Review Article

iMedPub Journals
http://www.imedpub.com

Insights in Enzyme Research
ISSN 2573-4466

2017
Vol. 1 No. 1: 5

DOI: 10.21767/2573-4466.100005

Cholesterol Oxidase: Source, Properties and Applications

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Citation: Devi S, Kanwar SS. Cholesterol Oxidase: Source, Properties and Applications. Insights Enzyme Res. 2017, 1:1.

Abstract

Cholesterol oxidase (CHOx), a FAD-dependent enzyme of the oxido-reductase family catalyzes the oxidation of cholesterol to cholestenone. CHOx is produced by a large number of bacterial species, and the actinomycetes being most prolific group. Being an enzyme of great commercial value, CHOx has drawn significant attention due to its use in determination of cholesterol level in various clinical and food samples and because of its novel applications in biosensors. In addition, the enzyme also finds application as an insecticide and in bio-catalysis for the synthesis of a number of steroids. Moreover, CHOx is also implicated in the bacterial pathogenesis and in the cardiovascular disease such as atherosclerosis and other heart diseases. These applications and pathological processes stress the need for screening and isolation of novel CHOx and enhancing knowledge about its structural and functional aspects. In present review we discuss the microbial sources, production including use of recombinant microbes, characterization, immobilization and major applications of CHOx.

Keywords: Cholesterol; Microbes; Cholesterol oxidase; Biosensors; Immobilization applications

Received: December 06, 2016; **Accepted:** January 04, 2017; **Published:** January 07, 2017

Introduction

Cholesterol oxidase (3β-hydroxysterol oxidase, EC 1.1.3.6) catalyzes the oxidation of cholesterol (cholest-5-en-3β-ol) to its 3-keto-4-ene derivative, cholestenone (cholest-4-en-3-one), with the reduction of oxygen to hydrogen peroxide (H_2O_2). However, some reported cholesterol oxidases from bacterial species oxidize cholesterol to 6β -hydroperoxycholest-4-en-3-one (HCEO) in place of cholest-4-en-3-one (CEO) [1]. Cholesterol oxidase is a flavin adenine dinucleotide (FAD)-dependent enzyme which exists in two forms, in the first form FAD cofactor is bound non-covalently to the enzyme (class I) and in the second form cofactor is linked covalently to the enzyme (class II) [2,3]. Cholesterol oxidases belong to flavin-dependent oxido-reductase superfamily and this superfamily is further divided into: glucose-methanol-choline (GMC) oxido-reductase and vanillyl alcohol oxidase (VAO) family. The type I cholesterol oxidases are the part of the GMC oxido-reductase family, and the type II cholesterol oxidases are assigned to VAO family [4].

Cholesterol oxidases are produced by microorganisms of both pathogenic and nonpathogenic nature such as *Mycobacterium* [5], *Brevibacterium* [6], *Streptomyces* [7], *Corynebacterium* [8], *Arthrobacter* [9], *Pseudomonas* [10], *Rhodococcus* [11], *Chromobacterium* [1] and *Bacillus* [12] species. In most of microorganisms, the CHOx is employed in the initial step of cholesterol metabolism. While in case of pathogenic bacteria CHOx acts as membrane-damaging factors and therefore contributes as a virulence factor in the pathogenicity of these bacteria [5,13]. In addition, *Streptomyces natalensis* produces a cholesterol oxidase (PimE) which acts as a signaling protein for the biosynthesis of an antifungal antibiotic, polyene macrolide pimaricin [14,15].

Screening and isolation of new CHOx producing bacterial strains are very important owing to their wide spectrum applications and commercial value. And keeping this in focus the enzyme has been isolated and purified from various microorganisms. CHOx is employed in the enzymatic assay of amount of cholesterol in food, serum and other clinical samples [16,17]. Cholesterol oxidase can also be used in the production of a variety of valuable intermediates employed for steroid drug production, such as 4-androstene-3, 17-dione (AD) and 1, 4-androstadiene-3,17-dione (ADD) which are main starting materials in the synthesis

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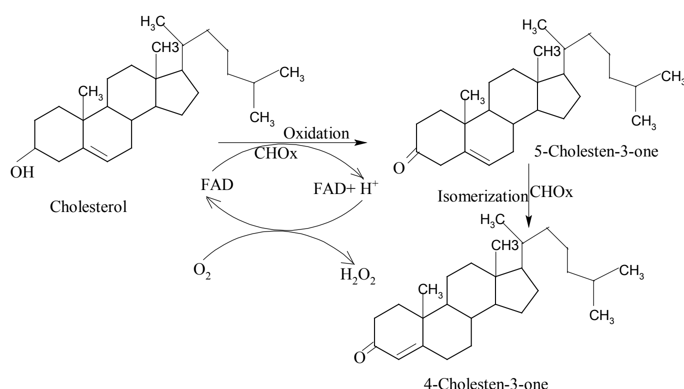
of anabolic drugs and contraceptive hormones [18]. The enzyme has also been shown to have larvicidal activity against cotton boll weevil (*Anthonomus grandis*) [19]. CHOx biosensors have found applications for detection of cholesterol level in various samples. Discovery of new microbial source(s) of CHOx with useful properties for applications in various fields continues to be of considerable interest despite the availability of commercial CHOx.

Mechanism of Action of Cholesterol Oxidase

CHOx catalyzes the oxidation of cholesterol to cholestenone (4-cholesten-3-one, CEO) in three consecutive conversions steps. In the first catalytic conversion occurs dehydrogenation of the OH-functional group at the 3-position of the steroid ring backbone with the loss of the 3 α-hydrogen and 3 β-hydroxy hydrogen (reductive half-reaction). The two resulting electron equivalents are transferred to the oxidized FAD enzyme cofactor that is converted to its reduced form in the process. In the next step, the reduced form of FAD cofactor reacts with molecular oxygen (O_2) to regenerate original enzyme in its oxidized form and hydrogen peroxide (H_2O_2) (oxidative half-reaction). In the final third step, the CHOx 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 CHOx from *B. cepacia*, *Pseudomonas* sp. and *Chromobacterium* sp. oxidizes cholesterol to HCEO [1,20]. 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 [21].

The reaction cascade followed by cholesterol oxidase in degradation of cholesterol is shown below:



Cholesterol oxidase structural properties and types

CHOx identified from various microbial sources show a few structural differences. A consensus sequence composed of repeating glycine residues (GXGXXG) followed by presence of aspartic acid or glutamic acid approximately 20 residues further is characteristic of a nucleotide-binding fold in most of the flavor enzymes [22]. In case of noncovalent form of CHOx, an almost identical consensus sequence of glycine moieties (G17-X-G19-X-G21-G/ A22) followed by a glutamate (E40) is present which shows the presence of nucleotide-binding fold. However, the covalently bound form of CHOx shows absence of this consensus sequence, indicating the probable absence of a nucleotide-binding fold [23]. Both forms of CHOx have been found to consist of one FAD-binding domain and one substrate binding domain. The cofactor binding motifs containing consensus sequence of glycine moieties facilitate hydrogen bond interactions by allowing a closer approach between protein main chain and phosphate oxygen atoms of the FAD cofactor [23]. In the covalent form of the enzyme, the diphosphate moiety is localized in a pocket made by the residues found between the third and fourth β -strands of a four-stranded β-pleated sheet [23]. Despite exhibiting structural differences both covalent and noncovalent enzyme forms possess a buried hydrophobic pocket for binding steroid ring backbone. Yue et al. after structural and mutational analysis of *Streptomyces* sp. SA-COO (class I enzyme) reported that His447 and Glu361 residues have implications in catalyzing the oxidation and isomerization reaction [24]. X-ray crystallographic structure determination of the *Brevibacterium sterolicum* class II enzyme has suggested that the FAD cofactor was covalently bound to an active-site histidine (His121) *via* the C8 α group of the flavin isoalloxazine ring [25]. This covalent bond is involved in the redox potential and it also contributes to the stability of the enzyme [26]. In addition, Glu475 and Arg477, located at the active-site cavity were suggested to constitute gate functioning in the control of oxygen access [27].

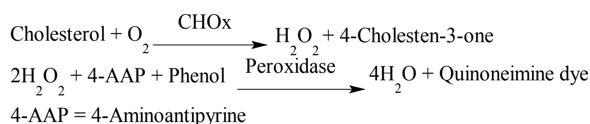
CHOx are broadly of two main types classified as class I and class II, in class I the FAD cofactor is bound non-covalently to the enzyme and in class II this cofactor is linked covalently to the [2,3]. Further, there does not seem to be any significant sequence homology between these two enzyme forms and thus both the forms appear to belong to different protein families [21]. The class I enzyme comes under GMC (glucose/methanol/choline) oxido-reductase family, while the class II enzyme belongs to the VAO (vanillyl-alcohol oxidase) family. Class I enzymes have been reported mostly from actinomycetes such as *Streptomyces* sp., *Brevibcterium, Rhodococcus* sp., *Arthrobacter*, *Nocardia* and *Mycobacterium* sp. while Class II enzymes have been identified in *Brevibacterium sterolicum, Rhodococcus erythropolis*, and in some Gram-negative bacteria such as *Burkholderia* sp., *Chromobacterium* sp. and *Pseudomonas aeruginosa*.

Analysis of cholesterol oxidase activity

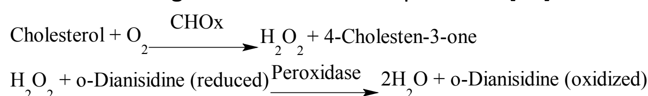
Literature shows various assay methods for determination of CHOx activity. These assay methods are employed for both qualitative and quantitative analysis of cholesterol oxidase activity. Cholesterol is metabolized by microorganisms both as carbon as well as energy source and cholesterol-assimilating microorganisms are generally considered to produce cholesterol oxidases. Cholesterol oxidase activity is detected extra-cellular in culture supernatant or in the whole cells of cholesterol-degrading bacteria. A colony-staining method based on the principle of Allain's method [28] is used for the screening of CHOx. In this method, filter paper squares that are dipped into a solution containing cholesterol, 4-aminoantipyrin, phenol and horseradish peroxidase are placed on bacterial colonies grown on an agar medium. CHOx activity of the test colonies is indicated by formation of quinoneimine dye which is red in color. For crude or purified CHOx, the activity determination assay method based on the same principle is used. In this method when

cholesterol is oxidized by CHOx to cholest-4-en-3-one with the simultaneous production of H_2O_2, which oxidatively couples with 4-aminoantipyrine and phenol in the presence of peroxidase to yield a chromogen with maximum absorption at 500 nm [28].

The stepwise reaction cascade promoted by CHOx to form hydrogen peroxide which subsequently converts 4-AAP to a chromogenic product (quinoneimine dye) is presented below:



In another assay, Nishiya et al. [29] have reported the use of cholesterol oxidase indicator plate for screening of CHOx producing colonies. These plates are prepared by adding cholesterol, Triton X-100, o-dianisidine and peroxidase to the agar medium. Oxidation of cholesterol by bacteria (as shown below) results into H_2O_2 production, which is utilized for oxidation of o-dianisidine with peroxidase and results in the formation of azo compound indicated by the medium color change into intense brown. Enzyme activity of crude or purified CHOx can be determined spectrophotometrically using peroxidase and o-dianisidine method. The H_2O_2 released during cholesterol oxidation is utilized to oxidize o-dianisidine a chromogenic substrate in a secondary reaction with horseradish peroxidase (HRP) with a resultant color change that is monitored spectrophotometrically at 500 nm [30]. These methods did not differentiate between CEO- and HCEO-forming CHOx. HCEO-forming CHOx is reported to form turbid halos around the bacterial colonies on an agar medium containing cholesterol due to the low solubility of HCEO. Therefore, this turbid halo formation can be applied for screening of HCEO forming cholesterol oxidase producer [31].



Production of Cholesterol Oxidase

Microbial strains producing cholesterol oxidase

CHOx is produced by several microorganisms that are found in remarkably differing environments. CHOx is produced by a bacterium in three forms: intracellular, extracellular and membrane bound. Turfitt [32] reported the enzyme for the first time from the bacterium *Rhodococcus erythropolis* (formerly *Proactinomyces erythropolis*). The most common microbial sources for production of CHOx are mainly *Arthrobacter* [33,34], *Bacillus* sp. [12,35], *Brevibacterium* [36], *Bordetella* [37], *Corynebacterium* [8], *Mycobacterium* [38,39], *Nocardia* [40,41], *Rhodococcus* [42,43] and *Streptomyces* [44-46]. Some Gram-negative bacteria such as *Burkholderia* [31], *Chromobacterium* [1] and *Pseudomonas* sp. [10] have also been reported to produce CHOx.

Physico-chemical parameters affecting enzyme production

Carbon source: Carbon source is one of major components in microbial fermentations which are used for cellular material synthesis and also as an energy source. The rate at which carbon source is used affects both, biomass formation and production of primary or secondary metabolites. Lee et al. reported cholesterol as the best carbon source and inducer for CHOx production by *Rhodococcus equi* no. 23 [47,48]. The effects of initial cholesterol concentration (0.52-1.08 $g.l^{-1}$) was also studied on cell growth and CHOx production and the optimal CHOx production was observed in the cultures having the initial cholesterol concentrations ranging from 0.8-1.08 $g.l^{-1}$ [48]. *Rhodococcus erythropolis* showed significant CHOx activity when grown in a mineral medium containing cholesterol as a sole carbon source [49]. Yazdi et al. investigated the effects of various steroids and sugars as carbon source on CHOx production and results revealed that cholesterol was best carbon source for CHOx production by *Rhodococcus equi* 2C [50]. For the maximum production of CHOx by *Streptomyces* sp. addition of 0.05% of cholesterol as inducer was found to be most suitable [51]. 0.15% (w/v) cholesterol concentration in the production medium is reported for optimum CHOx production from *Rhodococcus* sp. and *R. equi* 2C [42,50]. Moreover, Kreit et al. assessed some fatty acids on *Rhodococcus* sp. GK1 and found that hexanoate as sole carbon source yielded the highest CHOx production [42]. Cholesterol is also shown as the best carbon source for CHOx production by *Brevibacterium sterolicum* [6], *Corynebacterium cholesterolicum* [8], *Arthrobacter simplex* [34] and *Rhodococcus* sp. NCIM 2891 [52].

Nitrogen source: Application of an appropriate nitrogen source is one of the important factors for optimal production of CHOx. Lee et al. reported yeast extract at concentration of 0.4-0.5% w/v as the best nitrogen source for CHOx production by *Rhodococcus equi* no. 23 [47]. In another study, Yazdi et al. found that *Rhodococcus equi* 2C showed maximum production of enzyme with yeast extract at 0.3% w/v [50]. Highest production of enzyme by *Arthrobacter simplex* is reported in the presence of ammonium salts [34]. Ammonium chloride and ammonium acetate have been reported to show highest effect on CHOx production by *R. equi* no 23 [47] and *A. simplex* [34], respectively. Another study also reported that the combination of $(NH_4)_2HPO_4$ and yeast extract provided the highest enzyme production as compared to individual organic and inorganic components [52]. In another finding by Moradpour et al. it was observed that an organic nitrogen source (yeast extract) had more influence on CHOx activity than the inorganic nitrogen source namely $(NH4)_2SO_4$ [53]. The reason for this may be because organic nitrogen source contains most types of amino acids and growth factors for the growth of bacterium that could be metabolized directly by cells, hence promoting CHOx production [54].

Surfactants: Since cholesterol is insoluble in water, it often needs to be solubilized for its better dispersion and availability for microorganisms. Yazdi et al. studied effects of various surfactants, and amongst them Tween 80 and Tween 60 were observed to exhibit maximum effect on CHOx production while with the addition of Triton X100, the enzyme production was nearly inhibited [50]. Moradpour et al. found that after medium supplementation with Tween 20, *Streptomyces badius* showed significant increase in cholesterol oxidase production [53]. In contrast to this Doukyu et al. reported that CHOx production of *Nocardia erythropolis* and *Pseudomonas* sp. was relatively lower in the presence of detergents [1]. Increase in the enzyme

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production by addition of such surfactants might be due to the modification of plasma membrane permeability, which results in the enhancement of both uptake and exit of compounds from the cell. Furthermore, surfactants contribute to disperse cholesterol, which is quite water insoluble [55].

Effect of medium pH: Among the different physical parameters, pH of the growth medium plays a significant role by affecting microbial physiology and CHOx production. Being a significant factor, pH affects nutrient solubility and uptake, enzyme activity, cell membrane morphology and byproduct formation as well as its stability in the medium. Culture pH can have significant effects on both the rate of production and the synthesis of enzymes. The appropriate pH for maximum production of the CHOx can differ from that required for optimum growth of the CHOx producer. CHOx enzymes work best at a pH of 6.5-8.0 [21]. Yazdi et al. studied the effects of various initial pH values on *Rhodococcus* sp. and found that a pH set to 8 was optimum, whereas Lee et al. reported an initial pH of 7.0 as optimum for enzyme production from *Rhodococcus* sp. 2C [47,50]. While in contrast Moradpour et al. reported that pH of 6.5 supported the maximum production of CHOx [53]. In another investigation by Chauhan et al. an initial pH of 7.5 was found to be optimum for *S. lavendulae* for production of CHOx [54].

Effect of growth temperature: Cultivation temperature is known to be one of the various important operating parameters of fermentation processes as the microbial metabolic activity is well known to be sensitive to environmental temperatures. In addition to this it also affects media concentrations. The effects of cultivation temperature (34.2-39.8°C) on cell growth and CHOx production were investigated and it was observed that cell growth reached the same maximum value for all the cultivation temperatures tested, but the growth rate was higher at temperature above 37°C than that below 35°C. However, the optimal CHOx production occurred at 39°C [48]. Maximum CHOx was obtained at an incubation temperature of 30°C by *Streptomyces badius* [53]. Optimum yields of CHOx were achieved at 30°C in *Rhodococcus* sp., *Brevibacterium* sp. and *Bordetella* sp. [37,56,57].

Genetically Modified Organisms for Expression of Cholesterol Oxidase

Typical problems which are mainly encountered during any enzyme production are either low production or simultaneous production of other unwanted proteins. To overcome these problems one solution is to use genetically modified microorganisms in place of natural sources for the expression of desired protein. Despite being available commercially there is still large interest in new forms of CHOx with useful properties. The molecular weight range of the CHOx is 47-60 kDa [58] and most of the cholesterol oxidases contain approximately 550 amino acids with a 40 to 50 amino acids long signal peptide [11]. The CHOx from different microbial sources have been cloned and expressed successfully in appropriate host system [31,36,59-66].

First attempt for heterologous expression of CHOx was made by Molnar et al. The *Streptomyces* sp. SACOO CHOx gene was cloned and expressed in *Streptomyces lividans* but the enzyme activity was found to be very low, and the wild-type gene sequence failed in the expression of protein when *E. coli* used as a host system [67]. Sampson and Chen improved CHOx expression in *E. coli* from *Brevibacterium sterolicum* up to 60-fold by genetic modification and optimization of culture conditions [62]. To improve heterologous expression in *E. coli*, they replaced the codons of the first 21 amino acids with high expression *E. coli* codons and the mutant gene was expressed under the T7lac promoter [62]. A more or less similar strategy was adopted to overexpress the type II cholesterol oxidase from *B. sterolicum* [63]. CHOx encoding gene (choA) from *Chryseobacterium gleum* DSM 16776 was cloned into the pQE-30 expression vector and expressed heterologously in *E. coli* JM109 [66]. The expressed N-terminally His-tagged cholesterol oxidase (CgChoA) showed an optimum temperature of 35°C and a pH optimum of 6.75 with 15.5 U/mg maximum activities [66]. In another study, Doukyu and Nihei cloned and overexpressed the CHOx gene from *Pseudomonas aeruginosa* strain PA157 in *E. coli*. For the expression they used a putative cholesterol oxidase gene sequence from the genome sequence data of *P. aeruginosa* strain PAO1. An active form of expressed CHOx was obtained by growing recombinant cells at 10°C and purified enzyme showed optimal activity at pH 7.0 and a temperature of 70°C [68]. Chen et al. reported successful cloning and expression of CHOx encoding gene (*choAA*) from *Arthrobacter simplex* F2 in *E. coli* and *Pichia pastoris*. The deletion of the signal sequence from the gene was found favorable for the expression of extracellular CHOx by *P. pastoris* [9]. The CHOx gene from *Arthrobacter simplex* F2 was found to consist of 1653 base pairs and encoding a protein of 551 amino acids [9].

Purification Strategies for Cholesterol Oxidase

For developing new and efficient separation processes one must exploit effectively the differences in physicochemical properties of desired product from that of other contaminant components. Such properties include surface charge, surface hydrophobicity, molecular weight, affinity towards certain ligands, isoelectric point (pI), stability etc. The methods applied for the extraction and purification of CHOx are similar to those applied to other proteins, in general. The techniques used for extraction mainly rely on the nature of the enzyme, i.e., whether intracellular or extracellular. In case of extracellular enzyme, the enzyme is secreted from the cell into the growth medium and cell removal by differential centrifugation is often the only step required to obtain a good yield of active enzyme. Extraction of intracellular enzyme is comparably difficult because cell need to be lysed first for the removal of enzyme from within the cell. Extraction of CHOx *via* a non-ionic surfactant-based aqueous two-phase system is found to be very effective. In a study conducted by Ramelmeier et al. the effect of Triton X-114 solution above the cloud point was studied on CHOx from different bacterial sources. After optimization of this method a good recovery of the enzyme, over 70 and 90% in the detergent-rich phase for the extracellular and membrane-bound forms were found, respectively with simultaneous 10 to 20 fold concentration of the enzyme in single purification step [69]. In another similar study by Sojo et al. Triton

X-114 detergent was used for extraction of cell-linked CHOx. This same detergent was also used for purification and concentration of CHOx, which was found in the detergent rich phase with 11.6-fold purification and 20.3-fold concentration [70]. Minuth et al. achieved 5-fold purification and 4-fold concentration after adding the detergent pentaethyleneglycol mono *n*-dodecyl ether (C12EO5) to a non-clarified culture of *Nocardia rhodochrous* [71]. Ammonium sulphate fractionation is a common and very effective way to carry out the first purification step [1,6,46]. After ammonium sulphate fractionation, the purity of the enzyme can be improved further *via* chromatographic means. For CHOx, ion exchange and gel filtration chromatography are widely applied techniques [1,44,46,72]. The use of cholesterol affinity columns has led to efficient extraction schemes resulting in highly enriched enzyme extracts [10,35,73]. The CHOx produced by a *Chromobacterium* sp. strain DS-1 was purified 17.8-fold with 13% yield from culture broth by using DEAE-cellulose and butyl-Toyopearl 650S columns. The enzyme was reported to be a monomer with a molecular weight of 58 kDa after estimation by SDS-PAGE [1]. An extracellular CHOx enzyme was isolated from the *Streptomyces parvus* and purified 18-fold by ion exchange and gel filtration chromatography. Specific activity of the purified enzyme was found to be 20 U/mg with a 55 kDa molecular weight [46]. While, the molecular weights of the enzyme from *Streptomyces violascens* [74], *Brevibacterium sterolicum* [6] and *Schizophyllum commune* [75] were reported to be 61, 31 and 53 kDa, respectively. Rhee et al. purified an active enzyme from the culture supernatant of a *Pseudomonas* sp. with a yield of 70% by using cholesterylglycine-CM-cellulose column [76]. The CHOx enzyme produced by *Enterococcus hirae* was precipitated at 80% ammonium sulphate saturation and then purified by applying on Sephadex G-100 gel chromatography column with resultant yield of 79% and 2.3-fold purification. The molecular weight of this purified CHOx was found to be 60 kDa [72].

Xin et al. synthesized two chemical ligands by simulating the structure of natural coenzyme FAD of CHOx for affinity purification of the *Brevibacterium* sp. (DQ345780) enzyme, which was expressed in *E. coli* BL21 (DE3). After a single step of affinity purification by binding on the two sorbents, the protein recoveries were 9.2% and 9.7% with bioactivity recoveries of 92.7% and 91.3%, respectively. SDS-PAGE analysis of two affinity sorbents purified enzymes revealed approximately 95% purity [77]. In another affinity purification method extracellular CHOx was successfully purified from the culture broth of *Bacillus subtilis* by salting out with ammonium sulfate followed by purification with a riboflavin-affinity column chromatography [35]. Volonte et al. also reported single step purification for *Brevibacterium* CHOX expressed in *E. coli* by using Ni-affinity chromatography [63]. For the purification of recombinant *Brevibacterium* CHOx expressed in *E. coli* BL21 (DE3), an affinity protocol was developed using riboflavin and lumiflavin as the affinity ligands. The affinity separation provided 98% yield with riboflavin affinity column and 99.5% with HPLC Vydac C4 column *via* single step protein purification process [73].

Immobilization of Cholesterol Oxidase

Enzyme immobilization has drawn ample interest from both basic researchers as well as industries because of several commercial applications because the high cost of enzymes makes their use less economic. The enzyme immobilization is definitely a very effective way for enhancing the reusability and stability of enzyme in addition to reducing the overall cost of bio-catalytic processes [78]. Various types of methods have been used to immobilize enzyme: adsorption /carrier-binding, covalent binding, polymer entrapment and cross-linking [79]. The efficiency and stability of enzymes after immobilization are governed mainly by the support matrix and method used for their immobilization. Different matrix compositions and techniques have been used to achieve enzyme immobilization over the years [80]. Enzyme biosensors based on immobilized enzymes on a suitable matrix are gaining interest rapidly in the field of analytical technology [78,81]. The determination of cholesterol level in clinical and food samples is very important and for this, CHOx has been immobilized on various support matrices including polymeric membranes, hydrogels, sol-gel films, etc. for the development of suitable cholesterol biosensors [82-88]. Saxena and Goswami covalently immobilized CHOx onto the woven silk fiber produced by *Antheraea assamensis*. The immobilization was done using N-ethyl-N'-(3-dimethylaminopropyl) carbodimide and N-hydroxysuccinimide and the enzyme loading was reported to be 0.046 U cm^{-2} of silk fiber with a loading efficiency of 70% [89]. Yotova and Ivanov reported a method for individual and simultaneous covalent immobilization of CHOx and peroxidase to copolymer of acrylonitrile with acrylamide. After packing a small glass column with immobilized multienzyme complex it was used to develop a method for manual determination of cholesterol in foodstuffs [90].

There are different types of supports, which are available for effective covalent immobilization of CHOx mainly involving magnetic nanoparticles, hollow fiber dialyzers, perlite, silk mats, polyaniline films, alkylamine glass beads and mesoporous silica [83,87,89,91-93]. Gilles and Joseph immobilized CHOx onto Fe_3O_4 magnetic nanoparticles and the bound enzyme found to show better tolerance towards pH, temperature and substrate concentration [92]. In addition, Lin and Yang used polyacrylonitrile hollow fibers for covalently bonding the cholesterol oxidase *via* glutaraldehyde [94]. A CHOx from *Brevibacterium* sp. M201008 has been covalently immobilized onto functionalized sepharose particles that were activated with N-ethyl-N'-3-dimethylaminopropyl carbodiimide (EDC) with a consequent increase in the thermal, pH and storage stabilities of immobilized enzyme [95]. Yapar et al. immobilized CHOx in conducting network *via* complexation of chitosan with alginic acid. The immobilized CHOx maintained 63% of its initial activity after 44 measurements, showing an excellent operational stability [96]. Various types of nanoparticles mainly including metal and oxide nanoparticles have been used in fabricating electrochemical biosensors [85]. The magnetic fluorescent SiO_2 nanoparticles were used for CHOx immobilization, which were reported to improve thermal, storage and operational stabilities of immobilized CHOx [97].

Characterization of Cholesterol Oxidase

Cholesterol oxidases are produced from different microbial

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Table 1 Physicochemical properties of CHOx from microbial sources.

Sources	Optimum pH	Optimum temp (°C)	M. wt. (kDa)	Substrate specificity	Inhibitor(s)	Specific activity (Units/mg)	References
Arthrobacter simplex	7.5	50	57	Cholesterol, Ergosterol and Stigmasterol	$HgCl_2$, glutathione, *p*-chloromurcuribenzoate	3.6	[34]
Bacillus subtilis	7.5	37	105	Cholesterol	-	1.39	[35]
Bacillus sp.	6	60	36	Cholesterol, 5 α-cholestane, 5 α-cholestane-3 β-ol-7-one, coprostane, dihydrocholesterol, hecogenin, β-sitosterol and stigmasterol	-	7.6	[12]
Brevibacterium sterolicum	6.5	55	46.5	Cholesterol, pregnenolone and β-sitosterol	-	55.2	[36]
Bordetella sp.	7.0	37	55	Cholesterol	Ag^+ and Hg^+	20.8	[37]
Burkholderia cepaca ST-200	6.8-8.0	60	60	Cholesterol, β-Stigmasterol, Dehydroepiandrosterone, β-Sitosterol, β-Cholestanol and Epiandrosterone	-	16.9	[31]
Chryseobacterium gleum	6.75	35	60	Cholesterol	-	15.5	[66]
Corynebacterium cholesterolicum	7.0-7.5	40-42	57	Cholesterol, β-Stigmasterol, Dehydroepiandrosterone, Ergosterol, Pregnenolone and β- Sitosterol	$HgCl_2$ and $AgNO_3$	-	[8]
Chromobacterium sp. DS-1	7.0	65	58	Cholesterol,β-Stigmasterol, β-Sitosterol, β-Cholestanol Epiandrosterone, Dehydroepiandrosterone and Ergosterol.	-	13.9	[1]
Enterococcus hirae	7.8	40	60	-	$CoCl_2$ and $CdCl_2$	124	[72]
Rhodococcus sp.	7.0-7.5	40	55	Cholesterol, Stigmasterol, β-Sitosterol	-	-	[11]
Rhodococcus sp.	8.0	37	60		Hg^{2+}, Zn^{2+}, Cu^{2+} and Ag^+	35.64	[43]
Streptomyces fradiae	7	70	60	-	-		[45]
Streptomyces parvus	7.2	50	55	Cholesterol, Stigmasterol, Dehydroepiandrosterone, Ergosterol, Pregnenolone, β-Sitosterol, and β-Cholestanol.	Pb^{2+}, Ag^{2+}, Hg^{2+} and Zn^{2+}	20	[46]

Y-*Proteobacterium*	6.5	50	58	Cholesterol, β Stigmasterol, β-Sitosterol, and β-Cholestanol	-	14.4	[98]
Pseudomonas aeruginosa	7.0	70	59	Cholesterol, β-Cholestanol, β-Stigmasterol and β-Sitosterol	-	11.6	[68]

sources and their properties have also been studied extensively. The molecular weights of CHOx have been reported to be in the range of 47-60 kDa [21]. Microbial CHOx generally has neutral pH optima and possesses stability over a wide range of pH. The enzymes have temperature optima in the range 40-60°C [11,12,34,68]. The optimum temperature (70°C) of CHOx from *Streptomyces fradiae* was the highest among the enzymes reported so far [11]. Various properties of microbial CHOx (molecular weight, pH and temperature optima, effect of metal ions and detergents, substrate specificity) have been summarized **(Table 1)**.

Broader Applications of CHOx

Therapeutic uses

CHOx is used as an 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 and in human bile and in various food samples [98,99]. Normal cholesterol level in human blood is less than 200 mg/dL^{-1} 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 (hypercholesterolemia) 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 [100]. Cholesterol level in serum is generally determined by using an enzymatic assay [28,40]. 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. CHOx catalyzes oxidation of cholesterol to cholestenone with simultaneous release of hydrogen peroxide. The enzyme subsequently catalyzes the oxidative coupling of hydrogen peroxide 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 [101]. In recent years, various electrochemical biosensors are being designed by using immobilized CHOx for the determination of cholesterol in serum or food samples [82,88,102].

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 pesticides. Purcell et al. discovered a highly competent protein that can kill boll weevil (*Anthonomus grandis grandis* Boheman) larvae in the culture filtrates of *Streptomyces* sp. and identified this protein a CHOx [19]. Purified enzyme showed activity against boll weevil larvae at a 50% lethal concentration (LC_{50} of 20.9 $\mu g.ml^{-1}$), that is well comparable to the bioactivity exhibited by *Bacillus thuringiensis* proteins against other insect pests. The enzyme is involved in the lysis of the midgut epithelial cells of the larvae. Cholesterol or the related sterol at the membrane of the boll weevil midgut epithelium seemed to be accessible to the enzyme and is oxidized by CHOx, causing lysis of the midgut epithelial cells resulting in larval death [21]. The enzyme also shows insecticidal activity against lepidopteran cotton insect pests, including tobacco budworm (*Heliothis virescens*), corn earworm (*Helicoverpa zea*), and pink bollworm (*Pectinophora gossypiella*) [103]. Microbial insecticide proteins are very important for several pest control strategies employed in transgenic crops. Corbin et al. expressed the *Streptomyces* cholesterol oxidase gene in tobacco protoplasts [104].

Cell membrane structural studies

Cholesterol is one of the main constituents of eukaryotic cell membrane which is essential for maintaining cell membrane structure. Cholesterol oxidase is being used as a probe to investigate cholesterol interaction with phospholipids and the membrane structure of eukaryotic cell mainly the lipid rafts [105,106]. The lipid rafts are the domains in which cholesterol and saturated membrane lipids such as sphingolipids promote the formation of a highly ordered membrane structure [107,108]. Lipid rafts play roles in various cellular processes, including signal transduction, protein and lipid sorting, cellular entry by toxins and viruses, and viral budding [21]. Therefore, CHOx plays an important role in the study of the lipid raft functional aspects with regard to eukaryotic membrane.

CHOx as a potential new antimicrobial drug target

CHOx is produced by a wide variety of microorganisms including some life threatening pathogens such as *Rhodococcus equi, Mycobacterium tuberculosis* and *M. leprae* [109]. *Rhodococcus equi*, a Gram-positive bacterium infects young horses as well as humans as an opportunistic pathogen in immuno-compromised patients [5,110]. CHOx can be an interesting pharmaceutical target for treating bacterial infections. *In vitro* data suggested that during *R. equi* infection of the host cell, membrane lysis is facilitated by the induction of extracellular CHOx along with other candidate virulence factors [13]. The membrane-damaging activity of *R. equi* requires the presence of bacterial sphingomyelinase C, thus indicating that the CHOx substrate is not directly accessible to the enzyme in intact membranes [99]. It has also been reported that CHOx is also involved in the manifestation of HIV and nonviral prion origin (Alzheimer's) diseases [111]. The

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pathogenic bacteria utilize cholesterol oxidase for infection by converting the cholesterol of host cell membranes that alters 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. Therefore, and since no eukaryotic enzyme homologues exist, this type of bacterial CHOx falls into the scope of potential drug target for a new class of antibiotics which still remain to be explored [26].

Production of steroid hormones precursors

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 CO_2 and H_2O [21]. In the sequence of the cholesterol oxidation by microbial cells, 4-cholesten-3-one maybe oxidized with accumulation of the steroids 4-androstene-3, 17-dione (AD) and 1, 4-androstadiene-3,17-dione (ADD), which are important precursors of chemically synthesized hormones, or may be transformed to steroid intermediates [112].

Polyene macrolide pimaricin biosynthesis

Streptomyces natalensis CHOx which is a product of the *pimE* gene is involved in the biosynthesis of the polyene macrolide pimaricin [14]. This 26-member tetraene macrolide antifungal antibiotic is used in the food industry to prevent contamination of cheese and other nonsterile food with mold and also for treating the fungal keratitis because it interacts with membrane sterols (ergosterol is the major sterol found in fungal membranes), altering the membrane structure and causing leakage of cellular materials [99]. 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 external CHOx to the gene, disrupting culture triggered the pimaricin production. These results suggested the involvement of cholesterol oxidases as signaling proteins for polyene biosynthesis [21].

Cholesterol oxidase as a novel anti-tumor therapeutic molecule

CHOx catalyzes oxidation of cholesterol and has been used to track membrane cholesterol. Liu et al. reported that CHOx from *Bordetella* spp. made lung cancer cells both *in vitro* and *in vivo* to undergo irreversible apoptosis. CHOx 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 CHOx treatment also promoted the generation of reactive oxygen species (ROS). In addition, the CHOx treatment resulted in phosphorylation of JNK (c-Jun NH2-terminal kinase) and p38, downregulation of Bcl-2 (B-cell lymphoma/leukemia-2), up regulation 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 CHOx leads to irreversible cell apoptosis by decreasing cholesterol content and increasing ROS level. This indicates that the microbial CHOx may be a promising candidate for a novel anti-tumor therapy [113].

Cholesterol oxidase biosensors for different analytical assays

Cholesterol is an important analyte molecule and its detection is important for both clinical diagnosis and food analysis. Measurement of cholesterol concentration in blood is done routinely in medical screening and diagnosis. Moreover, estimation of cholesterol amount in various foods is vital for selecting a diet with optimum intake of cholesterol. Thus, there is a pressing need to develop new techniques for convenient and rapid determination of cholesterol levels in the blood and other samples. For this different electrochemical biosensors have been proposed. Biosensors with immobilized enzymes are getting huge interest in analytical technology as they facilitate the enzyme reuse as well as demonstrate the exclusive selectivity of the biological molecules and the processing power of modern microelectronics [81]. In the recent years, a variety of cholesterol biosensors have been developed [88]. Most of the reported cholesterol biosensors are based on amperometric technique besides, cholesterol biosensors based on photometric behaviors like luminescence, fluorescence and surface plasmon resonance (SPR) have also been reported [88,114,115]. Amperometric measurement of O_2 consumption or H_2O_2 production during cholesterol catalysis by CHOx is the frequently used strategy in cholesterol assay in the samples [116]. Various nanoparticles like metal nanoparticles (gold (Au), platinum (Pt), silver (Ag) NPs), metal oxide nanoparticles (zinc oxide (ZnO), iron oxide ($Fe3O_4$), cerium oxide (CeO_2), titanium oxide (TiO_2) NPs), carbon nanotubes (single-walled carbon nanotubes (SWCNTs), and multi-walled carbon nanotubes (MWCNTs) based materials have been exploited for designing cholesterol biosensor [117-121].

Conclusion

Cholesterol oxidase, a FAD-dependent oxido-reductase enzyme catalyzes the oxidation of cholesterol to cholestenone. CHOx is of ubiquitous nature in mammals however, it is also produced by a large number of microbes but prominently by bacterial species. As high levels of cholesterol and cholesteryl esters (hypercholesterolemia) have been found to be associated with cardiovascular disease, thus CHOx also alleviate symptoms of heart diseases. The enzyme finds ample applications in biosensor fabrication, steroids biotransformation and bio-control of insects, polyene macrolide pimaricin synthesis, and imparting virulence to *Rhodococcus equi, Mycobacterium tuberculosis* and *M. leprae*. The use of CHOx has also been recommended in lowering the cholesterol content of foods. Thus being a versatile enzyme, the newer microbial sources in nature need to be explored for synthesis of preferably extracellular CHOx, which is easy to purify, characterize and put for commercial use.

Acknowledgements

The authors are thankful to UGC, New Delhi for providing a grant under a research project (F. No. 43-89/2014(SR)) awarded to SSK, as well as Ministry of Science and Technology, Government of India, New Delhi for providing funding to the parent department. Further, the authors have no conflict of interest among themselves at their place of work or with the institution.

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