

Current Research in Microbiology

Chapter 5

Cholesterol Oxidases: Microbial Enzymes in Industrial Applications

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Abstract

Cholesterol oxidases are bifunctional flavoenzymes produced by diverse bacterial species. These enzymes catalyse the oxidation of steroid substrates containing a hydroxyl group at the 3 β -position of the steroid ring backbone. The enzyme exists in two main forms: one with the FAD cofactor embedded noncovalently in the enzyme; and one with the cofactor attached covalently to the protein. Cholesterol oxidase is of significant importance owing to its use in analysis of cholesterol amount in various clinical and food samples. In addition, the enzyme also acts as a larvicide biocontrol agent against many insects and is also involved in the biotransformation of a number of steroids. Moreover, cholesterol oxidase is also associated with some bacterial infections and thus can be explored as a potential new antimicrobial drug target to contain bacterial infections.

1. Introduction

The history of cholesterol oxidase dates back to over 70 years when search for cholesterol-degrading microorganisms was started and many species of *Mycobacterium* were observed to use cholesterol as a source of carbon and energy [1,2]. In eukaryotes, cholesterol is essential for maintaining cell membrane structure and for synthesizing a number of important compounds. Cholesterol is either derived exogenously or synthesized endogenously in the endoplasmic reticulum [3]. An abnormally high level of cholesterol in the blood leads to heart disease, in addition to many other severe health problems. Higher cholesterol levels (greater than 200 mg dL⁻¹) may cause hypertension, arteriosclerosis, lipid metabolism dysfunction, brain vessel thrombosis, nephritis, diabetes, jaundice and cancer. Alternatively, abnormally low cholesterol levels may cause hyperthyroidism, anemia and malabsorption [4,5]. Several

compounds such as statins, filipin and cyclodextrins may be used to manipulate cellular cholesterol [6]. Cholesterol oxidase is a bacterial enzyme that has proven to be very useful in biotechnological applications related to the detection of cholesterol and to the disruption of cholesterol-containing membranes. Cholesterol oxidase is commonly used to manipulate cholesterol levels of cells [7].

Cholesterol oxidase has many applications and one of most important one is its wide use for the detection of amount of cholesterol present in food, serum and other clinical samples through an enzymatic assay [8,9]. With the exception of glucose oxidase, this enzyme has become one of the most widely used biosensors in clinical applications [10]. Cholesterol oxidase is also used for the production of a variety of intermediates which are precursors for the synthesis of steroid hormones, such as 4-androstene-3, 17-dione (AD) and 1, 4-androstadiene-3,17-dione (ADD) [11]. Cholesterol oxidase plays a critical role in sterol catabolism by converting 3- β -hydroxysteroids to 3-oxo-4-enosteroids [10,12,13]. Cholestenone formed by catalysis of cholesterol by cholesterol oxidase is also an important synthetic intermediate in many steroid transformations. Previous studies have shown that it is effective against obesity, liver disease and keratinization [14]. Additionally, the enzyme has insecticidal effect against larvae of some insects [15], also used for membrane structure analysis and hypothesised to act as a signalling molecule for the biosynthesis of polyene macrolide pimaricin.

Cholesterol oxidase (3- β hydroxysterol oxidase, EC 1.1.3.6) is a member of flavin adenine dinucleotide (FAD) dependent enzymes super family and catalyzes the oxidation of cholesterol (cholest-5-en-3 β -ol) to its 3-keto-4-ene derivative, cholestenone (cholest-4-en-3-one), with the reduction of oxygen to hydrogen peroxide [16]. However, some bacterial cholesterol oxidases have also been reported to catalyse oxidation of cholesterol to 6 β -hydroperoxycholest-4-en-3-one (HCEO) in place of cholest-4-en-3-one (CEO) [18]. Cholesterol oxidase is a bacterial FAD-dependent enzyme which is found to exist in two different forms on the basis of bonding between enzyme and FAD cofactor. In first form the enzyme and its FAD cofactor are linked non-covalently (class I) and in second form cofactor is bound covalently to the enzyme (class II) [18,19]. Cholesterol oxidase is among the well-studied enzymes with prominent characteristics, structural features [20], broader biotechnological applications [21] and physiological functions [22,23]. Nonetheless, reports regarding cholesterol oxidase production, purification, molecular characterization and genetic analyses have been previously published [24].

Many bacteria belonging to the genera *Brevibacterium* [25], *Streptomyces* [26,27], *Corynebacterium* [28], *Arthrobacter* [29], *Pseudomonas* [30], *Rhodococcus* [31,32], *Chromobacterium* [17] and *Bacillus* [33,34] are reported to degrade cholesterol. Microbial cholesterol break down occurs by the degradation of its side-chain and the ring to acetyl CoA and propionyl CoA through a multi-step process and the enzyme which catalyzes the first step is

cholesterol oxidase. Most of steroids including cholesterol do not seem to be able to diffuse to the bacterial cytoplasm, and appears to be transported by specific uptake systems before being metabolized. In most of cholesterol degrading microorganisms, the cholesterol oxidase is employed in the initial step of cholesterol metabolism, while in case of pathogenic bacteria it acts as membrane-damaging factor contributing as a virulence factor and adds to the pathogenicity of these bacteria [35,36].

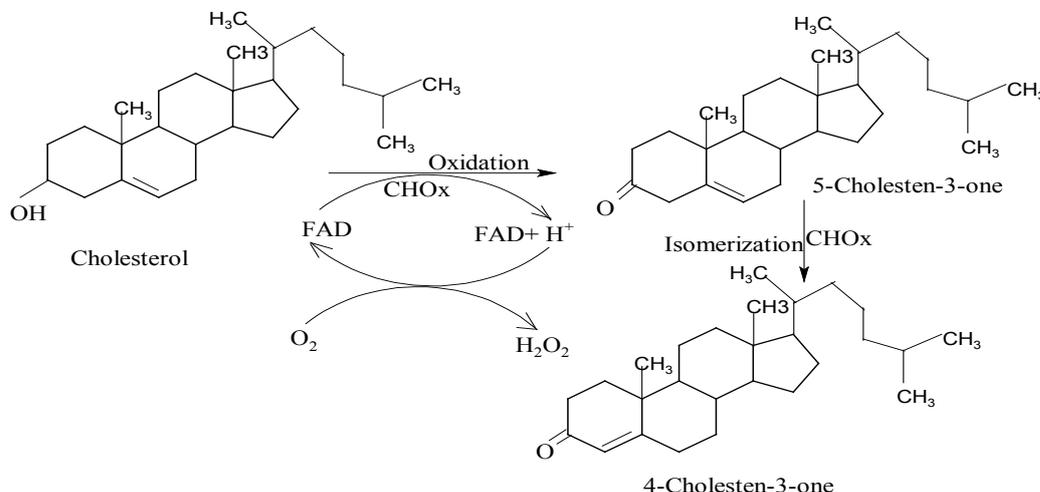


Figure 1: The reaction mechanism of cholesterol oxidase.

2. Sources of Cholesterol Oxidase

Cholesterol oxidase has been isolated from various bacterial sources, comprising both Gram-negative and Gram-positive bacteria [10,23]. Cholesterol oxidase producing bacteria produces it in three forms: intracellular, extracellular and membrane bound. Most of the microorganisms described as cholesterol oxidase producers, produce it in extracellular form which is eventually released into the fermentation medium. The cell-surface-linked form of enzyme is extractible with non-ionic detergents such as Triton X-100 (polyethylene glycol octylphenyl ether) and Lubrol PX (polyethylene glycol monododecyl ether) [37,38,39,40]. The most common microbial sources for production of cholesterol oxidase are *Arthrobacter* [41,42], *Bacillus* [33,43], *Brevibacterium* [44], *Bordetella* [45], *Corynebacterium* [28], *Mycobacterium* [46,47], *Nocardia* [48,49], *Rhodococcus* [32,38] and *Streptomyces species*. [26,50,51]. Some Gram-negative bacteria such as *Burkholderia* [52], *Chromobacterium* [17], *Enterobacter* [53] and *Pseudomonas species*. [30] have also been reported to produce cholesterol oxidase.

Cholesterol oxidase is a monomeric enzyme which exists in two forms classified as class I and class II, in class I the FAD cofactor is attached non-covalently to the protein and in class II this cofactor is bound covalently to the enzyme [54,55]. However these two classes of enzyme show same catalytic activity but do not have any significant sequence homology and thus appear to belong to different protein families [23]. Class I enzymes are part of the glucose-methanol-choline (GMC) oxido-reductase family and have been found mostly in actinomycetes such as *Streptomyces*, *Brevibacterium*, *Rhodococcus*, *Arthrobacte*, *Nocardia* and *Mycobacterium* spp. Whereas, Class II enzymes have been assigned to vanillyl alcohol oxi-

dase (VAO) family and have also been reported from *Brevibacterium sterolicum*, *Rhodococcus erythropolis* and some Gram-negative bacteria such as *Burkholderia* sp., *Chromobacterium* sp. and *Pseudomonas aeruginosa* [56]. Most of flavoenzymes contain a consensus sequence made up of repeating glycine residues (GXGXXG) continued by the presence of aspartic acid/ glutamic acid approximately 20 residues further is indicative of a nucleotide-binding fold [57]. The noncovalent form of cholesterol oxidase shows a nearly identical consensus sequence of glycine moieties (G17-X-G19-X-G21- G/A22) which is followed by a glutamate and confirms the presence of nucleotide-binding fold. However, the second form which exhibits covalent binding lacks this consensus sequence which indicates the probable absence of a nucleotide-binding fold [20]. Despite exhibiting structural differences both covalent and noncovalent enzyme forms possess a buried hydrophobic pocket for binding steroid ring backbone. Based on function, cholesterol oxidase contains two domains, the FAD-binding domain and the substrate-binding domain. There is no significant sequence identity between two types of enzymes resulting in different tertiary structure and kinetics mechanism [20,58]. It has been reported from different structural and kinetics studies that His447 and Glu361 residues act as main catalysts along with the conserved water molecule H₂O and Asn485 in type I cholesterol oxidase [59]. The 3-OH group of the steroid ring is linked to both the FAD cofactor and a bound water molecule *via* hydrogen bonding. The critical residues composing the active site of type II cholesterol oxidase from *Brevibacterium sterolicum* include Arg447, Glu475, Glu311, and Asn516 [54].

Cholesterol oxidase is thus a functional flavo enzyme which catalyzes the oxidation and isomerization of cholesterol to 4-cholesten-3-one in three successive steps (**Figure 1**). The first step results in the 3-OH group dehydrogenation with the loss of the 3 α - hydroxy and 3 β -hydroxy from the steroid ring backbone (reductive half-reaction). The two resulting electron equivalents are transferred to the oxidized FAD enzyme cofactor that is thence converted to its reduced form in the process. In the next step, the reduced form of FAD cofactor reacts with molecular oxygen (O₂) to regenerate original enzyme in its oxidized form and H₂O₂ (oxidative half-reaction). In the final third step, the cholesterol oxidase catalyzes isomerization of double bond in the steroid ring backbone, from Δ 5-6 to Δ 4-5, leading to final product formation. It has also been reported previously that cholesterol oxidase from *B. cepacia*, *Pseudomonas* spp., and *Chromobacterium* sp. oxidizes cholesterol to HCEO [17,60] The HCEO formation scheme differs only for a single step in which HCEO is formed from cholest-5-en-3-one, presumably by auto-peroxidation [23].

3. Properties of Cholesterol Oxidase

Cholesterol oxidases are among well studied enzymes and have been reported from several microorganisms. General range for molecular weight (Mr) of cholesterol oxidase is reported to be from 47–61 kDa. Cholesterol oxidases from different microbial sources are found

to be optimally active at neutral pH and are stable over a wide pH range. The enzymes have temperature optima in the range of 37–60°C (**Table 1**). Cholesterol oxidase from *Streptomyces fradiae* is reported to have highest optimum temperature of (70°C) among the enzymes reported [51]. Cholesterol oxidase produced from *Chromobacterium* sp. strain DS-1 is found highly thermostable retaining 80% of its original activity even at 85°C after 30 min [17]. Generally cholesterol oxidase does not require metal ions for its activity but in case of some enzymes, its activity was enhanced in the presence of metal ions [45,32]. Different chelating agents like EDTA, o-phenanthroline, and 8-hydroxyquinoline also do not seem to have any significant effect on the enzyme activity [17,61]. Since cholesterol is an insoluble compound, detergents as well as organic solvents are often added to the reaction solution to act as solubilizer(s). Cholesterol oxidase has been used for the optical resolution of non-steroidal compounds, allylic alcohols [62,63] and the bioconversion of a number of 3 β -hydroxysteroids in the presence of organic solvents [64,65]. Therefore, an organic solvent-tolerant cholesterol oxidase would be useful for these applications. Organic solvents often influence the cholesterol oxidase activity [49,66].

Table 1: Properties of cholesterol oxidases produced from different microbial sources.

Producer organism	Optimum pH	Optimum Temp. (°C)	Mr (kDa)	Temp. stability (°C)	Km (mM)	Specific activity (Units/mg)	References
<i>Brevibacterium sterolicum</i>	6.5	55	46.5	-	0.03	55.2	[44]
<i>Streptomyces aegyptia</i> NEAE 102	7.0	37	46	50	0.152	16.1	[27]
<i>Bacillus subtilis</i>	7.5	37	105	37	3.25	1.39	[33]
<i>Bordetella</i> sp.	7.0	37	55	50	0.556	20.8	[45]
<i>Chryseobacterium gleum</i>	6.75	35	60	-	0.50	15.5	[67]
<i>Rhodococcus</i> sp.	8.0	37	60	-	-	35.6	[32]
<i>Enterococcus hirae</i>	7.8	40	60	92	-	124.9	
<i>Streptomyces</i> sp.	7.2	50	55	65	0.02	20.0	[26]
<i>Chromobacterium</i> sp. DS-1	7.0	65	58	85	0.026.	13.9	[17]
<i>Burkholderia cepaca</i> ST-200	6.8-8.0	60	60	70	-	16.9	[52]
<i>Pseudomonas aeruginosa</i>	7.0	70	59	70	92.6	11.6	[68]

4. Substrates for Cholesterol Oxidase

Cholesterol oxidase is active on a large number of sterols with or without a C-17 alkyl chain. However, it is most active in the presence of cholesterol. Richmond (1973) reported that cholesterol oxidase from a *Nocardia* sp. was affected largely by the length of side chain [48]. Long side chains appeared to aid the orientation of the substrate with respect to the active enzymatic site. Allain et al., (1974) found that ergosterol, 5,7-cholestadien-3 β -ol, 20 α -hydroxycholesterol, 5 α -cholestan-3 β -ol and 7-cholesten-3 β -ol were oxidised at rates lower than that of the rate of cholesterol for the same enzyme [69]. In contrast to the enzyme from *Nocardia* sp., a cholesterol oxidase from *Brevibacterium sterolicum* was fairly reactive to substrates lacking a side chain [25]. The substrate specificity of cholesterol oxidase from *Streptomyces cinnamomeus* was studied [70] in oriented sterol monolayers and it was found that the cholesterol analogue 5 α -cholestan-3 β -ol was oxidized almost as fast as cholesterol itself. In addition, when the Δ -5 double bond in cholesterol was instead at the Δ -4 position and Δ -5 double bond was at the Δ -7 position (5 α -cholest-7-en-3 β -ol), the oxidation rate became slower. With C-17 side chain analogues of cholesterol, it was observed that the complete lack of the C-17 side chain (5-androsten-3 β -ol) or the insertion of an unsaturation at Δ -24 (desmosterol) or even an ethyl group at C-24 had no appreciable effects on sterol oxidation rate, implying that the enzyme did not recognize the side chain in oriented sterol monolayers [70]. Substrate specificity of a cholesterol oxidase from *Rhodococcus* sp. GK1 was examined and the enzyme was found to be most active with cholesterol (100%), β -sitosterol (70%) and stigmasterol (40%). Sterols with modified A-rings and B-rings or the 3 α -OH of cholic acid remained unoxidised [38]. Cholesterol oxidase from *Brevibacterium sterolicum* rapidly oxidized cholesterol, pregnenolone and β -sitosterol but was less reactive towards stigmasterol. However, it was found to be unreactive toward cholic acid, deoxycholic acid, 5 α -androstan-3 α ,17 β -diol and androsterone [44]. Except cholesterol, β -cholestanol was also oxidized at a high rate by most enzymes [23]. The double bond in the steroid ring between 5 and 6 positions does not seem to be essential for this enzyme activity and the sterols with the short side chain are oxidized at a low rate comparatively. Although the oxidation rates of pregnenolone by most enzymes were slow, the enzymes from *Chromobacterium* sp. DS-1, *Streptomyces* sp. SA-COO and *S. violascens* oxidized pregnenolone at a high rate [17,71]. *Chromobacterium* sp. DS-1 cholesterol oxidase oxidized most 3 β -hydroxysteroids. Interestingly, the cholesterol was the best substrate and β -cholestanol, pregnenolone and β -sitosterol were rapidly oxidized. In contrast, the β -stigmasterol, ergosterol, dehydroepiandrosterone and epiandrosterone were slowly oxidized. Epicholesterol was completely resistant to enzymatic oxidation [17].

Cholesterol is a commonly found steroid with a great importance in biology, medicine and chemistry as it plays an essential role as a structural component of animal cell membranes. Cholesterol is found normally in nature because of its high resistance to microbial degradation.

Owing to its complex spatial conformation and low solubility in water, cholesterol is a very resistant molecule to biodegradation. The high hydrophobicity and low volatility of cholesterol lead to a high absorption to solid phases. Because of high rate of persistence, cholesterol and some derived compounds such as coprostanol have been used as reference biomarkers for environmental pollution analysis [72]. Steroids, some of them derived from cholesterol constitute a new class of pollutants discharged into the environment as a result of human activity [73]. The potent metabolic activities of these compounds affect a large number of cellular processes and thus, their presence and accumulation in water-wastes and in certain ecological niches can affect the endocrine system of animals and humans [74]. Moreover, cholesterol is the main component of lanolin and this and other related sterols are natural contaminants fairly resistant to the anaerobic treatment that is carried out on the effluents from wool-processing industry [75]. 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 [76,77]. Hence flavin-mediated cholesterol oxidation is a process unique to microorganisms. It is generally assumed that the first reaction in the aerobic metabolism of cholesterol is its oxidation to cholestenone through two sequential reactions. That is, the oxidation of cholesterol to cholest-5-en-3-one followed by its isomerization to cholestenone both catalysed by cholesterol oxidase.

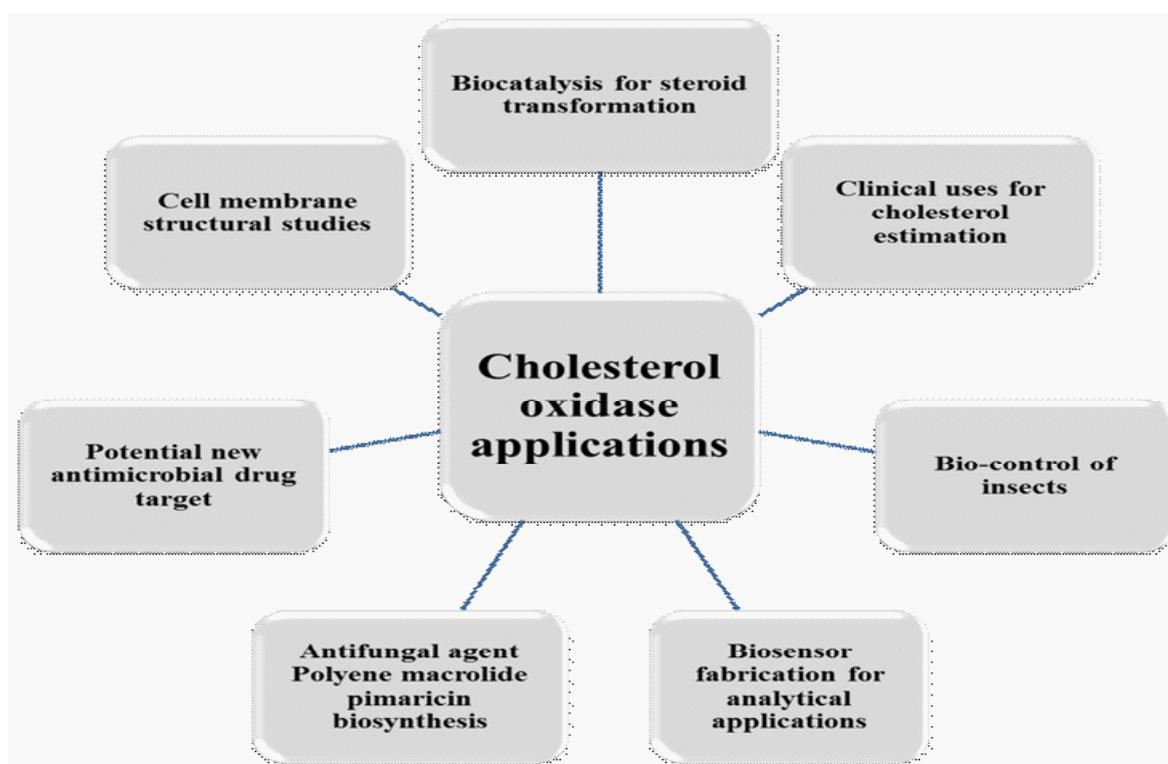


Figure 2: Broader applications of cholesterol oxidase.

5. Major Applications of Cholesterol Oxidase

The microbial oxidases have been reported from many bacterial species and diverse habitats. Invariably, cholesterol oxidases have been exploited in biotransformation's of steroids, biosensors, membrane structural studies, insect control & larvicidal drugs and antimicrobial drugs (**Figure 2**). The broader known applications of cholesterol oxidases are as follows;

5.1. Cholesterol biotransformation

Biotransformation's are structural alterations in a chemical compound which are catalyzed by microorganisms in terms of growing or resting cells or by isolated enzymes. Because of the high stereo- or regio-selectivity combined with high product purity and high enantiomeric excesses, biotransformation's can be technically superior to traditional chemical synthesis. If these features can be combined with economic benefits, biotransformation's become the functional part of new chemical processes for organic synthesis. Further advantages of biocatalytic processes are the mild and ecologically harmless reaction conditions (normal pressure, low temperature and neutral pH), which are important requirements for sustainability. 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 [78]. Cholesterol is metabolized by a large number of microorganisms through a complex metabolic pathway involving many enzymatic steps, first step involving the oxidation of the 3 β -hydroxyl group followed by the oxidation of the 17- alkyl side chain and the steroid ring system, finally degrading the entire molecule to CO₂ and H₂O [23]. In the sequence of the cholesterol oxidation by microbial cells, 4-cholesten-3-one may be oxidized with accumulation of the steroids AD and ADD, which are important precursors of chemically synthesized hormones or may be transformed to steroid intermediates [79]. *R. equi* DSM 89-133 was employed for the conversion of cholesterol and other sterols to androst-4-ene-3,17-dione and androsta-1,4-diene-3,17-dione and up to 82% of cholesterol was converted when the growth medium was supplemented with acetate [80].

Bioconversion of cholesterol to ADD and AD in cloud point system was studied [81]. 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* [82]. Biotransformation of sterols is initiated by modification of the 3 β -ol-5-ene- to 3-oxo-4-ene moiety. The role of cholesterol oxidase in this process has been elucidated. The cholesterol oxidase is not critical for sterol catabolism in the fast-growing AD-producing *Mycobacterium* sp. VKM Ac-1815D strain and the knock-out of cholesterol oxidase gene did not abrogate sterol ring-A oxidation [83]. Similar conclusions

were made earlier for *Mycobacterium smegmatis* mc2 155 [84]. In *Rhodococcus erythropolis* CECT3014, cholesterol oxidase was shown to be a major inducible extracellular cholesterol oxidase but its disruption did not alter cell growth on cholesterol [85]. However, in *Streptomyces virginiae* IB L-14, inactivation of cholesterol oxidase led to abrogate the oxidation of diosgenin to diosgenone and other 3-oxosteroids [86]. Targeted gene disruption of cholesterol oxidases in *Gordonia cholesterolivorans* CECT 7408T resulted in a mutant strain unable to grow on steroids. Two cholesterol oxidases, ChoM1 and ChoM2 were identified in *Mycobacterium neoaurum* NwIB and these were suggested to be essential for utilization of phytosterol as a carbon source [87]. Along with cholesterol oxidases, 3 β -hydroxysteroid dehydrogenases (3 β -HSDs) can catalyse 3 β -hydroxy group oxidation and $\Delta 5 \rightarrow 4$ isomerization in actinobacteria [84].

Mycobacterium smegmatis PTCC 1307 was used as a microbial agent to produce ADD and AD, 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 [88]. Wu and coworkers (2015) explored production of cholest-4-en-3-one by directly using cholesterol oxidase from *Rhodococcus* sp. in an aqueous/organic two-phase system and it was found that the conversion was more efficient in the biphasic system than in the aqueous or co-solvent system. After 48 h of reaction, the conversion rate reached 94.2% in the biphasic system and only 42.3% conversion was achieved in the aqueous system [14]. In another study biotransformation of cholesterol to ADD by cholesterol oxidase from *Chryseobacterium gleum* was studied and the growing cells produced 0.076 g ADD from 1 g cholesterol, which was equivalent to 10% molar conversion of cholesterol [89]. Whole-cells of the recombinant strains *Bacillus subtilis* 168/pMA5-choM1 and *B. subtilis* 168/pMA5-choM2 expressing choM gene encoding cholesterol oxidase from *Mycobacterium neoaurum* JC-12 were used as catalysts for the bioconversion of cholesterol to 4-cholesten-3-one and a percentage conversion of 67% and 83% was observed at 21 h [90]. *R. erythropolis* cholesterol oxidase was employed for the preparative oxidation of cyclic allylic, bicyclic and tricyclic alcohols and for the synthesis of several ergot alkaloids [63].

5.2. Therapeutic uses

Cholesterol oxidase is used as an efficient analytic tool for determining cholesterol in various samples; total and esterified cholesterol in serum or blood, from low-density lipoproteins to high-density lipoproteins, on the cell membrane, in gall stones, in human bile and in various food samples [21]. Normal cholesterol level in human blood is less than 200 mg/dL and lipoproteins contain cholesterol of which ~70% is in esterified form. High levels of cholesterol and cholesteryl esters (hypercholesterolemia) have been associated with cardiovascular disease such as atherosclerosis and other heart diseases, although lower levels (hypocholester-

olemia) may be associated with cancer, depression or respiratory diseases making determining of serum cholesterol concentration very important. Alzheimer disease risk is also reported to be related to hypercholesterolemia *via* involving oxidative stress mechanisms [91]. Cholesterol level in serum is generally determined by using an enzymatic assay [48,69]. As most of cholesterol in serum samples exists in an esterified form so prior incubation of serum with cholesterol esterase (EC 3.1.1.13) is required to release the free cholesterol. Cholesterol oxidase catalyzes oxidation of cholesterol to cholestenone with simultaneous release of H₂O₂. The enzyme subsequently catalyzes the oxidative coupling of H₂O₂ with a chromogenic dye which is determined spectrophotometrically. In addition to being used in the microanalysis of steroids in food samples it is also used for differentiating the steric configurations of 3-ketosteroids from the corresponding 3 β -hydroxysteroids [92]. Over the years, various electrochemical biosensors have been designed by using immobilized cholesterol oxidase for the determination of cholesterol in serum or food samples [93-95].

5.3. Insecticidal activity

Genetically modified plants which are able to control insect pests by producing insecticidal proteins (such as *Bacillus thuringiensis* toxin) are being used very widely to replace the use of synthetic pesticides. A highly efficient bacterial protein capable to kill larvae of boll weevil (*Anthonomus grandis grandis* Boheman) in the culture filtrates of *Streptomyces* sp. was identified as a cholesterol oxidase [15]. When this enzyme was used in purified form against boll weevil larvae, it showed good activity at lethal concentration of 50% (LC₅₀ of 20.9 μ g.ml), which is well comparable to the insecticidal bioactivity exhibited by *Bacillus thuringiensis* proteins. Cholesterol oxidase insecticidal activity is reported due to induction of lysis at the midgut epithelium of larvae upon ingestion. Cholesterol or some other related sterol at the boll weevil midgut epithelium membrane appeared to be available for oxidation by cholesterol oxidase, causing lysis of the midgut epithelial cells resulting in larval death [23]. The enzyme also showed insecticidal activity against lepidopteran cotton insect pests, including tobacco budworm (*Heliothis virescens*), corn earworm (*Helicoverpa zea*) and pink bollworm (*Pectinophora gossypiella*) [96]. Microbial insecticide proteins are very important in several pest control strategies employed in transgenic crops and cholesterol oxidase gene from *Streptomyces* sp. has been expressed in tobacco protoplasts [97].

5.4. Cell membrane structural studies

Cholesterol is the main sterol constituent of eukaryotic cell membrane essential for maintaining cell membrane structure and stability. The cell surface cholesterol can be determined accurately with cholesterol oxidase hence the surface area of a given cell can be estimated readily [98]. Cholesterol oxidase is used as a probe to investigate cholesterol interaction with phospholipids as cholesterol associates preferentially with sphingolipids in cholesterol-

rich lipid rafts areas of the membranes in eukaryotic cells [100,101]. The lipid rafts are the microdomains in which cholesterol and saturated membrane lipids such as sphingolipids promote the formation of a highly ordered membrane structure in comparison to the more disordered vicinity [102,103]. Lipid rafts play roles in various cellular processes, including signal transduction, protein and lipid sorting, cellular entry by toxins and viruses, and viral budding [23]. Therefore, cholesterol oxidase plays a significant role in the study of the functional aspects of lipid raft with regard to eukaryotic membrane.

5.5. Potential new antimicrobial drug target

A wide variety of microorganisms including some life threatening pathogenic bacteria such as *Rhodococcus equi*, *Mycobacterium tuberculosis* and *M. leprae* are reported to produce cholesterol oxidase [104]. *Rhodococcus equi* is a Gram-positive bacterium known for causing infection in young horses as well as in humans acting as an opportunistic pathogen in immunocompromised patients [36,105]. Cholesterol oxidase may possibly acts as an interesting pharmaceutical target for treating bacterial infections. *In vitro* studies advocated that during *R. equi* infection of the host cell, membrane lysis is facilitated by the induction of extracellular cholesterol oxidase along with other candidate virulence factors [35]. The membrane-damaging activity of *R. equi* requires the presence of bacterial sphingomyelinase C which suggested that the cholesterol oxidase substrate is not directly accessible to the enzyme in intact membranes [21]. It has also been reported that cholesterol oxidase is also involved in the manifestation of HIV and nonviral prion origin (Alzheimer's) diseases [106]. The pathogenic bacteria utilize cholesterol oxidase for infection by converting the cholesterol of membranes thus causing damage by altering the physical structure of the membrane. The emerging problem of antibiotic resistant bacteria and their abilities for rapid evolution have pushed the need to find alternative antibiotics which are less prone to drug resistance. Since no eukaryotic enzyme homologues exist, this type of bacterial cholesterol oxidase falls into the scope of potential drug target for a new class of antibiotics which still remain to be explored [107].

5.6. Polyene macrolide pimaricin biosynthesis

The *Streptomyces natalensis* cholesterol oxidase which is a product of the *pimE* gene plays an important role in the biosynthesis of the polyene macrolide pimaricin by acting as a signaling molecule [108]. This 26-member tetraene macrolide antifungal antibiotic is used in the food industry to prevent contamination of cheese and other non-sterile food with mold and also for treating the fungal keratitis because of its ability to interacts with membrane sterols especially ergosterol, resulting in the membrane structure alteration that causes leakage of cellular contents [21]. The polyene macrolide pimaricin gene is located in the center of the pimaricin biosynthetic cluster as the pimaricin production is completely blocked by the gene disruption which is recovered after gene complementation. The addition of purified *PimE* or

exogenous cholesterol oxidase to the gene disrupting culture can restore the pimaricin production. These findings suggested the involvement of cholesterol oxidases as signaling proteins for polyene biosynthesis [23].

5.7. Cholesterol oxidase as a novel antitumor therapeutic drug

Cholesterol oxidase catalyzes oxidation of cholesterol and has been used to track membrane cholesterol. Liu and coworkers (2014) reported that cholesterol oxidase from *Bordetella* species made lung cancer cells both *in vitro* and *in vivo* to undergo irreversible apoptosis. Cholesterol oxidase treatment inhibited phosphorylation of Akt (protein kinase B) and ERK1/2 (extracellular signal-regulated kinase 1/2) which was irreversible even after cholesterol addition. Further studies indicated that cholesterol oxidase treatment also promoted the generation of reactive oxygen species (ROS). In addition to this cholesterol oxidase treatment resulted in phosphorylation of JNK (c-Jun NH₂-terminal kinase) and p38, downregulation of Bcl-2 (B-cell lymphoma/leukemia-2), upregulation of Bax with the release of activated caspase-3 and cytochrome C likely due to production of hydrogen peroxide along with cholesterol oxidation. These findings suggested that cholesterol oxidase leads to irreversible cell apoptosis by decreasing cholesterol content and increasing ROS level indicating cholesterol oxidase may be a promising candidate for a novel antitumor therapy [109].

5.8. Probiotics

Probiotics are living microorganisms which upon ingestion in certain numbers exert health benefits on the host beyond inherent basic nutrition [110]. Though several *in vitro* and *in vivo* studies have proved that the administration of probiotics decreases serum/plasma total cholesterol, LDL-cholesterol and triglycerides or increases HDL-cholesterol but their hypocholesterolemic effects still remain controversial [111]. The species which have been found to exert cholesterol-lowering effects include genera like *Lactobacillus*, *Lactococcus*, *Enterococcus*, *Streptococcus* and *Bifidobacterium* [112]. Different mechanisms like co-precipitation of cholesterol with deconjugated bile, cholesterol binding to cell walls, integration of cholesterol into the cellular membranes during growth, production of short-chain fatty acids during fermentation and transformation into coprostanol are proposed for cholesterol lowering effects of probiotics [112-115]. Cholesterol oxidase, especially derived from bacterial cells plays a major role in the degradation of cholesterol in many fermented foods [116]. Khiralla, (2015) reported that *Leuconostoc mesenteroides* GMK03 could degrade cholesterol using the extracellular cholesterol oxidase and found to be tolerant to low acidity (p^H 2.5) and bile salts (0.3%), competent to adhesion to Caco-2 cells and have low antibiotic resistance, so could be considered as a promising probiotic strain [117]. A gene encoding cholesterol oxidase (choA) from *Streptomyces* [118] has been expressed in several species of probiotic bacteria including *Lactobacillus casei* [119], *Lactobacillus reuteri* [120] and *Streptococcus thermophilus* [121].

Rossi et al., (1998) reported the cloning of cholesterol oxidase from *Streptomyces* sp. into *Bifidobacterium* under its own promoter but without successful expression [122]. Park et al., successfully cloned and expressed cholesterol oxidase gene from a *Streptomyces* sp. in promoter and RBS (ribosome-binding site) of *Bifidobacterium longum* MG1 [123]. Bacterial degradation of cholesterol in cholesterol containing food may be beneficial for human health [124].

5.9. Cholesterol oxidase biosensors for analytical assays

Cholesterol is an important analyte molecule and its assay is important for the diagnosis and prevention of a number of clinical disorders and in food analyses. Moreover, quantification of cholesterol present in various foods is vital for selecting a diet with optimal intake of cholesterol. Thus, it is necessary to develop new techniques for easy and rapid estimation of cholesterol levels in various analytical samples [125]. Biosensors with immobilized enzymes are of huge interest in the various analytical procedures as they facilitate the enzyme reuse along with exclusive selectivity of the biological molecules and the processing power of modern microelectronics [126]. Over a period of time, a variety of cholesterol biosensors have been developed [95]. Most of the reported enzyme-based cholesterol biosensors are fabricated on amperometric technique besides, cholesterol biosensors based on photometric behaviors like luminescence, fluorescence and surface plasmon resonance have also been reported [95,127,128]. Amperometric measurement of O_2 consumption or H_2O_2 production during cholesterol catalysis by cholesterol oxidase is the frequently used strategy in cholesterol biosensors [129]. A cholesterol biosensor based on cholesterol oxidase-poly (diallyldimethyl ammonium chloride)-carbon nanotubes-nickel ferrite nanoparticles (ChOx-PDDACNTs-NiFe₂O₄NPs) was fabricated by using a single dropping step on a glassy carbon electrode surface [130]. Various nanoparticles like metal nanoparticles (gold (Au), platinum (Pt), silver (Ag) NPs), metal oxide nanoparticles (zinc oxide (ZnO), iron oxide (Fe₃O₄), cerium oxide (CeO₂), titanium oxide (TiO₂) NPs), carbon nanotubes (single-walled carbon nanotubes (SWCNTs), and multi-walled carbon nanotubes (MWCNTs) based materials have also been exploited for designing cholesterol biosensor [131-134].

6. Conclusion

Cholesterol oxidase has been reported from a large number of bacterial species and the actinomycetes being the most common group. Being an enzyme of great commercial value, cholesterol oxidase has drawn significant attention due to its wide spread use in determination of cholesterol level(s) in various clinical and food samples and because of its other novel applications like biocontrol of insects, polyene macrolide pimaricin synthesis, biocatalysis for the synthesis of a number of steroids and fabrication of biosensors. Biochemical and structural studies on cholesterol oxidase can play important roles in understanding of various catalysis aspects of different flavoenzymes as it belongs to flavoprotein oxidases. Thus cholesterol

oxidase is a versatile enzyme, the newer microbial sources in nature need to be explored for synthesis of extracellular cholesterol oxidase(s), which is/ are easy to purify, characterize and put for extended commercial uses.

7. References

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