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Cholesterol oxidase: Role in biotransformation of cholesterol

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ABSTRACT

Cholesterol oxidase (COX, E.C.1.1.3.6) catalyses the oxidation of cholesterol to 4-cholestene-3-one with the reduction of oxygen to hydrogen peroxides. COXs are secreted bacterial enzymes that catalyze the first step in the degradation of cholesterol. Some bacteria, such as *Mycobacterium*, *Rhodococcus* and *Nocardia* sp. produce an intracellular form of the enzyme that is membrane bound, while the enzyme from *Arthrobacter*, *Schizopyllum*, *Streptoverticillium*, *Brevibacterium* and *Streptomyces* is found in the extracellular fraction. These organisms play important roles in biotransformation and bioconversion of organic compounds. Bioconversion reactions are the subject of increasing interest in the pharmaceutical industry because of the demand for enantiomerically pure compounds. Bioconversion processes that involve enzymatic or microbial biocatalysts, when compared to their chemical counterparts, offer the advantages of high selectivity and mild operating conditions. Bioconversions may involve isolated and purified enzymes directly in free or immobilized form in order to enhance process stability. Medium engineering attempts to enhance the solubility of substrate and remove(s) the inhibition of product simultaneously by adding an inherently biocompatible and non-biodegradable ingredient to bioconversion medium. The extremely poor solubility of cholesterol as a substrate or steroids in aqueous media lowers the transformation rate and increase costs. The methods of enhancing steroid solubility in bioconversion media include substrate derivatization or micronization, ultrasonication or the use of detergents, water miscible co-solvents, cyclodextrins, polymers and liposomal aqueous biphasic media. Steroids like cholesterol are completely soluble in some organic solvents like benzene, toluene and butanol. Biphasic systems where in the microbial cells are present in the aqueous phase and steroids dissolved in the organic phase is considered an ideal system. In the present review article we try to discuss on the solvent tolerant properties and biotransformation capability of cholesterol oxidase producing different organisms of different species and their applications in different fields.

1. INTRODUCTION

In mammals cholesterol is more abundant in tissues, which either synthesize more or have more abundant densely-packed membranes, for example, the liver, spinal cord, brain and atheromata (arterial plaques). Cholesterol plays a central role in many biochemical processes, but is best known for its association with cardiovascular disease. COXs (cholesterol oxidase, EC 1.1.3.6) are secreted bacterial enzymes that catalyze the first step in the degradation of cholesterol. COX is part of a unique class of enzymes that are soluble proteins although they interact with highly insoluble substrate. In the first step, called the reductive half-reaction, the 3β-hydroxy group of the steroid ring system is oxidized to the corresponding ketone. Key to this conversion is the FAD (flavin adenine dinucleotide) cofactor, which become reduced in the process. In the second step the enzyme catalyzes isomerization of the double bond in the oxidized steroid ring system from the Δ5-6 position to Δ4-5 position, to give the final steroid product, cholest-4-en-3-one. In the final step of the enzyme reaction called an oxidative half-reaction; the reduced cofactor reacts with dioxygen and is thus reoxidized while O_2 is reduced to H_2O_2. Interest in these enzymes mostly relies in their utility in the determination of cholesterol in biological samples such as serum and foods, also in the bioconversion of a number of 3-hydroxysteroid in organic solvents and in reverse micelles [1].

Cell-free enzymes and microbial cells have been investigated for reduction of cholesterol level in foods [2-3] and for precursor's production in manufacturing pharmaceutical steroids from cheap sterols [4].

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Ca-alginate immobilized cells of *Streptomyces* sp. was studied for the production of COX [5], suggested that immobilized cells could be used for three consecutive fermentation cycles for COX production in higher quantities as compared with free cells. COX from *Bordetella* species (COX-B) led to irreversible cell apoptosis by decreasing cholesterol content and increasing reactive oxygen species (ROS) level. Hence, COX-B may be a promising enzyme for a novel anti-tumor therapy as reported [6].

1.1 Cholesterol

Cholesterol is essential for many metabolic processes occurring in the mammals. Cholesterol is a fatty compound involved in the transport of fat in the blood stream and is also a part of the structure of all cell membranes of tissues of the body. Only a small amount of this cholesterol comes directly from the food we eat and mammals are capable of synthesizing sufficient amount of cholesterol. Cholesterol is a steroid commonly found in nature with a great relevance in biology, medicine and chemistry, playing an essential role as a structural component of animal cell membranes. The ubiquity of cholesterol in the environment has made it a reference biomarker for environmental pollution analysis and a common carbon source for different microorganisms, some of them being important pathogens such as *Mycobacterium tuberculosis* [7].

1.2 Cholesterol biochemical functions

Cholesterol is produced by the liver and also made by most cells in the body. Little 'carriers' called lipoproteins carry cholesterol molecules in the blood. Cholesterol is being used by the body cells also. It is an essential component of several biochemical functions such as:

a) Formation and maintenance of cell membranes (helps the cell to resist changes in temperature and protects and insulate the nerve fibers).
b) Formation of sex hormones.
c) Help cellular metabolism to work efficiently; for example, cholesterol is essential for body to produce vitamin(s).
d) Aids in production of bile acids, which help the body to digest fat and absorb important nutrients.

The human body contains ~100 g of cholesterol. Most of this is incorporated in the membranes from which cells are constructed and thus cholesterol is an indispensable component of them. The insulating layers of myelin wound around neurons are especially rich in cholesterol. In far smaller quantities, but no less important, cholesterol is starting ingredient for the synthesis of the steroid hormones such as progesterone, estrogen, androgens (testosterone), glucocorticoides and mineral corticoids.

1.3 Chemistry of cholesterol

The chemical structures of cholesterol and cholesterone are virtually identical except that cholesterol is an alcohol (contains a hydroxyl or -OH group) and cholesterone is a ketone (the same oxygen is doubly-bonded to a carbon and has lost its -H). Cholesterone is said to be an "oxidized" form of cholesterol. Cholesterol is also the precursor from which the body synthesizes vitamin D. One of the major uses of the cholesterol is synthesis of bile acids. These are synthesized in the liver from cholesterol and these are secreted in the bile. They are essential in for the absorption of fats from the contents of the intestine. A clue to the importance of cholesterol is that most of the bile acids are not lost in the faeces but are reabsorbed from the lower intestine and recycled to the liver. There is some loss, however, and to compensate for this and to meet other needs, the liver synthesizes some 1.5-2.0 g of new cholesterol each day. This new cholesterol is eventually synthesized from the products of the fat metabolism.
Cholesterol consist of four fused rings, space and aliphatic side chain branched to the D ring at C-17, a hydroxyl group attached to a ring at C-3, and a double bond between C-5 and C-6 of B ring. This double bond makes both C-4 of A ring and C-7 of B ring on the same plane [Fig. 1].One may expects that both the C-4 and C-5 position should have an equal opportunity for an oxidative attack to occur. However, C-7 is indeed a common position for oxidants to react. In contrast, the attack rarely occurs at C-4 because of the possible shielding effect provided by the neighbouring hydroxyl group at C-3 and the trialkyl substituted C-5. Both the 20 and 25 C of the aliphatic side chain are at a tertiary position and are, therefore, more susceptible to oxidative attack than the other carbons.

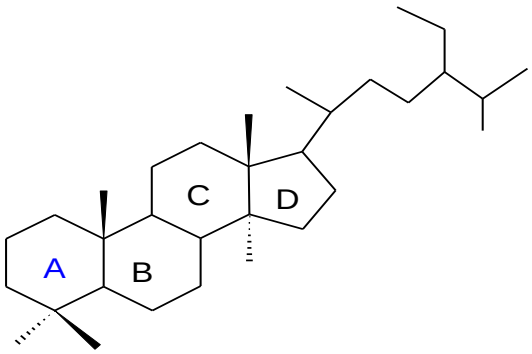

Fig. 1 chemical structure of cholesterol.

1.4 Cellular synthesis and regulation of cholesterol

Slightly less than half of the cholesterol in the body derives from the biosynthesis *de novo*. Biosynthesis in the liver accounts for approximately 10% and in the intestine approximately 15%, of the amount produced each day. Cholesterol synthesis occurs in cytoplasm and the microsomes from the two-carbon acetate group of acetyl-CoA. There are five major steps involved in the synthesis of cholesterol;

a) Acetyl-CoAs are converted to 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA).
b) HMG-CoA is converted to mevalonate.
c) Mevalonate is converted to the isoprene-based molecules, isopentanyl, pyrophosphate (IPP), with the concomitant loss of CoA.

d) IPP is converted to squalene.
e) Squalene is converted to cholesterol.

The cholesterol levels present directly regulate biosynthesis of cholesterol, though the homeostatic mechanisms involved are only partly understood. A higher intake from food leads to a net decrease in endogenous production, whereas lower intake from food has the opposite effect. The main regulatory mechanism is the sensing of intracellular cholesterol in the endoplasmic reticulum by the protein SREBP (sterol regulatory element-binding protein 1 and 2). In the presence of cholesterol, SREBP is bound to two other proteins: SCAP (SREBP-cleavage-activating protein) and Insig1. When cholesterol levels fall, Insig-1 dissociates from the SREBP-SCAP complex, allowing the complex to migrate to the Golgi apparatus, where SREBP is cleaved by S1P and S2P (site-1 and -2 protease), two enzymes that are activated by SCAP when cholesterol levels are low. The cleaved SREBP then migrates to the nucleus and acts as a transcription factor to bind to the SRE (sterol regulatory element), which stimulates the transcription of many genes. Among these are the low-density lipoprotein (LDL) receptor and HMG-CoA reductase. The former scavenges circulating LDL from the bloodstream, whereas HMG-CoA reductase leads to an increase of endogenous production of cholesterol [8]. The SREBP also pathway regulates expression of many genes that control lipid formation and metabolism and body fuel allocation. Cholesterol synthesis can be turned off when cholesterol levels are high, as well. HMG CoA reductase contains both a cytosolic domain (responsible for its catalytic function) and a membrane domain. The membrane domain functions to sense signals for its degradation. Increasing concentrations of cholesterol (and other sterols) cause a change in this domain's oligomerization state, which makes it more susceptible to destruction by the proteosome. This enzyme's activity can also be reduced by phosphorylation by an AMP-activated protein kinase. Because AMP, which is produced when ATP is hydrolyzed, activates this kinase it follows that cholesterol synthesis is halted when ATP levels are low.

2. CHOLESTEROL OXIDASE

Cholesterol oxidase is a flavo-protein that catalyses the oxidation and isomerization of steroids containing the β-hydroxyl group and double bond at C-5 of the steroid ring system. The enzyme has been used in the determination of serum cholesterol and in the clinical diagnosis of arteriosclerosis and other lipid disorders. In addition, it has been shown to be a potent paracide [9] and is currently been developed in the agriculture industry as a biological tool for pest control [10]. Furthermore, COX is an example of a soluble enzyme that interacts with a lipid bilayer to bind insoluble substrate. Structural and biochemical study on the enzyme containing the FAD-1 cofactor non-covalently bound to the protein have revealed the region of the enzyme involved with the lipid bilayer that had led to a possible mechanism for the membrane interaction [11-12]. In *Brevibacterium sterolicum*, COX was found to exist in two forms, one in which the FAD cofactor was non-covalently bound to the enzyme (BCO1) and the other in which the cofactor was covalently linked (BCO2).

Furthermore, some pathogenic bacteria require COX to infect their host macrophage, probably because of the ability of the COX to convert cholesterol to cholesterol-4-en-3-one in the membrane (Mechanism of reaction catalyzed by cholesterol oxidase, Fig: 2), [13]. COXs have been isolated from several sources other than *Streptomyces*, including members of the genera *Burkholderia* [14], *Rhodococcus* [15] and *Micrococcus* sp. [16]. The crystal structures of the enzymes from *Brevibacterium sterolicum* and *Streptomyces* sp. SACOO have been determined at 1.8A° and 1.5Å resolutions, respectively [17-19]. An increasing need for specific estimation of steroid in clinical samples has enhanced the importance and demand of COX in the pharmaceutical industry.

The enzyme can also be used in the production of precursors of hormonal steroids from cholesterol. COX is produced by two types of bacteria: (a) non-pathogenic bacteria, which utilize cholesterol as a carbon source; and (b) pathogenic bacteria, which require COX for infection of the host macrophage because of its ability to alter the physical structure of the lipid membrane by converting cholesterol into cholest-4-en-3-one. Both pathogenic and non-pathogenic bacteria up-regulate the expression of COX in the presence of cholesterol. The bacteria that convert cholesterol into cholesterone live in the digestive tract of most mammals, and cholesterone is found in their waste products. These bacteria use cholesterol as a carbon source and the oxidation reaction as a source of energy.

There is some evidence, suggests that the bacteria also use the cholesterone as a sensor for presence of fungi in the environment. The enzyme is found in microorganisms and studies have shown that for many bacteria the enzyme expression can be induced by the presence of cholesterol in the growth medium. Reports in the literature indicate that some bacteria, such as *Mycobacterium*, *Rhodococcus* and *Nocardia* sp. produce an intracellular form of the enzyme that is membrane associated [20-21] while the enzyme from *Arthrobacter*, *Schizopyllum*, *Streptoverticillium Brevibacterium* and *Streptomyces* is found in the extracellular fraction [22-23]. In addition to the differences in location of the enzyme, two molecule forms are also known and have been extensively characterized. COX is an interfacial enzyme as it binds transiently to the membrane surface during catalysis and can only access the substrate from the membrane phase. Other example of interfacial enzyme includes phospholipases [24-25].

In addition, features needed for isomerization have evolved within the same protein such that the enzyme is able to carry out this added catalytic step. Interestingly, bacterial-specific ketosteroid isomerases are known that carry out the isomerisation step independently of steroid oxidation [26]. The reason for having such a bifunctional (oxidation and isomerization reaction are carried out by the same enzyme) as well as monofunctional (when only isomerisation chemistry is carried out) enzyme in bacteria are not well understood.

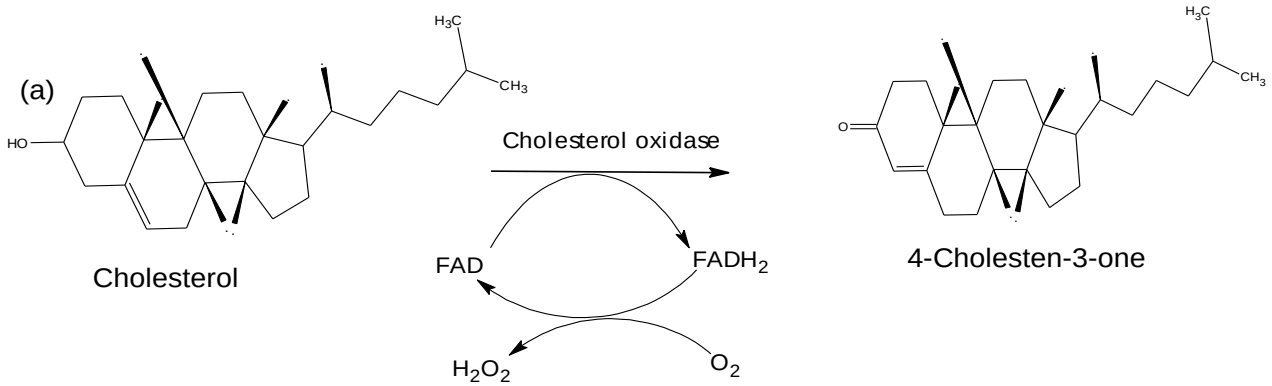

Fig 2: Mechanism of reaction catalyzed by cholesterol oxidase. (a) The cholesterol oxidase oxidizes cholesterol into 4-cholestene-3-one and H_2O_2; (b) H_2O_2 and O-dianisidine (reduced) with peroxidase enzyme oxidized O-dianisidine and releases water molecule. Part (b) shows the principle of an enzymatic assay in which H_2O_2 produced during a primary (a) reaction is used to oxidize O-dianisidine which is colorimetrically measured at A_{550}.

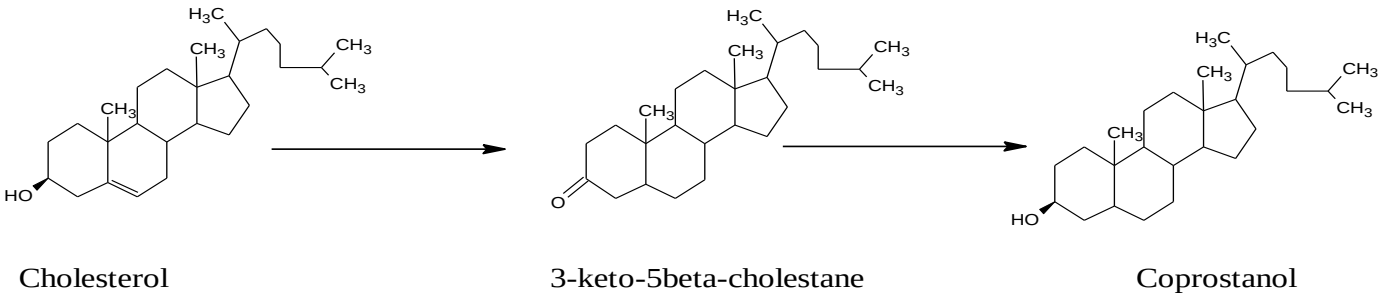

Fig. 3: Proposed pathway of reduction of cholesterol to coprostanol.

In eukaryotes, steroid oxidation and isomerization are important step(s) in the synthesis of a wide variety of steroid hormones that are carried out by NAD^+ dependent 3β-hydroxysteroid dehydrogenase as membrane-bound protein located in the endoplasmic reticulum and mitochondrion [27-30]. Hence flavin mediated cholesterol oxidation is a process unique to microorganisms.

In case of human gut microbiota, produces metabolites from a large range of molecules that host's enzymes are not able to convert. Among these molecules, two main classes of steroids, cholesterol and bile acids, denote two different examples of bacterial metabolism in the gut. Therefore, cholesterol is mainly converted into coprostanol, a non absorbable sterol which is excreted in the faeces. Moreover, this conversion occurs in a part of the human population only. Conversely, the primary bile acids (cholic and chenodeoxycholic acids) are converted to over twenty different secondary bile acid metabolites by the gut microbiota. The main bile salt conversions, which appear in the gut of the whole human population, include deconjugation, oxidation and epimerization of hydroxyl groups at C3, C7 and C12, 7-dehydroxylation, esterification and desulfatation [31]. Cholesterol (3β- hydroxyl-5-cholestene) and its bacterial biotransformation product (Fig 3) coprostanone (3β- keto-5β -cholestane) and coprostanol (3β- hydroxy-5β -cholestane) account for 95% of the total neutral steroid recovered from rat and human faeces.

3. BIOCONVERSIONS OF CHOLESTEROL

Bioconversions of hydrophobic compounds often meet with two serious obstacles: limited substrate accessibility to the biocatalyst as a result of the low aqueous solubility of most organics; inhibition or toxicity of both substrate and product exerted upon the microorganism [32]. Those problems also appear in biodegradation of the hazardous pollutants [33]. Integrated bioprocess [34] consisting of a bioreactor and a downstream unit, such as membrane bioreactor [35], was coupled in a way that guarantees the effective removal of the product inhibition to the microorganism. Medium engineering attempts to enhance the solubility of substrate and remove(s) the inhibition of product simultaneously by adding an inherently biocompatible and non-biodegradable ingredient to bioconversion medium. Many kinds of

medium systems have been developed, especially the organic-water two-phase system has been studied extensively [36-37]. Bioconversion of cholesterol to androst-1,4-diene-3,17-dione (ADD) and androst-4-ene-3,17-dione (4-AD) was carried out. Bioconversion of cholesterol to androst-1,4-diene-3,17-dione (ADD) and androst-4-ene-3,17-dione (4-AD) in cloud point system (CPS) was studied [38]. A mathematical model was presented based on the mass transfer rate and microbial growth rate. The calculations for cell concentration, substrate concentration and product concentration as functions of time were made. Some parameters, such as substrate concentration, mass transfer coefficient and maximum product inhibition concentration, indicated that the maximum product concentration is the most significant parameter in batch process. Adjusting substrate concentration, stirring speed and surfactant concentration validated the prediction. It showed the batch process of cholesterol bioconversion in CPS was controlled by inhibition of product. Adjusting surfactant concentration can diminish inhibition of product and increase final product concentration. The results also indicated that CPS could increase the substrate concentration of aqueous phase in batch process. The biocompatibility and substrate bioavailability in CPS was thus proved [39].

Most organic solvents are generally toxic to organisms. Although there are some microorganisms that can assimilate these toxic organic solvents, however in such case the concentrations of the solvents must be extremely low. Most microorganisms cannot grow in a medium containing large volumes of organic solvents. In recent years, however, several highly organic solvent-tolerant strains of *Pseudomonas aeruginosa*, *Pseudomonas fluorescence*, and *Pseudomonas putida* were isolated [40-42]. These strains thrive in *p*-xylene or toluene, which are highly toxic aromatic solvents. A *p*-xylene-tolerant mutant of *Escherichia coli* was isolated by mutation [43]. Extra-cellular COX from a mutant *Brevibacterium* sp. ODG-007, showed a strong capacity in bioconversion of yolk cholesterol to cholest-4-en-3-one, especially supplemented with NaCl and lipase C, as a yolk granule solubilizer. The bioconversion process was investigated first, to obtain basic information of the process and was further optimised by analysis of parameters, including COX concentration, dilution ratio and incubation time on the cholesterol conversion, employing response surface methodology (RSM) and central composite design (CCD). Under the optimum operational conditions; COX concentration of 5.39 $U.g^{-1}$ yolk powders, water: solid ratio of 3.54 and incubation time of 13.75 h, up to 85.6% yolk cholesterol was reduced and the concentration of the remaining cholestenone, an effective anti-obesity medicine in the product, was raised [44].

3.1 Toxicity of organic solvents towards microorganisms

The toxicity of chemicals like organic solvents can be described in different ways. The model of the so-called quantitative structure-activity relationship (QSAR) refers to the quantitative relation between the chemical structure of a molecule and its pharmacological, toxicological, ecological, chemical, biological, and physical effects. Sometimes the term QSPR can be found: that principle is limited to the relation between the molecule's structure and its physical and chemical properties. The discovery of drugs often involves the QSAR approach in order to identify chemical structures with potentially good inhibitory effects on specific targets and low toxicities. Here, of special interest is the prediction of log P as a crucial mean in identifying "drug-likeness". A model was created by researchers to calculate the log P values for a variety of compounds according to their atomic structure [45]. Chemical compounds with log P values ranging between 1 and 4 are regarded as being highly toxic for microorganisms. Below log P 1, the hydrophobicity of a compound is too low to enable entering the membrane; above log P 4, the substances are too poorly water-soluble and concomitantly too poorly bio available to cause any toxic effect. The higher the hydrophobicity or log P within this range of 1 to 4, the higher the compound's toxic effect on bacteria, following a systematic, nearly linear correlation. The chemical toxicity of compounds to microorganisms is often expressed as the concentration that causes 50 % growth inhibition, the so-called effective concentration (EC_{50}). It correlates negatively with the log P value [46-48,]. This correlation of hydrophobicity with toxicity was proven for a variety of solvent classes, like alkanes, alkanols, alcohols, aromatics and phenols.

3.2 Solvent properties and selection of organic solvent

Solvents are liquids that dissolve solid, liquid or gaseous compounds, without altering them chemically and resulting in a solution. In biotechnology, solvents enable and facilitate product recovery from aqueous reaction media. If a compound is suitable as a solvent for certain substrates and/ or products, is depending on its physico-chemical properties [Table 1], which are also determined by the solvent's molecular structure. The use of an organic solvent phase in biocatalytic applications can provide both kinetic and thermodynamic advantages. When a product is continuously removed by a solvent phase, a biocatalyst that is subject to decreased productivity due to end-product inhibition will show an increase in its activity.

Table 1: Desirable solvent characteristics [43].

1.	Favourable distribution coefficient for product and substrate
2.	High selectivity
3.	Low emulsion-forming tendency
4.	Low aqueous solubility
5.	Chemical and thermal stability
6.	Favourable properties for product recovery
7.	Non-biodegradability
8.	Non-hazardous
9.	Inexpensive
10.	Available in bulk quantity and
11.	Biocompatibility

For a successful implementation of this two-phase biotechnological approach in large scales, the proper solvent selection is absolutely crucial. An important parameter for the distribution of substrates and products in the organic phase is the hydrophobicity of the used solvent.

3.3 Effects of solvents on the membrane

The major target for the toxic effects of solvents in bacteria is the cell membrane. As soon as solvents accumulate in the cell membrane, they disrupt the membrane's integrity and lead to a loss of the membrane's function as permeability barrier to the environment, as protein and reaction matrix and as energy transducer what will concomitantly result in damage of cell metabolism, in growth inhibition or even in cell death [46]. It has been proven that a correlation existed between the toxicity to microorganisms on the one hand, and the hydrophobicity of a compound and it's partitioning into a lipid bilayer on the other hand [47].

Compounds with a log P value below 1 possess a low hydrophobicity and hardly partition into the membranes, which prevents them from being toxic to cells. Most compounds with a log P higher than 4 are very poorly water-soluble.

3.4 Solvent-tolerant bacteria

The toxic effect of suitable organic solvents on whole cells is a major drawback in their application in biotechnology and in the production of fine chemicals by whole-cell bio-transformations [48]. Most enzymes were found to be able to function in organic solvents [49]. Due to that, bioconversions with enzymes in the presence of organic solvents were established in the 1970s.

Nevertheless, two-phase bioconversions with whole cells using solvents with reasonable properties were still out of reach. This situation was changed in 1989, when researchers made a surprising discovery; they isolated a *Pseudomonas putida* strain, which was able to thrive in the presence of high concentrations of the toxic solvent toluene without being able to metabolise it. Later, a number of research groups reported tolerance towards solvents like xylene, styrene, and toluene for other species of *Pseudomonas* as well as for other genera, including *Flavobacterium*, *Bacillus* and *Rhodococcus* [41, 50].

This solvent-tolerance of microorganisms [51-74, Table 2] opened new possibilities in the quest for whole-cell biocatalytic production processes for toxic fine chemicals and in overcoming limitations in industrial biotransformation. The usage of solvent-tolerant microorganisms now allows the application of otherwise toxic solvents with the chemical properties needed for a successful implementation of whole-cell two-phase bioconversions [75]. Several two-phase bioconversions have now been established using solvent tolerant bacteria, overcoming toxic effects of substrates and products [76].

3.5 Mechanisms of solvent tolerance

Since the first report on solvent-tolerant bacteria, many research groups occupied themselves with the goal of investigating the mechanisms underlying the bacteria's ability to adapt to the presence of toxic solvents. "Adaptation" itself can be defined as changes in cell physiology and/ or composition in order to adapt to the environment, but without the means of genetic modifications (mutations). The investigation of the discovered solvent-tolerant microorganisms revealed a broad variety of potentially possibilities [Fig. 4] used by those bacteria in order to survive the otherwise toxic concentrations of organic solvents such as; rigidification of the cell membrane, change in the membrane's protein content/ composition, active excretion of the solvent, adaptation of the energetic status, changes in cell wall and outer membrane, modification of the cell surface properties, morphological changes, metabolization or transformation of the solvent.

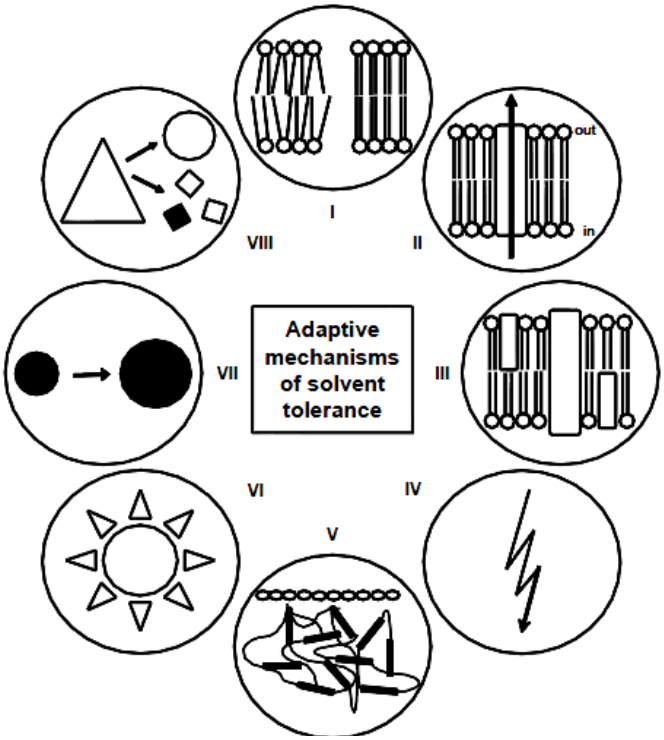

Fig. 4: Organic solvents/ lipophilic compounds. (I) Modification of the membrane's fluidity by increased structuring of the lipid bilayer membrane into the environment by energy-consuming transport systems (e.g. efflux pumps). (III) Modifications in the membrane proteins. (IV) Changes in the energetic status of the cell. (V) Changes in cell wall and outer membrane. (VI) Adaptation of the cell surface properties (surface charge and hydrophobicity). (VII) Morphological adaptation. (VIII) Transformation or degradation of the solvent [61-63].

In the cytoplasmic membrane, changes upon solvent presence at the level of the lipids and proteins have been observed. Those adaptation mechanisms enable the reestablishment of fluidity and rigidity of the cell membrane after being subjected to a solvent [77]. Four major adaptation targets on the membrane level were established:

1. degree of saturation of the fatty acids;
2. *cis/trans* isomerisation of unsaturated fatty acids;
3. composition of phospholipid headgroups;
4. dynamics of phospholipid turnover.

These adaptation mechanisms prevent the influx of solvents by decreasing the membrane permeability and fluidity.

Table 2: Selected organic solvent-tolerant bacterial strains in chronological order .

Organism	Tolerated solvents	Reference
Pseudomonas putida IH-2000	Heptanol and toluene	[51]
Pseudomonas putida PpG1 (mutant)	Toluene	[52]
Pseudomonas putida Idaho	Dimethylphthalate and toluene	[53]
Pseudomonas aeruginosa ST-001	Heptanol and toluene	[54]
Pseudomonas putida S12	Dimethylphthalate and toluene	[55]
Flavobacterium DS-711	Benzene and toluene	[56]
Bacillus DS-994	Benzene and toluene	[57]
Pseudomonas aeruginosa PAO1161	Xylene and hexane	[58]
Pseudomonas aeruginosa LST-03	Toluene	[59]
Pseudomonas putida DOT-T1	Toluene	[60]
Sphingomonas aromaticivorans B0695	Toluene, naphthalene, xylenes, *p*-cresol, fluorene, biphenyl and dibenzothiophene	[61-62]
Arthrobacter ST-1	Benzene	[63]
Pseudomonas LF-3	Toluene	[67]
Pseudomonas mendocina LF-1	Dimethylphthalate	[68]
Pseudomonas mendocina K08-1	Toluene	[69]
Rhodococcus strain 33	Benzene	[70]
Bacillus	Toluene	[71]
Pseudomonas putida GM62, GM73 *Pseudomonas* sp. strain GM80	Toluene	[72]
Bacillus cereus strain R1	Toluene	[73]
Chlorella vulgaris	Isopropanol	[74]
Rhodococcus opacus	Benzene, toluene, styrene, xylene, ethylbenzene, propylbenzene, octane and decane	[75]
Staphylococcus sp. strain ZZ1	Toluene	[76]

4. APPLICATIONS OF CHOLESTEROL OXIDASE IN BIOTRANSFORMATION

COX is used in the treatment of bacterial infections because of its ability to alter the physical structure of the cell membrane due to the conversion of cholesterol into cholest-4-en-3-one. Gene disruption studies indicated that COX is the main membrane-damaging factor, contributing to the pathogenicity of the microbes *in vivo*. Mutational analysis indicated that COX membrane damaging factor imparts to haemolytic reaction elicited by *Rhodoccocus equi* in the presence of sphingomyelinase C-producing bacteria, such as *Listeria ivanivii*, *Bacillus cereus* and *Staphylococcus aureus* [77].

COX is used in the microanalysis of steroids in the food specimens for determining the stearic configuration of 3-ketosteroid from their corresponding 3β-hydroxysteroids.COX with *choEt* and peroxidase was co-immobilized onto electrochemically polyaniline films. These polyaniline-enzyme films characterized using spectroscopic techniques, have been used to fabricate a cholesterol biosensor [78]. They have also co-immobilized COX and *choEt* onto tetraethylosilicate (TEOS) sol-gel film to act as a cholesterol biosensor. Cholesterol detection is important in clinical and food analysis. Different electrochemical biosensors have been proposed for cholesterol detection [79-80].

4.1 Biotransformation of cholesterol

The importance of microbial biotransformations became a reality when the 11a-hydroxylation of progesterone by a *Rhizopus* species was patented in 1952 for the production of steroid drugs and hormones [81]. Since then, microbial transformation of steroids has been used for therapeutic and commercial value [82].

Microbial bioconversion has been focused mainly in steroid hydroxylation, D1 dehydrogenation and sterol side chain cleavage that associated to chemical synthesis steps, have enhanced the large-scale production of natural and modified steroid analogues. The use of whole cells instead of enzymes is preferred as the production costs are lower and it is possible to perform multi-steps conversions with a single biocatalyst [83]. The manufactured steroid compounds have a wide range of therapeutic applications as anti-inflammatory, immunosuppressive, diuretic, anabolic, contraceptive agents, breast and prostate cancer and anti-obesity agents, among others. 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. Some steroid molecules are also called neurosteroids due to their role as memory enhancers, inducers of endocrine response to stress, anxiolytic agents, anticonvulsants, antidepressives and neuroprotective effect [84].

The ability of some microorganisms, represented by *Mycobacterium*, *Corynebacterium* and *Arthrobacter* to utilize sterols as sole sources of carbon and energy, was first discovered and found that sterol ring structure and the side chain were metabolized by different mechanisms. In 1969 cholesterol decomposing ability of 1589 microbial strains was examined. The production of substantial amounts of 17-ketonic compounds without appreciable degradation of the steroid nucleus by *Mycobacterium* sp. NRRL B-3805 and B-3683 was reported. In 1976 the latter was shown by Conner to be able to convert tall oil sterols to C_{19} steroids. *Mycobacterium fortuitum* was used to convert sitosterol to androst-4-ene-3, 17-dione and other intermediates [85]. Microbial transformation of polish tall oil was performed using *Mycobacterium sp*. MB 3683 [86]. Testosterone production using *Mycobacterium sp*. NRRL B-3683 and isolation of a new mutant for sterol biotransformation were reported by research groups from Cuba [87-88]. They also studied the effect of glucose and lactose on the steroid biotransformation by the same strain [89]. *Mycobacterium smegmatis* PTCC 1307 (CIP 73.26)

was used as a microbial agent to produce androsta-1,4-diene-3,17-dione (ADD) and androst-4-ene-3,17-dione, two useful precursors in the synthesis of steroid drugs. The side chain of cholesterol, as the substrate, was selectively cleaved in the presence of five enzyme inhibitors. An intermediate structure with intact side chain, cholest-4-ene-3-one, was also detected and purified [83]. *Mycobacterium smegmatis* was found to be efficient in cleaving the side chain of sterols selectively in the presence of enzyme inhibitors. The formation of seven low molecular weight degradation products of progesterone by *Mycobacterium smegmatis* was reported in 1965. Scientist esterified cholesterol to succinate of 26-OH-4-cholestene-3-one and 26-OH-1, 4-cholestadiene-3-one using *Mycobacterium smegmatis*. They also hydrogenated 19-nortestosterone enantiomers by the same microorganism. As reported by a German patent, *Mycobacterium smegmatis* was used to prepare 6-OH-3-oxo-Δ1,4-steroids [84]. In 1981, immobilized preparations of *Mycobacterium smegmatis* were used to produce 4-androstene-3,17-dione from sterols [85]. *Mycobacterium smegmatis* PTCC 1307 degrades cholesterol and the biotransformation products were identified as androsta-1,4-diene-3,17-dione(ADD), androst-4-ene-3,17-dione (AD), and an intermediate steroid with intact side chain, cholest-4-ene-3-one. AD and ADD are suitable precursors in the synthesis of steroid hormones such as testosterone and estrogens.

Nocardia sp. was isolated from an exotic soil of the northwestern Himalayas and was capable of selectively cleaving the side chain of sterols (cholesterol and phytosterol) yielding androstane steroids An extracellular production of 1,4-androstadiene-3,17-dione (ADD) was observed in the fermentation medium. The conversion studies were carried out with a cholesterol concentration ranging from 0.3 to 3 g/l, but the fermentation conditions in biotransformation experiments gave the maximum yields (theoretical yield was 90 %) at 0.5 g/l cholesterol concentration with pH 7.2 in the presence of Tween 80 concentration 2 g/l; in addition, effects of the media were also studied [86].

Microbial selective side chain cleavage of the sterols (β-sitosterol, stigmasterol, cholesterol) is the only biological method available for the production of ADD and AD [87]. AD and ADD, belonging to the family of 17 ketosteroids, have served as potential substrates for the production of sex hormones, anticoncipients, and antiphlogistics as well as blood pressure regulating agents [88-91]. AD has been the starting material for the preparation of androgens and anabolic drugs and more recently for the production of spironolactone [91-92]. ADD has served as a precursor for estrogens and other contraceptive agents. To meet this increasing demand, more than 60% of the raw materials for the steroid drugs are produced by the selective microbial side chain cleavage of sterols [93], which are abundantly present in the form of phytosterols (a plant origin) and cholesterol (an animal origin).

Production of AD and ADD as the side chain cleavage product of sterols (β-sitosterol, stigmasterol, cholesterol, ergosterol, and campesterol) has been reported from various bacteria and fungi genera *Arthrobacter*, *Bacillus*, *Rhodococcus*, *Pseudomonas*, *Mycobacterium*, *Corynebacterium*, *Nocardia*, *Clostridium*, *Micrococcus*, *Brevibacterium*, *Streptomyces*, *Serratia*, *Protoaminobacter* and *Fusarium* [89, 94].The conversion of cholesterol to AD and ADD from soil isolates, *Rhodococcus* sp. [94] and *Micrococcus roseus* RJ6 [95] has highlighted the preferential use of cholesterol as a substrate. ADD yield from cholesterol was reported, using two-step microbial transformation [96]. Cholesterol was initially converted to cholestenone by *Arthrobacter simplex* U-S-A-18. Cholestenone was prepared directly from the fermentation broth of *A. simplex* and converted to ADD by *Mycobacterium* sp. NRRL B-3683. Conversion of AD to ADD has been reported in mixed culture of *Mycobacterium-Nocardioides* [97]. ADD yield by *Mycobacteria* sp. was enhanced by the addition of fresh cultures of *Nocardioides*. The 1, 2-dehydrogenase activity from *Nocardioides simplex* NCIMB 8929 was used for the conversion of AD to ADD. Fermentation of cholesterol and its conversion to AD was carried out using the mycobacterial strain, *Mycobacterium* sp. MB3683.

Research of potentially useful steroids has highlighted the need for the isolation of microorganisms capable of performing the required transformations. Many reports on the isolation of microorganisms capable of producing AD and ADD by utilizing cholesterol as a substrate have been reported in the literature [98]. The enrichment of a bacterial population as a selective mechanism for isolating strains capable of utilizing cholesterol as sole source of carbon and energy is in agreement with the earlier observations [89]. Compared to earlier studies [96-97], present studies report the isolation of a novel isolate which was capable of producing ADD as a single isolate when transformation was terminated at 96 h, and the yields of ADD and AD by *Nocardia* sp. were higher using cholesterol as a substrate rather than β-sitosterol. The low conversion yield of stigmasterol as a substrate has been explained due to presence of the C-22 double bond having a depressing effect on degradation activity of microbial strains [98]. Cholesterol as a substrate has good properties for microbial side chain oxidation [98], and the steps involved for the conversion of sterols to AD and ADD are: oxidation of the 3β-hydroxy group to the keto group; oxidative cleavage of the aliphatic side chain of sterol; isomerisation; and dehydrogenation. A newly isolated *actinomycete*, *Gordonia neofelifaecis* (NRRL B-59395) from the faeces of *Neofelis nebulosa*, was used to selectively degrade the side-chain of cholesterol. The intermediates were purified and characterized. Quantitative analysis of the accumulated metabolites from cholesterol side-chain cleavage was conducted during the biotransformation. The results showed that the profile of accumulated intermediates was different from those of other reported microorganisms. Among the five metabolites, androsta-1,4-diene-3,17-dione (ADD) was the main product of the side-chain degradation, with a high conversion rate (87.2%), indicating its potential for industrial production of ADD. At the end of transformation, the substrate cholesterol was completely consumed. The effect of some factors on the bioconversion was also investigated. This was the first report regarding cholesterol side-chain cleavage using bacteria belonging to *Gordonia* [99].

Table 3: Different microbial sources used for the biotransformation of cholesterol.

Microbes	Source	Biotransformation	Reference(s)
Pseaudomonas sp. Strain ST-200	humus soil	cholesterol to 6β-hydroxycholest-4-en-3-one	[102]
Arthrobacter simplex U-S-3011	soil from industrial area	cholesterol to cholestenone	[103]
Mycobacterium smegmatis PTCC 1307	commercially obtained	cholesterol to androsta-1,4-diene-3,17-dione (add) and androst-4-ene-3,17-dione	[104]
Mycobacterium sp.	commercially obtained	cholesterol to androsta-1, 4-diene-3, 17-dione (add) and androsta-4-ene-3,17-dione (ad)	[105]
Bacillus subtlis AF333249	organic solvent mono-phasic system	cholesterol	[106]
Gordonia neofelifaecis (NRRL B-59395)	faeces of *Neofelis nebulosa*	androsta-1,4-diene-3,17-dione (add) and androst-4-ene-3,17 dione (ad)	[107]
Lactobacillus helveticus	commercially obtained	androsta-1, 4-diene-3, 17-dione and androst-4-ene-3, 17 dione	[108]
Nocardia sp.	exotic soil of the northwestern himalayas	1,4-androstadiene-3,17-dione (add) and 4-androstene-3,17-dione (ad)	[109]
Brevibacterium sp. ODG-007	commercially obtained	yolk cholesterol	[110]
Lactobacillus bulgaricus	fermented dairy food (rabaddi)	cholesterol to testosterone	[111]
Chryseobacterium gleum	isolated from clinical source	cholesterol to androsta-1,4-diene-3,17-dione (add)	[112]
Nocardia sp.	exotic soil northwestern himalayas	cholesterol to 1,4-androstadiene-3,17-dione (add)	[87]

In recent years, the genus *Gordonia* has received much attention for its potential use in industrial and environmental biotechnology. Many species of *Gordonia* are able to degrade toxic environmental pollutants, such as rubber, benzothiophene, dibenzothiophene, hydrocarbon and phenol [100-101]. The species of *Gordonia* are widely distributed in various environments such as soil, activated sludge, biofilm, industrial wastewater, oil-producing wells and mangrove rhizosphere. However, side-chain cleavage of cholesterol has not been reported in these organisms. Many microorganisms have been reported [Table 3] to be capable of transformation sterols into androsta-1, 4-diene-3,17-dione (ADD) or androst-4-ene-3,17-dione (AD), however investigation of the side-chain degradation process of steroids and its industrial application were confined to several bacteria [102]. In *Pseudomonas* where the most elaborate investigation on cholesterol side-chain degradation was conducted, 8 intermediates were obtained [103], the first reaction of cholesterol catabolism is 3b-hydroxysteroid oxidation resulting in formation of cholest-5-en-3-one, catalysed by 3b-hydroxysteroid dehydrogenase in *Pseudomona* sp., while the first reaction was catalyzed by cholesterol oxidase in *Gordonia neofelifaecis*, since cholesterol oxidase activity was detected in the culture broth. The transformation of cholest-4-en-3-one to cholesta- 1,4-dien-3-one was catalysed by 3-ketosteroid 1,2-dehydrogenases, while in most microorganisms, cholesta-1,4-dien-3-one has not been detected [103]. The 3-oxobisnorchola-1,4-dien-22-oic acid is an intermediate of the degradation process from cholesta-1,4- dien-3-one to ADD, but no intermediate from cholest-4-en-3-one to AD was obtained, suggesting that the main pathway of cholesterol side-chain degradation is through cholesta-1,4-dien-3-one and 3-oxobisnorchola-1,4-dien-22-oic acid in *Gordonia neofelifaecis*. Cholesterol oxidase has applications in blood serum and food cholesterol determination, manufacturing of reduced cholesterol diet. The mechanisms by which the organisms remove cholesterol though are not yet clear. *In vitro* studies have suggested several possible mechanisms including enzymatic deconjugation of bile salts, microbial transformation of cholesterol to coprostanol, cholesterol binding to the bacterial cell wall, and direct assimilation of cholesterol [104]. The growing cells of *Lactobacillus helveticus* assimilated cholesterol and showed high intracellular COX like activity, which has not been previously reported [105]. Study demonstrated the assimilation and intracellular degradation as a cholesterol-lowering mechanism in *lactobacilli*.COX is also implicated in the manifestation of some of the diseases of bacterial (tuberculosis), viral (HIV) and non-viral prion origin (Alzheimer's). These applications and disease mechanisms have promoted the need of screening, isolation and characterization of newer microbes from diverse habitats as a source of COX to learn more about its structural and functional aspects [121].

5. CONCLUSION

Steroid transformation is of great importance in the pharmaceuticals industry. Cholesterol analogues are therapeutically more active. Since pharmaceuticals uses of steroids are numerous, sterol conversions by microbial systems are more effective in steroid and drug industries. The extremely poor solubility of cholesterol as a substrate or steroids in aqueous media lowers the transformation rate and increase costs. The methods of enhancing steroid solubility in bioconversion media include substrate derivatization or micronization, ultrasonication or the use of detergents, water miscible co-solvents, cyclodextrins, polymers and liposomal aqueous biphasic media. Steroids like cholesterol are completely soluble in some organic solvents like benzene, toluene and butanol. Biphasic systems where in the microbial cells are present in the aqueous phase and steroids dissolved in the organic phase is considered an ideal system. Unfortunately, the major problem remains that most bacteria and their enzymes are inactivated or destroyed in the presence of common/ toxic organic solvents. Thus it is extremely desirable that the microbes contain relevant enzymes in sufficient amounts and in high specific activities, even in the presence of the generally destructive non-polar phase. This may be possibly accomplished by using organic-

solvent tolerant bacteria having desired enzyme activities. The use organic media in biocatalytic processes can hardly be conducted (if at all) in aqueous solution because of extremely low solubility of substrate and/or unfavourable shift of the reaction equilibrium in water. However, isolation and use of solvent-tolerant microbes make an environmentally friendly green alternative to the use of conventional chemical catalysts.

6. REFERENCES

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