Review

MICROBIAL CHOLESTEROL OXIDASE IT'S CHARACTERISTIC'S AND WIDE SPECTRUM APPLICATION

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ABSTRACT: Due to increasing the cholesterol related disorder like cardiovascular diseases, atherosclerosis or coronary heart diseases and also management of hypercholesterolemia. CHO has become second largest diagnostic enzyme after glucose oxidase in clinical industry. Endophytes are endo-symbionyts that lives asymptomatically inside the living tissue of a plant without showing any apparent signature of their presence. Endophytic fungi are considered to be lucrative source of bioactive metabolites. **Sterols**, also known as steroid alcohols, are a subgroup of the steroids and an important class of organic molecules. They occur naturally in plants, animals, and fungi, with the most familiar type of animal sterol being cholesterol. Cholesterol is vital to animal cell membrane structure and function and a precursor to fat-soluble vitamins and steroid hormones.Cholesterol oxidase (CHO) is a monomeric flavoprotein containing FAD that catalyzes the first step in cholesterol catabolism. This bifunctional enzyme oxidizes cholesterol to cholest-5-en-3-one in an FAD-requiring step, which is then isomerized to cholest-4-en-3-one with the release of H_2O_2. CHO is has diversified application viz- agriculture, analytical, industrial, pharmaceutical and as a biosensor. CHO is one of the key enzymes in microbial sterol metabolism. The interest in this enzyme is due to its industrial importance and its wide utilization in the determination of cholesterol in blood serum and food (Parra *et al*., 2007), and to its assumed potential in the manufacture of diets with reduced cholesterol (Chenfeng *et al.,* 2002).

KEY WORDS: *Cholesterol Oxidase*, Microbial Sterol, *Endophytes,*

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1. INTRODUCTION:

1.1. General

In eukaryotes, cholesterol is essential for maintaining cell membrane structure and for synthesizing a number of important compounds. Moreover, improper maintenance of cholesterol concentrations can severely affect the physiological function of an organism. Microbial Cholesterol Oxidase (CHO) is water soluble interfacial enzyme. CHO (EC 1.1.3.6) is Flavoenzyme which catalyzes the oxidation of cholesterol and converts 5-cholesten-3 –ol into 4 cholesten-3-ones (Murooka, *et al*., 1986).

Cholesterol + O_2 -> cholest-4-ene-3-one + H_2O_2 (1)

H_2O_2 + cholesterol probe -> resorufin (λmax at 570nm) (2)

Due to increasing the cholesterol related disorder like cardiovascular diseases, atherosclerosis or coronary heart diseases and also management of Hypercholesterolemia CHO is become second largest diagnostic enzyme after glucose oxidase in clinical industry.

Coronary heart disease (CHD) is the greatest killer of human being in modern age. It causes 25% to 35% deaths in most of the developing countries. In India deaths due to CHD is increased from 1.17 million in 1990 to 1.59 in 2000 and is expected to be 2 million by 2010.

CHO has various other applications like agriculture, analytical, industry, and pharmaceutical sectors and so on. It has great commercial value. For instance, CHO can be used for enzymatic determination of cholesterol in serum when coupled with cholesterol esterase (COE-301, COE-311, COE-313) in clinical analysis, biological insecticide and precursors for steroid hormones (Bell, *et al.,* 1998). CHO can also help to degrade dietary cholesterol which is indirectly related to cardiovascular diseases and hence to improve human health (Kaunitz, H.1978). The first report on cholesterol oxidation in *Rhodococcus erythropolis* (formerly *Proactinomyces erythropolis*; Turfitt 1948), microbial cholesterol oxidation has been reported from many microorganisms. First report the crude preparation of cholesterol oxidase from a soil *Mycobacterium* (Stadtman *et al*. 1954).

This enzyme can be produced by a bacterium in three forms:intracellular, extracellular and membrane bound. Due to the wide spectrum applications of CHO, screening and isolation of bacterial strains producing extracellular form of CHO are of great importance (Yazdi, *et al.,* 2001). CHO is one of the key enzymes in microbial sterol metabolism. The interest in this enzyme is due to its industrial importance and its wide utilization in the determination of cholesterol in blood serum and food (Parra *et al*., 2007), and to its assumed potential in the manufacture of diets with reduced cholesterol (Chenfeng *et al.,* 2002). Indeed, dietary cholesterol degradation is considered as a means of protection against atherosclerosis. Other possible applications of CHO are in the production of precursors for the chemical synthesis of pharmaceutical steroid hormones (Alexander *et al.,* 1995), and for its insecticidal activity that is vital for pest control strategies employing transgenic crops (Shen *et al*. 1997). CHO has been isolated from several microbial sources, including species of *Arthrobacter* (Wenhsiung *et al*., 1988), *Bacillus* (Kim *et al.,* 2002), *Brevibacterium* (Pornpen *et al*. 2006), *Nocardia* (Sojo *et al*., 1997), *Pseudomonas* (Isobe *et al*., 2003), *Rhodococcus* (Elalami *et al.,* 1999), *Streptomyces* (Pornpen *et al.*, 2006) and *Schizophyllum* (Fukuyama *et al.,* 1979). In this study, CHO producing organisms were isolated from the waste of a regional oil mill

and characterize by microbiological methods and evolutionary relationship with relevant organisms were established.

Cholesterol and its oxides have been detected in a variety of foods and foodstuffs, especially egg, milk, meat and their processed products (Baticz and Tomoskozi 2002). The first step of cholesterol degradation is performed by cholesterol oxidase (Aparicio and Martín 2008). Bacterial degradation of cholesterol in cholesterol-containing foods may be beneficial for human health to avoid atherosclerosis or coronary heart disease. Cholesterol oxidase-producing microorganisms, including *Arthrobacter* (Chen *et al.* 2006), Brevibacterium (Fujishiro et al. 1990), *Corynebacterium, Mycobacterium* (Brzostek *et al.* 2007), *Nocardia* (Richmond 1973), *Rhodococcus* (Yazdi *et al.* 2008), Streptomyces (Fukuda *et al.* 1973), and *Streptoverticillium*, have been isolated from a variety of quite different environments. The enzyme has also been found in several Gram-negative bacteria such as Burkholderia (Doukyu and Aono 2001), *Chromobacterium* (Doukyu *et al.* 2008), and Pseudomonas (Doukyu and Aono 1998).

A cholesterol oxidase from eukaryotic microorganisms such as *Basidiomycetes* and *Schizopyllum* (Fukuyama and Miyake 1979) has also been reported.

1.2. Structure of Cholesterol Oxidase:

The structure of cholesterol combines the backbone of a steroid and an alcohol and is found in the bloodstream (Fig. 1). This important biomolecule maintains membrane fluidity and acts as a precursor in the production of Vitamin D and hormones. Recent studies have correlated high cholesterol levels (in conjunction with an increased low-density lipoprotein concentration) with atherosclerosis and myocardial infarction (Weinruch 2007). Low cholesterol levels (hypocholestrolemia) indicate a weakening immune system (Shor Posner G 1993). Clinicians and scientists measure cholesterol content of human serum to monitor patient health and prevent cholesterol related diseases.

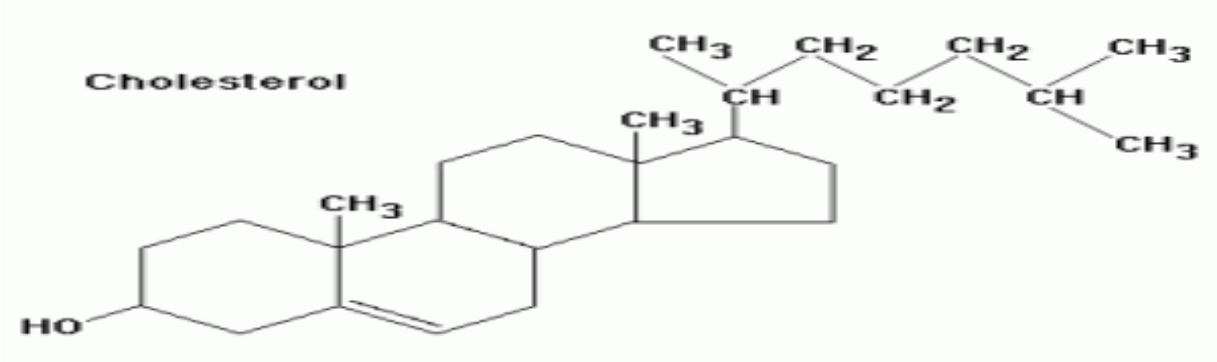


Figure 1: Structure of cholesterol.

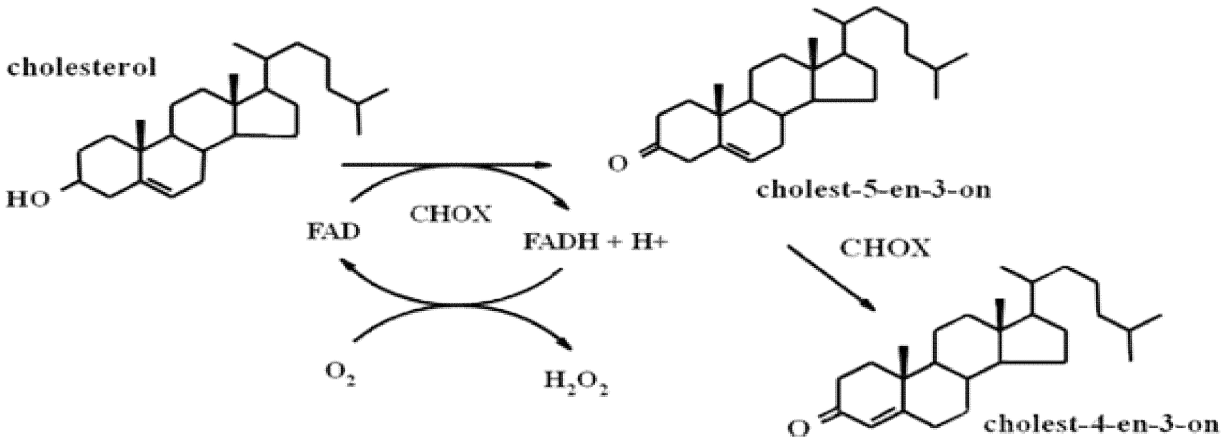


Figure 2: Cholesterol Oxidase Mechanism

There are two forms of cholesterol oxidase, one containing the FAD cofactor non-covalently bound to the enzyme (class I) and another containing the cofactor covalently linked to the enzyme (class II; Croteau and Vrielink 1996; Sampson and Vrielink 2003). These two enzymes have no significant sequence homology. These two forms of enzymes belong to different protein families. The class I enzyme belongs to the GMC (glucose/methanol/choline) oxidoreductases family, while the class II enzyme belongs to the VAO (vanillyl-alcohol oxidase) family, a group of enzymes which contain a fold proposed to favor covalent flavinilation. B. sterolicum possesses both class I and class II enzymes. Class I enzymes have been identified mostly in

Actinomycetes such as *Streptomyces spp., B. sterolicum, Rhodococcus spp.,* and *Mycobacterium spp.* Comparison of amino acid sequences from some of the class I enzymes has been reported (Navas *et al.* 2001). These sequences contain a consensus sequence for FAD binding, Gly-X-Gly-X-X-Gly, in the N-terminal region of the mature cholesterol oxidases (Ohta *et al.* 1991). The structural and mutational analysis of Streptomyces sp. SA-COO (class I enzyme) has revealed that His447 and Glu361 residues are implicated in the activity for the oxidation and isomerization (Yue *et al.* 1999). These residues are also conserved in the sequences of various class I enzymes. The phylogenetic tree analysis based on the amino acid sequences of the enzymes, including hypothetical proteins, showed that class I enzymes can be further divided into two groups (class I-1 and I-2;). Unlike the class I-1 enzymes, the class I-2 enzymes lack a signal sequence, suggesting a cytoplasmic localization (Navas *et al.* 2001). Class II enzymes have been identified in *B. sterolicum, R. erythropolis*, and gram-negative bacteria such as *Burkholderia spp.*, *Chromobacterium sp.*, and *Pseudomonas aeruginosa* showing similarity (43% to 99%) to one another. The structure of the *B. sterolicum* class II enzyme has been determined by X-ray crystallography and refined to high resolution (Coulombe *et al.* 2001). The structure suggested that the FAD cofactor was covalently bound to an active-site histidine (His121) via the C8α group of the flavin isoalloxazine ring. This covalent bound is implicated in the redox potential and contributes to the stability of the enzyme (Caldinelli. *et al.* 2005). In addition, Glu475

and Arg477, located at the active-site cavity, were suggested to constitute gate functioning in the control of oxygen access (Piubelli *et al.* 2008). These amino acid residues are conserved in the sequence of the class II enzymes. By the phylogenetic tree analysis, it was found that there are at least two groups of class II enzymes, one group consisting of the Actinomycetes enzymes (class II-1) and the other consisting of gram-negative bacteria (class II-2)

3 D Structure of CHO:

Cholesterol oxidase (CHO) from *B. sterolicum* has been studied by X-ray crystallography and the crystal structure of the enzyme was determined at a resolution of 1.8A, which provided a complete structural description of the enzyme (Vrlelink, *et al* 1991). The features of subtrate binding to dehydroisoandrosterone, a competitive inhibitor of cholesterol oxidase, have also been analyzed by X-ray crystallography and the catalytic mechanism was discussed (Li, *et al* 1993). The amino acid sequence of CHO from *Streptomyces* sp. is highly homologous to that of CHO from *B. sterolicum (59.2%* identity). In particular, residues composing the active site and the FAD-binding site are highly conserved between CHO from *Streptomyces* sp. and CHO from *B. sterolicum*. On the basis of the amino acid sequence homology between the two enzymes, a model of the three-dimensional structure of CHO was constructed (Murooka, *et al* 1998). The initial model was refined by energy minimization using the steepest descent method followed by the conjugate gradient method (Fujli,*etal*1994).

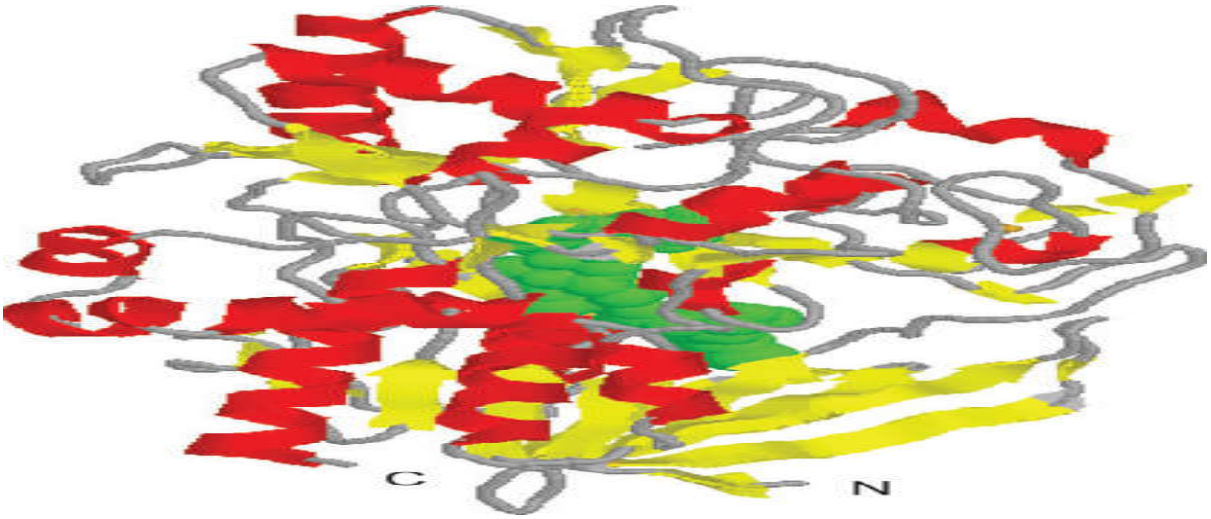


Figure 3: ***Secondary structure representation ofPimE CO from Streptomyces natalensis. TheFAD cofactor is shown in green as a spacefillmodel. The figure was made with the ProteinExplorer 2.80 program.***

1.3. Properties of cholesterol oxidases:

Cholesterol oxidases from several microorganisms have been extensively studied. Various properties of microbial cholesterol oxidases (viz. molecular weight, pH, temperature, stability, and inhibition characteristics) are summarized below. The molecular weights of theCholesterol oxidases have been reported to be in the range of 47–60 kDa. Most cholesterol oxidases are secreted into the growth medium. However, intracellular or membrane bound enzyme cholesterol oxidases have been reported from Mycobacterium (Wilmanska *et al.* 1995) and

Rhodococcus (Kreit *et al.* 1994; Wilmanska *et al.* 1995; Sojo *et al.* 1997). R. erythropolis produces both membrane-bound and extracellular cholesterol oxidases (Sojo *et al.* 1997).

pH Activity of the Cholesterol Oxidases:

Microbial cholesterol oxidases generally have their optimum pH – 7 (Neutral pH). But some species having optimum pH 6 (*Rhodococcus equi)* are slightly acidic and their pH range is generally (4-10).

Table 1: Various Micro Organisms and their specific pH:

Organism	Minimum	Maximum	Optimum	Reference
Brevibacterium sp.	5	7.5	7	(Terezinha J.G. Salva *et al* 2000).
Pseudomonas aeruginosa.	5	9	7	(Jasim and Diwan 2010)
Streptomyces fradiae	5	9	7	(Tabatabaei Yazdi *et al* 1999)
Bordetella sp.	4	10	7	(Y. Lin *et al*.2010)
Bacillus licheniformis	6	8	7	(Lachlan Mac *et al.,* 2000)
Rhodococcus sp.	5	9	7	(Lashkarian *et al*.2010)
Rhodococcus equi.	4	8	6	(Salman Dinakar M *et al* 2013)

Temperature Activity of the Cholesterol Oxidases:

The optimum temperature of cholesterol oxidase production is 30°C-50°C. The heat stability of the enzyme is of a great advantage in clinical uses. Optimum temperature of the *Streptomyces fradiae* formaximum enzyme activity is 70°C (Terezinha J.G. Salva *et al* 2000) which is higher than optimum temperature of *Brevibacterium* sp. 50°C. Thermostability of an enzyme is very important property for the industrial application. Thus some group (Yoshiaki *et al*, 1997; Murooka *et al*; 1998) carried out work on strain improvement of *Streptomyces* sp. by mutagenesis for the production of Thermostable CHO. *Streptomyces fradiae* is reported to produce CHO with optimum stability 70°C.

Table 2: Various Micro Organisms and their specific temperature:

Organism	Minimum	Maximum	Optimum	Reference
Brevibacterium sp.	35°C	65°C	50°C	(Terezinha J.G. Salva *et al* 2000).
Pseudomonas aeruginosa.	25°C	65°C	35°C	(Jasim and Diwan 2010)
Bordetella sp.	30°C	70°C	50°C	(Y. Lin *et al*.2010)
Rhodococcus sp.	20°C	50°C	35°C	(Lashkarian *et al*.2010)
Rhodococcus equi.	25°C	65°C	45°C	(Salman Dinakar M *et al* 2013)
Bacillus licheniformis	25°C	45°C	35°C	(Lachlan Mac *et al.,* 2000)
Nocardia rhodochrous	13°C	44°C	30°C	(W. H. Liu *et al*., 1987)
Streptomyces fradiae	25°C	90°C	70°C	(M.Tabatabaei Yazdi *et al* 2000)

Effect of metal ion on Cholesterol Oxidase:

Metal ions are rarely required for cholesterol oxidase activity. Chelating agents, including EDTA, ophenanthroline, and 8-hydroxyquinoline, did not show a significant inhibitory effect on the enzyme activity (Tomioka *et al*. 1976; Kamei et al. 1978; Inouye *et al*. 1982; Isobe *et al*. 2003; Doukyu *et al.* 2008).

Table-3: Effect of divalent metal ions on the enzyme

Divalent metal ions	Relative activity (%)
Control	100
Ca2+	98
Cu2+	96
Mg2+	100
Ni2+	99
Fe2+	98
Pb2+	65
Ag2+	55
Hg2+	50
Zn2+	74
Mn2+	130

Effect of Organic Solvent on Cholesterol Oxidase:

Organic solvents as well as detergents are also employed to solubilize the steroids. Cholesterol oxidase has been used for the optical resolution of non-steroidal compounds, allylic alcohols (Dieth *et al*. 1995; Biellmann 2001), and the bioconversion of a number of 3β-hydroxysteroids in the presence of organic solvents (Kazandjian *et al.* 1986; Khmelnitsky *et al.* 1988; Doukyu *et al*. 1996). Therefore, an organic solvent-tolerant cholesterol oxidase would be useful for these applications. Organic solvents often influence the cholesterol oxidase activity (Cheetham *et al.*

1982; Pollegioni *et al.* 1999). It has been reported that organic solvents with low log P values inactivate most enzymes (Laane *et al.* 1987). Pollegioni *et al.* examined the stability of cholesterol oxidases from *Streptomyces hygroscopicus* and *B. sterolicum* in the presence of various concentrations of isopropanol (Pollegioni *et al.* 1999). The activity of the *B. sterolicum* enzyme is rapidly inactivated, whereas the *S. hygroscopicus* enzyme retained 70% of the initial activity after 5 h in the presence of 30% propan-2-ol at 25°C. We also examined the stability of various cholesterol oxidases in the presence of organic solvents (Doukyu *et al.* 2008). Commercially available cholesterol oxidases, including *Streptomyces sp*., *Cellulomonassp*., *Nocardia sp.,N. erythropolis*, and *P. fluorescens*, were inactivated by the addition of 50% volume of dimethylsulfoxide, methanol ethanol, acetone isopropanol, ethyl acetate, or butanol after incubation for 24 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.

Stability of Cholesterol Oxidase:

The enzyme was stable from 4 °C to 65 °C temperature. The optimum temperature at pH 7.2 was 50°C. The enzyme retained about 68%, 66%, and 46% of its activity after incubation for 30 min at 55°C, 60°C, and 65°C, respectively. However, the enzyme lost almost all (86%) activity after 30 min at 75 °C. No detectable change in activity occurred when the enzyme was stored in buffered Triton X-100 (0.3%) for 6 months at 4 °C. Among the CHO from Streptomyces sp., thermal stability of *S. parvus* (temperature optimum at 50 °C) was second highest after *Streptomyces fradiae* (temperature optimum at

70 °C) [Yazdi, *et al* 2001]. Reported pH optima for most of bacterial CHO range between 7.0 and 7.5 [Nishiya, Y. (1997).; Uwajima, *et al* 1974].

Ca2+, Cu2+, Mg2+, Ni2+, and Fe2+ do not exert any effect on the enzyme activity. However, Pb2+, Ag2+, Hg 2+, and Zn2+ reduced the activity to 65%, 55%, 50%, and 74%, respectively. And DTT and β-Mercaptoethanol decreased the enzyme activity, suggesting that disulfide linkage is essential for the enzyme activity. Addition of Mn2+ activated the enzyme by 30%. Inhibition by Ag2+ and Hg2+ were completely prevented by the addition of glutathione (10 mM) in the assay mixture [Wang, *et al* 2008]. This suggested that –SH group may be involved in the catalytic activity of CHO from *S. parvus* Cho from *Pseudomonas* sp. COX629 and γ-Proteobacterium were also found to be activated by Mn2+ [Lee, *et al* 1989,; Isobe, *et al* 2003]. The enzyme showed good stability in the presence of a wide range of detergents. The enzyme activity was highest in Triton X-100 and Triton X-114 at 50°C. At a low concentration of Triton X-100 (0.03–0.5%), CHO activity increased whereas at higher detergent concentrations the opposite effect occurred. Presence of only 0.1% SDS fully inactivated the enzyme where as 100% activity was observed in presence of sodium cholate.

Table-4: Enzyme stability in detergents and organic solvents

Reagent group	Reagent (0.5%)	Relative activity (%)
Detergents	Control	100
Tween-80		150
Triton X-100		450
Triton X-100 (1%)		78
Triton X-114		325
Sod. cholate		100
SDS		0
β-Mercaptoethanol		54
DTT		31
Organic solvents (10%)		
DMSO		99
Methanol		110
Ethanol		150
Ethyl acetate		105
Acetone		60
Isopropanol		250
Chloroform		15
Benzene		90

Table no 5: CHO activity produced by different microorganisms.		
Microorganism	**Activity (U/ml)**	**References**
Streptomyces badius.	2.38	Z.moradpour, *et al* 2012
Brevibacterium sp.	1.4834	Yang and Zhang 2012
Pseudomonas aeruginosa.	1.71	Jasim and Diwan 2010
Streptomyces sp.	2.82	Parekh *et al* 2012.
Micrococcus sp.	3.68	kanchana *et al*, 2011
Bacillus subtilis SFF34	3.140	A. Kumar, *et al* 2000.
Enterobacter sp. COX 8 – 9	0.434	A. Brzostek, *et al* 2007
Streptomyces lavendulae	1.140	Z, Liao, *et al* 2004.
NCIM 2499		
Arthrobacter simplex	1.50	R. C. Srivastava, *et al* 2000.
Bacillus sphaericus	0.050	R. Foster, *et al* 2000.
Rhodococcus sp.	0.290	S. Brahim, *et al* 2001.
Rhodococcus sp.	0.240	M. T. Yazdi, *et al* 2001.
Pseudomonas sp. ST-200	0.198	N. Doukyu, *et al* 1989.
Rhodococcus sp. *R*14-2	1.50	T. Yao and K. Takashima, 1998.
Brevibacterium sp. DGCDC	1.285	M. H. Ropers, *et al* 2001.
Rhodococcus sp. GKI	0.380	A. Elalami, *et al* 1999.

1.4. Market Value of CHO:

Due to the low production of the CHO enzyme the market value of this enzyme is very high.For the maximize the yield the initial avenue pursued was the enhancement of cell production via Alterations of growth medium. Takagi *et al*. examined the effect of oleic acid adsorption onto the cell surface of *Schizopyllum commune* on yields of cholesterol oxidase. It was found that cholesterol oxidase production depended on the amount of oleic acid in the culture broth, and that it was the level of insoluble oleic acid, rather than the soluble component adsorbed onto the cell, that was directly influencing the formation of cholesterol oxidase. In addition, the optimum extent of COD production was correlated with increases in the rate of agitation.

2. SOURCES OF CHOLESTEROL OXIDASES:

CHO was first isolated from *Nocardia erythropolis* by Turfitt in 1944 and is produced by many microorganisms present in the environment, belonging to the genera *Arthrobacter, Corynebacterium, Nocardia, Mycobacterium, Schizopyllum, Brevibacterium, Streptomyces, Rhodococcus, Burkholderia, Pseudomonas* and *Proteobacterium*. Due to its low yield and high demand, the production costs of this enzyme are relatively high. Among the bacterial strains, which are CHO producers, strains of *Rhodococcus equi* were shown to produce high levels of CHO; however, the pathogenic nature of these microorganisms and the long production time (60–72 h), required for CHO production, have limited their commercial

application. In 2002, Lv *et al* reported that mutant Brevibacterium could produce CHO at levels of 1285 U/L after 36 h of cultivation. Later, Wang *et al*. demonstrated that *Rhodococcus* sp. R14-2 could produce extracellular CHO at a high level (1500 U/L) in an optimized medium after 60 h of cultivation. However, there are few reports on cholesterol transformation by species of the genus *Bordetella,* and there are no reports on the isolation of CHO from these species.

Table 6: List of Cholesterol oxidase producing Bacteria:

List of Extracellular Bacteria producing CHO:

Bacteria.	**Location.**	**pH.**	**Reference:**
Actinomyces sp.	Extracellular	6.0-10.0	Cheetham *et al* 1982
Arthrobacter simplex.	Extracellular	7.0-8.0	Petrova, *et al* 1979
Brevibacterium sterolicum.	Extracellular	6.5	W. H. Liu, *et al* 1988;
Bacillus sp.	Extracellular	6.0-7.0	N. Croteau and A. Vrielink, 1996 L.Motteran, *et al* 2001
Nocardia erythropolis	Extracellular	6.0-9.0	Y.Shirokane, *et al* 1977
Pseudomonas sp.	Extracellular	7.0	B. C. Buckland,*et al* 1971;
Rhodococcus equi.	Extracellular	8.0	Bokoch, *et al* 2004
Streptomyces violascens	Extracellular	7.0	T. L. Johnson *et al* 1991
Streptomyces sp.	Extracellular	5.0-5.5	Fukuyama and Miyake, 1979
Streptomyces griseocarneus	Extracellular	7.0	L. N. Sampson *et al* 2003
Gamma Proteobacterium Y-134	Extracellular	7.0	K. Isobe, *et al* 2003

List of Intracellular Bacteria Producing CHO:

Bacteria.	**Location.**	**pH.**	**Reference.**
Arthrobacter rhodochrous	Intracellular	7.0	N. Doukyu, 2009.
Actinomyces lavendulae	Intracellular	6.0-10.0	Cheetham *et al* 1982
Corynebacterium cholesterolicum	Intracellular	8.0-9.0	C. H. Rhee, *et al* 2002
Mycobacterium sp.	Intracellular	6.0-8.0	T. C.Stadtman, *et al* 1954
Nocardia rhodochrous	Intracellular	6.0-6.5	N. Doukyu, 2009.
Nocardia erythropolis	Intracellular	6.0-9.0	Y.Shirokane, *et al* 1977
Nocardia rhodochrous	Intracellular	8.0-9.0	L. Motteran, *et al* 2001
Streptomyces violascens	Intracellular	7.0	T. L. Johnson *et al* 1991
Streptomyces sp.	Intracellular	5.0-5.5	Fukuyama and Miyake, 1979
Streptomyces griseocarneus	Intracellular	7.0	L. N. Sampson *et al* 2003

Table 7: List of Cholesterol oxidase producing Fungi:			
Fungi:	**Location:**	**pH:**	**Reference:**
Shizophyllum commune	Extracellular	5.0	H. Fukuda, *et al* 1973

3. APPLICATION OF CHOLESTEROL OXIDASE:

Table no -8: Various application of the CHO producing microorganism:		
Source of CHO	**Function's**	**Reference's**
Nocardia sp.	Cholesterol measurement in blood serum	J. C. Vidal, *at al* 2003
Streptomyces badius. clinical samples.	Cholesterol concentration in food, serum 2012	Z.moradpour, *et al* and other
Streptomyces sp.	Insecticidal activity	M. K. Ram, *et al* 2001
Bacillus licheniformis.	For Clinical uses.	Lachlan Mac *et al.,* 2000
Streptomyces fradiae.	Thermostable	M.Tabatabaei Yazdi *et al* 2000
Chromobacterium sp. Detergent tolerance.	Thermostable, Organic and	Doukyu, *et al* 2008
Micrococcus sp.	For clinical and commercial purpose	kanchana *et al*, 2011
Rhodochorus erythropolis	Biocatalysis, oxidations of cyclic allylic, bicyclic and tricyclic alcohol to synthesize Several ergot alkaloids.	M. R. Pourjavid, *et al* 2009

CHO is a diagnostic enzyme since it helps in determining the cholesterol content of serum from the patients, in the cell membrane of erythrocytes (patzer *et al.*1978), in human bile and gall stones. Wide and varied uses of CHO clinical, agricultural and biotechnology industry display the ever growing demand of this enzyme. Various strategies have been applied to get higher yields of CHO from diverse microbial sources.

3.1. CHO Use in Clinical Diagnosis:

Cholesterol esters will be hydrolyzed by cholesterol esterase. Cholesterol will be oxidized into cholest-4-en-3-on and $H_2O_2$by bacterial cholesterol oxidase. H_2O_2 in the presence of phenol and amino-4-antipyrin forms a complex of red colorshowing absorption maximum between 505 nm.

Cholesterol ester **Cholesterol Esterase**cholesterol + fatty acids

Cholesterol **Cholesterol Oxidase**cholest-4-en-3-on + H_2O_2

H_2O_2 + phenol + amino-4-antipyrin **Peroxidase** quino neimin + 2 H_2O

CHO is a useful analytic tool for determining cholesterol in various samples: (a) total and esterified serum; (b) from low-density lipoproteins to high-density lipoproteins; (c) on the cell membrane of erythrocytes (and of other cells and cellular

compartments); and (d) in gall stones and in human bile. Normal human blood serum contains less than 5.2 mm (200 mgÆdL)1) of cholesterol; in plasma, lipoproteins contain cholesterol and about 70% is esterified by fatty acids. Determining the concentration of serum cholesterol is fundamental in the assessment of a variety of diseases (e.g. in atherosclerosis and other lipid disorders) and for estimating the risk of thrombosis, myocardial infarction, etc. The risk for Alzheimer disease is also related to hypercholesterolemia via mechanisms involving oxidative stress: this disease is characterized by the accumulation of amyloid b-peptide (a 39–43 amino acid peptide) in the neocortex, which is connected to peroxidative damage. The amyloid b-peptide forms complexes with Cu2+ ions, which oxidize cholesterol into cholest-4-en-3-one, thus mimicking the activity of CHO. In fact, brain tissues from Alzheimer disease patients had a cholest- 4-en-3-one content _ 2-fold higher than brain tissues from controls [Puglielli L,*at al* 2005].

3.2. CHO Use in Agricultural Field:

Genetically modified plants that produce insecticidal proteins (e.g. the *Bacillus thuringiensis* toxin) are now available to control insect pests of several major crops. In 1993, from a random screening of > 10000 filtrates from microbial fermentations, Monsanto Co. (St Louis, MO, USA) discovered a highly efficient protein in culture filtrates that killed boll weevil (Anthonomus grandis Boheman) larvae [Purcell JP, e*t al* 1993]. Histological and biochemical studies identified the protein as CHO: purified CHO is active against boll weevil larvae at a 50% lethal concentration (LC50) of 20.9 lgÆmL)1, which is comparable to the bioactivity of *B. thuringiensis* proteins against other insect pests. The CHO gene from the *Streptomyces* sp. Strain A19249, exhibits 85% DNA sequence identity and 89% amino acid sequence similarity with known Streptomyces sp. SA-COO (CHO gene). In addition to boll weevil, several lepidopterans were negatively affected by the presence of CHO at a dietary concentration of 0.001%. Adding the products of cholesterol oxidation by CHO (i.e. cholest-en-3-one and hydrogen peroxide) to the diet, and pretreating the diet with the enzyme, excluded insecticidal effects caused by the ingestion of toxic compounds. However, the boll weevil larvae are acutely sensitive to ingested CHO because it induces lysis at the midgut epithelium. Boll weevil adults are insensitive to ingested CHO, although the fecundity of adult females was greatly reduced if 50 lgÆmL)1 of the enzyme was present in the diet [Greenplate JT, e*t al* 1995]. CHO reduced subsequent oviposition (up to 83% in eggs laid) and larval survival (97% reduction as compared to controls) because of poorly developed ovaries and few developing oocytes. CHO was expressed in transformed tobacco plants, and the synthesis levels in leaf tissues routinely ranged from approximately 5–50 lg of enzyme per g fresh weight. In the absence of a chloroplast- targeting sequence, CHO production resulted in severe abnormalities in plant development and fertility. When produced as a fusion with a chloroplast-targeting peptide, synthesis of the mature and the full-length enzyme did not cause the deleterious phenotypic effect observed with untargeted CHO [Corbin DR, *et al* 2001]. Transgenic leaf tissues expressing CHO exerted insecticidal activity against boll weevil larvae. When produced in the cytosol, or when targeted to chloroplasts, CHO metabolizes phytosterols in vivo. Transgenic plants expressing CHO in cytosol accumulated low levels of

saturated sterols (stanols), while the transgenic plants expressing chloroplast-targeted CHO maintained a greater accumulation of stanols and appeared phenotypically and developmentally normal.

3.3. CHO Use as Insecticide:

We tested over 10000 filtrates from microbial fermentations for insecticidal activity against major pests.(Purcell *et al* ;1993, 1994b) . Two Streptomyces culture filtrates (Monsanto isolates A19241and A19249) killed boll weevil larvae in feeding studies and the characterization studies describe above indicated that the active components were proteins (Purcell *et al*,; 1993). Subsequent purification identified the presence of a major protein of M, 52,500 in chromatography fractions which correlated with boll weevil mortality (Purcell *et al*,; 1993) and amino acid sequencing of the purified protein showed a high degree of homology with cholesterol oxidase from *Streptomyces* sp. (Ishizaki *et al*,; 1989). The identity of the protein was confirmed by its ability to oxidize cholesterol in an in vitro enzymatic assay.(Gallo,;1981). Since its introduction to Brazil, 19 years ago, the boll weevil (*Anthonomus grandis* Boheman, 1843) has become the most damaging pest of cotton (*Gossypium hirsutum* L.) crop. Before its introduction, Brazil was the world's largest exporter of cotton fibers; nowadays, the production has been dramatically reduced and isinsufficient, even, to satisfy the annual national demand of 850,000 ton (Beltrão, 1999). Research has produced several technologies aiming to minimize the damage caused by this pest, e.g. earliness and short cycle cultivars. The principal control, however, is obtained through the application of chemical insecticides. The drawbacks of this approach are high costs of the insecticides and the relatively narrow window during the crop's development when insecticide application is effective. This is due to the fact that boll weevil eggs are deposited within the cotton squares and bolls and the larvae develop entirely within those structures. If insecticide application is not made at the right time, the reduction in yield can amount to over 70% (Gabriel *et al*., 1986). Besides, the indiscriminate and incorrect use of insecticides has lead to extremely negative consequences, affecting humans directly and/or indirectly via the agricultural ecosystems (Almeida, 2000). Several research centers have made efforts to search and evaluate new insecticidal proteins suitable for boll weevil control in particular lectin and amylase and proteinase inhibitors; however, according to Greenplate *et al.* (1995), these molecules retard growth and increase the time of development of the parasite, but seem to afford little acute toxicity. As an alternative, Purcell *et al*. (1993, 1994) tested several microbial filtrates for insecticidal activity against boll weevil and found that two *Streptomyces* culture filtrates killed boll weevil larvae in feeding studies. It was found that the active component was cholesterol oxidase. In neonate and 2nd instar larvae fed on diets containing this enzyme, the epithelial cell layer of the midgut was disrupted at low doses and complete lysed at high ones. Greenplate *et al.* (1995) showed the effect of cholesterol oxidase on fertility and egg viability using artificial bolls treated with a 50 mg/mL of enzyme solution, and concluded that cholesterol oxidase might represent an alternative to the insecticides currently used for boll weevil control.

3.4. CHO Uses in Virulence:

CHO is an interesting pharmaceutical target for treating bacterial infections. *R. equi* is a Gram-positive coccobacillus that resides within macrophages of the host. It is a common soil

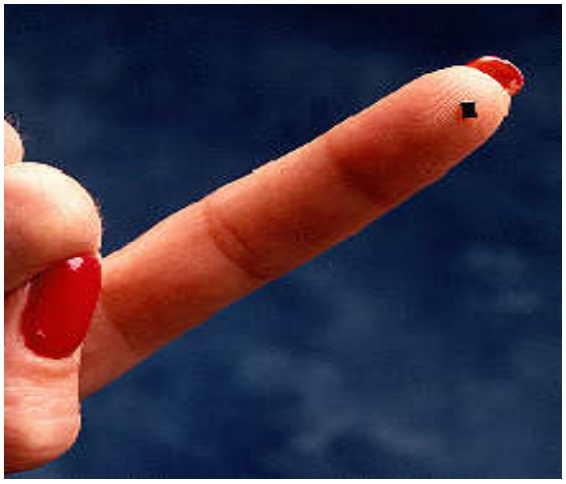
organism that frequently infects young horses; the most common manifestation is a chronic suppurative bronchopneumonia with abscess formation and cavitary pneumonia. The clinical manifestations of *R. equi* infections are different: the most frequent form is severe pyrogranulomatous pneumonia.

3.5. CHO as Biosensor:

Biosensor is one such product of biotechnology that is becoming increasingly popular in fields like environmental monitoring [M. Mascini, 2001; M. Mascini,], bioterrorism [M. Mascini, 2001; F M Burkle, 2003], food analyses [R. Eden-Firstenberg,*et al* 1988] and most importantly in the area of health care and diagnostics [Malhotra *et al* 2003]. This rapidly expanding field has an annual growth rate of 60 %, with major impetus from the health-care industry (30% of the world's total analytical market) supported with other analytical areas of food & environmental monitoring including defense needs [Mart Chaplin].

With rising healthcare costs and to improve patient care, diagnostic laboratories have been challenged to develop new tests that are reliable, cost–effective and accurate and to optimize existing protocols by making them faster and more economical. Although there are number of commercial successes, but most successful to date is the glucose biosensor [Brouwers,*et al*, 2005] for routine monitoring of glucose in blood by individuals suffering from diabetes.

The success of an enzyme biosensor relies on how well an enzyme bonds to a desired biosensor surface and remains active during a desired application. Immobilization of a bio receptor onto a matrix plays an important role for desired thermal stability. Biological molecules including enzymes, antibodies, organelles, etc., can be immobilized in a thin layer at a desired transducer surface by using different



This "medical telesensor" chip on a fingertip can measure and transmit body temperature

(Courtesy: Oak Ridge National Laboratory, ref. 32).

methods. Both the choice of support material and immobilization method can influence enzyme activity and operational stability of a biosensor. The conventional approaches for enzyme immobilization on electrode surfaces include adsorption, cross-linking with glutaraldehyde, covalent attachments using carbodiimide chemistry and entrapment/ encapsulation of sensing agents within polymeric gels or carbon paste [B. Kuswandi, *et al* 2001; D. Avnir, *et al* 1994].

In 2010, a new electrochemical biosensor was introduced [P. Norouzi, *et al* 2010] for cholesterol determination that combined a Fourier transformation continuous cycle voltmeter [FFTCCV] technique in a flow injection analysis [P. Norouzi, e*t al* 2010; Q. C. Shi and T. P. Zhi, 2005]. This was the first application of FFTCCV method for cholesterol biosensor based on CHO. The CHO was immobilized onto MnO_2 nano-particles and multi-walled carbon-nanotubes (MWCNTs) and placed on a glassy carbon electrode surface using nafion. This was first time

when a very high-sensitivity and low detection limit detector was used for cholesterol biosensor on MWCNT/ MnO2 NPs modified electrode. Biosensor showed longterm storage stability up to 30 days, 92.3% sensitivity was retained after 54 days; afterwards there was a decrease in sensitivity due to the loss of catalytic activity. A surface plasma resonance based biosensor for simple, label-free, highly selective and sensitive detection of cholesterol employing the flavo-enzyme CHO as a sensing element has been proposed [R. Gehlot, *at al* 2008]. The CHO along with bovine serum albumin was immobilized onto the gold-plated sensor chip covalently attached with 11-mercaptoundecanoic acid. The analyte brought changes in the sensor surface that could be monitored in real time. The biosensor was specific for cholesterol and showed no significant interference from potent molecules such as ascorbic acid and uric acid.

A novel amperometric cholesterol biosensor immobilize with CHO on electrochemically polymerized polypyrole-polyvinlyulphonate (PPy-PVS) film entrapped on platinum electrode was fabricated [F. Yıldırımoglu, *et al* 2009]. Until now, different conducting polymers have been used for immobilization of bio-molecules such as polyacetylene, polythiohpene (PPy), polyindole and polyaniline [S. Çete, *et al* 2006; G. Li, Y. Wang and H. Xu, 2007]. A PPy-PVs composite membrane was used because of its unique featured "charge controlled membrane" in which the fixed charge were electrochemically checked by an internal electronic state [S. Çete, *et al* 2006]. Mostly, cholesterol biosensors have been used in biochemical analysis owing to their good selectivity, low cost, small size, fast response and long term stability. The cited literature based on cholesterol biosensors appeared to be mainly focused on diagnosing disorders [M. V. Mendes, *et al* 2007,; J. C. Vidal, *et al* 2003,; M. H. Yang, *et al* 2005; M. M. Kalayil, *et al* 2010.].

3.6 CHO use as Probe of cytoplasmic membrane cholesterol:

CHO from different microbial sources has been long used as a cholesterol-probing biocatalyst, in investigations regarding cholesterol distribution in the cytoplasmic membrane of differentiated cells (JADO, et al 1993,; ZAGER, et al 2000), cholesterol trafficking in intestinal cells (FIELD, et al 1998), cholesterol intracellular movement (CHERRADI, et al 1996), and the relations that exist between cholesterol and other functional molecules, as for example membrane receptors (PANG, et al 1999,; NGUYEN, et al 2003). In fact, the literature is replete of this sort of investigations that have well contributed to our understanding of the relation of macromolecules with cholesterol, physiological transformation of cholesterol, and the significance of disorder in this metabolism at the clinical level.

3.7 CHO Use as Probiotics:

Probiotics are living microorganisms, which upon ingestion in certain numbers exert health benefits on the host beyond inherent basic nutrition (Guarner and Schaafsma, 1998). Although many *in vitro* and *in vivo* studies have shown that the administration of probiotics reduces serum/plasma total cholesterol, LDL-cholesterol and triglycerides or increases HDL-cholesterol, their hypocholesterolemic effects remain controversial (Ooi and Liong, 2010). The species with cholesterol-lowering effects studied include genera as *Lactobacillus, Lactococcus,Enterococcus*, *Streptococcus* or *Bifidobacterium* (Pereira and Gibson, 2002). Probiotics have been suggested to reduce cholesterol via various mechanisms that do

not necessarily imply the transformation of cholesterol by the probiotic strains, e.g. enzymatic deconjugation of bile acids by bile-salt hydrolases of probiotics (Lambert *et al*., 2008), assimilation of cholesterol (Pereira and Gibson, 2002), co-precipitation of cholesterol with deconjugated bile (Liong and Shah, 2006), cholesterol binding to cell walls of probiotics (Liong and Shah, 2005), incorporation of cholesterol into the cellular membranes of probiotics during growth (Lye *et al*., 2010a), production of short-chain fatty acids upon fermentation by probiotics in the presence of prebiotics (De Preter *et al*., 2007) and conversion into coprostanol (Lye *et al*., 2010b). As stated above, the most frequent transformation of cholesterol in the intestinal anoxic habitats is a biogenic reduction of its double bond yielding coprostanol, which is directly excreted in faeces (Li *et al*., 1995; Harder and Probian, 1997). Many intestinal fermenting bacteria are able to catalyse this reaction (Groh *et al*., 1993; Freier *et al*., 1994) decreasing the amount of absorbed cholesterol and leading to a reduced concentration in the physiological cholesterol pool (Ooi and Liong, 2010). Lye and colleagues (2010b) evaluated the conversion of cholesterol to coprostanol by strains of lactobacilli such as *L. acidophilus,L. bulgaricus* and *Lactobacillus casei* detecting both intracellular and extracellular cholesterol reductases in these strains, indicating possible intracellular and extracellular conversions of cholesterol to coprostanol. Cholesterol reductase has been also directly administered to humans to convert cholesterol to coprostanol in the small intestines to reach a bloodstream cholesterol-lowering effect (Ooi and Liong, 2010). One of the best-studied mechanisms proposed for the cholesterol-lowering effects of probiotics is the enzymatic deconjugation of bile acids by bile salt hydrolases (Begley *et al*., 2006). Bile, a water-soluble end-product of cholesterol in the liver that consists of cholesterol, phospholipids, conjugated bile acids, bile pigments and electrolytes, is released into the duodenum upon ingestion of food. When it is deconjugated by bile salt hydrolases-producing strains, bile acids are less soluble, being no absorbed by intestines and eliminated in the faeces. Cholesterol is then used to synthesize new bile acids as a homeostatic response, resulting in a hypocholesterolemic effect of probiotics (Begley *et al*., 2006; Ooi and Liong, 2010). The cholesterol-lowering effects are also attributed to the ability of probiotic strains to bind cholesterol, a process that is growth-dependent and strain-specific. However, although growing cells remove more cholesterol than dead cells, the heat-killed cells can still remove cholesterol from media, indicating that some cholesterol is bound to the cellular surface (Usman and Hosono, 1999). During the probiotic growth, the presence of cholesterol increases the concentration of saturated and unsaturated fatty acids, leading to increased membrane strength and subsequently higher cellular resistance towards lysis (Lye *et al*., 2010a; Ooi and Liong, 2010).

3.8. CHO Use in Decrease of diet cholesterol intake:

Raised serum cholesterol level has been considered as a main factor that leads to the development of atherosclerosis and cardiovascular diseases. As a result, the need to reduce dietary cholesterol intake has been considered in official policies, and removal of cholesterol from cholesterol rich foods has become an attractive research objective. In this sense, and since about the end of the eightieth years, laboratory reports have described the use of cholesterol-

degrading microorganisms or isolated enzymes, in order to reduce cholesterol content in egg yolk or milk (Johnson, *et al* 1990), both are of large public consumption. CHO from different microbial sources was active on cholesterol either in milk (Xianshing, *et al* 1990) or egg yolk (Christodoulou, *et al* 1994). Besides, the CHO gene from a *Streptomyces* strain was cloned and expressed in *Streptococcusthermophilus*, a species usually used in the production and ripening of yogurt and several cheese varieties (Somkuti, *et al* 1997). Heterologous expression of CHO gene in alimentary microorganisms should be considered as a worth strategy for food cholesterol modification, since 4-cholesten-3-one, the enzyme reaction product, could not be absorbed by the epithelial cells in the intestinal tract, and seems to be nontoxic. Generally and regardless the biological method for reducing food-cholesterol content, the processed product has to be ascertained for safety consumption, nutritional and organoleptic characters. In this, the process has to obey to the legislation under consideration.

3.9. CHO in Sterol conversion:

3-keto-4-en-derivatives of sterols, products of CHO activity, are needed for laboratory purposes and, in some cases, for the selective cleavage of their side chain, a process leading to the tetracyclic nucleus, which is industrially useful for obtaining final active steroids. An example of the selective cleavage of the cholesterol side chain by two selected microorganisms was described (Lee, *et al* 1993). The microorganisms separately used, in growing cultures, were a CHO expressing species of *Arthrobacter* for cholesterol conversion to 4- cholesten-3-one, and *Mycobacterium*sp. for the side chain cleavage of the last product. Two procedures, concerned with the use of CHO from *Brevibacterium* sp. were reported by the Upjohn Laboratories for the synthesis of steroids. The first one was elaborated for the synthesis of 7α-hydroxycholest-4-en-3- one (Alexander, *et al* 1995), which occupies a pivotal position in the dual pathways for bile acid biosynthesis. Availability of this compound is necessary to verify the correlation of its serum concentration to liver cholesterol 7α-hydroxylase level. CHO was used at the final step of the procedure to convert 3β, 7α-dihydroxy-cholest-5-ene. The second procedure was described for the direct stereoselctive synthesis of 7β-hydroxytestosterone, a known product of androgen metabolism,starting with 5-androstene-3β, 7β, 17β-triol (Labaree, *et al* 1997). Because CHO is poorly active with this substrate, its C8-ester at C-17 was used to mimic the bulky and hydrophobic side chain of cholesterol. At the final step of the procedure, 7β-hydroxytestosterone-17- caprylate, resulting in by CHO activity, was treated with porcine lipase to remove the acid moiety. Microbial CHO, free or immobilized, can be used for commercial conversion of cholesterol or plant sterols to the Corresponding 3-keto-4-ene structures. Besides, cells of *Rhodococcus* species, or other closely related strains, can be efficiently used for the conversion, since the contact of enzyme-substrate is direct due to the external enzyme location. However, any conversion process has to prevail over the insolubility of steroids in water, which causes difficulty in application. There are several means to keep sterols and their transformation products in pseudo soluble states for procedures Using CHO-containing cells or isolated enzymes. These means comprise (i) aqueous/organic solvent two-phase system (Liu, *et al* 1996),(ii) phospholipid liposomes (Goetschel*et al* 1992), (iii) reverse micelles formed of surfactant and solvent

(Gupte, *et al* 1995,;Kane,*et al* 2000), and (iv) aqueous media using natural or chemically modified cyclodextrins (Alexander, et al 1995,; Jadoun, *et al* 1993). Nonionic detergent micelles with isolated cholesterol oxidase, or water miscible solvent (e.g. 2-propanol at 20%, v/v) can be also useful for sterol transformation. If cells were used, addition of iron-complexing agent, a 9α-hydroxylase inhibitor, such as 2, 2'-dipyridyl, would be required, in certain cases, to prevent transformation of 3-keto-4-ene steroids to other.

4. CONCLUSION:

Cholesterol Oxidase has diversified applications. Due to increase in the cholesterol related disorder like cardiovascular diseases, atherosclerosis or coronary heart diseases and also management of hypercholesterolemia, CHO has become second largest diagnostic enzyme after glucose oxidase in clinical industry. It has become an enzyme of great interest today.

Due to the low production of the CHO enzyme the market value of this enzyme is very high.

So researchers improve the yield of cholesterol oxidase by optimization and new techniques.

CHO has a much diversified application in field of clinical, industrial, agricultural, biosensors etc. which have increased the interest in exploring various natural habitats for discovering newer and potential microbial sources which give high yields of CHO.Being the promising area for enzyme isolation still fungi has remained unexplored area in case of cholesterol oxidase. The only known fungi are *Schizophyllum commune*(Fukuyama and Miyake 1979). And after that no studies was further executed.

The sterol biosynthetic pathway has provided a rich source of targets for commercially important bioactive molecules (Bellamine et al. 2001). The sterol biosynthesis in fungi show well defined patterns. Ergosterol (ergosta-5,7,22-trien-3b-ol) is major sterol present in fungi- ascomycetes and basidiomycetes (Weete 1973; Nes and McKean 1977; Nes and Nes 1980; M_janelle et al. 2000). The more primitive mastigomycetes typically produce no ergosterol, but contain cholesterol and C29 4-desmethylsterols including 24-ethylcholesterol (Nes and Nes 1980; Grandmougin-Ferjani et al. 1999). In contrast the zygomycetes appear to be a transitional taxon and include ergosterol and non-ergosterol producers (Weete and Gandhi 1997; Grandmougin-Ferjani et al. 1999). Zygomycetous fungi such as Mortierellaalpina are now mass cultured for their arachidonic acid content (e.g. Bajpai et al. 1991).

Cholesterol is predominate in most primitive fungi (i.e.,*Chytridiomycota).* Where ergosterol is present but not predominate, other C-28 sterols, presumably ergosterolprecursors, accumulate to varying degrees depending on the species and growth conditions (Nichols. S.D;1998)

Endophytes are endo-symbionyts that lives asymptomatically inside the living tissue of a plant without showing any apparent signature of their presence. Endophytic fungi are considered to be lucrative source of bioactive metabolites.

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