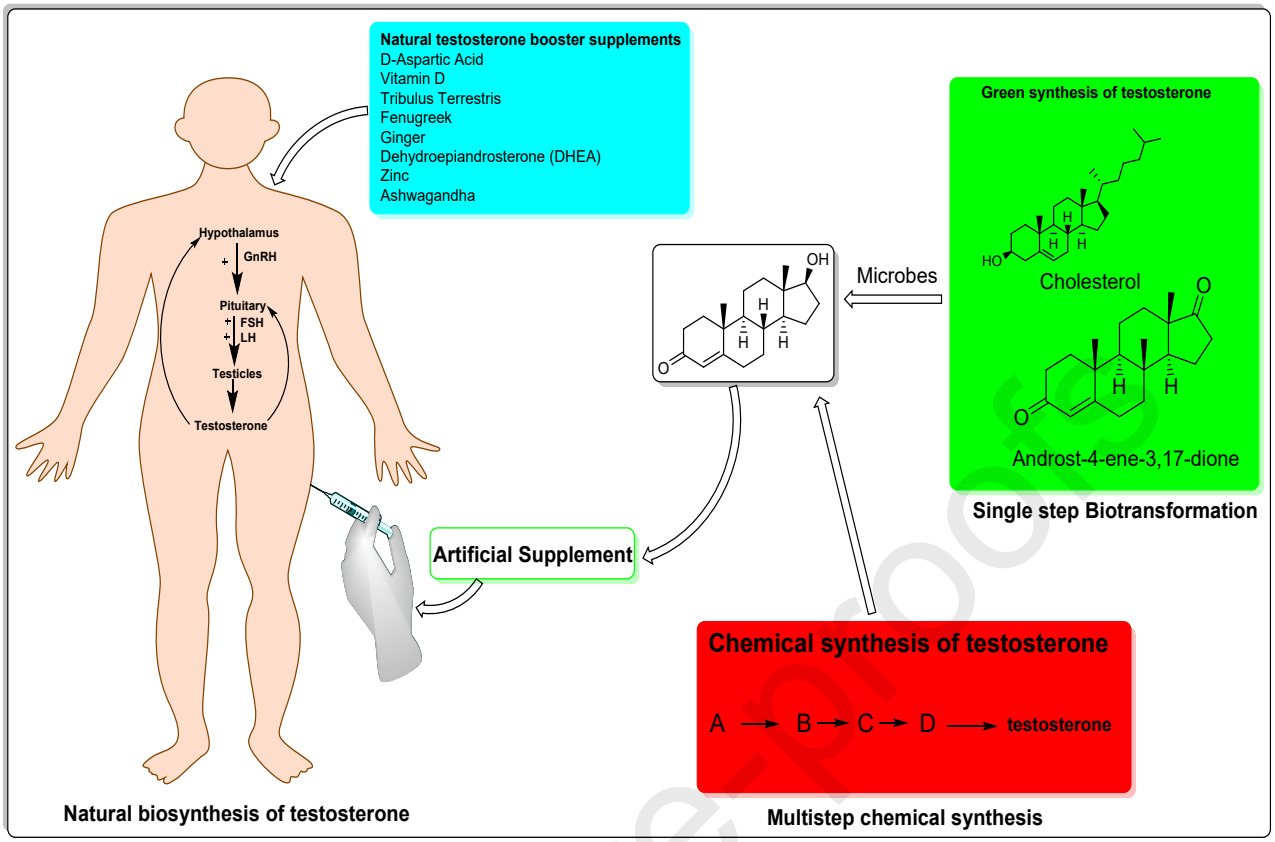

Fig. 3. Overall production of testosterone

3. Production of testosterone by microbial biotransformation

Many steroids and sterols have been reported (Table. 1) from microbial bioconversion since past half century for producing steroid analogues [1, 37]. These conversions include various reactions like hydroxylation, methoxylation, esterification, acylation, halogenation, isomerization, dehydrogenation/reduction, and hydrolyzation or side-chain cleavage at all carbon atoms of the four basal ring structure of steroids except C-10 and 13 [2]. Chemical cleavage is not appropriate/ suitable for the modification of basic steroid ring structures since they are sensitive to it [38]. Reagents such as sulphur trioxide, pyridine, and selenium dioxide are often required for chemical synthesis which contributes towards the hazardous ill-effects to the environment due to their disposal issues which makes chemical synthesis un-eco-friendly [39]. This shows that the transformation through microbes is more appropriate and advantageous over chemical synthesis as it gives higher conversion rates, higher specificity, runs in mild pressure and temperature conditions, thus being an effective alternate to chemical production [1, 2]. Different microorganisms have been studied for production of TS and its derivatives that are not readily accessible by chemical synthesis [16] such as

filamentous fungi [17], yeasts [18], bacteria [19] and plants [20]. They have also shown synthesis of TS from enzymatic reduction of AD by 17β-HSD [21].

As per the reports by Wang et al. the microbial transformation of cholesterol by a strain of *Mycobacterium* sp. grown in 4% glucose supplemented nutrient broth resulted in the formation of TS [40]. Various other studies by Llanes et al. and Hung et al. using different *Mycobacterium* strains also revealed TS as a principal produce of biological transformation [41, 42]. Borrego et al. studied about the conversion of cholesterol to TS by *Mycobacterium* sp. MB-3638 using saline medium with glucose and urea to significantly increase the yield of TS. The bioconversion was about 98% in 72 h of cultivation till 144 h and superior to the ones reported earlier [3]. However, Fernández-Cabezón et al. used engineered *M. smegmatis* for TS production by cloning and overexpressing two genes encoding microbial 17β-hydroxysteroid: NADP 17-oxidoreductase, from the bacterium *Comamonas testosteroni* & fungus *Cochliobolus lunatus* with *M. smegmatis* wild type and a genetically engineered AD producing mutant as the host strains. These recombinant strains were able to produce TS from AD with high yield when grown in minimal medium with cholesterol as substrate and glycerol as carbon and energy source. The bioconversion was achieved approx. 80% in 69h [5]. Using mutants of *Mycobacterium* sp. treated with ethyl methane sulfonate or mitomycin C, Egorova et al. did the production of AD as a major product from sitosterol with a yield of 70-75% and *Mycobacterium* sp. NRRL B-3683 mutant having the capability to convert the produced AD into TS [43, 44]. Reduction of AD to TS mediated by yeast was investigated by Singer et al. in an aqueous media with natural and chemically modified Cyclodextrins [45, 46]. Thus, for the synthesis of pharmacologically active TS, AD is an important initiating compound. Liu and Lo biotransformed cholesterol by *Mycobacterium* sp. in a single step process to reduce AD into TS resulting 51% of molar conversion rate after 120h of cultivation by glucose metabolism in fermentation culture [47]. A single step microbial biotransformation was carried out by Lo et al. for the production of TS from phytosterol by a mutant ST2, derived from phytosterol assimilating and AD producing, *Mycobacterium* sp. B-3805S. A synthetic medium having phytosterol (0.1%), glucose (2%) and peptone (1%) with increased DO initially, favoured side chain degradation of phytosterol to AD. Later, its decrease to zero resulted in reduction of AD to TS with 31% conversion ratio of phytosterol to TS via AD [6]. Another *Mycobacterium* mutant strain VKM Ac-1816D was studied by Egorova et al. with high levels of 17-HSD activity for the redox conversion of C-19 steroids AD, ADD, TS, etc. It was capable to biotransformed sitosterol (5g/L) into TS with 50-55%

molar yield. The multienzyme oxidation of side chain and reduction of 17-keto group of 3, 17-di-ketoandrostene by 17-HSD, results in the formation of TS from β-sitosterol [4]. Using *Lactobacillus bulgaricus* in an aerated fermenter, Kumar et al. performed the biotransformation of cholesterol for production of TS by reducing AD in a glucose supplemented medium with maximum accumulation of 1.56mmol/l in 96h using 4% glucose [48]. Microbial production of TS was carried out by Faramarzi et al. by transformation of AD from a fungus, *Aspergillus terreus* PTCC 5283 which resulted in formation of two metabolites, TS and testololactone after incubation of 3 days at 27°C [49]. Earlier, these two metabolites were isolated by Mostafa & Zohri [50] from progesterone side-chain biodegradation in *Aspergillus flavus.* Transformation of progesterone was also done by some zoosporic fungi for the production of TS and testololactone as per the reports of Khallil & Mostafa [51]. Using live cells of *Mycobacterium* sp. NRRL B-3683, Lee and Liu reported the production of ADD from cholesterol with maximum productivity of 0.19 g/1 per day and 77% molar conversion was achieved when 1.0 g/1 of cholesterol added into the reaction medium [52]. Earlier, Oshima et al. reported the use of immobilized cells for ADD production [53]. To selectively degrade the side-chain of cholesterol by biotransformation, newly isolated actinomycete, *Gordonia neofelifaecis* (NRRL B-59395) was used by Liu et al. for producing ADD. The highest conversion rate of 87.2% was shown through side chain degradation by completely consuming cholesterol [54]. Guevara et al. metabolically engineered *Rhodococcus ruber* Chol-4 for the production of TS from AD with 61% molar conversion by use of glucose as cofactor regenerator while 91% of TS was extracellularly recovered after 3 days of cell biotransformation [55].

Therefore, manipulations in TS forming substrates through biotransformation by microbes as well as improvement through genetic engineering techniques can be carried out for the production of TS hormone at higher scale to cope up with its necessity in the pharmaceutical market for synthesis of potent steroid drugs.

Table 1.

Microorganisms reported for the biotransformation of sterols for the production of steroids and their analogues.

Micro-organism	**Substrate**	**Product**	**Reference**
Mycobacterium sp.	Cholesterol	TS	[40, 41, 42]

Mycobacterium sp. MB-3638	Cholesterol	TS	[3]
Mycobacterium sp. NRRL B-3683 mutant	AD	TS	[43]
Engineered *M. smegmatis*	17β-HSD, Cholesterol, AD	TS	[5]
Mycobacterium sp.	Cholesterol, AD	TS	[47]
Lactobacillus bulgaricus	Cholesterol, AD	TS	[48]
Yeast	AD	TS	[45]
Aspergillus terreus PTCC 5283	AD	TS, Testololactone	[49]
ST2 mutant derived from *Mycobacterium* sp. B-3805S	Phytosterol, AD	TS	[6]
Aspergillus flavus	Progesterone	TS, Testololactone	[50]
Zoosporic fungi	Progesterone	TS, Testololactone	[51]
Mycobacterium sp. mutant	Sitosterol	AD	[44]
Mycobacterium mutant strain VKM Ac-1816D	17β-HSD, β Sitosterol	TS	[4]
Mycobacterium sp. NRRL B-3683	Cholesterol	ADD	[52]
Gordonia neofelifaecis (NRRL B-59395)	Cholesterol	ADD	[54]

4. Pharmacological activity of testosterone

From the last few decades, steroid compounds are amongst the widely marketed chemicals by pharmaceutical industries due to their diverse physiological roles in human bodies [1]. The binding of steroid hormones to their respective intracellular receptors, acting as transcription factors has traditionally been associated with their therapeutic action for the regulation of

gene expression [54]. The steroid compounds manufactured exhibit a wide range of therapeutic properties like immunosuppressive, diuretic, anti-inflammatory, anabolic, progestational and contraceptive agents [1, 56]. TS is used as a male birth control agent, for development of male sex organs as well as for the treatment of menopause in men [25] while muscle growth and maintenance are promoted by its anabolic properties. Some common problems in males like loss of lean body and bone mass, weakness, lethargy, low energy, depression, decreased libido, frailty and impotence are associated with production of lower level of TS in body than the normal levels [57]. A potent androgen produced from TS by 5α-reductase is DHT responsible for prostate growth. For treating benign prostate hyperplasia and prostatic hypertrophy, inhibitors of DHT expression are used [2, 25, 58]. Presence of TS at constant levels and an intact stable stroma helps in the normal functioning and maintenance of glandular structure in adult prostates because slight modifications in TS and stroma can lead to prostate carcinogenesis [23, 59]. Numerous microbial isolates have been known to produce a wide range of modified steroid derivatives which are pharmacologically active by modifying them through hydroxylation at different ring positions. Thus, these microbial enzymes are of great significance to steroids [60]. In 2004, an intramuscular TS undecaonate steroid compound entered into the market and achieved a great response for the treatment of hypogonadism. Initially it was given orally but gained more popularity after injectable preparations were made using tea seed oil as a vehicle by Chinese investigators. Normal range of serum levels were observed in volunteering hypogonadal men for many weeks [61] and since then, 1000mg ampoule preparations have been licensed in many countries with latest approval of 750mg by the Food and drug administration [10].

5. Role of fermentation in production of testosterone

Fermentation has played an essential role in the production of natural products from natural resources. Presently, the use of microbial transformed processes is widely accepted by the pharmaceutical industries. Therefore, applying fermentation process for the production of TS can help reduce the excess time taken in chemical reactions; avoid laborious process, non-specific, multistep reactions, harsh reaction conditions; and production of unwanted side-by-products, etc. Various microorganisms have been engineered successfully for TS biosynthesis using metabolic engineering. As seen in Fig. 4 representing different ways leading to steroid synthesis, the conversion of cholesterol to TS by whole cell would be an important approach for TS biosynthesis [1, 15].

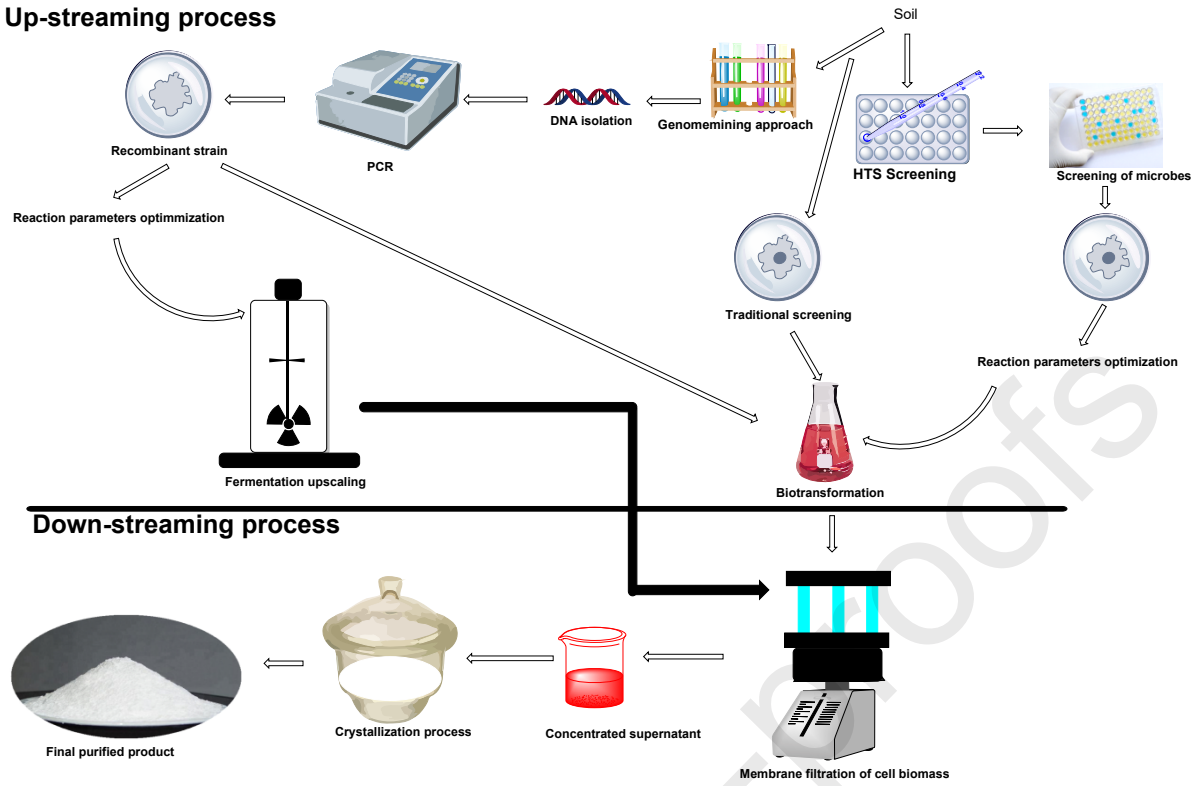

Fig. 4. Illustration of fermentation process for the production of steroids.

Therefore, in order to produce TS on a larger scale, techniques like metagenomic engineering, HTS of efficient strain must be performed before the fermentation process. These techniques can help in TS production on large scale making industrial processing lead towards green synthesis for betterment of the environment [62].

6. Conclusion

To conclude, TS is a pharmaceutically significant, principal male steroid with high therapeutic value. Many chemical synthesis methods have been developed for steroid production but due to asymmetric centres in TS molecule, its synthetic synthesis becomes a difficult and non-specific process. Presently microbial transformation is trending among the pharmaceutical industries therefore, to alternate the chemical route, microbial biotransformation is preferred as a suitable method for TS production providing high specificity, efficient yield and an emerging step towards green synthesis.

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Declaration of competing interest

The authors report no declarations of interest.

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Highlights

- **Microbial biotransformation provides alternate route for synthesis of testosterone.**
- **Microbial biotransformation provides green synthesis of steroids**
- **Cholesterol is an essential skeleton molecule for the production of TS**