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Microbial conversion of steroid compounds: recent developments

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Abstract

Steroid compounds can be ranked among the most widely marketed products from the pharmaceutical industry. Highly specific reactions are required to produce functionalized compounds with therapeutic use and commercial value. The complexity of steroid molecules renders the use of biocatalysts particularly interesting, due to the high regio- and stereo-selectivity of the reactions to be performed. These characteristics, together with the mild conditions required for bioconversions, led to the development of high yield biological production processes, which are more environmentally friendly than their chemical synthesis counterparts, a feature that is presently a major concern of industrialists. Although some of these bioconversions are well-established, efforts are ongoing in order to increase the efficiency of the existing processes as well as to identify new potentially useful bioconversions. In this work an overview of recent developments on the use of microorganisms for steroid production is presented.

Keywords: Steroid compounds; Biotransformation; Cell immobilization; Non-conventional media

1. Introduction

The production of steroid drugs and hormones is one of the best examples of the successful application of microbial technology in large scale industrial processes. Steroids are structurally derived from cyclopentanoperhydrophenantrene (sterane) (Fig. 1). The research efforts in this field were triggered around 1950, with the announcement of the pharmacological effects of cortisol and progesterone, two endogenous steroids, and with the identification of the 11α-hydroxylation activity of a *Rhizopus* species, a decisive step in the development of the practical synthesis of steroids with useful biological activity [1]. Several microbial bioconversion of steroids and sterols have been reported ever since [2–8], focusing mainly on steroid hydroxylations, Δ^1-dehydrogenation and sterol side-chain cleavage. These biotransformations, mostly associated to chemical synthesis steps, have provided adequate tools for the large scale production of natural or modified steroid analogues. The latter are currently favored when compared to their natural counterparts due to some therapeutic advantages, such as an increased potency, longer half-lives in the blood stream, simpler delivery methods and reduced side effects. The preferential use of whole cells over enzymes as biocatalysts for the production of these pharmaceutical derivatives mostly results from the costs of the latter enzyme isolation, purification and stabilization [9]. The manufactured steroid compounds have a wide range of therapeutic purposes, namely as anti-inflammatory, immunosuppressive, progestational, diuretic, anabolic and contraceptive agents [1–4,6,7,10–12]. They have also been successfully applied for the treatment of some forms of breast and prostate cancer and osteoporosis, as replacement agents in the treatment of adrenal insufficiencies [13,14], in the prevention of coronary heart disease [15,16], as anti-fungal agents [17,18], as active ingredients in anti-obesity agents [19], and in the inhibition of HIV integrase, prevention and treatment of infection by HIV and in the treatment of declared AIDS [20]. Recently, a steroidal glycoside, torvoside H, isolated from the fruits of *Solanum turvum*, exhibited anti-viral activity on herpes simplex virus type 1 [21]. The therapeutic action of steroid hormones has been traditionally associated to their binding to the respective intracellular receptors, which act as transcription factors in the regulation of gene expression [22]. In the last decade, however, considerable evidence has emerged suggesting that some steroids, such as dehydroepiandrosterone (DHEA) and dehydroepiandrosterone sulfate (DHEAS) [23], progesterone, pregnenolone and their sulfate derivatives [24,25],

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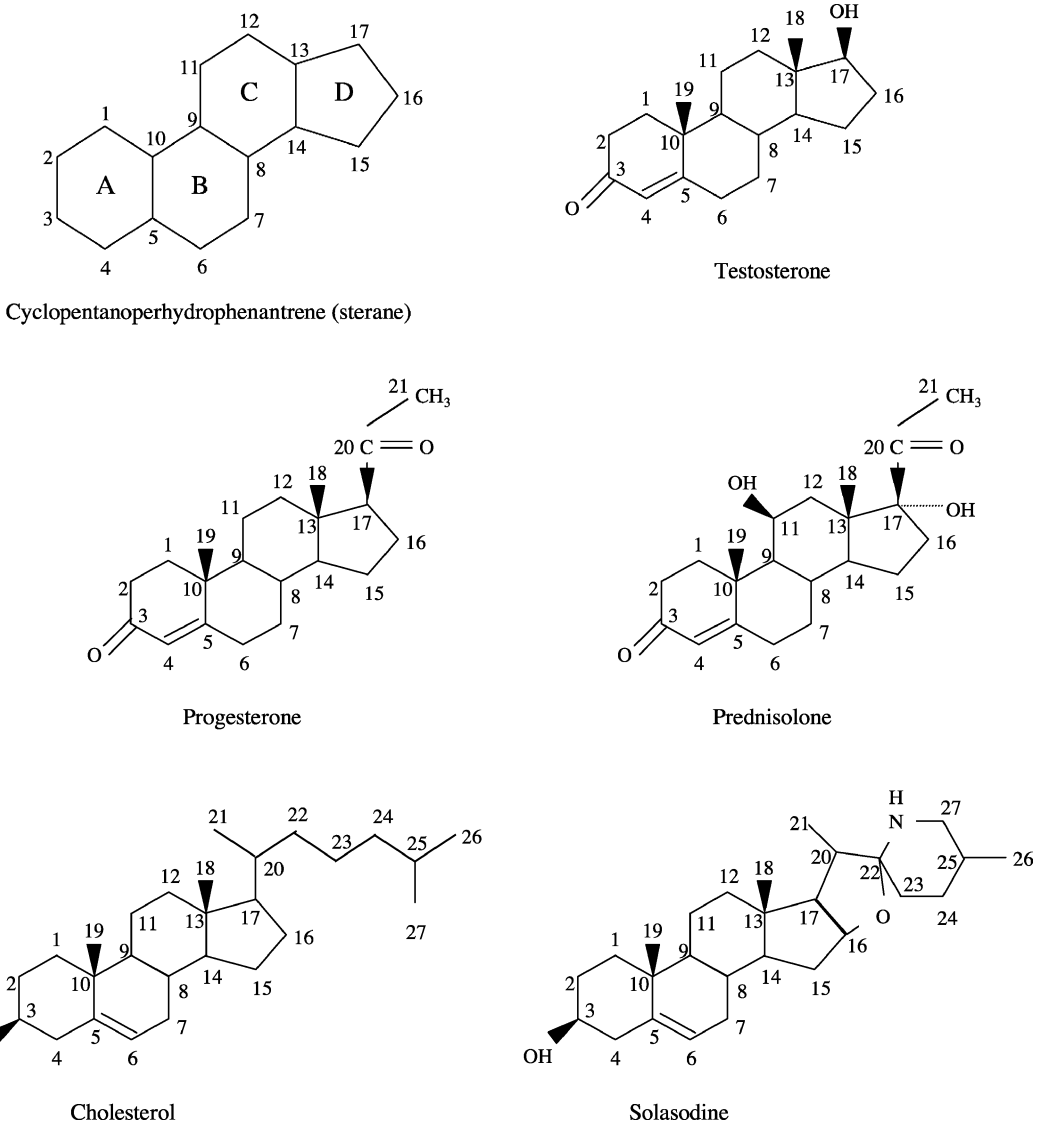

Fig. 1. Sterane, the parent ring structure of steroid compounds. Also shown are the structures of some representative steroids.

17β-estradiol, allopregnanolone and synthetic alphoxolone (3α-hydroxy-5α-pregnan-3,20-dione) and ganaxolone (3α-hydroxy-3β-methyl-5α-pregnan-20-one) [24], could behave as allosteric modulators of neurotransmitter receptors, therefore altering neuronal excitability through direct interaction with the cell surface [22]. Such steroids, synthesized by glial cells and present in high concentrations in the central or peripheral nervous systems, are designated neurosteroids [24,26]. Recent research work has highlighted the role of neurosteroids as memory-enhancers [27,28], as inducers of endocrine response to stress [24], as anxiolytic agents [24], as anticonvulsants [29] and antidepressives [24], as well as their neuroprotective effect [24], particularly against oxidative stress-induced damage [30] or from DNA damage in the brain induced by exposure to endogenous and environmental mutagens [31]. Progesterone and pregnenolone have also been shown to reduce damage affects from spinal cord injury [32,33]. Progesterone has also been shown to play an important role in myelin repair, following nerve lesion [26,34]. In addition to their action at the neurotransmission level, neurosteroids can also affect gene expression through classic interaction with intracellular nuclear receptors [24]. Another reported application of steroid-like compounds consists of the use of steroidal cationic lipids as a tool for gene delivery. The DNA/lipid complexes used contained the plasmid pCMV-Luc, encoding firefly luciferase, and were expressed in mouse melanoma cells [35]. Although research developments have led to the identification of a vast array of potentially useful steroid compounds, possibly exceeding 5000, efforts are continuously being made both for the discovery of novel steroid compounds and the isolation of microorganisms capable of performing the required steroid transformations. In addition to production cost issues, the preferential use of whole cells over enzymes for the

production of these chemicals is increasing, resulting from the necessity of co-factor regeneration and the possibility of multi-step conversions with a single biocatalyst [9]. The improvement of the steroid biotransformation activity of currently used strains, as well as the manipulation of metabolic pathways of sterol producing strains through genetic engineering techniques, are presently being carried out, together with research efforts towards an increased understanding of the mechanisms of sterol transport across biocatalyst membranes. Current developments at the level of process productivity include rational design of fermentation media, improvements in the solubility of lipophilic substrates and biocatalyst immobilization, easing downstream processing and the setting up of continuous reaction processes.

2. Chemical versus biochemical processes

The complex structure of the steroid molecule requires complicated, multi-step schemes for the chemical synthesis of steroid compounds. It often involves the preparation of intermediate derivatives with protected groups and their subsequent regeneration, once the intended reaction has occurred, limiting the overall process yield and making it expensive and time consuming. Furthermore, the basic ring structure of some steroid derivatives is sensitive to cleavage by a wide variety of chemicals [36]. Chemical synthesis also requires the use of reagents such as pyridine, sulfur trioxide or selenium dioxide which are hazardous to the health of production staff and constitute a serious environmental disposal problem [37,38]. Microbial steroid conversions are performed in mild temperature and pressure conditions and can provide an efficient alternative to chemical synthesis for the development of manufacturing processes, once the limitations often encountered of unsatisfactory productivity and/or purity levels of the conversion products are overcome. The steroid industry thus couples the chemical and biological approaches taking advantage of the best aspects of each. A global overview, where this is evidenced, is given in Figs. 2–5.

3. Production of steroid precursors: raw materials and sterol side-chain cleavage

Phytosterols, together with sapogenins such as diosgenin, hecogenin and solasodine, are established in the pharmaceutical industry as starting materials for the production of steroid hormones (Fig. 2). Stigmasterol, β-sitosterol and campesterol are the most commonly used phytosterols, since the major pathways in advanced vascular plants leads to a sterol content profile mainly constituted by these three sterols [41]. The development of economically feasible processes based on these substrates results both from their low cost and from the ease of their transformation into steroid intermediates. Stigmasterol and crude sitosterol (a mixture of β-sitosterol and campesterol) are extracted mainly from soybeans and separated by crystallization [1]. Stigmasterol is usually chemically oxidized to progesterone [7,42], since its C22 double bond has a depressing effect on the specific degradation activity of commonly used microbial strains [43]. Cholesterol, another employed starting material, obtained from animal fats and oils, such as lard, beef tallow, milk fat or fish oil [44] and sitosterol are microbially cleaved to 17-ketosterols, mainly 4-androstene-3,17-dione (AD), 9α-hydroxy-4-androstene-3,17-dione (9α-OH-AD) and 1,4-androstadiene-3,17-dione (ADD) [1–7]. However, the production of testosterone from cholesterol using a single strain, either *Mycobacterium* sp. NRRL B-3805 [45,46] or *Lactobacillus bulgaricus* [47], has also been reported. The metabolism of glucose supplemented to the fermentation medium provided the reducing power, NADH, required to reduce AD to testosterone [46]. The effect of glucose supplementation to the bioconversion medium for the reduction of AD to testosterone, as well as the need for controlling the pH of the same medium, was also highlighted by Llanes et al. [48]. The metabolic pathways of the side-chain cleavage of β-sitosterol, campesterol, cholesterol and their 19-hydroxy derivatives and 3β-acetoxy-19-hydroxycholest-5-ene, with *Mycobacterium* sp., *Rhodococcus* sp. and *Moraxella* sp. have been proposed [49–53]. Other phytosterols, such as lanosterol [54], lanosterol derivatives [55] and ergosterol [56] have proved to be promising substrates for steroid production. Some examples of sterol side-chain cleavages by microorganisms are given in Table 1.

In recent years cheaper sources of sitosterol have been looked for in order to further reduce production costs [7]. Sitosterol was successfully isolated from tall-oil, a waste effluent from the paper industry, and a purified fraction of the isolate was microbially transformed to steroid intermediates in yields comparable to the use of pure sitosterol [61]. A procedure for the isolation and purification of a sitosterol-rich phytosterol mixture from pulping soap, a by-product upstream from tall-oil, was recently reported [15]. Wastes from the sugar cane industry were also found to provide a source for an easily bioconvertible β-sitosterol/stigmasterol-rich mixture, once a *Mycobacterium* mutant strain with a side-chain cleavage activity non-inhibited by stigmasterol was isolated [62]. The affinity of microbial biocatalysts for different sterol substrates, already evidenced in the pioneer reports by Marshek et al. [63], was recently further highlighted in the work of Ahmad et al. [64], Slijkhuis and Marx [65] and Barthakur et al. [66]. Thus, the effective use of sterol mixtures isolated from wastes for steroid synthesis clearly depends on the final mixture composition.

Other efforts have been made in order to enhance the productivity of organisms producing steroid precursors. Oversynthesis of ergosterol during continuous fermentation of *Candida maltosa* [67] and of β-sitosterol, campesterol, stigmasterol in cultures of flax [68] was achieved through optimization of fermentation parameters. Optimization of steroidal sapogenins accumulation in callus cultures of

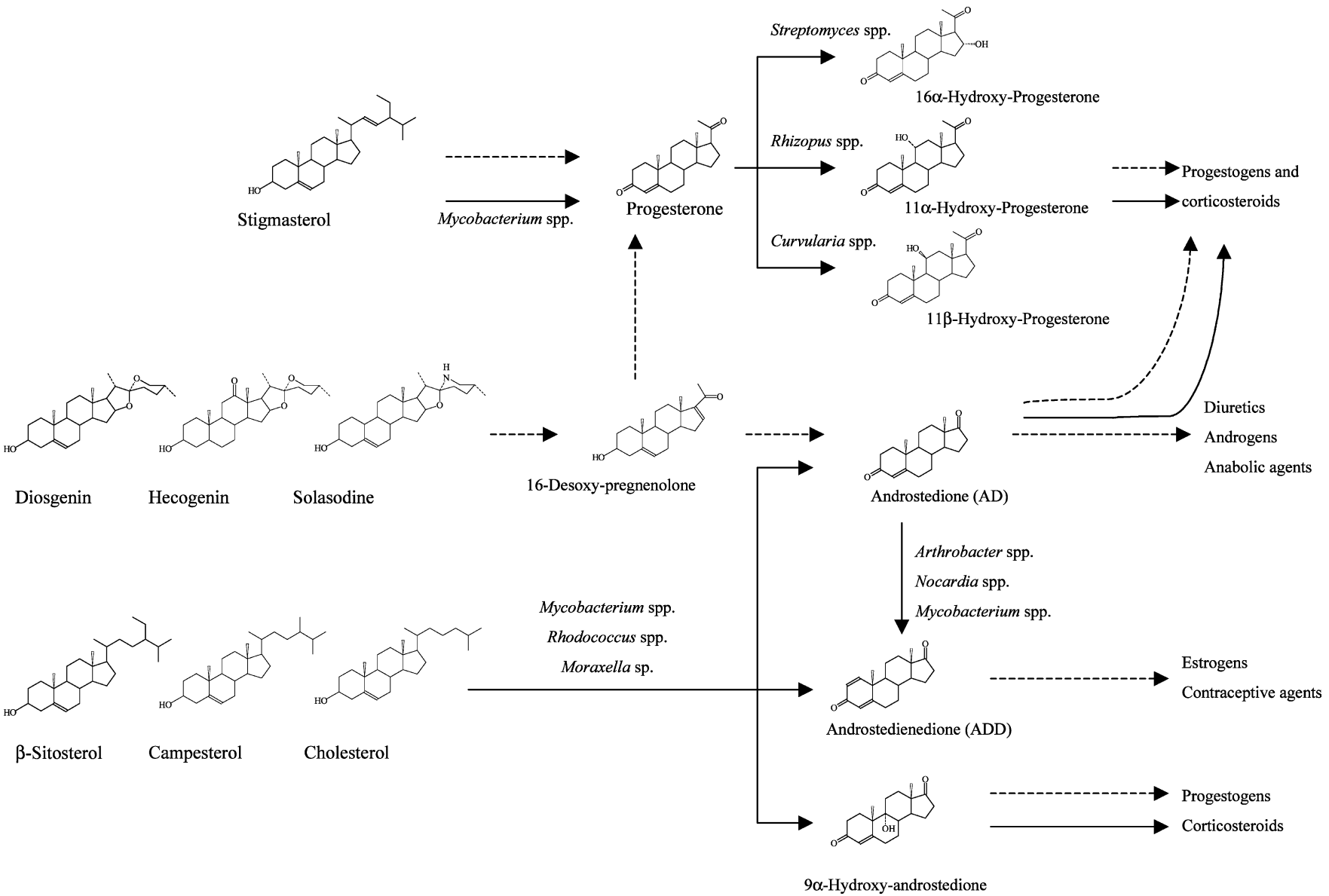

Fig. 2. An overview of steroid production, from raw materials to finished products. Full lines indicate bioconversion whereas dashed lines indicate chemical transformation [1–7].

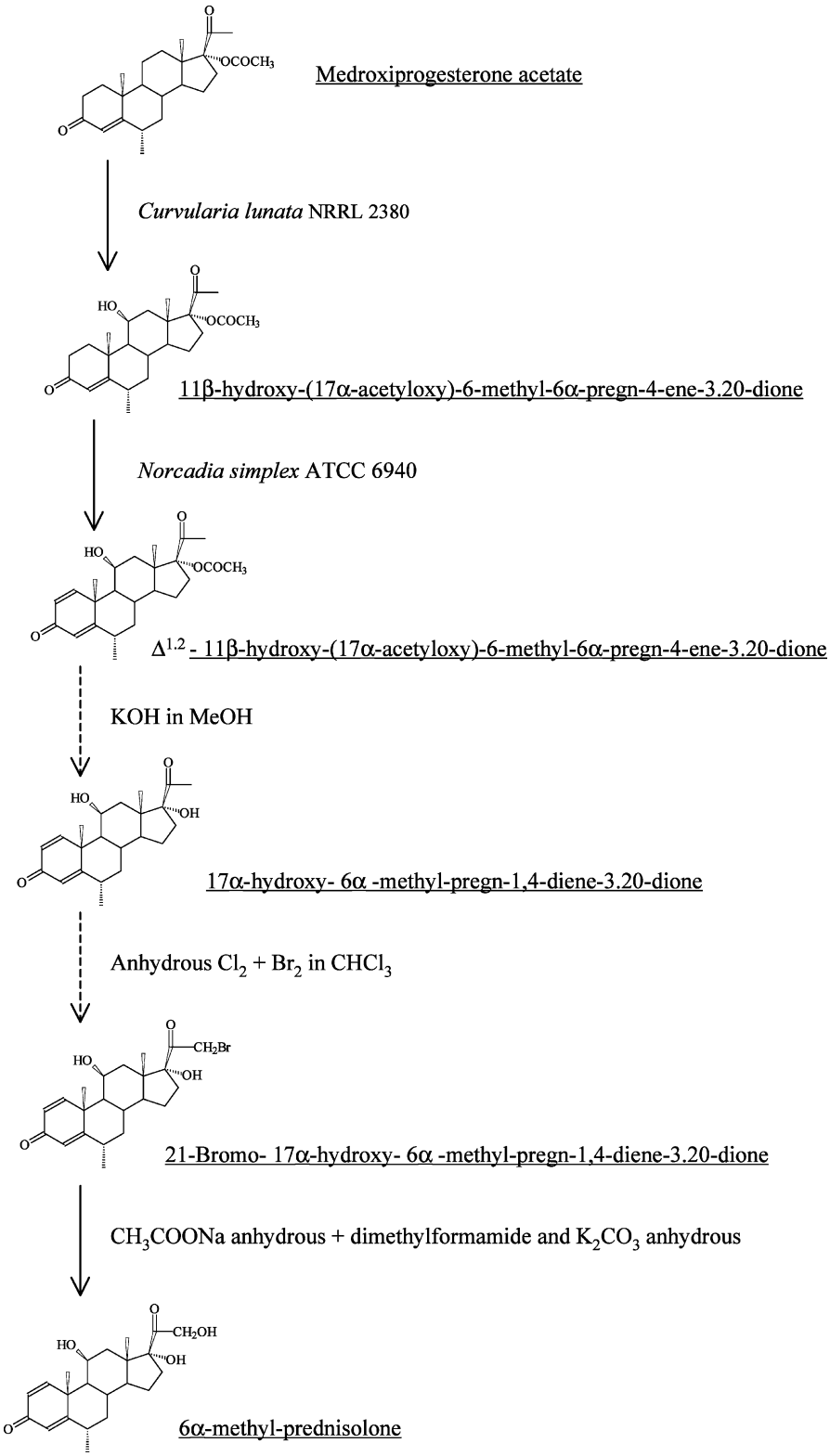

Fig. 3. Combined chemical and microbial methods for steroid production: manufacturing 6α-methyl prednisolone. Full lines indicate bioconversion whereas dashed lines indicate chemical transformation [38].

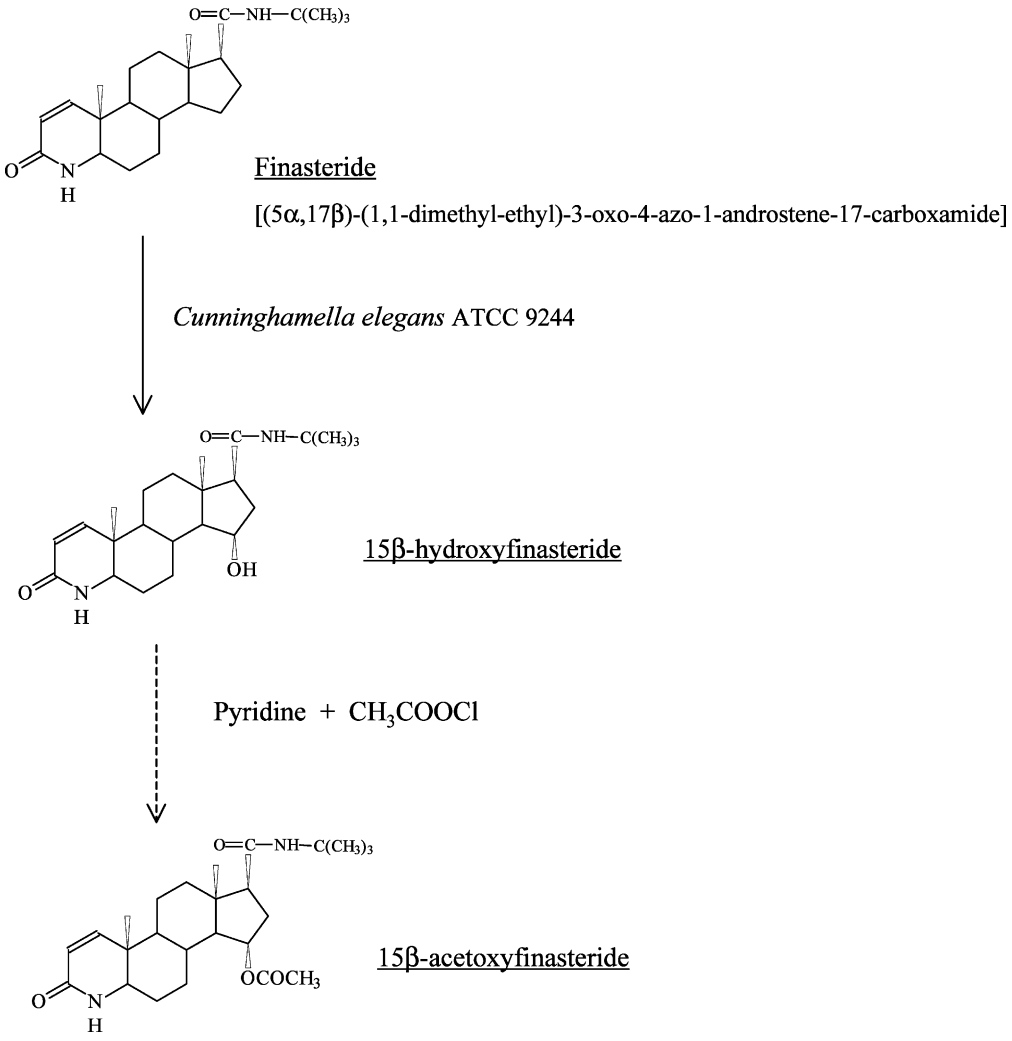

Fig. 4. Combined chemical and microbial methods for steroid production: manufacturing 15β-acetoxyfinasteride. Full lines indicate bioconversion whereas dashed lines indicate chemical transformation [39].

Table 1
Fermentative production of 17-ketosteroids

Substrate ($g l^{-1}$)	Microorganism	Main product	Molar yield (%)	Reference
Lanosta-7,9(11)-dien-3β-ol (0.25)	*Mycobacterium* sp. NRRL B-3805	4,8(14)-Androstadiene-3,17-dione	30	[55]
3β-Acetoxy-19-hydroxycholest-5-ene (0.5)	*Moraxella* sp.	Estrone	15	[53]
Cholesterol (1.0)	*Mycobacterium* sp. NRRL B-3805	Testosterone	51	[46]
Ergosterol (0.3)	*Mycobacterium* sp. NRRL B-3805	4-Androstene-3,17-dione	35	[56]
	Mycobacterium sp. NRRL B-3683	1,4-Androstadiene-3,17-dione	30	[56]
α-Sitosterol (1.0)	*Mycobacterium* sp. NRRL B-3805	4-Androstene-3,17-dione	25	[57]
	Mycobacterium sp. NRRL B-3683	1,4-Androstadiene-3,17-dione	20	[57]
β-Sitosterol (1.0)	*Mycobacterium* sp. NRRL B-3805	4-Androstene-3,17-dione	90	[58]
β-Sitosterol (5.0)	*Mycobacterium* sp. VKM Ac-1815D ET1	4-Androstene-3,17-dione	72	[59]
Phytosterols (10)	*Mycobacterium* MB 3683	4-Androstene-3,17-dione	90	[60]
Phytosterols (30)	*Mycobacterium* MB 3683	4-Androstene-3,17-dione	80	[60]

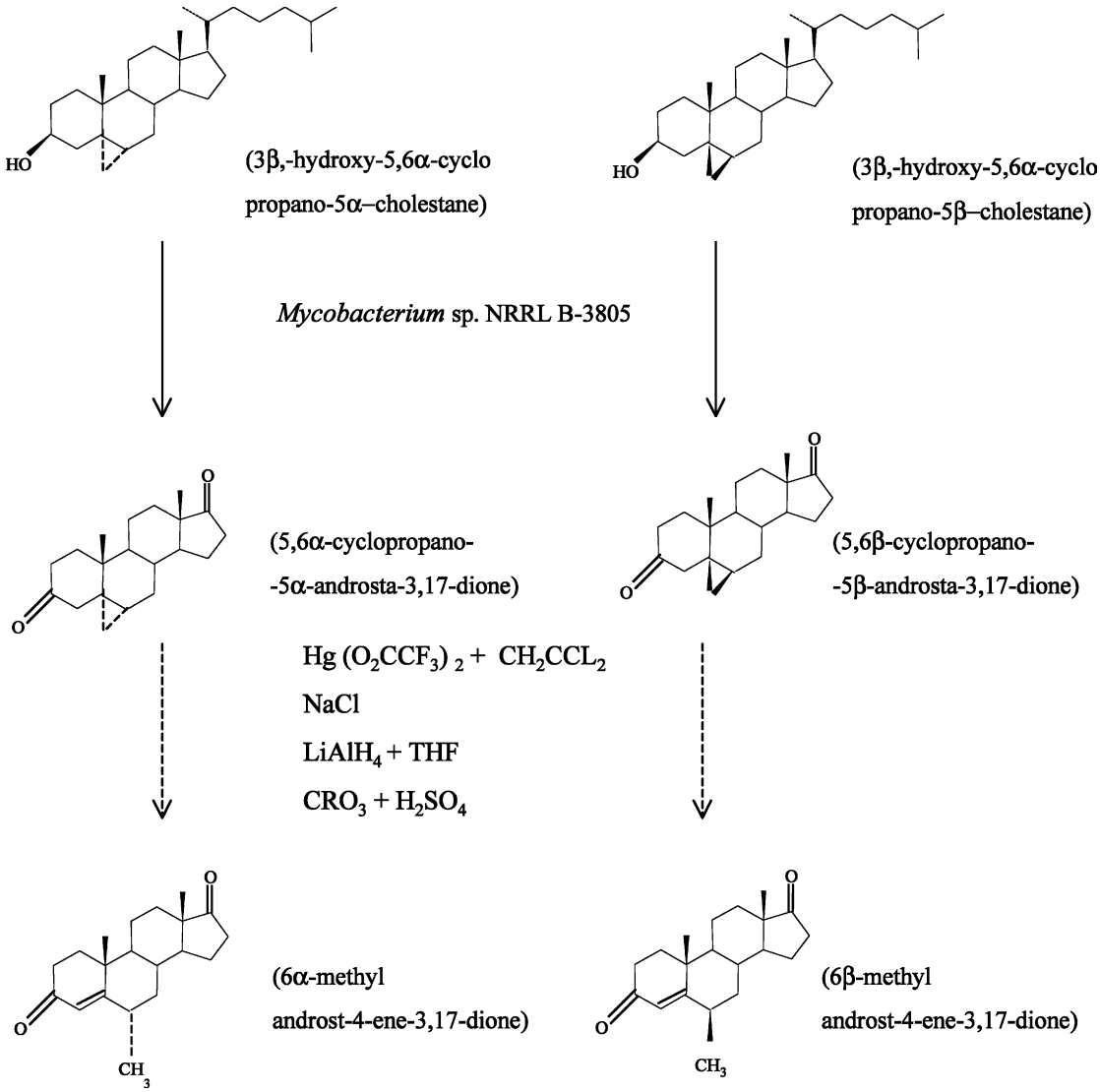

Fig. 5. Combined chemical and microbial methods for steroid production: a route to 6α-methyl steroids. Full lines indicate bioconversion whereas dashed lines indicate chemical transformation [40].

Agave amaniensis, through the evaluation of the effect of several ions, was performed using a central composite design [69]. Increasing the concentration of sucrose and phosphate added to modified Murashige and Skoog medium from 85 to 255 mg phosphate l^{-1} and from 20 to 40 g sucrose l^{-1}, led to shifts in the metabolic pathways of *Dioscorea deltoidea* cells from diosgenin (3.98 mg g^{-1} dry cell weight) to sitosterol (3.48 mg g^{-1} dry cell weight) [70].

4. Hydroxylation

Hydroxylations are possibly the most widespread type of steroid bioconversion. Hydroxylations can be used to build intermediates for further chemical synthesis, by offering access to otherwise inaccessible sites of the steroid molecule, or to provide the steroid molecule with the adequate structure for therapeutic applications [2,8]. Microorganisms able to hydroxylate steroids in positions C1 to C21 and in position C26 have been reported [2–8,71]. 11α-, 11β-, 15α and 16α-hydroxylations are currently established processes in the steroid industry [7,72] mainly for the production of adrenal cortex hormones and their analogues. 11α-, 11β- and 16α-hydroxylations are usually performed using *Rhizopus* spp. or *Aspergillus* spp., *Curvularia* spp. or *Cunninghamella* spp., and *Streptomyces* spp., respectively [2–8]. The oxygen group in position C11 is regarded as essential for anti-inflammatory action [8], whereas 16α-hydroxylated steroids have increased glucocorticoid activity [16]. Nevertheless, replacement of the 11β-hydroxyl group by a chlorine atom did not lead to a loss of affinity for the glucocorticoid receptor, thus enabling the manufacture of effective compounds for the treatment of skin disorders [16]. 11α-Hydroxylation is also used in the production of

an intermediate in the synthesis of Desogestrel, a contraceptive drug [72]. A detailed study on the effects of the morphology and physiology of *Rhizopus nigricans* pellets in the 11α-hydroxylation of progesterone was performed [73]. A repeated batch procedure revealed that the best maintenance of bioconversion activity resulted from pellets harvested at the active growth cultivation phase, cultured with high stirring speed from a low inoculum size. High conversion yields (above 90% for a substrate concentration of $0.3\,g\,l^{-1}$) were thus observed in two successive batch biotransformations. Conversion yields above 95% were observed in the 11α-hydroxylation of estr-4-en-3,17-dione (ED) and canrenone (CR) using *Aspergillus ochraceus*, for initial substrate concentrations ranging from 10 to $25\,g\,l^{-1}$ [74,75]. Surprisingly, however, the bioconversion yields obtained with substrates with purity levels between 90 and 95% exceeded by 15–20% those obtained with 98–100% pure substrates. The effect of medium composition on the formation of by-products, during the 11α-hydroxylation of 13-ethyl-4-gonene-3,17-dione by using *Aspergillus awamori*, was investigated and a mathematical model for estimating product yields was developed [76]. The 15α-hydroxylation of 13β-ethyl-4-gonene-3,17-dione, leading to an intermediate in the production of Gestoden, a contraceptive drug, has also been extensively studied [77,78]. The bioconversion was preferably performed by nor-AD induced *Penicillium raistrickii* [77]. The 15β-hydroxylation of finasteride by *Cunninghamella elegans* ATCC 9244 with a product yield of about 80% was reported [39]. The hydroxylated-finasteride derivative is a useful intermediate in the production of 4-aza-steroids, which are efficient inhibitors of testosterone 5-reductase. This enzyme is responsible for the reduction of testosterone to dihydrotestosterone, which is ultimately related to malignant conditions leading to prostate cancer. The 9α-hydroxylation of steroids also leads to intermediate compounds useful in the pharmaceutical industry, namely as starting materials for the production of 9α-halogen corticoids [8] and to 11-keto structures [7]. Resting *Rhodococcus* sp. cells have been used as catalysts for the bioconversion of AD to 9α-hydroxy-AD (9α-OH-AD) [79–81]. The increase in steroid transformation activities of non-induced *Rhodococcus* sp. cells was shown to depend on the contact time with the inducer substrate and to follow a consecutive pattern, 9α-hydroxylation activity developing earlier than Δ^1-dehydrogenation activity [80,81]. A strategy based on the addition of protein synthesis blockers to the growth medium before a significant development of the undesired Δ^1-dehydrogenase activity was thus proposed, as a means to improve the effectiveness of the 9α-hydroxylation process [80,81]. A process for the 7α-hydroxylation of DHEA and pregnelone (PRG) using *Fusarium moniliforme* mycelia has been recently presented [82]. The 7α-hydroxylated derivative of DHEA is claimed to be useful in certain cancers and Alzheimer disease therapies, to increase immune response and as an anti-obesity agent [82], besides presenting anti-glucocorticoid action [82,83]. Most of these therapeutic uses are shared by the 7α-hydroxylated derivative of PRG [82]. The 7α-hydroxylation enzyme complex is mainly microsomal and its constitutive activity is low [83], with bioconversion yields below 2% [84]. Induction with DHEA proved effective for both biotransformations and allowed product yields of 80 and 65% for the 7α-hydroxylated derivatives of DHEA and PRG, respectively, both from a $0.4\,g\,l^{-1}$ substrate concentration [82]. The higher affinity of the 7α-hydroxylase of *F. moniliforme* to DHEA as compared to other 3-hydroxysteroids, namely PRG, epiandrosterone and estrone, was further evidenced by Cotillon and Morfin [84]. Lower bioconversion yields and specific initial reaction rates, as well as higher Michaelis constants, were observed when the latter steroid molecules were assayed as substrate, as compared to DHEA [84]. Production of 7α-hydroxyestradiol using a Cyp7b steroid hydroxylase is also claimed [85]. The use of 7α-hydroxy steroids in the development of products for the diagnosis and therapy of neuropsychiatric or immune disorders has been claimed [85]. 7α-Hydroxy steroids may also play a role in the immune response [86].

7β-Hydroxylation of finasteride by *Mortierella isabellina* has also been reported, however, a low (20%) product yield was observed [39]. The hydroxylated derivative can lead to useful products in prostate cancer therapy.

The sites of biocatalyst action in steroid hydroxylation were shown to be dependent on the stereochemistry of the substrate [87]. *Cephalosporium aphidicola* preferably hydroxylated 13β-methyl steroids in positions C11α and C14α, whereas 13α-methyl steroids were hydroxylated in positions C1α and C7α [87]. The relation between the structural features of different steroid compounds and the site-specific 14α-hydroxylation of steroids was investigated. Progesterone was the most adequate substrate for the production of 14α-hydroxylated derivatives using *Thamnostylum piriforme* [88]. Structural features also played a major role in the hydroxylation of 3α,5-cycloandrostanes using *C. aphidicola* [36].

Compelling evidence has been gathered identifying cytochrome P450 as responsible for steroid hydroxylation reaction in both bacteria and filamentous fungi (Table 2). However, each given whole-cell hydroxylation of steroid molecules is often hampered by the formation of by-products, a drawback which could possibly be overcome if a detailed knowledge of the mechanism of the metabolic pathway of the bioconversion could be obtained. In a work focusing on the 16α-hydroxylation of progesterone with *Streptomyces roseochromogenes*, Berrie et al. observed the formation of a secondary dihydroxylated by-product, 2β,16α-dihydroxyprogesterone, produced in a 1:3.6 ratio to the main product, 16α-hydroxyprogesterone [94]. Using a reconstituted protein system, obtained from purified cell extracts *S. roseochromogenes*, composed of cytochrome P450, together with a ferredoxin-like redoxin and a flavin-containing redoxin reductase, two electron transfer proteins, these authors were able to change the ratio

Table 2
Some examples of steroid hydroxylation reactions promoted by cytochrome P450

Steroid hydroxylation reaction	Microorganism	Reference
7α	*Fusarium moniliforme, Phycomyces blakesleeanus*	[82–84,89]
11α	*Aspergillus ochraceus, Aspergillus fumigatus, Rhizopus nigricans*	[90–92]
15α	*Penicillium raistrickii*	[93]
16α	*Streptomyces roseochromogenes*	[94]
6β	*Bacillus thermoglucosidasius*	[95]
11β	*Cochliobolus lunatus*	[96]
15β	*Bacillus megaterium*	[97]

of 2β,16α-dihydroxyprogesterone to 16α-hydroxyprogesterone to 1:10 [98]. This outcome was obtained provided the redoxin was purified from cells grown in the absence of progesterone. If otherwise, an isoform of the redoxin, formed as a result of progesterone dependent post-translational modification, led to the decline in the 16α-monohydroxy- to 2β,16α-dihydroxyprogesterone ratio.

5. Δ^1-Dehydrogenation

The introduction of a 1,2-double bond in the A ring of corticoids enhances their affinity for the glucocorticoid receptor, which, coupled to a synergistic effect with the 3-keto-Δ^4 group reducing its metabolic degradation rate, leads to compounds with increased therapeutic potency [10]. Microbial Δ^1-dehydrogenation is usually performed with whole cells. Since this is a co-factor-dependent reaction, the continuous regeneration of the necessary co-factor is ordinarily accomplished by the active cell machinery.

Δ^1-Dehydrogenation of 6α-methyl-cortisol to 6α-methyl prednisolone using *Arthrobacter globiformis* resting cells has been shown to follow a substrate inhibition type kinetics, where oxygen is a second substrate and the inhibition effect is exerted at the level of electron transfer along the respiratory chain. A mathematical model, based on multiple substrate inhibition, provided a good fit to the experimental data [99].

The use of a genetic algorithm for the optimization of a synthetic culture medium for the production of *Arthrobacter simplex* cells with high hydrocortisone-Δ^1-dehydrogenase activity, led to a 100-fold increase in the specific activity, based on dry biomass, as compared to currently reported data [100].

6. Aqueous or non-conventional media

The solubility in aqueous media of steroid and sterol compounds is usually below 0.1 mM and 1 μM, respectively [101]. This considerably limits the productivity of the biotransformation systems, where the initial substrate concentration hardly ever exceeds 10–20 mM. Furthermore, powdered substrates added to the fermentation medium tend to clump and are difficult to disperse [102]. Thus, the overall biotransformation rate of steroids in aqueous media was considered to depend not only on the specific activity of the biocatalyst, but also on the dissolution rate of the solid substrate [103,104]. Assuming a pseudo-steady-stationary state the rate of substrate dissolution and the biotransformation rate could be equated Eq. (1) [104].

$$r(s) = KA(S^* - s) \quad (1)$$

In Eq. (1), K is a mass transfer coefficient, A the total effective area for mass transfer (considered to be directly proportional to the concentration of solid substrate), S^* the solubility of the substrate, s the substrate concentration in solution and $r(s)$ is the rate of biological substrate consumption.

Conventional approaches to minimize this mass transfer limitation include the use of micronized substrates [60,105–107], and the feeding of substrates in the form of saturated solutions in water-miscible solvents, such as dimethylformamide [37,55,57,73,108–111], methanol [94], acetone [112–114] or 1,2-propanediol [115]. The amount of co-solvent added usually has to be kept below 1.5–5% (v/v), to prevent biocatalyst deactivation. Other feeding strategies are the use of substrate suspensions in surface active agents such as Tween 80 [62,65,116], Triton X-100 [117], lecithins [58,112], polypropylene glycol [60], silicone [60], and the addition of cyclodextrins with the formation of substrate inclusion complexes [118–123]. In the latter case, the resulting biocatalytic activity was shown to depend on the chemical structure of the cyclodextrin and the mode of its addition [121]. Complex formation between steroid drugs and cyclodextrins can be obtained for adequate concentrations of these chemicals, and results in the lowering of the observed hydrophobic nature of the steroid [124]. However, although the formation of such complexes facilitates sterol solubilization, it can be expected that the adsorption and uptake properties of the complex in biological systems differ from those of the uncomplexed drug. Vegetable oils are often used as solubilizing agents in microbial fermentations, however its use was found to be detrimental to the yield of AD in mycobacterial degradation of sitosterol [60].

Recently, it was observed that the overall specific bioconversion rate of sitosterol side-chain cleavage could be enhanced if cell wall defective mycobacterial cells were used, clearly suggesting that the permeation rate of the substrate through the cell envelope could be the rate limiting process [125]. Using *Mycobacterium vaccae* or derived strains, enhanced sterol penetration through the cell envelope was achieved when vancomycin [126], glycine [127], lecithin [58] and polycations, such as protamine, polymyxin B nonapeptide or polyethyleneimine [128], were added to the fermentation medium. Vancomycin and glycine tampered with the cell wall peptidoglycan layer [125], by shifting the molar

ratios of some of the constituents, namely increasing the muramic acid to diaminopimelic acid molar ratio, indicating a reduction in the cross linking level between peptide units [126,127]. Glycine was also responsible for changing the relative proportion of mycolic acids to other lipids in the strain used [127]. Lecithin interfered with the fatty acid profile [58,128] of the cell envelope. As a result, the relative specific activity of specific sterol side-chain cleavage to AD and ADD of the treated cells could be increased by up to three-fold. Protamine, on the other hand, was recently shown to alter the relative proportion of the non-covalently bound lipids of the outer leaflet of the mycobacterial cell wall bilayer, through a shift in their fatty acid composition [128]. Irrespective of the cell wall structure affected by the added chemical, its action resulted in the disturbance of the integrity and fluidity of the cell wall bilayer. This led to an enhancement in the production of 17-ketosteroids from β-sitosterol [58,126,127], with an up to three-fold increase in the specific side-chain cleavage cell productivity, even though a decrease in biomass production was usually observed [126–128]. Changes in the composition of the mycobacterial cell wall have also been shown to result from the nature of the carbon source used for cell growth [129]. Cultivation on either glycerol or fructose led to an enhancement in the hydrophobic nature of the cells, as a result of the paraffin synthesis, as compared to what was observed with glucose, when no paraffins were quantified. On the other hand, glycerol-grown cells had a considerably thinner cell wall (a reduction of around 40%) as compared to glucose- and fructose-grown cells, an effect which still requires further studies. Although these observations on the effect of the carbon source on the mycobacterial cell wall nature were not correlated to sterol side-chain cleavage activity in the paper by Borrego et al. [129], they could be related to the increased product yields reported by Dias et al., observed when glycerol-grown cells were used to cleave a sitosterol-rich fraction of tall-oil to AD, as compared to fructose-grown cells [130]. Rajkhowa et al. brought further evidence on the influence of the nature of the cell wall in the mechanisms of sterol uptake, while evaluating the growth of *A. simplex* SS-7 in the presence of β-sitosterol [131]. Although the production of an extracellular sterol-pseudosolubilizing protein during cell growth was observed, its role was found unable to fully account for the rate of sterol uptake, since the rate of sterol pseudosolubilization was 14 times smaller than the rate of sterol uptake during normal growth. Only under stress conditions the mechanism of sterol pseudosolubilization could account for substrate uptake. It was thus considered that in such conditions substrate uptake occurs primarily through cell adhesion to the sterol particles. The cell wall composition of cells grown in the presence and absence of sterol was compared, the former showing a lipid content to be more than double than the latter, whereas the carbohydrate and protein contents were similar. The high cell wall lipid content was considered to impart high cell-surface hydrophobicity, thus helping cells to adhere to the hydrophobic sitosterol particles. Furthermore, the fatty acid profile of the cell wall lipid of sterol-grown cells showed a high percentage of long chain length fatty acids, unlike the cells grown in the absence of sterol. Unidentified fatty acids, tentatively related to mycolic acids, which have been reported to be involved in the use of other water-insoluble hydrocarbons by microbial cells [132], were also found among the lipidic fraction of the cell wall of sterol-grown *A. simplex* SS-7 cells.

A more radical approach to cope with the low water solubility of steroid-like compounds has been the use in the bioconversion medium of a water-immiscible organic phase as substrate carrier and for in-situ product recovery [2–7,133,134]. The organic solvent must be non-toxic to the biocatalyst, while providing high solubility of the substrate and product and adequate water/organic partition coefficients. Solvents with a $\log P$ value in excess of 4 usually result in the best compromise between these requirements [133], although cell activity predictions based solely on the $\log P$ value of the solvent may not be totally accurate [135]. Furthermore, adequate hydrodynamic conditions and phase volume ratio (organic volume/aqueous volume) values have to be defined, in order to enhance mass transfer and thus increase the overall reaction rate, as observed in the cholesterol oxidation to cholestenone and in the Δ^1-dehydrogenation of a cortisol derivative, both using resting *A. simplex* cells [136,137]. Work performed on the Δ^1-dehydrogenation of cortisol and derivatives with whole *A. simplex* cells highlighted the primary target of the solvent toxic action as the cell's respiratory chain, rather than the Δ^1-dehydrogenase itself [138,139]. Thus, artificial electron acceptors are required in order to bypass the respiratory electron transport chain and allow continued activity [138–140]. The organic–aqueous two-liquid phase approach was also followed by Krook and Hewitt in the experimental set-up claimed for the production of 6-methyleneaneandrosta-1,4-diene-3, 17-dione from 6-methyleneandrost-4-ene-3,17-dione, using whole cells of *A. simplex* [141]. Conversion yields in excess of 99% were reported, for an initial substrate concentration of $60\,g\,l^{-1}$, using potassium phosphate buffer (pH 8.8) as aqueous phase and toluene as organic phase, present in a volumetric aqueous:organic phase ratio of 5:95. Menadione was used as exogenous electron carrier and catalase was used for peroxide scavenging.

Organic–aqueous two-liquid phase systems have recently found application in multi-enzyme steroid bioconversions, using resting *Mycobaterium* sp. NRRL B-3805 cells for the bioconversion of sitosterol to 17-keto-steroids [135,142]. Low catalytic stability was reported, although deactivation could not be attributed to the presence of the organic solvent, dioctyl phthalate, but more probably to the depletion of the cellular oxidative potential, since incubation of the biocatalyst in nutrient media between successive batch bioconversion runs improved its operational stability [143]. Water-immiscible organic solvents were also used in the development of experimental set-ups for the stereospecific

oxidation of 3β-hydroxysteroids [144], cholesterol [145] and cholesterol analogues [146] using growing *Pseudomonas* sp.

Organic–aqueous two-liquid phase bioconversions are usually carried out in simple agitated vessels [147,148]. However, Oda et al. effectively used an agar plate anaerobic interface bioreactor for the reduction of methyl 7-ketolithocholate to methyl ursodeoxycholate using whole cells of *Eubacterium aerofaciens* [149]. The experimental set-up consisted basically of an agar plate, onto which a microbial culture was inoculated, covered by a film of the organic phase containing the substrate. Among several organic solvents evaluated for use as a compatible organic phase, dihexyl ether was selected as the most adequate, allowing a product yield in excess of 80%, for an initial substrate concentration of $5\,g\,l^{-1}$. When the bioconversion was performed in aqueous media hardly any product was formed, due to the low solubility and toxicity of the substrate [150]. Reactor shaking ($40\,strokes\,min^{-1}$) only increased product yield by around 15%, suggesting that mass transfer was not significantly enhanced by convective flow [149]. The reduction of methyl 7-ketolithocholate thus proceeded efficiently without shaking. These authors also highlighted the need for buffering the aqueous phase, so as to overcome the decrease in pH caused by accumulation of organic acids, by-products formed during metabolism of mono- and disaccharides present in the growth medium. Mannitol was effectively used as supplement to this medium, increasing the product yield by 2.4-fold. The slow metabolism of mannitol to fructose prevented excessive formation of toxic organic acids through the glyceraldehyde 3-phosphate/pyruvate pathway, while allowing the required production of glyceraldehyde-3-phosphate to reduce $NADP^+$, therefore enabling the regeneration of the active form of the hydroxysteroid dehydrogenase [150].

The encapsulation of steroids in liposomes provides a tool to overcome the undesirable side effects associated with the use of organic solvents. This was effectively used in the cortisol dehydrogenation to prednisolone by free *A. simplex* cells [151].

7. Cell immobilization

Immobilized cell systems have been commonplace in steroid bioconversion investigations [2–7], the protective microenvironment provided proving a major advantage, particularly when a separate organic phase is present [152,153]. Mass transfer limitations due to inadequate partition of the lipophilic substrates into hydrophilic gels have been reduced through the use of polyurea-coated alginate beads, in the 11α-hydroxylation of progesterone with *A. ochraceus* cells in organic–aqueous two-liquid phase systems [152]. The outcome was a six-fold increase in the overall reaction rate as compared to the use of uncoated supports. The activity enhancement in microbial cells immobilized on sintered glass Raschig rings, used for the conversion of pregnenolone triacetate into prednisolone in aqueous media, as compared to cells immobilized on DHEA–dextran-modified sirane supports, has been associated to the hydrophobic character of the Raschig rings matrix, which favored substrate partition from the aqueous medium [106]. Polyvinyl alcohol (PVA) entrapped *A. globiformis* cells were used for the Δ^1-dehydrogenation of cortisol, 6α-methyl cortisol and progesterone [154]. Specific activity was strongly dependent on the bead diameter, which ranged from 1.0 to 6.5 mm, when progesterone and particularly 6α-methyl cortisol were used as substrates. Cortisol dehydrogenation, on the other hand, was hardly influenced by the bead diameter, suggesting that, in this case, the overall reaction rate was kinetically controlled. A six-fold reduction in the relative activity of cortisol dehydrogenation was observed when immobilized cells were used, with respect to free cells. However, a high operational stability, allowing 31 bioconversion cycles without loss of activity, together with a high degree of cell retention and mechanical stability were observed. *A. globiformis* cells immobilized in polyacrylamide gel were used for the bioconversion of $5\,g\,l^{-1}$ microcrystalline cortisol in 100 l aqueous media, with a productivity of $0.6\,g\,l^{-1}\,h^{-1}$ [107]. Celite-immobilized mycobacterial cells have been effectively used for sitosterol side-chain cleavage in organic medium, provided an adequate hydration layer is present, allowing AD and ADD yields in excess of 90% for an initial substrate concentration of $5\,g\,l^{-1}$. Although the use of small diameter support particles (from 0.067 to 0.193 mm) apparently allowed kinetic control of the bioconversion, the solvent toxicity effects became more evident [152,155,156].

The combination of sintered glass-immobilized *Flavobacterium dehydrogenans* and *A. simplex* cells with *Curvularia lunata* free mycellium has been used for the production of prednisolone from pregnenolone triacetate, with continuous substrate supply in aqueous medium [68]. The most convenient pathway for the bioconversion involved the saponification of the substrate with *F. dehydrogenans*, and subsequent hydroxylation with *C. lunata* and Δ^1-dehydrogenation with *A. simplex*. Immobilized cultures of *C. lunata* and *Nocardia simplex* performed the 11β-hydroxylation and Δ^1-dehydrogenation of medroxyprogesterone acetate to produce an intermediate of 6α-methyl prednisolone. The two-step bioconversion was carried out in a single packed-bed reactor or in two packed-bed reactors run in sequence, operated in either order [98].

Immobilized mixed cultures of *C. lunata* spores and *Mycobacterium smegmatis* cells have been used for the two-step conversion of cortexolone into prednisolone in a single-stage fermentation by Ghanem et al. [108]. Conversion efficiencies in excess of 90% were reported, for an initial substrate concentration of $0.1\,g\,l^{-1}$. A decrease in conversion efficiency was observed if repeated batch-wise biotransformations were carried out. This was particularly noticeable if the conversion medium was composed of solely distilled water, as compared to diluted nutritive media, which could be due to the absence in the former of salts and nutrients.

These are required to sustain cell viability and enzymatic productivity and allow co-factor regeneration, their lack ultimately leading to cell lysis. Incubation in nutrient media, after a given number of consecutive batch bioconversions runs, was thus successfully used to restore biocatalytic activity. This approach was also followed by Koshcheenko et al. while developing an effective set-up for repeated batch-wise bioconversions of cortisol to prednisolone using immobilized *A. simplex* cells [157]. Operation in continuous mode provided a more elegant way to overcome the decrease in activity observed in repeated batch-wise bioconversions of cortexolone to prednisolone [108]. The continuous operation led to a prednisolone average conversion yield of 53%, as compared to 23% for the batch mode. This improvement in the retention of catalytic activity observed when continuous operation was used instead of the repeated batchwise mode was more significant in the Δ^4-reduction of ADD, performed by polyacrylamide–hydrazide-immobilized cells of *Clostridium paraputrificum* [158]. Conversion yields in excess of 95% were maintained for around 100 h, whereas in repeated consecutive bioconversions of 5 h each, a decrease in conversion yield of about 30% per incubation was reported. The lower efficiency of the consecutive batchwise mode of operation was related to the oscillations in the metabolic state of the cells [158]. The combination of an adequate medium composition and the lack of fluctuations in environmental conditions was also suggested as an adequate approach to maintain a stable level of catalytic activity [159].

The dehydrogenation of AD entrapped in multilamellar vesicles using *A. simplex* cells immobilized in calcium pectate was recently evaluated, as compared to the use of aqueous media [160]. AD was fully transformed in 2 h irrespective of the bioconversion medium for substrate concentrations ranging from 0.3 to 0.45 mM. However, when the substrate concentration was increased to 0.63 mM, only 50% conversion was achieved after 3 h of incubation in aqueous medium, whereas total bioconversion was achieved in 2 h in liposomal medium. The same result was obtained in this medium when the substrate concentration was increased to 1 mM. No significant differences in the bioconversion time course were observed when free cells were used in the liposomal medium, clearly suggesting an increase in substrate uptake when this bioconversion medium was used.

8. Strain manipulation and screening for new strains and novel steroid-like compounds

Screening and isolation of active microbial strains for steroid bioconversion is presently an important part of the research and development effort in the steroid drug industry. A wide array of microorganisms was screened by Manosroi et al. for the 11β-hydroxylation and Δ^1-dehydrogenation steps. Among the isolates, *Cunninghamella blakesleana* SRP1 and *Bacillus sphaericus* SRP 1 exhibited high 11β-hydroxylation and Δ^1-dehydrogenation activities, respectively allowing a cortisol yield of 62% from cortexolone, and a subsequent prednisolone yield of 67% [71].

Screening of mold cultures to transform steroids has also been carried out and the fungus *Circinella* sp. IOKh 1220 was found to be capable of introducing the 9α-hydroxyl group into pregnenolone, with a 48% conversion yield, and without structural modification of the ring A [161].

Agrobacterium sp. M4 isolated from soil samples was found to efficiently degrade cholesterol, with cholestenone as primary metabolite [162].

The ability of *Bacillus* strains to hydroxylate AD was recently reported for the first time by Schaaf and Dettner [163]. Two *Bacillus* species isolated from the foregut of the dytiscid water beetle *Agabus affinis*, and designated HA-V6-3 and HA-V6-11 were able to metabolize AD (0.2 g l^{-1}) to yield 6β-OH-AD as major product. Total substrate conversion was observed after 24 h of incubation when the latter strain was used. Strain HA-V6-3 showed lower activity, since a considerable amount of the substrate was still present in the culture extract after 48 h of incubation. 14α, 11α- and 7α-OH-AD were produced by both strains as secondary products, but apparently only strain HA-V6-11 yielded a dihydroxylated product (6β,14α-OH-AD).

Manosroi et al. tested several strains, among culture collection strains and isolates, and different modes of operation to perform the bioconversion of cortexolone to prednisolone. The highest prednisolone yield was obtained by the sequential use of free *Cunninghamella echinulata* ATCC 8688a for 11β-hydroxylation and alginate entrapped *B. sphaericus* TCC 13805 for Δ^1-dehydrogenation [164].

Several fungal strains were tested for the 15α-hydroxylation of 13β-ethyl-4-gonene-3,17-dione. A product yield of 76.5% was observed when *Fusarium nivale* cells were incubated in the presence of 4 g l^{-1} substrate. The 15α-hydroxylase of *F. nivale* cells was inducible by norethisterone [72].

Micrococcus roseus RJ6 has been identified as an effective biocatalyst for the degradation of cholesterol to AD and ADD. The enzymatic pathway is apparently expressed by genes located in a 10 kb plasmid, concluded after plasmid curing resulted in the loss of sterol degradation activity [165].

Aiming at obtaining a strain useful for the industrial production of corticoid intermediates, Dogra and Qazi isolated bacteria from soil and animal faeces and evaluated a particular isolate, identified as *M. roseus*, for specific sterol degradation activity [166]. This isolate was matched to *Micrococcus* strains with cholesterol degradation activity to ADD and exhibited an enhancement of around 5% in ADD yield, as compared to the most effective control strain, which provided a 60% ADD yield, in a 3-day bioconversion run. The degradation activity of the isolated strain was shown to be plasmid-encoded, while the control strains exhibited absence of plasmid DNA, thus indicating that their sterol degradation activity was encoded in the genome.

Screening of microorganisms able to grow on bile acids and in the presence of organic solvents led to the isolation

of *Pseudomonas putida* ST491 [167]. When grown in $5\,g\,l^{-1}$ litocholic acid in the presence of diphenyl ether, 60% of the substrate was cleaved to AD (75% yield), with ADD (17% yield) and pregna-1,4-dien-3-on-20-al (8% yield) as secondary products. If the organic solvent was replaced by Triton X-100 in the aqueous conversion medium, 40% of the substrate was converted to ADD alone [167].

Fermentation of testosterone and pregnenolone using the fungus *Botrytis cinerea* yielded hydroxylated metabolites 7β,17β-dihydroxyandrostan-3-one (73% yield) and 3β,11α,16β-trihydroxypregne-5-en-3-one (39% yield), respectively, as major products, from a $0.25\,g\,l^{-1}$ initial substrate concentration [168].

The ability of fungal strains *Exophiala jeanselmei* var. *lecaniicorni* and *Ceratocystis paradoxa* to perform bioconversions on various steroid substrates was evaluated by Porter et al. [169]. The incubation of the former strain in the presence of 3β-hydroxyandrost-5-ene-17-one ($2\,g\,l^{-1}$) yielded 3β,17β-dihydroxyandrost-5-ene and 3β,6β-dihydroxy-5β-androstan-17-one (about $0.2\,g\,l^{-1}$ each product). Such microbial hydration of an alkene to produce an alcohol is rarely observed. This strain was also able to cleave the side chain of several pregnanes to C_{19} hydroxy- and keto-steroids. *Ceratocystis paradoxa* was also able to yield 3β,17β-dihydroxyandrost-5-ene ($0.06\,g\,l^{-1}$) from 3β-hydroxyandrost-5-ene-17-one ($2\,g\,l^{-1}$). Both ascomycetes showed ability to transform 3-keto-Δ^4-steroids by the reduction of the α,β-unsaturated system.

First literature report on the biotransformation of substrates by the deuteromycetes *Fusarium oxysporum* var. *cubense* focused on its use for steroid biotransformation [170]. This fungus effected 7α-hydroxylation on 3β-hydroxyandrost-5-en-17-one ($15\,g\,l^{-1}$) in around 40% yield. It also performed 15α-hydroxylation on testosterone and on pregn-4-en-3,20-dione in almost quantitative yields and on estrone, the latter, however, in rather small yield. Another deuteromycetes, *Colletotrichum musae*, was also examined for its potential on steroid bioconversion, since it had never been used to effect biotransformations with any substrate [170]. Although this fungus was able to metabolize 3β-hydroxyandrost-5-en-17-one to 3β,17β-dihydroxyandrost-5-ene, testosterone to ADD and 17α,21-dihydroxypregn-4-en-3,11,20-trione to 17α,20,21-trihydroxypregn-4-en-3,11-dione, the product yields were rather low suggesting the need for improved fermentation conditions.

Site selective oxygenation of steroid molecules is frequent in filamentous fungi, although scarce in bacterial strains [171]. A thermophilic bacterium, *Bacillus stearothermophilus*, was incubated in the presence of progesterone ($50\,mg\,l^{-1}$), to produce three monohydroxylated metabolites, 20α-hydroxyprogesterone, 6β-hydroxyprogesterone and the rare 6α-hydroxyprogesterone, in relative percentage yields 60.8, 21.0 and 13.6, respectively. A new metabolite, 9,10-seco-4-pregnene-3,9,20-trione, was also isolated in a relative percentage yield of 3.7 [171].

Ergosterol is the end product in the sterol biosynthetic pathway in *Saccharomyces cerevisiae* [172]. By deleting the *ERG5* gene, responsible for sterol C22 desaturase, and expressing in the same yeast the bovine mature forms of adrenoredoxin reductase, adrenodoxin, side-chain cleavage cytochrome P450, type II human 3β-hydroxysteroid dehydrogenase-isomerase and *Arabidopsis thaliana* Δ^7-sterol reductase, a genetically engineered *S. cerevisiae* strain able to produce pregnenolone and progesterone from galactose was obtained by Duport et al. [173]. Modified yeast strains, expressing at least one of the enzymes involved in the biodegradative pathway leading from cholesterol to cortisol, and containing an inactive acetyl-CoA pregnenolone acetyl transferase, were claimed [174]. The modified yeast strains were also claimed to oxidize in vivo endogenous or exogenous sterols, with the production and possible isolation of 3β-hydroxysteroids, or exogenous steroids, such as pregnenolone, with production and possible isolation of 3-keto-Δ^4-steroids [174].

Recently, Lamb et al. identified a sterol biosynthetic pathway in *M. smegmatis* [175]. These authors had previously proposed the hypothesis of existence of such a metabolic pathway in mycobacterial cells, after ascertaining a significant number of homologies between the genome sequence of *Mycobacterium tuberculosis* and that coding for the sterol biosynthetic enzymes of *S. cerevisiae*. The development of a steroid cell-factory, based on the approach followed by Duport et al. [173] could be envisaged.

Mycobacterium sp. VKM Ac-1815D clones with altered resistance to coupled antibacterial agents, namely to nalidixic acid and gentamycin and to rifampicin and to gentamycin, were able to cleave sitosterol ($5\,g\,l^{-1}$) giving AD as a major product, with a molar yield of 69–71% [53]. The parent strain performed the same cleavage, but with a molar yield of AD of 64%. The mutants were further treated with either ethyl methane sulfonate or mitomycin C. The mutant strains were still able to cleave the side chain of sitosterol giving molar yields of AD of 68–71%. Unlike the initial strains, however, the mutated clones were able to use 3,17-diketosteroids, namely AD and 9α-OH-AD. This possibly indicated regeneration in the key enzymes, 9α-hydroxylase and $\Delta^{1,2}$-dehydrogenase.

Other approaches focus on increased microbial synthesis of sterols and steroid precursors. Thus, overexpression of a truncated *HMG1* gene in a *S. cerevisiae* strain led to a slight increase in ergosterol levels [9]. An enhancement in the level of 4-desmethyl sterols by at least 10% in the seeds of plants was claimed by Harker et al. [176]. This was achieved through the use of a gene expressing a non-feed back inhibited HMG-reductase. A method developed to leach the seeds in order to obtain an oil containing these sterols, which are useful in the prevention of coronary diseases, is also claimed by these authors.

Recombinant cytochrome P450 was expressed in *Escherichia coli* and the transformed cells were used for the hydroxylation of progesterone to DHEA. The bioconversion

system developed is claimed to be industrially useful [177]. *Schizosaccharomyces pombe* cells transformed with a vector plasmid encoding human aldosterone synthase was used for the production of aldosterone, corticosterone and 18-hydroxy-corticosterone [162].

The deletion of a nucleotide sequence encoding the 3-ketosteroid-Δ^1-dehydrogenase of *Rhodococcus erythropolis* RG1-UV29, a natural steroid-degrading microorganism, was claimed by van der Geize et al., as a tool to obtain an effective strain to produce 9α-OH-AD from AD [178]. Inactivation of two genes, *kstD1* and *kstD2*, was necessary to eliminate 3-ketosteroid-Δ^1-dehydrogenase activity, thus preventing the degradation of the steroid nucleus [178,179]. This allowed the accumulation of the desired product in high yields (80–100%), from 10 to 20 g l^{-1} initial substrate concentrations, in bioconversion runs of about 20 h [176], a noticeable enhancement in productivity when compared to results published so far [79,80].

The isolation of novel steroid-like compounds, with potential therapeutic applications, from natural sources, has also been an active field of research. Herbarulide, a ketodivinyllactone lactone steroid, was isolated from the crude fermentation extract produced by the endophytic fungus *Pleospora herbarum*, which exhibited antimicrobial, herbicidal and algicidal activity [180]. Marine organisms have also proven to be a rich source of interesting new steroid metabolites. Six new steroids, designated yonarasterols A through F, were isolated from *Clavularia viridis*, a soft coral from the Okinawa sea [181]. A new ecdysteroid, 2β,3β,14α-20β-tetrahydroxy-22α-(2-hydroxyacetiloxy)5β-colest-7-en-6-one, was isolated from the Caribbean sponge *Iotrochota birotulata* [182]. 3β,5α,6β,7α-Tetrahydroxy-cholest-8(9)-en-11-one and 3β,5α,6β-trihydroxy-cholest-8(9)-en-7,11-dione, two incrustasterols isolated from *Dysidea incrustans*, and containing the unusual $\Delta^{8,9}$-11-keto functionality, were proved to be cytotoxic against human renal carcinoma and melanoma cell lines [183]. A survey on new spongal steroids is beyond the scope of this work, but detailed information on this subject can be found in a recently published review [184].

9. Perspectives

Expected developments in biological production of steroid compounds range from the identification of novel and improvement of existing biocatalysts to the improvement of the biotransformation process.

The recent technological developments, associated with combinatorial biocatalysis will allow faster and wider screening of new steroids, to ultimately generate libraries of therapeutically relevant compounds. Novel steroid-like compounds are also increasing the field of their applications. Non-aqueous biocatalysis will benefit from the knowledge gathered on the response mechanisms of microbial cells to the presence of organic solvents. The understanding of these defense mechanisms, combined with recombinant DNA technology may lead to the development of highly effective and stable biocatalysts for use in non-conventional media. Genetic manipulation is expected to lead to biocatalysts with constitutive, rather than inducible expression of activity, as well as with enhanced activity levels. Directed evolution and DNA shuffling rapidly emerging technologies can lead to the development of tailored, highly selective, enzyme activities, able to perform the intended bioconversion in virtually any suitable medium. The development of efficient methods for in vitro coenzyme recycling will expand the use of enzyme preparations in steroid bioconversions. Rational design of fermentation/bioconversion media, combined with the developments in process monitoring may lead to the implementation of robust, highly effective biotransformation processes.

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