Review of Literature
REVIEW OF LITERATURE

Steroids are saturated hydrocarbons containing a minimum of seventeen carbon atoms, in a system of rings, three six membered and one five membered. The basic structure of these four rings is known as cyclopentanoperhydrophenanthrene (6). (CPPP).

All the steroids, sterols, cortisones and related compounds are either modification of these structures or there is a addition of a side chain or a group at one place or the other. The steroids of medicinal importance are prepared from diosgenin or sterol by combination of chemical or microbiological processes.

Steroids are physiologically active compounds of complex structure. Naturally occuring steroids possess remarkable hormonal properties. They include hormones of adrenal cortex [cortisones(7), cortisols (8), corticosterone(9)] the progestational hormone [progesterone (10)], androgen or male hormones, [testosterone(11), Dihydroxytestosterone (12)] and female sex hormone [Estradiol(13), Estrone(14)].
Adrenal Cortex Hormones:

Cortisone (7)

Cortisol (8)

Corticosterone (9)

Progestational Hormone:

Progesterone (10)
Androgen Hormone:

Testosterone (11)

Dihydroxytestosterone (12)

Female Sex Hormones:

β-Estradiol (13)

Estrone (14)

Sterols are steroid alcohols containing a hydroxyl group at carbon 3 of ring A and a branched aliphatic chain of eight or more carbon atoms at carbon - 17. They occur either as free alcohols or as long chain fatty acid esters of the hydroxyl group at carbon -3. The basic structure of sterols consists of saturated tetracyclic hydrocarbons perhydrocyclopentanophenanthrene.

The use of microbes for the conversion of steroidal molecules can be traced back to 1937, when for the first time Mamoli and Vercellone (1937 a, b) converted 5-androsten - 3, β -ol - 17-one (15) to androstendiendione (16) to testosterone (17) by the use of a yeast culture.
However this biotransformation was superceded by efficient non-enzymatic method. The capacity of Mycobacterium strains to utilize sterols as sole carbon source was recognised (Nagasawa et al. 1969). However, nothing came of these early observations.

Through inadvertent use of yeast cultures contaminated by bacteria, (Mamoli and Vercellone 1938) it was demonstrated that bacteria were also capable of transforming steroids. Thus at the opening of World War II, it was recognised that microbial transformation of steroids were possible, the early reports having established interconversions of ketones and alcohols, double bond isomerisations and reductions (Nagasawa et al. 1970). These early observations set the stage for post war studies in which for the first time the two major microbial transformations of continuing
biotechnology importance were recorded with cholesterol as substrate, both a -7 dehydrogenation by an *Azotobacter* sp. (Horvath and Kramli 1947) and a 7-hydroxylation by *Proactinomyces roseus* were described (Kramli and Horvath 1948, 1949).

A second independent line of investigation of the microbial metabolism of sterols to degraded product systematically initiated by Turfitt (1944, 1948) began at the same time and developed ultimately to commercial processes in which cholesterol and phytosterol are oxidised to useful C-19 steroid intermediates.

The discovery in 1949 of the powerful anti-inflammatory activity of cortisone, led to great interest in this group of steroids. Indeed, the first applications of biotechnology to the preparation of useful steroids were directed to making these adrenal steroids. (Nagasawa et al.1970).

The first successful application of biotechnology to such effort did not yet utilize micro-organisms but involved the perfusion of bovine adrenal glands. The report of Hechter et al. 1949 that perfused adrenals transformed cortisone (18) to corticosterone (19) by specific enzymic 11β-hydroxylation clearly pointed directions and taken up directly by several pharmaceutical companies (G.D. Searle and Co. 1979, Nagasawa et al. 1970)
The most important chapter in the history of microbial transformations has had to with the synthesis of the hormones of the adrenal gland and their more powerful and therapeutically selective synthetic analogues.

A classical demonstration of the unity of biology was made with the dramatic announcement by Peterson and Murray 1952 that *Rhizopus arrhizus* could convert progesterone (20) to 11α-hydroxyprogesterone (21).

With the discovery that the side chain of naturally occurring sterols such as cholesterol (22), β-sitosterol (24) or campesterol (25) can be degraded selectively by micro-organisms (Soehngen, 1913),
Natural sterols commonly used as starting materials for steroid drug synthesis

Cholesterol (22)  
Stigmasteryl (23)

β-Sitosterol (24)  
Campesterol (25)

Ergosterol (26)  
Deoxycholic Acid (27)

bacterial genera represented by *Arthrobacter*, *Nocardia*, *Pseudomonas*, *Mycobacterium* and *Corynebacterium* shot into prominence because of their ability to utilize sterols as sole source of carbon and energy.
This biotransformation methodology has attracted much attention leading to the development of several useful processes (Ahmed et al. 1992). The complete degradation of these molecules is of no commercial interest (Kieslich 1985).

Whitmarsh (1964) tried to slow down the break down of cholesterol (28) (with Nocardia species) by addition of an organic inhibitor, hoping thus to isolate intermediates which might give an indication of the route by which this breakdown proceeds. The inhibitor used was 8-hydroxyquinoline and it was then possible to detect 3-keto-\( \Delta^4 \)-bis-norcholanic acid (29) as well as very small amount of \( \Delta^{1,4} \)-bis norcholanic acid(30) in acid extracts of the culture medium. In the neutral fraction small amounts of 4-androstene-3, 17-dione(31) and 1, 4-androstanediene-3, 17-dione (32) were detected.

Thus, the use of inhibitor was to prevent attack of the ring sytem since if the ring is attacked the starting material is converted into a worthless product of non-steroid character.

Arima et al. (1969) reported cholesterol decomposing ability of 1589 microbial strains. 236 strains from actinomycetes, bacteria, moulds and yeasts were reported of oxidizing cholesterol (33) into cholestenone (34). Cholesta-1, 4-diene-3 one (35) was produced by 5 strain of Streptomyces. The complete decompositon of cholesterol molecule was observed in the genera : Arthrobacter, Bacillus, Brevibacterium, Corynebacterium, Microbacterium, Mycobacterium, Nocardia, Protaminobacter, Serratia and Streptomyces. \( \alpha, \alpha' \)-dipyridyl and arsenite inhibited decomposing enzymes giving rise to cholestenone, Cholesta 1, 4-diene-3-one (35) and an intermediate probably devoid of sterol side chain.
Cholesterol (33)

\[ \text{Cholest-4-ene-3-one (34)} \]

\[ \text{Androst-4-ene-3, 17-dione (36)} \]

\[ \text{Androsta-1, 4-diene-3, 17-dione (37)} \]

\[ \text{3 Hydroxy-9, 10 secoandrosta-1, 3, 5 (10), triene-9, 17-dione (38)} \]
Nagasawa et al. (1969, 1970) described selective cleavage of the side chain of various sterols such as campesterol (25), \( \beta \)-sitosterol (24), stigmasterol (23) and 7-dehydro-cholesterol giving rise to ADD by depressing breakdown of steroid skeleton with \( \alpha, \alpha \)-dipyridy1 or arsenite and microbial degradation pathway of cholesterol was postulated.

The pathway exemplified in cholesterol is considered to be the general degradation of sterols by decomposing microorganisms (Arima et al. 1969). It was further demonstrated that ADD thus formed from sterols was converted into 3-hydroxy-9, 10-seco androsta-1, 3, 5 (10) triene-9, 17-dione (38).

The side chains of sterols such as cholesterol, campesterol, \( \beta \)-sitosterol, stigmasterol, 7-dehydrocholesterol, ergosterol (26) and \( \beta \)-cholestanol were selectively cloven affording ADD but their converting yields into ADD were found substantially influenced by their structural difference (Nagasawa et al. 1970). The cleavability decreased along the following order:

- pregnenolone > lithocholic acid > cholesterol > campesterol, \( \beta \)-sitosterol > stigmasterol > 7-dehydrocholesterol > \( \beta \)-cholestanol > ergosterol.

Nagasawa et al. (1970 b) reported microbial transformation of sterols yielded \( C_{19} \) steroid intermediates: androst-4-ene-3, 17-dione (ADD), androsta-1, 4-diene-3, 17-dione (ADD), androst-4-ene-17 \( \beta \)-ol-3-one, androsta-1, 4-diene-17 \( \beta \)-ol-3-one and 5\( \beta \)-androstan-3\( \alpha \),17\( \beta \)-diol respectively in the degradation of cholesterol by Arthrobacter simplex in presence of 0.8 mM \( \alpha, \alpha \)-dipridyl.
Nagasawa et al. (1970 c) stated the ability of accumulating androsta-1, 4-diene-3, 17-dione (ADD) in the digestion of cholesterol by *Arthrobacter simplex* IAM 1660 using 167 compounds and (i) chelating agents (ii) Ni^{2+}, Co^{2+}, Hg^{2+}, As^{3+}, Sb^{3+}, Bi^{3+}, Cd^{2+}, SeO_{3}^{2-} and AsO_{4}^{2-} ions and (iii) redox dyes were found effective for ADD accumulation. Ionic state of the chelating agent was unfavourable for ADD accumulation but inactive ethylaenediamine tetraacetic acid could be turned effective with aid of surface active agents and pencilline. Lipophilic structure of the chelating agents was required probably for its penetration through the cell membrane. The target process of the ADD accumulating agents was inhibiting 9α-hydroxylation.

Marsheck et al. (1972) described microbial degradation of cholesterol and plant sterols to produce androsta-1,4-diene-3, 17-dione and androst-4-ene-3, 17-dione. The first microbiological production of 20α-hydroxy methyl pregna-1, 4-diene-3-one was reported by two newly isolated bacteria designated *Mycobacterium* sp. NRRLB-3683 and *Mycobacterium* sp. NRRLB-3805. These mycobacteria produce substantial amounts of 17 ketonic compounds without appreciable degradation of the steroid nucleus. No ring degradation inhibitory agents were necessary.

An important goal of microbial side chain cleavage is to produce C_{19} or C_{22} steroids with intact steroid nucleus (Kieslich 1985).

Selective removal of side chain requires blocking of the enzymes responsible for ring cleavage viz C-1(2) dehydrogenase and for 9α-hydroxylase (Ahmed et al. 1992). To meet this end three different approaches have been employed:
i) Structural modification of the substrate, thus preventing enzyme attack on the ring system.

ii) Inhibition of 9α-hydroxylase by chemical means. Such as complexing agents for Fe²⁺.

iii) Mutation of the micro-organisms.

**Chemical Versus Biochemical Processes:**

The chemical synthesis of steroid compounds required complicated, multistep schemes. It also requires preparation of intermediate derivatives for the protection of particular groups, their subsequent groups and their subsequent regeneration. Thus making the chemical synthesis expensive and time consuming. At the same time the basic ring steroid derivatives are sensitive to cleavage by a variety of chemicals (Mahato and Banerjee, 1985, Mahato and Majumdar, 1993). Chemical synthesis also requires the use of reagents such as pyridine, sulphur trioxide or selenium dioxide which are hazardous to the health of human beings and constitute a serious environmental disposal problem (Weber et al. 1995, Roberts et al. 1996). 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. The steroid industry thus couples the chemical and biological approaches taking advantage of the best aspects of each. As alternative to chemical methods, the microbial removal of aliphatic side chain of phytosterols offers a promising method for use of these sterols and has been studied for sometime (Mahato and Mukherjee 1984, Mahato and Majumdar 1993, Fernandes et al. 2003). The microbial removal of side chain of phytosterols offers a promising method, and processes for the conversion of sterols to 17 keto-steroids.
have been developed and are also in commercial use (Smith et al. 1984).

**Raw Materials:**

Marsheck et al. (1972) had stated that utilization of soyabean sterols, \( \beta \)-sitosterol, campesterol and stigmasterol were an economical source of intermediate for steroid manufacture and had been considered for supplementing or supplanting the commercial processes from diosgenin obtained from *Barbasco* root and other sources. Makino et al. (1974) described the transformation of cholesterol to ADD using wastes discharged from wool washing as substrate, which were hydrolysed by calcium oxide before addition to a mixed culture of *Arthrobacter simplex* and *Nocardia corallina*, they obtained, a total yield of 32% AD and ADD after 48 hrs. of fermentation.

Martin (1977), stated that primary raw material for producing steroid drugs by partial synthesis serves diosgenin from *Dioscorea* sp. Stigmasterol and cholesterol from plant and animal sources respectively. (Petrow 1969). As a result of shortage of diosgenin the conversion of plant sterols (phytosterols) such as soyabean sterols (\( \beta \)-sitosterol) and congeners admixed with substantial amounts of stigmasterol or tall oil sterols, [about 17 different compounds of which sitosterol and campesterol comprise about 80% (Cooner and Rowe, 1976)] has attracted much attention and several useful processes have been developed. Compared to soyabean sterols, tall oil sterols were transformed with similar efficiency. Similarly residues of press mud containing \( \beta \)-sitosterol and campesterol were used successfully for C-19 steroid production (Coto et al. 1985, Martinez et al. 1987, Quinata et al.1985, Srivastava 1985) Srivastava and Srivastava (1985)
stated that it is difficult to meet the increasing demand for the steroidal drugs which are manufactured from diosgenin and solasodine because of scarcity of raw material (*Dioscorea and Solanum* sp.)

Vadalkar et al. (1985) used sulphitation press mud samples collected from the sugar factories from Punjab, which was extracted with organic solvents to obtain the crude wax which was further fractionated to recover the sterol mixture. He isolated bacteria from animal faeces, garden soil and compost. In their studies they used sugarcane sterols which were added in the isolation medium in varied concentrations. They isolated an effective strain which could produce ADD where as Srivastava and Srivastava (1985) extracted sterols from the dried sulphitation mud collected from sugar factories of Gorakhpur and Deoria by the method of Bose and Gupta (1961). This concentration in the purified mixture was measured by a Liberman-Burchard reaction (Katayama et al. 1974) with a standard curve drawn from β-sitosterol (Sigma) and extra-pure cholesterol (BHD, India). Sterols (overall concentration of 0.1% with 94% purity) extorted from press mud were suspended in the phosphate salt medium with a homogenizer for isolating the bacterial strain by enrichment culture techniques. The bacterium *Arthrobacter globiformis* grew well on sterols and transformed them into ADD, a precursor of steroidal drugs and hormones.

Venkatramesh et al. (1987) stated that the major pathway in advanced vascular plants leads to a sterol content profile mainly constituted by the phytosterols, stigmasteryl, β-sitosterols and campesterol. These phytosterols are the precursors of many pharmaceutically active steroids (Martin et al. 1997, Jain et al. 1980). Stigmasteryl
has been a suitable raw material for chemical synthesis of pregnane derivatives (Szentirmai, 1990) on an industrial scale because the C-25 double bond promotes the chemical degradation of the side chain of the steroid skeleton. Sitosterol now represents one of the most economical, inexpensive raw materials. More than 60% of the raw materials for steroid drugs are produced by selective side chain cleavage of sterols and the bioconversion process is the most economical way to obtain steroid compounds as primary products for chemical synthesis producing sexual hormones, anticonceptients, antiphlogistics and blood pressure regulating agents.

Hogg et al. (1992) reported that stigmasterol and crude sitosterol, extracted mainly from soyabees and separated by crystallization. Stigmasterol is usually chemically oxidized to progesterone (Kieslich 1985, Mahato and Garai, 1997) since its C-22 double bond has a depressing effect on specific degradation activity of commonly used microbial strains (Goswami et al. 1984). Isolation of sitosterol from tall oil, a waste effluent from the paper industry, and a purified fraction of the isolate was microbially transformed to steroid intermediates (Szykula et al. 1991). Wastes from sugarcane industry was a source for an easy bioconvertible β-sitosterol/stigmasterol rich mixture, once a *Mycobacterium* mutant strain with a side chain cleavage activity non-inhibited by stigmasterol was isolated, reported by Perez et al. (1995).

Sitosterol occurs widely in the plant kingdom alongwith other phytosterols e.g. stigmasterol and campesterol. Its major sources are cotton seed oil, tall oil and sugarcane wax. Large quantities of sitosterol accumulate during the isolation of stigmasterol from
soyabean. Its availability on large scale has evoked interest in its use in steroid drug synthesis (Kieslich, 1985). Cheaper sources of sitosterol were looked for in order to further reduce production costs (Mahato and Garai 1997). Other approaches focus on increased microbial synthesis of sterols and steroid precursor. Thus overexpression of a truncated HMG1 gene in a *Saccharomyces cerevisiae* strain led to slight increase in ergosterol levels (Bortolini et al. 1997). Vinarov et al. (1997) reported oversynthesis of ergosterol during continuous fermentation of *Candida maltosa*. Cunha et al. (1997) had achieved β-sitosterol, campesterol, stigmasterol in culture of flax through optimization of fermentation parameters.

Rojas et al. (1999) stated that increasing the concentration of sucrose and phosphate added to modified Murashige and Skoog medium from 85 to 225 mg phosphate l⁻¹ and from 20 to 40 gm sucrose l⁻¹ led to shift 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). Andrijany et al. (1998/1999) studied the optimization of steroidal sapogenin accumulation in callus cultures of *Agave amaniensis*, through the evaluation of the effect of several ions, using a central composite design. Other efforts have been made in order to enhance the productivity of organism producing steroid precursors.

Sarangthem and Srivastava (1997) reported presence of phytosterols in *Bamboo*. Sarangthem et.al (1998) stated further that no work had been done regarding the involvement of micro-organisms, in accumulating phytosterols in the fermenting process. Sarangthem and Singh (2001, 2003) reported an increase of phytosterol content
from 0.12 to 0.62% dry weight after the fermentation of the succulent shoot slices of *Bambusa balcooa* Roxb. The fermenting microbial strains *Bacillus subtilis*, *B. licheniformis*, *B. coagulans* and *Micrococcus luteus* isolated and characterised for the first time from these fermented samples. Further extraction of crude phytosterols was done from these fermented bamboo shoot slices. The isolated β-sitosterol was then subjected to microbial conversion using *B. subtilis* yielding a considerable amount of androsta diene dione in the presence of metal chelate inhibitor (0.1% α,α-dipyridyl).

Tianwei et al. (2003) studied the fermentation parameters in cultivation of ergosterol by *Saccharomyces cerevisiae* and reported that ergosterol yield could be enhanced to 1160 mg/l when dissolved oxygen was kept at 12% (± 1%). Shen-Ping et al. (2003) reported eight ergostane type sterols from the fungus *Catathelasma imperiale* and three of their derivatives.

Soyabean extract residue (scum), a waste of soyabean oil production was converted to 9α OH (AD) androst-4-ene-3, 17-dione by *Mycobacterium* sp. VKMAc-1817d, was studied by Donova et al. (2005). The content of transformable sterols (sitosterol, stigmasterol and campesterol) in scum was estimated at approximately 14%. The yield of 9α-OH-AD molar yield approximately 65% was reached at 60 hr, from the scum preparation containing 10g dm⁻³ transformable sterols. The process productivity was comparable with that of high quality of sterol of wood origin (tall sitosterol). Another similar type of work was demonstrated by *Mycobacterial* cells to convert phytosterols from sugarcane mud into androst-4-ene-3, 17-dione (AD) and androsta-1, 4-diene-3, 17-dione (ADD) (Perez et al. 2005).
Microbial Sterol Metabolism:


Ahmed et al. (1992) stated that the steroid ring structure and the side chain are metabolised by different mechanisms. These enzymatic reactions do not follow a given order but occur simultaneously and independently. Thus, if the ring structure of the substrate was modified, the enzyme normally involved in the degradation of the ring system was unable to catalyse the fission; if however, the side chain was modified, the enzyme responsible for its degradation was unable to cleave side chain of the substrate.

Cleavage of Side-Chain: The pathway of the C-17 sterol side chain degradation during the microbial conversion of cholesterol to 17-ketosteroids has been elucidated by Sih and co-workers (Sih et al. 1965, 1968, Tai et al. 1970, 1970a). In contrast to mammalian systems where 17-ketosteroids are formed via cleavage of the C17-C20 bond, micro-organisms shorten the side chain of sterol by a mechanism similar to the β-oxidation of fatty acids. The activity of enzymes of the fatty acid β-oxidation system is influenced by the chemical structure of the substrate (Szentirmai, 1990). Thus, cholesterol is step-wise converted from a C27-steroid via C24 and C22 carboxylic acid (39) intermediates to a C18 steroid (40) with the production of 2 molecules of propionic acid and one molecule of acetic acid. These end products have been observed for various genera including Arthrobacter, Bacillus, Brevibacterium, Mycobacterium, Nocardia, Pseudomonas,
R—H = Cholesterol
CH₃ = Sitosterol

Oxidase

isomerase

Dehydrogenase

Androsta-1, 4-diene-3, 17-dione

The first reaction of the microbial side chain cleavage is hydroxylation of C-26 and its subsequent oxidation to steroid carboxylic acid. A C-26 steroid acyl-Co A is formed next by the action of a specific acyl-Co A synthetase. A multiple-enzyme complex completes the degradation of C-26 steroid acyl-Co A to Δ⁴-cholenic acid Co A and propionic acid. This enzyme complex contains an FMN dependent steroid acyl-Co A oxidase, a hydratase, a beta-hydroxyacyl-Co A dehydrogenase and a thiolase. Since this complex is highly unstable during purification it has led to the assumption that the units are only loosely associated by hydrophobic forces (Chen 1985). When sitosterol or campesterol is used as substrate, carboxylation at C-28 is carried out prior to separation of C_{25-26-27} as propionic acid (Fujimoto et al. 1982) from the steroid molecule. This steroid enoyl-CoA carboxylase contains (Chen 1985, Fujimoto et al. 1982) biotin and uses HCO₃⁻ as a second substrate (Chen 1985, Fujimoto et al. 1982). After the reverse-aldol lyase reaction, propionic acid or acetic acid are separated from the sitosterol or campesterol derivative respectively before Δ⁴-cholenic acid is formed (Chen 1985).

The multi-enzyme reaction sequence is repeated for the degradation of Δ⁴ cholenic acid, yielding C_{22} steroid bis nor chol-4-enic acid (BNC) and acetic acid. The conversion of C_{22}-steroid to a C_{19} steroid (40) proceed via a different mechanism and can also be carried out
anaerobically. Therefore, a C-17(20) dehydrogenation and C-17 hydration followed by an aldolytic fission is thought to be responsible for the formation of C_{19} steroid such as androst-4-ene-3, 17 dione (AD) and androsta-1, 4-diene-3, 17-dione (ADD) and another molecule of propionic acid (Tai et al. 1970, Sih et al. 1965, 1967). Overall side chain of 8 carbon atoms is converted to 2 molecules of propionic acid and one molecule of acetic acid. When the side chain of sitosterol or campesterol is degraded, an extra molecule of propionic or acetic acid is formed from HCO_{3} and C_{28-29} or C_{28} respectively.

Different microbes have atleast two pathways for cleavage of side chains. Horvath and Kramli (1947) isolated 6 methyl heptan-2-one from fermentation of cholesterol indicating a direct cleavage between C-17 and C-20 sterols. Bhattacharya et al. (1984), also indicated that a similar pathway exists in *Pseudomonas convexa*. However, this is a rare pathway and most organisms prefer to use step-wise degradation-terminal oxygenation of 19-hydroxy cholesterol followed by progressive β-oxidation.

**Enzyme Inhibitors for selective cleavage:**

The key enzyme in steroidal ring fission, the 9α-hydroxylase, has been found to be a monoxygenase consisting of several proteins forming an electron transfer chain. Some of these proteins contain Fe^{2+} as an essential metal ions. Removal or replacement of these ions results in complete inactivation of the enzymatic activity. Numerous processes for the selective side chain cleavage of sterols employing enzyme inhibitors have been developed. The most effective inhibitors are listed in Table-1 α, α-dipyridyl, 1, 10-phenanthroline and 8-hydroxyquinoline have been used most
frequently. Since the compounds are toxic or at least decrease the growth rate of micro-organisms they are added after the cultures have grown for sometime. In the presence of enzyme inhibitors, cholesterol as well as β-sitosterol, campesterol, stigmasterol and their mixtures can be effectively converted to \( C_{19}, C_{22}, \) or \( C_{27} \) steroids. (Steinert et al. 1987, Srivastava et al. 1983, Srivastava et al. 1985, Coto et al. 1985 and Martin 1977).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Mechanism of action</th>
</tr>
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<tbody>
<tr>
<td>α, α - Dipyridyl</td>
<td>Chelating agents for Fe(^{2+})</td>
</tr>
<tr>
<td>1, 10 - Phenanthroline</td>
<td></td>
</tr>
<tr>
<td>8 - Hydroxy quinoline</td>
<td></td>
</tr>
<tr>
<td>5 - Nitro - 1, 10 - phenanthroline</td>
<td></td>
</tr>
<tr>
<td>Cupferron</td>
<td></td>
</tr>
<tr>
<td>Diphenylthiocarbazone</td>
<td></td>
</tr>
<tr>
<td>Diethylthiocarbamate</td>
<td></td>
</tr>
<tr>
<td>Isonicotinic acid hydrazide</td>
<td></td>
</tr>
<tr>
<td>Xanthogenic acid</td>
<td></td>
</tr>
<tr>
<td>0 - Phenylenediamine</td>
<td></td>
</tr>
<tr>
<td>4 - Isopropyltropolone</td>
<td></td>
</tr>
<tr>
<td>Tetraethylthiuram sulfide</td>
<td>Metal ions replacing iron blocking SH-functions</td>
</tr>
<tr>
<td>( \text{Ni}^{2+}, \text{CO}^{2+}, \text{Pb}^{2+}, \text{SeO}_3^{2-}, \text{AsO}_2^{-} )</td>
<td>Redox</td>
</tr>
<tr>
<td>Methylene blue</td>
<td>Redox reaction</td>
</tr>
<tr>
<td>Resazurine</td>
<td></td>
</tr>
<tr>
<td>n-Propanol</td>
<td></td>
</tr>
</tbody>
</table>

Processes Employing Enzyme Inhibitors: Inhibition of microbial decomposition of cholesterol with metabolic inhibitors revealed that the addition of iodoacetate, \( \alpha, \alpha\)-dipyridyl, or arsenite caused
the accumulation of cholestenone and cholesta-1, 4-dien-3-one. Although some differences were observed in the inhibitory effect of the chemicals on microbial strains, p^CM^B causes the accumulation of the intermediates on *Arthrobacter simplex*, NaF on *Brevibacterium lipolyticum* and NaHSO\(_3\) on *Streptomyces tanashiensis* (Arima et al. 1969). These chemical were presumed to block an enzyme cleaving a sterol side chain. Wix et al. 1968 screened 59 strains of *Mycobacteria* and found that *Mycobacterium phlei* and *M. smegmatis*, *M. fortuitum*, *M. thermophilis* and *M. butyricum* converted 4-cholesten-3-one to ADD in yield of up to 55% in the presence of 8-hydroxy-quinoline. They also found that 1, 10-phenanthrolone, 5-nitro-1, 10 phenanthrolone, \( \alpha \), \( \alpha \)-dipyridyl and cupferron were effective in accumulating ADD. Numerous other steroids and sterols can also be transformed to ADD by *M. phlei*. The substrate concentration (cholesterol) could be increased to 1g/liter without any loss of yield. Later Tomorken\( \gamma \) et al. 1969 found that ADD can also be formed from 4\( \beta \), 5\( \beta \)-epoxycholestan-3-one.

Van der Waard et al. (1968) found that 104 mg Ni\( ^{++} \) per liter was optimum for the conversion of cholesterol to ADD. \( \beta \)-sitosterol, campesterol and stigmasterol were transformed under similar conditions though the yield was low as compared to cholesterol. In addition to Ni\( ^{++} \), presence of Co\( ^{++} \), Pb\( ^{++} \) and Se\(_2\)O\(_3\)\(^{--} \) resulted in accumulation of ADD.

Processes in which enzyme inhibitors are employed are summarized in Table 2. In addition to enzyme inhibitors, many different chemicals such as oils or adsorbents have been found to increase the yields of 17-ketosteroids. The selective inhibition of steroid ring fission is
optimised with respect to the nature of the enzyme inhibitor, its concentration and time of addition to the culture.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Product</th>
<th>Microorganism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2, 2'-Dipyridyl 1, 10-phenanthroline</td>
<td>1, 4-androstadiene-3, 17-dione (ADD) and</td>
<td><em>Mycobacterium sp.</em></td>
<td>Wix et al. 1968</td>
</tr>
<tr>
<td>8-hydroxy-quinoline</td>
<td>other androstane derivatives</td>
<td><em>A. simplex</em></td>
<td>Shah 1980</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Pseudomonas sp.</em></td>
<td>Iida 1985</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Rhodococcus sp.</em></td>
<td>Zajaczkowska 1988</td>
</tr>
<tr>
<td>AD, ADD</td>
<td></td>
<td><em>M. phlei</em></td>
<td>Saunders 1986</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. globiformis</em></td>
<td>Srivastava 1982</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Nocardia sp. 29</em></td>
<td>Martin 1976</td>
</tr>
<tr>
<td>Chelating agents and active carbon</td>
<td>ADD</td>
<td><em>Mycobacterium sp.</em></td>
<td>Mitsubishi 1978, 1979</td>
</tr>
<tr>
<td>Chelating agents and Styrene-divinyl</td>
<td>ADD, ADD, 23, 24 -dinocholanic acids</td>
<td><em>Mycobacterium sp.</em></td>
<td>Schering Ag.</td>
</tr>
<tr>
<td>benzene copolymers</td>
<td></td>
<td><em>NRRL B-3683</em></td>
<td></td>
</tr>
<tr>
<td>Chelating agents and fats, oils,</td>
<td>ADD, 1-dehydrotestosterone</td>
<td><em>A. simplex</em></td>
<td>Upjohn Co. 1978, Mitsubishi, 1978</td>
</tr>
<tr>
<td>glycerides</td>
<td></td>
<td><em>Brevibacterium lipolyticum</em></td>
<td>Upjohn Co. 1978</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>M. phlei</em></td>
<td>Upjohn Co. 1978</td>
</tr>
<tr>
<td>Inorganic ions</td>
<td>AD, ADD, other androstane derivatives</td>
<td><em>Mycobacterium sp. KNGSF 70</em></td>
<td>Vanderwaard 1969</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>A. simplex</em></td>
<td>Nagasawa 1970</td>
</tr>
<tr>
<td>Redox dyes</td>
<td>ADD</td>
<td><em>A. simplex</em></td>
<td>Nagasawa 1970</td>
</tr>
<tr>
<td>Propanol</td>
<td>AD, ADD, and other derivatives</td>
<td><em>Pseudomonas sp. NCIB 105</em></td>
<td>Owen 1983</td>
</tr>
</tbody>
</table>

AD - Androst-4-ene-3, 17-dione
ADD - Androsta-1, 4-diene-3, 17-dione
The preferred organisms used in such process are *Arthrobacter simplex*, *Nocardia* sp, *Brevibacterium lipolyticum* and *Corynebacterium* sp, but also strains belonging to the genera *Bacillus*, *Microbacterium*, *Serratia*, *Streptomyces* and *Pseudomonas* have been used occasionally. Mixed cultures of *A. simplex* and *Nocardia corallina* or *Pseudomonas aeruginosa* have been described to show enhanced productivity (Martin 1977).

α, α-dipyridyl, 1, 10-phenanthroline and 8-hydroxyquinoline are the preferred chelating agents. They are used in the concentration range 0.1-1 mM. In the fermentation of free sterol, chelating agents such as α, α-dipyridyl or phenanthroline, EDTA and other sequestering agents, 8-hydroxyquinoline and lipophilic micellar sequestering of substrates have been generally used with moderate success. But these reagents also invariably decrease the activity of iron containing C-26 hydroxylase resulting in sluggish metabolism. So far the mutational studies have been promising but are yet to lead to viable commercial process for C-19 steroids (Rehm and Reed, 1980).

Inorganic ions or redox dyes have been employed only very rarely. Adjustment of the optimal concentration of the chelating agent in the fermentation broth is essential for high yields but very difficult to achieve. Iron or other metal ions in the culture medium react with these compounds and neutralize their inhibitory effect on the enzymatic activity, as they are no longer available for complexing of intracellular protein bound Fe$^{2+}$. Therefore, the components of the culture broth have to be controlled rather exactly, especially if complex ingredients such as cornsteep liquor or molasses are used.
Specific suppression of breaking the steroid skeleton could yield the steroids deprived of the cumbrous sterol side chain. Three principle methods of preventing degradation of the steroid skeleton were deviced.

i) By chelating agents.

ii) By Ni\(^{2+}\) and Co\(^{2+}\) ions.

iii) By converting into phenolic and 6, 19-oxido (cyclo) steroids resistant against the microbial attack.

The effects of these compounds on the microbial degradation of cholesterol could be grossly classified to:

i) Depression of 3-hydroxyldehydrogenase, leaving starting cholesterol.

ii) Depression of \(\Delta^1\)-dehydrogenase, resulting cholest-4-en-3 one.

iii) Depression of the cleaving activity of sterol side chain, accumulating cholest-4-en-3-one and cholesta-1, 4-dien-3, 17-dione.

iv) Depression of breaking the steroid skeleton, thus accumulating C\(_{19}\) steroids.

The effects (i), (ii) and (iii) were observed distributed in a relatively wide range of compounds but the effect (iv) often accompanied by (i), (ii) and (iii) was limited to (a) chelating agents (b) Ni\(^{2+}\) and Co\(^{2+}\) (c) Hg\(^{2+}\), Cd\(^{2+}\), As\(^{3+}\), Sb\(^{3+}\), Bi\(^{3+}\), AsO\(_2\)\(^{-}\) and SeO\(_2\)\(^{2-}\) ions and (d) redox dyes as shown in Table-2.

Labedeva et al. (1972) observed that ADD was obtained from cholesterol, using *Proactinomyces asteroides*, in the presence of Co\(^{3+}\), \(\alpha\), \(\alpha\)-dipyridyl or 8-hydroxyquinoline. According to Awata et al. (1974) mixed cultures of *Arthrobacter simplex* and *Norcardia corallina* showed enhanced productivity. Cholesterol (2g/liter) was
converted to 21% androstane compounds in the presence of $\alpha$, $\alpha$-dipyridyl by 1:1 mixture of these strains. Okamoto and Teramoto (1974) found that certain antibiotics such as pencillin G, carbomycin, polymyxin B, streptomycin, chloramphenicol, neomycin and kanamycin caused 2 fold stimulation in the conversion of cholesterol to ADD, by mixed cultures, in the presence of $\alpha$, $\alpha$-dipyridyl. This increase was due to increased permeability of the cell membrane.

In order to trap the accumulating 17-ketosteroids it seemed promising to use hydrophobic organic resins known to adsorb steroids very efficiently. Addition of Amberlite XAD-2 and similar resins enhanced the ADD yields 2 to 3 fold. Studies on the mechanism of stimulation by these adsorbents indicated that $\alpha$, $\alpha$-dipyridyl was also adsorbed causing decreased toxicity of this compound although the capability to trap Fe$^{2+}$ was not hindered (Martin and Wagner 1976). However the main effect seems to consist in the selective adsorption of 17-ketosteroids because of micelle formation of the substrates and for this reason are not adsorbed—Presumably, the stimulation of the transformation by addition of active carbon is based on similar mechanism. Vadalkar (1980) reported presence of 1mM $\alpha$, $\alpha$-dipyridyl for the bioconversion of pregenenolone to androsta-1, 4-diene-3, 17-dione by Arthrobacter simplex.

Ahmed et al. (1991, 1991a) had reported Rhodococcus equi DSM 89-133 capable of cleaving side chain selectively in presence of 0.3mM $\alpha$, $\alpha$-dipyridyl. Maximum production of C-19 steroids was of the order 27 mole % of the initial cholesterol level in the medium. Perez et al. (2001) reported that enzyme inhibition by Ni$^{2+}$ seems to be effective in Arthrobacter cells for detection of androstadienedione
(ADD), as well as androstenedione (AD) and testosterone (TES) in cholesterol biotransformation experiments.

**Conversion of Sterols with Modified Structure:**

Dodson and Muir (1961) converted 19-hydroxy androst-4-en-3, 17-dione into estrone by a *Pseudomonas* sp. and Sih and Rahim (1963) observed the same reaction with *Nocardia restrictus*.

![Chemical structure diagram](image)

19-hydroxy-4-androstene-3, 17-dione (41)  

*Pseudomonas* sp.

Estrone (42)

Sih et al. (1965) thought it possible to aromatize a 19-nor or 19-hydroxysterol with the concomitant degradation of side chain to a 17-ketone. They prepared 19-hydroxy-4-cholestene-3-one and converted it to estrone in 8% yield by *N. restrictus*. A new isolate *Nocardia* sp. CSD 10 converted the above compound into estrone in 30% yield and 19-hydroxy-4-stigmasten-3-one into estrone in 10% yield.

Sih et al. (1965) and Lee (1967) converted cholesterol (43) to 3β-acetoxy-19-hydroxy-4-cholestene (44) and converted the latter into estrone (45) with *Nocardia* sp. CSD 10 in 72% yield. 3β-acetoxy-19-hydroxy-5-cholestene can be prepared by chemical means. The entire process, cholesterol to estrone, can be carried out in overall yield of 50% and is illustrated in the following figures :-
The complete methodology has been worked out to a high degree of perfection by Ciba group (Kalvoda et al. 1963). These developments have made the microbial process of estrone from sterols a formidable competitor against the synthetic process. Based on the observation of Sih and collaborators that functionalisation of $C_{19}$ group protects the steroid nucleus from microbial degradation, several other research groups studied the conversion of related compounds by microorganisms. Processes using substrate modification for the selective degradation of sterol side chain are summerised in Table-3.
Table 3
Selective cleavage of side chain chain with modified substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>Microorganism</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>19-Hydroxysterols</td>
<td>Estrone</td>
<td><em>Nocardia restrictus</em> ATCC 14887</td>
<td>Sih et al. 1965</td>
</tr>
<tr>
<td>19-Norsterols</td>
<td></td>
<td><em>Nocardia sp.</em> ATCC 19170</td>
<td></td>
</tr>
<tr>
<td>3-Hydroxy-19-nor-(\Delta^{1,3,5})-sterols</td>
<td></td>
<td><em>Mycobacterium phlei</em></td>
<td></td>
</tr>
<tr>
<td>19-Hydroxy-4-cholesten-3-one</td>
<td>Equilin, Equilenin</td>
<td><em>Mycobacterium sp.</em></td>
<td></td>
</tr>
<tr>
<td>19-hydroxy-(\Delta^{4,7})-sterol</td>
<td></td>
<td><em>M. phlei</em></td>
<td>Jankov et al. 1986</td>
</tr>
<tr>
<td>3-hydroxy-19-nor-(\Delta^{1,3,5(10)})-sterols</td>
<td>Estrone</td>
<td></td>
<td>Goswami et al. 1982</td>
</tr>
<tr>
<td>3-hydroxy-19-nor-(\Delta^{1,3,5(10)}, 7(8))-sterols</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3β-acetoxy-5α-chloro (fluoro)-6β-19-oxido-sterols</td>
<td></td>
<td><em>Mycobacterium sp.</em></td>
<td>Denot et al. 1967</td>
</tr>
<tr>
<td>3β-acetoxy-5α-bromo 6β-19-oxidosterols</td>
<td>5α-Bromo-6β, 19-oxido androstane-3, 17-dione</td>
<td><em>Nocardia sp.</em> ATCC 19170</td>
<td>Sih et al. 1968</td>
</tr>
<tr>
<td>3α, 5α-cyclosterols</td>
<td>3α, 5α-cyclo-androstan-17-one</td>
<td><em>M. phlei</em> KNGSF 70</td>
<td>Martin et al. 1976</td>
</tr>
<tr>
<td>4 hydroxycholestenone</td>
<td>3β-hydroxy, 5α-androstan-4, 17-dione</td>
<td><em>M. phlei</em></td>
<td>Tomorkeny et al. 1975</td>
</tr>
<tr>
<td>4-cholestone-3-(o-carboxy-methyl)-oxime</td>
<td>4-Androstene-17-one-3-(o-carboxy methyl)-oxime</td>
<td><em>M. phlei</em></td>
<td>Atrat et al. 1981</td>
</tr>
<tr>
<td>3β-19-Diacetoxycholesterol</td>
<td>Estrone</td>
<td><em>M. phlei</em></td>
<td>Jankov et al. 1986</td>
</tr>
<tr>
<td>19-Iodo-cholesterol</td>
<td>No transformation</td>
<td><em>M. phlei</em></td>
<td></td>
</tr>
</tbody>
</table>

In most microorganisms the side chain elimination is believed to take place before aromatization can occur, as 19-nor-3-hydroxy-cholesta-1,3,5(10) triene is not metabolized (Sih et al. 1965). On the other hand, Afonso et al. (1966) reported that the side chain can be eliminated after ring A aromatisation. The metabolism of 19, 6β-oxidosteroids gave good yields of the corresponding 6β, 19-oxido androst-4-ene-3, 17-dione. Deghenghi et al. (1967), Abbott et al. (1979) reported 19-hydroxy-cholesta-4,7-diene-3-one gave estrone.
Available evidence indicates that the reduction of C-7(8) double bond is a pre-requisite for complete removal of cholestane side-chain and aromatization of ring A. However, *Mycobacterium* sp. using 19 hydroxy-4, 7-cholestadiene-3-one yielded a mixture of estrone (20%), equilin (16%) and traces of equilenin. Similarly 19-nor cholesta-1, 3, 5(10) 7 and 8 tetraene-3-ol were transformed to equilenin and traces of equilin by *Corynebacterium simplex* and to equilenin by *Nocardia rubra* (Denet et al. 1967). It is believed that the presence of the 19-hydroxy methyl group prevents 9α-oxygenation. This is true of most strains excepting in *Corynebacter* which produces $\Delta^{9-11}$ estrone, presumably from the 9α-hydroxy intermediates. 3β-acetoxy-5α-chloro-6β, 19-oxidocholestane (46) has also served as substrate for microbial side chain cleavage. *Nocardia* sp. ATCC 19170 was capable of converting it (46) to 6β, 19-oxido androst-4-ene-3,17-dione (47) with an yield of about 36% in 80 hr.

\[
\text{H}_2\text{COC} \quad \text{Cl} \quad \text{H}_2\text{COC} \quad \text{Cl}
\]

3β-Acetoxy-5α-chloro-6β, 19-oxido cholestane

(46)

\[
\text{Nocardia sp.} \quad \text{ATCC 19170} \quad \text{Nocardia sp.} \quad \text{ATCC 19170}
\]

6β, 19-oxidoandrost-4-ene-3, 17-dione

(47)

Conversion efficiency with the strain ATCC 19170 for 3β-acetoxy-19-hydroxy-5-cholestene which can be prepared from cholesterol in three chemical steps was about 70%. 19-norcholesta-1, 3, 5 (10) triene-3-Ol (48) was not oxidized by this strain but prolonged
incubation (240 hrs.) with *N. restrictus* ATCC 14887 yielded estrone (49) in small amounts, reported by Charney and Herzog (1967).

Chemical modification of steroids have met with success in the prevention of ring degradation. Particularly noteworthy is the fermentation of 3α, 5α, cyclo-6β-hydroxy sterols and their methyl ethers to yield the corresponding 17-keto derivatives in 60% yield in *Mycobacterium phlei* KNGSF 70. (Shirasaki et al. 1969) The 6β, 19-oxido derivatives of sterols have also been converted to 17-oxo steroids by *Arthrobacter sp.* (Van der Waard et al. 1970) Successful side chain elimination from 3β-methyl ether of sterols as well as 4β - 5β oxide cholestan-3-one was observed in the presence of enzyme inhibitors (Ramadevi 1976).

Substrate modification of a completely different nature had been found to prevent breakdown of the steroid ring system when organisms capable of utilizing cholesterol are used C19 steroids could also be prepared by combining different methods of substrate structure modifications.
Growth of *M. phlei* on 4-hydroxy-4-cholestene-3-one (50) yielded 3β and 3α-hydroxy-androstane-4, 17-dione (51) and a further reduced compound, 3β, 4α-dihydroxy-5α-androstane-17-one (52) (Tomorkeny et al. 1975).

Buki et al. (1976) reported preparation of dehydroepiandrosterone (54) from 3β-alkoxy cholest 5-enes (53) and 3β-alkoxy stigmast-5-ene by aerobic fermentation with *M. phlei*. 
The fermentation products are modified likewise and cannot serve as substrates for the enzymes which subsequently attack the ring structure. (Bhome et al. 1980, Dehgeng i et al. 1967, Denot et al. 1967).

Horhold et al. (1979) had reported production of 4-androstene-3, 17-dione (AD) from a cholesterol derivative such as cholest-4-ene-3-(O-carboxymethyl)-oxime by *M. phlei*.

For strains of *Mycobacterium*, cholesterol-3-carboxy methylxime derivatives (55) have been described as ideal substrates with respect to solubility and transformation rates (Bohme et al. 1980); the resulting androstan-3-oxime (56) can be saponified to the corresponding ketone under acidic condition (Atrat et al. 1981).

![Chemical structures](image)

Kolyvanova et al. (1982) reported that cholesterol has been converted to ADD, cholesta 1-4-diene-3-one and cholest-4-ene-3-one by *Arthrobacter simplex* and the resulting ADD has been separated from cholestanes by adsorbing it on active carbon. Strains of *Mycobacterium flavum, M. mucosum* and *M. lacticola*, metabolize
19-hydroxysitosterol and its acetate to 19 hydroxy androst-4-ene-3, 17-dione and estrone.

Kuraray et al. (1983) reported preparation of 12β-hydroxy androsta-1, 4-diene-3, 17-dione (57) from deoxycholic acid (58) or its salts using the strains *Pseudomonas stutzeri*.

Bhattcharya et al. (1984) reported conversion of cholesterol derivatives 3β-methoxy cholest-5-ene, 3β-chlorocholest-5-ene, 3, 5-cyclocholestan-6-one and potassium cholesteryl sulphate, by *Moraxella sp.*, to the corresponding 17 keto steroids in 10-15% yield in shake flasks. Klimashina et al. (1989) reported the substrate specificity of biotransformation enzymes of culture *Nocardia erythropolis*. It was established that delta 22 bond in the side chains of 3 sterols of microbial origin of ergosterol, ergosta-5, 7-dien-3 beta-ol and ergosta-7, 22-dien-3 beta-ol and delta 7 bond slows, and delta 5 bond of cholesterol makes impossible cleavage of side chain of sterols.

Madyastha et al. (1994) reported the microbial conversion by *Moraxella sp.* of 3β-acetoxy-19-hydroxy cholest-5-ene (6 gm) into
estrone (712 mg) a major metabolite. Minor metabolites identified were 5α-androst-1-en-19 ol-3, 17-dione (33mg), androst-4-en-19 ol-3, 17-dione (58 mg), androst-19 ol-3, 17-dione (91 mg).

**Conversion of Sterols by Mutants:**
Mutagenic treatment has been employed for improving the efficacy of sterol side chain cleaving bacteria. Such mutants are biochemically blocked at 9α-hydroxylase and/or C-1(2) dehydrogenase expression level i.e. unable to produce either both the enzymes or at least 9α-hydroxylase. Such mutants can degrade sterol side chain selectively without the necessity of modification of the substrate or addition of the enzyme inhibitors (Wovcha et al. 1978, Cargile et al. 1974).

Mutants blocked at different levels were isolated from *Mycobacterium fortuitum* ATCC 6842 after treatment with N'-methyl-N'-nitro-N-nitrosoguanidine (NTG). Each of the mutant was found to efficiently converting β-sitosterol and other sterols to products useful as intermediates in the manufacture of medically important steroids.

Further mutation of *M. fortuitum* NRRL B-8119 yielded strain SCM-1 which could not degrade sterol side-chains that were branched at C-24. Conversion of β-sitosterol (59) by this mutant resulted in the accumulation of 9α-hydroxy-27-nor-4-cholesten-3, 24-dione (60), whereas cholesterol (61) was degraded mainly to 9α-hydroxy-4-androstene-3, 17-dione (62) (Wovcha et al. 1978).
The available information on the use of mutants is, however, limited because most such data is patented (Mitsubishi Chem Ind. Ltd. 1978, 1979, Richter Gedcon Vesgyeszeti Gyar, 1983, Sankyo Co. Ltd. 1966, 1967, Sih 1984, Upjohn Co. 1978, 1980, Wisconsin Alumini Res. Found, 1965, 1966). Imada et al. (1979) reported preparation of ADD (64), AD (65), 17β-hydroxy androsta-1, 4-diene-3-one (66) and testosterone(67) from cholesterol (63) by a mutant of *Arthrobacter simplex* (MC-1-0803) and has also been patented.
The most interesting structures with partially degraded side chain are, 20-carboxy and 20-hydroxy-methyl-4-pregnene-3-ones and their corresponding 1, 2-dehydro derivatives. Thus, such acids can be
readily converted into progesterone, 1, 2-dehydro progesterone or 17α-hydroxy-1, 2-dehydro progesterone and therefore useful starting material for production of corticosteroids (Neuland 1979). Although yields averaging 80% have been described, the best process appears to be based on the utilization of a mutant of Corynebacterium sp. DSM 1444 with the resulting bioconversion of 3g/l cholesterol (68) to 2.5 g 20-carboxy-pregna-1, 4-dien-3-one (69) in 72 hr (Schubert et al. 1978).

![Chemical structures](image)

R-H=cholesterol
CH₃=sitosterol
(68)

20-carboxy-pregna-1, 4-diene-3-one
(69)

12β-hydroxy-pregna 1, 4-diene-3-one
(70)

12α-hydroxy-pregna 1, 4-diene-3-one
(71)

Process for the production of 20-hydroxymethyl compounds were patented but economic considerations required selectively high yields (Mitsubishi et al. 1979).
However, 20-carboxy pregnane derivates were isolated as intermediates in various procedures (Hill et al. 1982, Sharmah et al. 1989). 9, 10-seco-derivatives such as 3-hydroxy-9-oxo-9, 10-seco-pregna-1,3, 5 (10)- triene-20-carboxylic acid formed by A. simplex mutants are useful intermediates in the production of Vitamin D₃.

Therefore partial degradation of sterol side chain has been of principal interest. However, mutants chol 73 DSM 1444 and ATCC 31385 from Corynebacterium sp. are capable of converting cholesterol nearly quantitatively into 3-oxo-23, 24-dinorcholesterol-1, 4-diene-22-oic acid (Hill et al. 1982). Knight and Wovcha (1984) reported dinorchorlanic acids, partially degraded side chain structure, are frequently found in small amounts, when sterols are transformed by micro-organisms. As for process in which enzyme inhibitors have been employed, certain additives to the fermentation broth also stimulate bioconversion by various mutants. For instance, addition of small amounts of vegetable oil such as soyabean, egg yolk, lecithins and cyclodextrins enhanced C₁₉ steroid yields from sterols by mutants of Arthrobacter, Mycobacterium and Nocardia (Martin 1977, Hesselink et al. 1989). The formation of C₂₂ steroids was stimulated by compounds containing boron. Although adsorbents such as styrene-divinyl benzene resins increase C-19 steroid yield with enzyme inhibitors, they also stimulate product formation with the mutants. Therefore, a major effect appears to be selective adsorption of C₁₉ steroids as a results of formation of micelles of sterols. With Mycobacterium sp. NRRLB-3683, C₁₉ steroid yields of more than 80% were obtained in the presence of 5g Amberlite XAD-2 g per lit. and 7g Tween 20 per lit.
The technique of transposon mutagenesis has been successfully used for the production of hydroxy-3-oxo-1, 4-pregnanadiene-20-carboxylic acids (hydroxy OPDC) from bile acids (Park et al. 1987). Transposon induced mutants of the *Pseudomonas* PS 8-1 obtained by using Tn5 and Tn10 accumulated three groups of catabolites from bile steroids. The catabolites accumulated were:

1. hydroxy-3-oxo-1, 4-pregnanadiene-20-carboxylic acids (hydroxy OPDC).
2. hydroxy-3-oxo-5β-cholanic acids
3. hydroxy-3-oxo-4-cholenic acids.

All mutants isolated reverted at frequencies ranging from $10^{-6}$ to $10^{-8}$ per cell plated. One Tn5 induced mutant that accumulated hydroxy OPDC type products was stabilized such that reversion frequency was reduced from $10^{-7}$ to $10^{-8}$ per cell plated. This stabilized strain could be used for 96 hr. without appreciable product loss. The concentration of upto 1% bile acid could be transformed to OPDC type products with yields of over 80%. Microbial production of OPDC from sitosterol has also been patented (Iida et al. 1987) *Corynebacterium* K-3, a mutant from *Corynebacterium* 320-6-2-1 was cultured in a medium containing a sitosterol - campesterol mixture for production of OPDC, the product OPDC was obtained in 56.9% yield.

Choi and Lee (1989) reported a process for the improvement in yield of ADD from cholesterol. By using UV and/or NTG (N-methyl-N'-nitro-N-nitrosoguanidine) as mutagens, a mutant of *Brevibacterium lipolyticum* was developed which converted cholesterol to ADD with a yield greater than 60%. A comparative study of mutagenesis of a strain of *Nocardia* sp. with NTG, Ethidium bromide (EB) and UV
light was undertaken by Jiang 1990. It was observed that NTG and UV were better mutagens than EB. The optimal dose of NTG was 1000 μg ml⁻¹ and the UV was 2-3 min. After treatment the strain showed a conversion rate of > 30% which was initially only 5.8%.

Perez et al. (1995) reported isolation and partial characterization of new mutant for sterol biotransformation in Mycobacterium sp. This new sterol biotransforming mutant was isolated from NRRLB-3683 Mycobacterium sp. after nitrosoguanidine mutagenesis. The mutant showed an enhanced ability to biotransform stigmasterol into 17 ketosteroids compared with parental strain. Barathakur et al. (1996) reported steroid transformation by mutants of Mycobacterium sp. with altered response to antibiotics. Borrego et al. 2001 studied the three steroidal precursor producing mutants of Mycobacteria preserved by freeze-drying for ten years. Egrova et al. (2002) reported production of androstenedione using mutants of Mycobacterium sp. After treatment with ethyl methane sulfonate or mitomycin C, mutants were obtained that retained the ability to produce AD from sitosterol with a molar yield of 70-75%. A mutant strain was reported to effectively reduce 3, 17-diketosteroids at C-17. The 17β-hydroxysteroid dehydrogenase activity of its crude extract was twice as high as that found for the parent organism. This approach offered the possibility of obtaining improved labelled biocatalysts for AD or testosterone production from sterols.

**Newer Techniques for selective transformation:**

Microbial transformations of steroids have often met with two serious obstacles:

i) A limited substrate accessibility to the biocatalyst as a result
of low aqueous solubility of most steroids.

ii) Toxicity of both substrate and product exerted upon the micro-organisms. Attempts have been made to overcome these limitations by the use of surfactants and water-miscible or immiscible solvents. (Hesselink et al. 1989, Bar 1989, Goswami et al. 1983).

However, the introduced surfactants or organic solvents may partly or fully inactivate the biocatalyst. So the inherently non-toxic ingredients viz. biocompatible cyclodextrins can be added to the culture medium to eliminate these shortcomings. Spasov et al. (1983) observed that the cyclodextrin inhibited the precipitation of the substrate and the time required for the transformation could be lowered. Further, crystallographic monitoring of microbiological steroid transformations has been reported. Cyclodextrins are well known for their remarkable ability to complex organics and form water soluble and insoluble inclusion compounds (Szejtli 1988). This property has been applied in facilitating and enhancing microbe mediated transformations of toxic or water insoluble organics (Uvasdy et al. 1983, Hesselink et al. 1989, Bar et al. 1989). Hesselink et al. (1989) stated that the microbial side-chain cleavage of sterols is a slow process because of their low solubility of substrates and products and their low transport rates to and from cells. They have demonstrated that the addition of cyclodextrins substantially enhance the conversion of cholesterol, sitosterol and Δ^4-cholestenone to a mixture of AD and ADD by Mycobacterium sp. NRRL B-3683 in a purely aqueous fermentation system. For cholesterol, β-cyclodextrin gave the best results whereas γ-cyclodextrin was the best clathrate for β-sitosterol and Δ^4-cholestenone biotransformation. A 2:1 molar ratio of cyclodextrin:
substrate was found to be optimal in all cases giving rise to 1.7-3.0 fold increase of the specific side chain cleavage activity. It was observed that the cyclodextrins had no influence on cell growth. Application of the optimal conditions resulted in the enhancement of molar yield of ADD from 35-40% to over 80% in the presence of cyclodextrins.

Horhold et al. (1990) reported that styrene-divinyl benzene copolymers such as Amberlite XAD-2 resins were capable of adsorbing C-19 and C-22 steroids, selectively as sterols form micelles in aqueous suspension and thus not absorbed. The addition of such polymers has been reported to result in 2-3 fold increase in yields of C-19 steroids. An intensive and systematic investigation of the oxidation of cholesterol to cholest-4-ene-3-one by Rhodococcus erythropolis was undertaken by Jaudon and Bar (1993). The biotransformation was studied in a stirred bioreactor. They found that addition of substrate with either β or γ-cyclodextrin led to a limited enhancement effect. The microbial oxidation of β and γ-cyclodextrin complexes of cholesterol was totally inhibited. In contrast, the alkylated cyclodextrins, dimethyl-, trimethyl- and hydroxypropyl-β-cyclodextrin exhibited a remarkable enhancement of the microbial oxidation, irrespective of their mode of addition.

Sedlaczek et al. (1999) reported increase in the formation of androstene derivatives from side chain degradation of β-sitosterol by Myobacterium sp. NRRLB 3683 in the presence of glycine. As the sterol transformation is carried out inside the cell, higher product accumulation could indicate the faster diffusion of highly hydrophobic substrate through the cell wall permeability barrier. Cell wall
preparations were obtained to analyse the effect of glycine on peptidoglycan components. Peptidoglycan is known to be the target for glycine action. In glycine treated preparations, the molar ratio of diaminopimelic and muramic acid, the marker compound of tetrapeptides and glycan strands respectively was about 60% lower than in the control. This indicates a possible reduction in cross linking between peptide units and the destruction of peptidoglycan. Unexpectedly, glycine also caused changes in the relative proportion of mycolic acids to other lipids occurring in the strain used for this study. The enhancement of β-sitosterol side chain degradation is likely to result from disturbing the integrity of cell wall components responsible for permeability barrier in *Mycobacteria*.

**Strain Improvement:**

Strain improvement is a complex and multidisciplinary activity and an excellent review on this subject has been covered by Rowland (1984). Some progress in this direction has been made is amply demonstrated by reports dealing with the physiology of side-chain cleavage and evidence for the plasmid mediated degradation of steroids in at least 37 different bacterial strain (Tenneson et al. 1979, Szentirmai 1990). Attempts are made for improvement of strains, modification of substrates and manipulation of media, temperature and period of incubation in order to pursue microbial transformation of steroids for development of processes for exploitation by the drug industry and also for preparation of newer potentially useful steroid analogues which are otherwise inaccessible (Mahato et al. 1985). Kirby and Usdin (1985) reported the isolation and restriction mapping of a mini-plasmid from the actinomycete *Nocardia corallina*. The plasmid pKU 100 isolated was a ccc DNA molecule, 2.7 kb in length.
with a wide variety and a number of unique restriction sites making it suitable for developing as a cloning vector.

Sharmah et al. (1989) isolated *Arthrobacter oxydans* 317 which was incapable of steroid ring degradation interestingly, this was a plasmid cured strain and was unable to accumulate 4-androstene-3, 17-dione from β-sitosterol; this mutant also lacked the enzyme Δ¹-dehydrogenase but was able to hydroxylate C₁⁹ steroids at C-17 position. Furthermore, this strain was able to degrade side chain of β-sitosterol partially. Hesselink et al. (1989) constructed the total DNA gene bank of *Mycobacterium sp.* NRRLB-3683 in the cosmid pLAFRI in order to identify genes responsible for steroid transformation. After *in vitro* packaging by the phage λ, *Escherichia coli* HB 101 or XJS 5037 were infected, resulting in a gene bank of 32,000 recombinants. Recombinant plasmids were conjugated to *Pseudomonas testosteroni* and conjugants were screened for growth on steroids. Based on this technique 22 positive recombinants have been isolated. Novel steroid intermediates were produced by 8 *P. testosteroni* recombinants. One of these recombinants showed significant nucleus degradation. It was observed that *E. coli* strains carrying same *Mycobacterium* DNA inserts could not transform cholesterol nor did they degrade the steroid nucleus. Clearly *E. coli* was not suitable host for such conversions. Their study showed that genes coding for some essential steps in cholesterol degradation are possibly organised in a single operon on the 12.45 kb fragments. The use of hybrid bacteria has already been patented for steroid production by GBF (Germany) 1979. The above scenario clearly points towards more basic knowledge of the metabolic and genetic understanding of side chain cleavage system before recombinant DNA research could result in
structural improvement of microbial sterol transformations (Hocknull 1989, Konard 1983).

Dutta et al. (1992) reported that plasmid determines 1(2) dehydrogenation and 9α-hydroxylation of steroid ring structure in *Arthrobacter oxydans*. Furthermore the metabolic blocks in the degradation of β-sitosterol by a plasmid cured strain were reported. The strain could form androsta-1, 4-diene-3, 17-dione from 3-oxo-23, 24 bis-norchol-1, 4-dien-22-oic acid to a limited extent. The existence of metabolic blocks to 3-oxochol-4-en-24-oic acid to 3-oxo-23, 24 bis-norchol-4-en-22-oic acid and further conversion to AD by the plasmid cured strain 317 A1 was suggested. Dogra and Qazi (1999) identified *Micrococcus roseus* RJ 6 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. Dogra and Qazi (2001) aiming at obtaining a strain useful for the industrial production of corticoid intermediates, isolated bacteria from soil and animal faeces and evaluated a particular isolate identified as *M. roseus* for specific sterol degradation activity. This isolate was matched to *Micrococcus* strains with cholesterol degradation activity to yield 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 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.
Vidal et al. 2001 reported selection of phytosterol biotransforming strains from *Mycobacterium sp.* using a high concentration of β-sitosterol (14g β-sitosterol per lit.) as the unique source of carbon. During 6 months, the bacterial cultures were transferred successively. It was observed that after seven transfers, the *Mycobacterium sp.* MB-3683 and the *Mycobacterium fortuitum* B-11045 increased their biotransformation capacity from 20% to 64% and from 34% to 55% respectively. The products in the highest proportion identified for each trial were androstenedione and androstadienedione. Their results suggested that the high substrate concentration could be a selective mechanism to obtain strains more efficient in the biotransformation of β-sitosterol into steroidal bases. The extraction of the biotransformation products was made with methanol and ethyl acetate (1:1). The qualitative and quantitative analysis was made by means of thin layer chromatography and gas-liquid chromatography.

The deletion of a nucleotide sequence encoding the 3 keto-steroid Δ¹ dehydrogenase of *Rhodococcus erythropolis* RG1-UV 29, a natural steroid degrading microorganism, by Van der Geize et al. (2001) as a tool to obtain an effective strain to produce 9α-OH-AD from AD. Inactivation of two genes kst D1 and kst D2, was necessary to eliminate 3-keto-steroid Δ¹ dehydrogenase activity, thus preventing the degradation of steroid nucleus (Van der Geize et al. 2000). This allowed the accumulation of the desired product in high yields (80-100%) from 10 to 20 g per lit. initial substrate concentrations, in bioconversion runs of about 20hr (Harker et al. 2001), a noticeable enhancement in productivity, when compared to results published so far (Angelova et al. 1996 and Mutafov et al. 1997)
Perez et al. (2003) had stated resistance to androstanes as an approach for androstandienedione (ADD) yield enhancement in industrial mycobacteria. They had stated that the resistance to ADD of industrial mycobacteria was demonstrated as a valuable approach to increasing ADD yield in sterol fermentations. Colonies growing at 1 mg per ml ADD in culture medium after nitrosoguanadine mutagenesis showed a differential behaviour in respect to parentals in cholesterol biotransformation. In the presence of exogenous ADD, a substantial depletion of ADD production was observed in parented strains B 3683 and Ex 4, whereas it was unaffected and even increased in resistant colonies. An apparent reduction from ADD to androstandione and testosterone was also noticed. Furthermore, the ADD resistance phenotype may be related to the increase in steroid 1, 2 dehydrogenase activity. Perez et al. (2003) had reported ADD yield in fermentation from cholesterol using mixed cultures *Mycobacterium-Nocardiodes*. They stated that ADD yields in fermentation from cholesterol using mycobacteria may be limited by a certain amount of AD, also produced in fermentation broths, which can interfere further crystallization and purification process. As in general it has been demonstrated that for AD over 10%, the interference is highly remarkable. In order to face this problem the 1, 2 dehydrogenase activity from NCIB 8929 *Nocardiodes simplex* was used for AD conversion to ADD in cholesterol fermentation by an AD producing mycobacterial strain, *Mycobacterium sp. MB 3683*. After 96 hr. of an initial biotransformation by mycobacteria, *Nocardiododes* young cultures were added and 18 hr later, all the AD produced was transformed to ADD. If *Nocardiodes* culture was previously induced with AD 0.05 mg/ml for 5 hr., a better response was noticed.
Brzostek et al. (2005) reported that the catabolic potential for sterol degradation of fast-growing mycobacteria was well known. However, no genes or enzymes responsible for the sterolid degradation process have been identified as yet in three species. One of the key enzymes required for degradation of the steroid ring structure is 3-ketosteroid delta super (1)-dehydrogenase (ksdD). The recent annotation of the *Mycobacterium smegmatis* genome (TIGR database) revealed six ksdD homologues. Targeted disruption of MSME G 5898 (ksdD-1) gene, but not the MSME G 4855 (ksdD-2) gene, resulted in partial inactivation of cholesterol pathway and accumulation of intermediate 4-androstene-3, 17-dione. This effect was reversible by the introduction of the wild type ksdD-1 gene into *M. smegmatis* Delta ksdD-1 or overexpression of ksdD-2. The data indicated that ksdD-1 is the main ksdD in *M. smegmatis* but that ksdD-2 is able to perform the cholesterol degradation process when over produced.

**Immobilization:**


A comparison of side chain cleavage activity of immobilized cells is presented in Table-5.
<table>
<thead>
<tr>
<th>Microorganism</th>
<th>Support</th>
<th>Substrate</th>
<th>Product</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nocardia</td>
<td>DEAE-Cellulose</td>
<td>Cholesterol</td>
<td>C$<em>{19}$ and C$</em>{22}$</td>
<td>Atrat et al. 1980b</td>
</tr>
<tr>
<td>erythropolis</td>
<td>Ionic cross-linked</td>
<td></td>
<td>steroids</td>
<td></td>
</tr>
<tr>
<td></td>
<td>polymer gel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polyethylene</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>maleic acid anhydride</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Polyacrylamide gel</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycobacterium phlei</td>
<td>Polyacrylamide gel</td>
<td>3, 3-Ethylene</td>
<td>Corresponding side chain</td>
<td>Atrat et al. 1980</td>
</tr>
<tr>
<td>IMET SG 1026</td>
<td>cholest-5-en-4- (0- carboxy methyl) - oxime</td>
<td></td>
<td>4-cholesten-3- cleaved derivatives</td>
<td></td>
</tr>
<tr>
<td>M. smegmatis</td>
<td>IMET H 124</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. phlei</td>
<td>Polyacrylamide gel</td>
<td>&quot;</td>
<td>C$_{19}$-Steroids</td>
<td>Borman et al. 1983</td>
</tr>
<tr>
<td>IMET GS 1026</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhodococcus equi</td>
<td>Polyacrylamide gel</td>
<td>Cholesterol</td>
<td>C$_{19}$-Steroids</td>
<td>Ahmad et al. 1990</td>
</tr>
<tr>
<td>DS M89-133</td>
<td>Alginate</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Celite beads</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Agar-agar</td>
<td>19-Hydroxy Cholesteryl acetate</td>
<td>Estrone</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Adyopolated polystyrene</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5
Side chain cleavage efficiency of immobilized bacterial cells

<table>
<thead>
<tr>
<th>Micro-organisms</th>
<th>Immobilized / Free Cells</th>
<th>Efficiency (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mycobacterium phlei ISMET SG 1026</td>
<td>Immobilized (Polyacrylamide gel)</td>
<td>96</td>
<td>Atrat et al. 1981</td>
</tr>
<tr>
<td></td>
<td>Free Cells</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Immobilized</td>
<td>50-60</td>
<td>Atrat et al. 1980</td>
</tr>
<tr>
<td>M. fortuitum DSM 1134</td>
<td>Immobilized (Chitosan/E 100)</td>
<td>24</td>
<td>Steinert et al. 1987</td>
</tr>
<tr>
<td></td>
<td>Immobilized (Silicone)</td>
<td>10</td>
<td>Steinert et al. 1987</td>
</tr>
<tr>
<td></td>
<td>Free Cells</td>
<td>68</td>
<td>Steinert et al. 1987</td>
</tr>
<tr>
<td>Rhodococcus equi. DSM 89-133</td>
<td>Immobilized (Alginate)</td>
<td>74</td>
<td>Ahmed et al. 1990</td>
</tr>
<tr>
<td></td>
<td>Immobilized (Polyacrylamide gel)</td>
<td>60</td>
<td>Ahmed et al. 1991</td>
</tr>
<tr>
<td></td>
<td>Free cells</td>
<td>80</td>
<td>Ahmed et al. 1991</td>
</tr>
<tr>
<td></td>
<td>Immobilized (Porous celite bead)</td>
<td>71</td>
<td>Ahmed et al. 1990</td>
</tr>
</tbody>
</table>

Immobilized cells of Mycobacterium phlei were able to degrade side chain of 4-cholesten-3-(O-Carboxy-methyl)-oxime to 4-androstene-17-one-3(O-carboxy-methyl)-oxime in a continuous system over a period of more than 40 days. In another study, influence of polymerising matrix on side chain cleavage activity of M. phlei was studied by Atrat et al. (1980) which demonstrated that conditions of polymerisation including number of cells incorporated and temperature of the reaction substantially affected side chain cleavage (Atrat et al. 1980a). The half life of immobilized cells has been found to be several fold higher than the free cells (Srivastava et al. 1982). Atrat et al. (1980b) compared several immobilization techniques for side chain cleavage.
by *M. phlei* and *M. smegmatis* of two cholesterol derivatives namely 3, 3-ethylenedioxycholest-5-en and 4-cholesten-3(O-carboxymethyl)-oxime. *Mycobacterium phlei* IMET SG 1026 was suitable for oxime derivative with a conversion efficiency of 50-70%. Immobilized *M. phlei* cells exhibited higher transformation stability in comparison to free intact cells (Atrat et al. 1980b). *Nocardia erythropolis* was immobilized on several carrier amongst which polyethylene malic acid anhydride exhibited higher activity yielding C-22 and C-19 products (Atrat et al. 1980a). To overcome the solubility problem cholest-4-en-3-(O-carboxymethyl) oxime which is more soluble was degraded smoothly by polyacrylamide encapsulated *M. phlei* to the corresponding androst-4-en-3-(O-carboxymethyl) oxime-17-one (Madyastha et al. 1984). Krishna Rao et al. (1986) immobilized *Moraxella* sp. on several matrices including adepolylated polystyrene, polyacrylamide gel and agar-agar; the latter retained highest side chain cleavage activity. Flygare et al. (1987) reported a new approach for immobilization of *Mycobacterium* sp. for side chain cleavage of cholesterol to AD and ADD. This approach involved adhesion of magnetic materials of submicron size to the bacterial surface. This was based on laboratory prepared magnetic oxide that had been derivatized with hydrophobic octyltrichlorosilane (OTCS) or 3-amino-propyl triethoxysilane (ADS) or trimethyl chloro silane (TMCS) or coupled with OTCS and TMCS both. Among the above preparations magnetic oxide derivatized with OTCS showed best properties. It is interesting to note that magnetic materials exhibited no adverse effect on the transforming capacity of the cells and the activity of immobilized bacteria was comparable to or even better than activity of free cells.
In a recent study, Ahmad et al. (1990, 1990a) immobilized Rhodococcus equi for side chain cleavage of cholesterol. They observed that immobilized cell had at least five times higher half-life than the free cells. Rhodococcus equi immobilized on to porous celite beads retained an activity profile comparable to free cells; however, biomass loading appeared to be a limiting factor (Ahmad et al. 1990). As indicated by emerging literature this immobilization methodology appears promising (Klein et al. 1990). The lower efficiency of the consecutive batchwise mode of operation was related to the oscillations in the metabolic state of the cells.

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 5g per lit. 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 (Dias et al. 1994, Fernandes et al. 1998, Llanes et al. 2001).

The improvement in the retention of catalytic activity, when continuous operation was used, instead of the repeated batchwise mode was more significant, in the Δ4-reduction of ADD, performed by polyacrylamide-hydrazide immobilized cells of Clostridium paraputrificum. Abramov et al. (1990) further reported that conversion yields in excess of 95% were maintained for around 100 hrs, whereas in repeated consecutive bioconversions of 5 hrs a decrease in conversion yield of about 30% per incubation was observed.
Industrial Application:

Steroids are of vital importance for biotechnology based pharmaceutical industry because of their life saving nature. (Abbott et al. 1978).

According to an estimate of the early 1980s (Mahato and Majumdar 1993), the production of steroids drugs and hormones to the value of $400 million involved biotechnological procedures. Bhattacharya et al. (1984), estimated, the current turnover of estrone based oral contraceptives in India is Rs. 4 crore per annum. Although methods were developed in several laboratories for total synthesis of estrone and steroid analogues, the Indian steroid industry largely depended on imported estrone, as it finds the cost involved in large scale manufacture abnormally high.

If side chain cleavage activity becomes economically feasible in monophasic or biphasic systems at the industrial scale, it would be a highly attractive proposition. In this context, the possibility of side chain cleavage of sterols in organic media has been highlighted by Ahmad et al. (1990), Srivastava et al. (1985).

Schmid (2001) stated that a well established industrial application of biocatalysis is the biotransformation of steroids, the products of which are used in contraceptives and other steroid hormone derivatives. 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 multistep conversions with a single biocatalyst (Fernandes et al. 2003).
The steroid drug production industry demands more than 2000 tons of natural sterols annually (Szentirmai 1990) and there is an increasing need for cheap and available sterol containing raw materials. 'Sterol constituent' represents one of the major factors determining the price of the product C$_{17}$-Ketosteroid, and minimization of the cost is needed. Attempts have been made to obtain C$_{17}$-Ketosteroids (androstenedione-AD and androstadienedione-ADD) from the wastes of the pulp and paper industry, neutral and upgraded neutral fraction of tall oil products, without isolation and purification of phytosterol.

The world wide market for AD and ADD is estimated to be approximately U.S $ 750\times10^6$ annually (Biojournal 2000). Processes become cheaper according to lower costs for obtaining such raw materials or substrates (Fernandes et al. 2003). For instance, Forbes Medi-Tech has developed an innovative fermentation technology that converts plant sterols, isolated from tall oil (Kutney 1998), into pharmaceutical fine chemicals, essential for the production of various pharmaceutical steroids such as contraceptive agents and anti-inflammatory.

Gibewonyo et al. (1990) at Merck Sharp and Dohme Research Laboratories, Rahaway, New Jersey developed a large scale continuous substrate feed process for the biotransformation of simvastatin by *Nocardia sp*. They described a process of microbial hydroxylation of simvastatin by a *Nocardia sp.* for the preparation of cholesterol lowering drug. By conversion efficiencies of 22-25% with the ratio of desired product/side products of 0.7 were obtained by this process.