2.1. Overview

Cholesterol oxidase (cholesterol: oxygen oxidoreductase, EC 1.1.3.6) Chox is a flavoenzyme (Flavin Adenine Dinucleotide) FAD-dependent enzyme. It catalyzes the oxidation of cholesterol (cholest-5-en-3β-ol) in the presence of molecular oxygen to form cholesten-4-en-3-one (or 4-cholesten-3-one) and hydrogen peroxide (H_2O_2) (Smith and Brooks, 1974; MacLachlan et al., 2000). However, in some cases like the Chox produced by *Burkholderia cepacia* strain ST-200, *Pseudomonas spp*., and *Chromobacterium sp*. strain DS-I has been found to oxidize cholesterol to form 6β-hydroxyperoxycholest-4-en-3-one instead of cholest-4-en-3-one produced by a majority of Chox (Doukyu and Aono, 1999; Doukyu et al., 2008; Doukyu, 2009). This flavoenzyme contains a single molecule of FAD as the redox cofactor which may be covalently (Coulombe et al., 2001) (Lim et al., 2006) or non-covalently (Pollegioni et al., 1999; Gadda et al., 1997) bound to the enzyme depending on the microbial species to which they belong. In one special case, the flavin moiety is FMN, although its evidence is still not convincing (Watao et al., 2003).

2.2. Sources of Cholesterol oxidase

Chox is produced by many prokaryotic microorganisms including bacteria, actinomycetes, and some fungi found in a quiet diversifying environment. Chox is a microbial enzyme produced by several microorganisms since long, including *Nocardia rhodochrous* (Buchland et al., 1975), *Arthrobacter simplex* (Liu et al., 1988), *Pseudomonas sp.* (Noriyaku and Rikizo, 1998), *Actinomyces lavendulae* (Petrova et al., 1981), (Shirshova et al., 1992), *Streptomyces fradiae* (Yazdi et al., 2008). However, no report of plants or mammalian sources of Chox is available to date. Among the microbial producers of Chox many microorganisms are pathogenic. In the past decade, there are

various other reports of Chox production by *Bordetella* (Song et al. (2010), *Brevibacterium* (Yang and Zhang, 2012), *γ-proteobacterium* (Isobe et al. (2003), *Mycobacterium* (2012), *Rhodococcus* (2010), and *Streptomyces* (Tomioka et al., 1976; Toyobo Corp. Ltd., 2000; Praveen et al., 2011, Varma and Nene, 2003; Tripathi et al., 2013; Yazdi et al. 2001).

Actinobacteria are another group of bacteria gaining importance in Chox production. In recent years, apart from other microorganisms, there has been a remarkable increase in Chox production and purification from *Streptomyces sp.* The major underlying reasons are its non-pathogenicity, high enzyme production, and wide-ranging applications in the field of clinical pathology, food industry, agricultural importance, and biosensors.

2.3. *Streptomyces* Cholesterol oxidase

Streptomyces are common soil bacteria belonging to the genus *Actinomycetes*. They are supposed to be the connecting link between bacteria and fungi. They grow as mycelium and produce spores. *Streptomyces* are non-pathogenic microorganisms well known for antibiotic and secondary metabolites production due to which *Streptomyces* strains are considered to be industrially important (Beppu 1995, Berdy 1980).

Streptomyces are mostly the producers of extracellular Chox, however, very few have been reported for intracellular Chox production. *Streptomyces* Chox has been reported to be superior for serum cholesterol assay as compared to the Chox from *Nocardia* and *Pseudomonas* because of lower reagent cost, stability, and longer shelf life of *Streptomyces* Chox (Lolekha and Jantaveesirirat, 1992). In the enzymatic endpoint method of serum cholesterol assay, *Streptomyces* showed accurate results near the clinical decision cut-points for serum cholesterol assay among the 4 different sources of Chox; *P. fluorescens*, *Cellulomonas*, and *Brevibacterium* tested (Lolekha et al., 2004).

2.4. Mechanism of Cholesterol oxidase Action

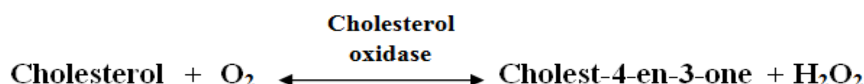
Chox catalyzes three chemical conversions, which are listed below - an overview of the enzymatic steps is presented in **Fig 2.1.**

- The first catalytic conversion, called the reductive half-reaction, is the dehydrogenation of the alcohol function at the 3-position of the steroid ring system. The resulting two redox equivalents are transferred to the (oxidized) flavin cofactor that becomes reduced in the process.
- In the second catalytic step, the reduced flavin reacts with dioxygen to regenerate the oxidized enzyme and hydrogen peroxide (H_2O_2); the oxidative half-reaction.
- Finally, the oxidized steroid undergoes an isomerization of the double bond in the steroid ring system, from Δ^{5-6} to Δ^{4-5}, to form the final product cholest-4-en-3-one. In general, this isomerization reaction occurs faster than the release of the intermediate, cholest-5-en-3-one, from the enzyme. The specificity of Chox for various substrates derived from the cholestane skeleton has been the object of several studies, in particular those describing newly discovered Chox (Massey and Ganther, 1965). While the dehydrogenation of the CH–OH function at position 3 of the cholestane is retained. Most low molecular mass alcohols (e.g. propan-2-ol) are substrates that can reduce the enzyme within 1 h at a concentration of 1M (Muller et al. 1973). This highlights the concept that Chox is an alcohol oxidase adapted to accommodate the bulky cholestane frame.

2.5. Flavo-enzyme - The Role of Flavin Moiety in Catalysis

Chox is a flavoenzyme containing the FAD prosthetic group as an obligatory part of its activity. The Flavin moiety (isoalloxazine) is the characteristic feature of all Chox reported to date. Generally, it is found as a FAD cofactor but in one specific case, the

Reaction involved



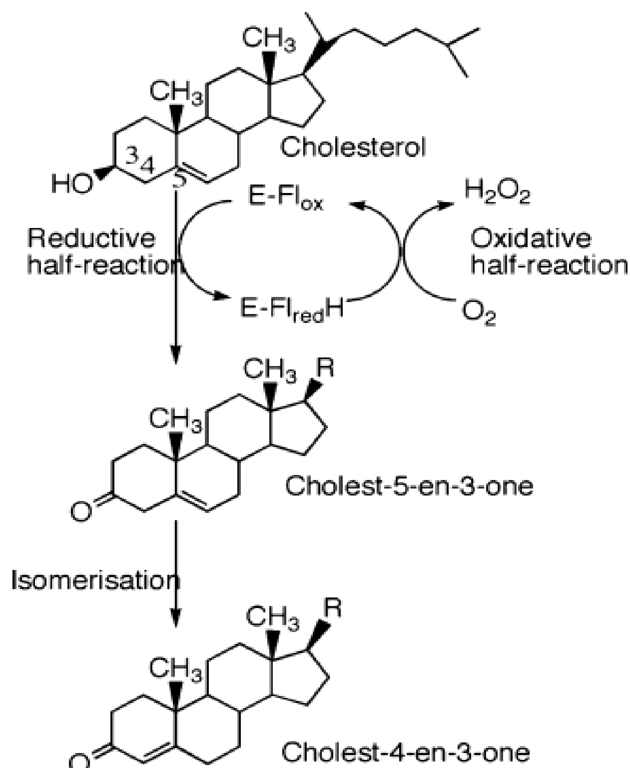


Fig 2.1 Reaction steps catalyzed by cholesterol oxidase (Chox). The terms 'Reductive' and 'Oxidative' half-reaction refer to the changes of the redox state of the bound flavin coenzyme. Cholest-5-en-3one does not occur in the free form under normal catalytic conditions. Its conversion to the final product, cholest-4-en-3-one, is faster than its formation. [Fig adapted from Vrielink and Ghisla, 2009]

flavin moiety has been found to be FMN, although its evidence is still not substantial (Watao et al., 2003). This flavin moiety present in the active site of the Chox acts as a redox catalyst and helps in the shuttling of electrons or redox equivalents during the catalysis.

Chox mediated catalysis of cholesterol involves three chemical conversions as described by Vrielink and Ghisla (Vrielink and Ghisla, 2009). In the first catalytic step

(reductive half-reaction), the dehydrogenation of the alcohol present at the 3rd position of the steroid ring system results in two redox equivalents that are received by the FAD cofactor (oxidized); the FAD cofactor concomitantly gets reduced in this process. In the second step (oxidative half-reaction), the reduced FAD reacts with molecular oxygen and gets oxidized itself, releasing the oxidized enzyme and hydrogen peroxide (H_2O_2). In the third and final step, the oxidized steroid undergoes an isomerization, from Δ^{5-6} to Δ^{4-5}, in which an unstable intermediate cholest-5-en-3one is converted to form the final product cholest-4-en-3-one.

2.6. Forms of Cholesterol oxidase

The FAD may be covalently or non-covalently bound to the enzyme, based on which Chox has been broadly classified into two classes.

2.6.1. Type - I Cholesterol oxidase

Those Chox in which the FAD cofactor is non-covalently but tightly linked to the enzyme are known as Type I. They belong to GMC (Glucose/ Methanol/ Choline) oxidoreductase family. They have been identified mainly in *Actinomycetes* such as *Streptomyces sp.*, *Brevibacterium sterolicum*, *Rhodococcus* sp. and *Mycobacterium* sp. These enzymes contain a consensus sequence Gly-X-Gly-X-X-Gly for FAD-binding at the N-terminal region of the mature Chox.

This class can be subdivided into the following two subclasses based on the presence or absence of a signal sequence for localization of Chox:

- Class-I-1, which has a signal sequence
- Class-I-2, which does not have any signal sequence (cytopasmic localization)

2.6.2. Type - II Cholesterol oxidase

Those Chox in which the FAD cofactor is covalently linked to the enzyme is categorized as Type-II. They belong to VAO (Vanillyl alcohol oxidase) family, containing a fold that favors covalent flavinilation. They have been identified in *Brevibacterium sterolicum*, *Rhodococcus erythropolis,* and gram-negative bacteria such as *Burkholderia* sp., *Chromobacterium* sp. and *Pseudomonas aeruginosa*.

This class can be subdivided into the following two subclasses on the basis of its source:

- Class-II-1, which consists of actinomycetes enzymes
- Class-II-2, which consists of gram-negative bacteria enzymes

2.7. Applications of Cholesterol oxidase

2.7.1. Diagnosis

The major application of Chox is the determination of total serum cholesterol in human serum samples which helps in the assessment of various life-threatening diseases like atherosclerosis, estimation of the risk of thrombosis and cardiovascular disease (Ernst et al. 2002), and other lipid disorders. Since most of the cholesterol present in serum samples is esterified, incubation with cholesterol esterase (EC 3.1.1.13) is needed to yield free cholesterol which is frequently oxidized by the Chox. Finally, the horseradish peroxidase enzyme (EC 1.11.1.7) catalyzes the oxidative coupling of the hydrogen peroxide produced with an indicator dye (4-aminoantipyrine and phenol) to form chromogen (a red colored quinoneimine dye) and this is the reason why most methods use the coupling of H_2O_2 to indirectly measure the cholesterol.

2.7.2. Insecticidal Agent

In higher eukaryotes, Chox interacts with the membrane lipid bilayer in order to bind its steroid substrate and this action yields cholest-4-en-3-one which significantly alters the structural integrity of the membrane (K.B. Ghoshroy et al., 1994). Therefore, the enzyme disrupts the midgut epithelial membrane when it is ingested by larvae of some Coleoptera ex. *Anthonomus grandis* (boll weevil) and Lepidoptera ex. *Heliothis virescens*, *Helicoverpa zea, Pectino phragossypiella*) thus exhibiting potent insecticidal activity (D.R. Corbin et al., 2001) and is used as an insecticidal and larvicidal agent in agricultural biotechnology (D.R.Corbin et al., 1994).

2.7.3. Analysis of the sterol content in foods

The ability to use 3β-hydroxysterols as substrates have permitted its use for the determination of cholesterol and other sterols in foods. Currently, most methods are based on the use of biosensors (Basu et al., 2007; Wu and Choi., 2003). Particular interest is being paid to the development of enzymatic assays for the detection of phytosterols given their cholesterol-lowering properties (R.A. Moureau et al., 2003).

2.7.4. Transformation of steroids

Chox has broad substrate specificity, so they are used in the transformation of steroids that are intermediates for the synthesis of steroid hormones or other pharmaceuticals steroids. In principle, Chox can oxidize any 3β-hydroxy steroids to the corresponding ketones, which are important precursors of chemically synthesized hormones (D. Labaree et al., 1997; L.W.Guo et al., 2003)

2.7.5. Other applications

Because Chox is the first enzyme in the microbial degradation of cholesterol, the

enzyme has also been investigated for the reduction of cholesterol levels in foods (H.Aihara et al., 1988; C.L.V et al., 2002). Also, given its broad substrate specificity, the enzyme has been used for the optical resolution of non-steroidal compounds such as allylic alcohols (J.F.Biellmann, 2001; S.Dieth et al, 1995).

2.8. Microbial Sources of Cholesterol oxidase

Turfitt (1944) was the first to isolate Chox from *Nocardia erythropolis*; he showed the oxidation of cholesterol from Chox by *Proactinomyces spp*. (Turfitt, 1944, 1946, 1948). Schatz et al. (Schatz et al., 1949) isolated a soil *Mycobacterium,* and Stadtman et al. (Stadtman et al., 1954) isolated the product 4-cholesten-3-one from incubation with Chox (from this *Mycobacterium*). Since then, Chox is widely produced by several bacterial and actinomyces sp. from quite differing environments. However, there is only one report of Chox production by fungi i.e. *Schizopyllum commune* (Fukuyama & Miyake, 1979), on the other hand, there are no reports on Chox production from plant and mammalian sources. The cholesterol assimilating microorganisms and the cholesterol-degrading bacteria are considered to produce Chox. The cholesterol assimilating microorganisms are involved in the production of membrane-bound Chox, mostly pathogenic. Chox producing microorganisms produce both extracellular and intracellular/membrane-bound Chox. Most of them produce cell-bound Chox which needs additional efforts in the extraction process. Various microorganisms producing Chox along with their extracellular/intracellular, pathogenic/non-pathogenic nature and nature of their prosthetic group (covalently bound/non-covalently bound) are listed in **Table 2.1**.

Table 2.1 Microorganisms producing cholesterol oxidase and their properties

Name of Microorganism	Classification	Extracellular/ Intracellular	Pathogenic/ Non-Pathogenic	FAD cofactor	Reference
Arthrobacter	Bacteria	Intracellular/ membrane	Pathogenic	Non-covalently bound (Class I)	Wilmanskaet al. (1988)
Nocardia erythropolis	Bacteria	Intracellular membrane	Pathogenic	-	Richmond (1973); Atrat (1992)
Nocardia rhodochrous	Bacteria	Intracellular membrane	Pathogenic		Buckland et al. (1976)
Rhodococcus sp.	Bacteria	Both Extracellular and Membrane-bound	Pathogenic	Non-covalently bound (Class I)	Watanabe et al. (1986); Watanabe et al. (1989); Kreit et al. (1992)
Corynebacterium	Bacteria	Extracellular			Shirokane et al. (1977)
Mycobacterium	Bacteria	Intracellular/ membrane bound	Pathogenic		Schatz et al. (1949); Smith et al (1995)
Brevibacterium sterolicum	Actinomyces	Extracellular	Pathogenic	Covalently bound	Uwajima et al (1973), Ohta et al (1991)
Streptoverticillium cholesterolicum	Actinomyces	Extracellular	Non-pathogenic		Inouye et al (1982)
Pseudomonas	Bacteria	Extracellular	Pathogenic		Rhee et al (1991)

Schizopyllum commune	Fungi	Extracellular		Covalently bound flavin	Fukuyama & Miyake (1979)
Streptomyces sp.	Actinomyces	Extracellular	Non-pathogenic	Non-covalently bound	Yazdi et al. (2001); Tomioka et al. (1976); Toyobo Corp. Ltd. (2000); Praveen et al. (2011); Varma and Nene (2003); Tripathi et al. (2013); Chauhan et al.(2009)
γ-Proteobacterium	Bacteria	-	-	FMN cofactor*	Isobe et al. (2003)
Chromobacterium sp. DS-1	Bacteria	-	-	-	Doukyu et al. (2008)
Bordetella sp.	Bacteria	Extracellular	Pathogenic	-	Song et al. (2010)
***Streptomyces sp*. SA-COO**	Actinomyces	-	Non-pathogenic	Non-covalently bound	Pollegioni et al. (1999)**;** Gadda et al. (1999)

Note: All Chox found in different microorganisms contain covalently or non-covalently bound FAD prosthetic group for their activity. *γ-Proteobacterium Y-134*, is an exception as it was found to contain covalently bound FMN as a cofactor.

2.9. Fermentative Production of Cholesterol oxidase

Because of the moderate productivity of Chox by the various microorganisms used and the additional requirement of adding cholesterol to culture media to induce/enhance production, the cost of Chox remained relatively high (Sojo et al. 1997; Kreit et al. 1994). The yield of Chox production was enhanced by altering the composition of the production medium and physical parameters of culture conditions. The culture conditions for Chox production by different microorganisms have been presented in **Table 2.2**. Attempts have been made for the cloning and expression of Chox gene from different sources for the improvement of the Chox producing strain that would be effective for over-production of Chox for commercial applications (Solaiman and Somkuti 1995; Murooka et al. 1991). In case of the comparative study between the *Arthrobacter simplex* (Lieu et al. 1983), *Mycobacterium cholesterolicum* (Stadtman et al., 1954), and *Rhodococcusequi* (Watanabe et al. 1989) which produce a maximum level of Chox in the presence of 0.3, 0.05, and 0.1% cholesterol, respectively, the maximum levels of Chox production have been reported to be 1.50 U/ml by *A. simplex* (Liu et al. 1983), 0.05 U/ml by *B. sphaericus* (Suh et al. 1993) and 0.29 U/ml by *Rhodococcus sp.* (Park et al. 1998). *Bacillus subtilis SFF34* could produce much higher levels of Chox (3.14 U/ml) than other strains. *A. simplex*, *B. sterolicum*, *R. equi,* and *Streptomyces sp.* have been known to produce their maximum level of Chox after 96 h, 72 h, 100 h, and 40 h of cultivation, respectively. *B. subtilis* SFF34 could produce its maximum level of Chox after 24 h of incubation. There are other noteworthy methods by which the enzyme was unexpectedly screened (Kamaie et al. 1978) as they devised a method of screening anti-cholesterol substances based on the antagonism between polyene antifungal antibiotics and cholesterol against yeast.

Table 2.2 Culture Conditions for the Production of Cholesterol oxidase by Different Microorganisms

S.No	Name of Microorganism	Culture Conditions					Production (Unit/ml)	References
		Fermentation time (h)	Temp. (°C)	pH	RPM	Seed Age		
1	*Rhodococcus equi No. 23*	30	37	7.5	300	-	0.34	Chou et al. (1999)
2	*Bordetella sp.*	96	37	-	260	48	1.7	Song et al. (2010)
3	*Streptomyces lavendulae* NCIM 2499	72	30 ± 2	7.0	180	48	1.67	Annapure et al. (2009)
4	*γ-Proteobacterium*	14	30	8.0	120	-	-	Isobe et al. (2003)
5	*Brevibacterium sp.*	36	30	7.5	-	-	1.483	Yang et al. (2012)

2.10. Purification of Cholesterol oxidase

The procedures applied for the extraction and purification of Chox are similar to those applied to enzymes, in general. However, subtle differences in the extraction and purification procedures are generally required in order to obtain a sufficient yield of the enzyme from Chox producing bacteria to improve yield and/or clarification. Many researchers have performed DEAE ion-exchange chromatography, hydrophobic ionic chromatography, affinity chromatography. Only a few pieces of literatures are available on the partitioning of Chox in aqueous two-phase systems (ATPS).

2.10.1. Partitioning in Aqueous Two-Phase System

Extraction using ATPS has been recently used as a simple, fast, cost-effective, and eco friendly downstream processing method for the recovery of biomolecules. ATPS has been successful to a large extent in overcoming the limitation of conventional organic-aqueous

extraction and hence applicable for the extraction and purification of biomolecules (Walter et al., 1991; Albertsson 1986; Zaslavasky, 1995). ATPS has about 80-90% water and provides a biocompatible environment for biologically active molecules (Chen and Lee, 1995). Cells are immobilized on one phase of ATPS and the essential product is separated towards the other phase. Polymer/salt systems require lesser time to separate and show better selectivity for protein extraction than polymer/polymer systems and are hence preferred for industrial application. A simple dialysis method is applied for the recovery of biological proteins from the salt phase. Several reports are available for the construction of phase diagram for a number of biomolecules (Bamberger et al., 1984; Walter et al., 1991; Albertsson, 1986; Diamond and Hsu. 1992; Zaslavasky,1995). The composition of the top and bottom phase in the binodal curve was determined gravimetrically in terms of phase volume ratios (V_t/V_b). The identical composition for top and bottom phase has been obtained at the plait point of the binodal curve. The distance between the composition of the top and bottom phases in a binodal curve is termed as tie-line length (TLL), which affects viscosity, density, and interfacial tension between the phases. In case of the PEG/Salt ATPS system, the concentration of PEG in the top phase increases with longer TLL, which results in higher viscosity in the top phase. On the other hand, with longer TLL, the salt concentration increases in the bottom phase which has hardly any effect on the viscosity of the bottom phase. Hence, the viscosity difference between the top and bottom phases increases as the TLL increases. In contrast to the effect of TLL on viscosity, the variation in the density of the top phase is very small in comparison to density in the bottom phase. This is due to the fact that, as the TLL becomes longer, the phases become closer to solutions of pure PEG in the top and pure salt in the bottom. PEG concentration

has a small effect on density, whereas salt concentration affects the density much more strongly. As TLL increases, the difference between the top and bottom phase composition becomes greater, and hence the interfacial tension increases (Juan et al., 2012). The phase formation of ATPS is affected by the concentration of externally added salts, different molecular weights of PEGs, hydrophobicity, and concentration of the polymers (Walter et al., 1991; Albertsson, 1986' Diamond and Hsu, 1992; Banik et al., 2003).

The efficient parameters of partitioning like the partition coefficient of the desired protein, volume ratio are required to be optimized in such a way that the cell debris is partitioned into one phase and the desired protein partitioned into another phase (Abbot et al., 1990; Hustedt et al., 1985). The partition coefficient of a biomolecule is defined as the ratio of the equilibrium concentration of the protein in the top phase to that in the bottom phase. The ionic nature of the protein, characteristics of the polymer, phase composition, system pH, and temperature affect the partition coefficient of biomolecules (Banik et al., 2003; Pandey and Banik, 2011). The residual amount of PEG along with the salt can be removed by ultra-filtration (Hustedt et al., 1985) or using chromatographic column based on adsorption/ hydroxyapatite/ ion-exchange/ affinity chromatography (Albertsson, 1986).

2.11. Properties of Cholesterol oxidase from Different Sources

Chox from several microorganisms has been extensively studied. Various properties of microbial Chox viz. molecular weight, pH and temperature optima, stability, and inhibition characteristics were determined in several pieces of literature. The molecular weight of the Chox has been reported to be in the range of 47 – 60 kDa. Most Chox are secreted into the growth medium. However, intracellular or membrane-bound enzyme Chox has been reported from *Mycobacterium* and *Rhodococcus. R.erythropolis* produces both membrane-bound and extracellular Chox.

2.11.1. pH and Temperature Optima and Stability Profiles of Cholesterol oxidase

Microbial Chox generally has neutral pH optima and possesses stability over a wide range. The enzyme has temperature optima in the range of 40°C - 60°C. The optimum temperature (70°C) of Chox from *Streptomyces fradiae* is the highest among the enzymes reported so far. Chox from *Chromobacterium sp. strain DS-1* is highly thermostable. The thermal stability of the *DS-1 enzyme* was compared with commercially available Chox from various bacterial sources such as *Streptomyces sp*., *Cellulomonas sp*., *Nocardia sp*., *Nocardia erythropolis*, *Pseudomonas fluorescens*, and *B. cepacia ST-200*. All of these commercial enzymes lost most of their activity after incubation for 30 min at 60 – 80°C. Exceptionally, the enzyme from strain *DS-1* retained 80% of its original activity even at 85°C after 30 min. These results and the survey of the thermal stability of the other reported enzymes show that the *DS-1 oxidase* exhibits the highest thermal stability among all Chox reported so far. The thermal stability of *Streptomyces* Chox was improved by random mutagenesis (Nishiyaet al.1998). The half-life of a multiple mutant (S103T, V121 A, and R13 5H) showing the highest stability was 52.2 min at 60°C, while that of the wild type was 7.8 min. The *DS-1* enzyme retained more than 90% of its original activity after incubation for 240 min at 60°C. Therefore, the thermal stability of the *DS-1* enzyme is much higher than that of the multiple mutants from the *Streptomyces* enzyme.

2.11.2. Effect of metal ions and chemical reagents

Metal ions are generally not required for Chox activity. Chelating agents, including EDTA, o-phenanthroline, and 8-hydroxyquinoline, did not show a significant inhibitory effect on the enzyme activity. In many cases, Chox activity is markedly inhibited by an SH inhibitor, Hg^{2+}, or Ag^{+}. By contrast, Ag^{+} scarcely influenced the activity of the enzyme

from strain *DS-1*. $FeCl_3$ and $FeSO_4$ remarkably inhibited the activity of the enzyme from *S. violascens*. $CuSO_4$ significantly inhibited the activity of the enzyme from *Streptoverticillium cholesterolicum*. The addition of p-chloro mercuric benzoate partially reduced the activity of the enzymes from *A.simplex* and *B.sterolicum*. The activities of the enzymes from *Pseudomonas sp*. Chox629 and *γ-Proteobacterium* were partially activated by the addition of Mn^{2+}.

2.11.3. Effect of detergents

Since cholesterol is an insoluble compound, detergents are often added to the reaction solution to act as a solubilizer. In addition, the monitoring of high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol in serum is important for the diagnosis of hyperlipemia or atherosclerotic diseases. Therefore, several methods for the separation of HDL or LDL cholesterol with various detergents have been devised. Since detergents influence the enzyme activity and often inactivate the activity of Chox and most of the other enzymes; a Chox with high activity and stability in the presence of a wide range of detergents is expected to improve the differential assay method for HDL and LDL cholesterol in serum. A detergent-tolerant Chox was reported from *γ-Proteobacterium Y-134*. The Y-134 enzyme retained more than 80% of its original activity in 0.5% Triton X-405 and sodium cholate after incubation for 1 h at 60°C. In this experimental condition, commercially available enzymes from *Nocardia* and *Brevibacterium* lost most of their activities. The DS-1 enzyme previously discussed also showed stability in the presence of 0.5% detergents at various temperatures when compared with commercially available enzymes of *Streptomyces sp*., *Cellulomonas sp*., *Nocardia sp*., *N.erythropolis,* and *P. fluorescens*. All Chox tested were stable in Tween 20, Triton X-100, Triton X-405, sodium cholate, and sodium polyoxyethylene alkyl ether

sulfate (Emal 20CM) after incubation for 1 h at 30°C. Commercially available enzymes were completely inactivated by the addition of ionic detergents such as sodium dodecyl sulfate (SDS) or sodium lauryl benzene sulfonate (LBS) after incubation for 1 h at 30°C. By contrast, the DS-1 enzyme was relatively tolerant to SDS and LBS. In addition, the treatment with sodium dodecyl sarcosinate or Emal 20CM completely inactivated all of the enzymes except the DS-1 enzyme after 1 h at 60°C. The DS-1 enzyme was relatively tolerant to these detergents even at 60°C.

2.11.4. Effect of organic solvents

Organic solvents are also used to solubilize steroids. Chox has been used for the optical resolution of non-steroidal compounds, allylic alcohols, and the bioconversion of a number of 3β-hydroxysteroids in the presence of organic solvents. Therefore, an organic solvent-tolerant Chox would be useful for these applications. Pollegioni et al. examined the stability of Chox from *Streptomyces hygroscopicus* and *B. sterolicum* in the presence of various concentrations of isopropanol. The activity of the *B. sterolicum* enzyme is rapidly inactivated, whereas the *S. hygroscopicus* enzyme retained 70% of the initial activity after 5 h of incubation with 30% propanol at 25°C. The stability of Chox obtained from various sources was examined in the presence of different organic solvents. Commercially available Chox, including *Streptomyces sp.*, *Cellulomonas sp.*, *Nocardia sp.*, *N. erythropolis,* and *P. fluorescens* were inactivated by the addition of 50% volume of dimethyl sulfoxide, methanol, ethanol, acetone, isopropanol, ethyl acetate, or butanol after incubation for24 h at 37°C. By contrast, DS-1 and *B. cepacia* ST-200 enzymes were stable in the presence of all solvents except for acetone.

2.12. Commercial availability

The cost of Chox is relatively very high as compared to other enzymes. Some of the major underlying reasons are its low productivity, intracellular localization or membrane-bound nature of enzyme, the pathogenicity of the producer organism, the addition of inducer to enhance the production, and lack of inexpensive inducer. Due to the inducible nature of Chox, its productivity remains very low by the Chox producer bacteria inspite of the cost of medium components incorporated for its production. Commercial culture media such as corn-steep liquor necessitate the addition of cholesterol (or similar compound) to induce and enhance the production. The addition of cholesterol in commercial culture media adds to the cost of production media, however, no other inducer has been reported to date. Moreover, most of the Chox producing bacteria are highly pathogenic and have ruled out commercial developments. *Rhodococcus equi* showed the highest level of Chox activity rather than any of the known Chox producing bacteria, but at the same time, the enzyme Chox is a crucial part of its pathogenicity, thus limiting its commercial production (Linder and Bernheimer, 1997). Similarly, other microorganisms producing Chox so far, including *Mycobacterium*, *Pseudomonas*, *Bordetella* and *Brevibacterium* are pathogenic in nature. In this context, *Streptomyces* has proven to be a harmless culture because it is non-pathogenic. *Streptomyces* is well suited for the commercial production of Chox and is also best suited for diagnostic application for the detection of cholesterol in serum and other clinical samples (Lolekha and Jantaveesirirat, 1992). The Biochemical properties of Chox purified from different *Streptomyces sp.* are listed in **Table 2.3.**

Table 2.3 Biochemical Properties of *Streptomyces* Cholesterol oxidases

***Streptomyces* *sp*.**	**Mol. wt (kDa)**	**pH optima**	**pH stability[a]**	**Temp. optima (°C)**	**Thermal stability[b]**	**Inhibitors**	**Reference**
Streptomyces fradiae	60	7.0	pH 4-10 (4°C, 4 h)	70	10% (70°C, 1 h)	-	Yazdi et al. 2001
Streptomyces violascens		7.0		50		$HgCl_2$ and $AgNO_3$	Tomioka et al. 1976
Streptomyces sp. SA-COO	58	6.5-7.0	pH 4-8 (25°C, 20 h)	45-50	<50% (55°C, 15 min)	$HgCl_2$ and $AgNO_3$	Toyobo Corp. Ltd. 2000
Streptomyces parvus	55	7.2	pH 4-11	50	46% (65°C, 30 min)	Pb^{2+}, Ag^{2+}, Hg^{2+}, Zn^{2+}	Praveen et al. 2011
Streptomyces lavandulae NCIM 2421			pH 6-10 (30°C, 1h)		65% (50°C, 30 min)	-	Varma and Nene. 2003
Streptomyces sp.	62	7.0	pH6-8 (4°C, 24 h)	37	100% (30-40°C,120 min)	Ba^{2+} and Hg^{2+}	Tripathi et al. 2013
Streptomyces hygroscopicus	53	-	-	-	-	-	

[a]Incubation conditions are shown in the parantheses

[b]Relative residual activities calculated as a percentage of the original enzyme activity are shown in the column. Incubation conditions are indicated in the parentheses.