Studies on Production, Characterization and Application Potential of Cholesterol Oxidase from *Rhodococcus* sp. NCIM 2891

A Thesis

Submitted by

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For the award of the degree

Of

Doctor of Philosophy

Department of Biosciences & Bioengineering
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October, 2014

Dedicated to

My

Beloved Parents

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STATEMENT

I do hereby declare that the matter incorporated in this thesis is the result of investigations carried out by me in the Department of Biosciences & Bioengineering, Indian Institute of Technology Guwahati, India, under the guidance of **Prof. Pranab Goswami**.

In keeping with the general practice of reporting scientific observations, due acknowledgements have been made wherever the work described is based on the findings of other investigators.

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Certificate

This is to certified that the thesis entitled "**Studies on Production, Characterization and Application Potential of Cholesterol Oxidase from *Rhodococcus* sp. NCIM 2891**" that is being submitted by **Seraj Ahmad** (Roll No. 08610616) for the award of the degree of Doctor of Philosophy is an authentic record of the results obtained from the research work carried out under my supervision in the Department of Biosciences & Bioengineering, Indian Institute of Technology Guwahati, Assam, India.

The results incorporated in this thesis have not been submitted to any other University or Institute for the award of any degree.

Dr. Pranab Goswami
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October, 2014

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ABSTRACT

Cholesterol oxidase (EC 1.1.3.6) is a flavoenzyme that in most cases catalyses the oxidation of cholesterol to 4-cholesten-3-one (CEO, cholestenone). The product cholestenone has been used as a precursor for the synthesis of hormones and many intermediate steroidal compounds which are eventually used to produce anabolic drugs and contraceptive. Classical (one-variable-at-a-time) and statistical methods (Plackett-Burman and Central composite design) were used to optimize growth medium for the production of cholesterol oxidase from *Rhodococcus* sp. NCIM 2891. Cholesterol oxidase activities from the classically and statistically optimized media were 0.75 and 3.25 U/ml, respectively. The statistically optimized medium had 4.33- and 9.7-fold higher enzymatic activity than the classically optimized and unoptimized basal medium, respectively. The ratio of enzyme production to cell growth rate was 29-fold higher in our statistically optimized medium than in the basal medium, indicating that the enzyme production could be classified as mixed type of growth. Cell-bound cholesterol oxidase accounted for 90.68±2 % of the total enzymatic activity of the growth medium. Interactions between the cholesterol oxidase-inducing substrate cholesterol and medium growth substrates yeast extract and $(NH_4)_2HPO_4$ significantly enhanced the production of cell bound cholesterol oxidase. Our results validate the statistical approach as a potential technique for achieving the large-scale production of cell-bound cholesterol oxidase from *Rhodococcus* sp. NCIM 2891.

The cell-bound cholesterol oxidase from the *Rhodococcus* sp. NCIM 2891 was purified 3-fold by Diethylaminoethyl-Sepharose chromatography. The estimated molecular mass (SDS-PAGE) and K_m of the enzyme were ~ 55.0 kDa and 151 µM, respectively. Fe^{2+} and Pb^{2+} at 0.1 mM of each acted as inhibitors, while Ag^{+}, Ca^{2+}, Ni^{2+} and Zn^{2+} activated the enzyme at similar concentration. The purified cholesterol oxidase was immobilized on

chitosan beads by glutaraldehyde cross-linking reaction and immobilization was confirmed by Fourier Transform Infrared spectroscopy, Scanning Electron Microscopy and Energy Dispersive X-ray analysis. The optimum temperature (45 °C, 5 min) for activity of the enzyme was increased by 5 °C after immobilization. The both free and immobilized cholesterol oxidases were found to be stable in many organic solvents except for acetone. The biotransformation of cholesterol (3.75 mM) with the cholesterol oxidase immobilized beads (3.50 U) leads to ~ 88 % milli molar yield of cholestenone in a reaction time of 9 h at 25 °C. The immobilized enzyme retains ~ 67 % activity even after twelve successive batches of operation. The biotransformation method thus, shows a great promise for the production of pharmaceutically important cholestenone.

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LIST OF ABBREVIATIONS

ABTS	2,2'-azino-bis[3-ethylbenzothiazoline-6-sulfonic acid] diammonium salt
AD	Androst-4- ene-3,17-dione
ADD	Androsta-1, 4-diene-3, 17-dione
AKRs	Aldo-keto reductases
ANOVA	Analysis of variance
BSA	Bovine serum albumin
CCD	Central composite design
CD	Circular dichroism
CEO	4-cholesten-3-one
CFU	Colony forming unit
CHAPS	Sodium cholate and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
Da	Dalton
DEAE	Diethyl amino ethyl
DMSO	Dimethylsulfoxide
DTT	Dithiothretol
EC	Enzyme commission
EDTA	Ethylenediamine tetraacetic acid
EDX	Energy dispersive X-ray
FAD	Flavin adenine dinucleotide
FDB	1-fluoro-2,4-dinitrobenzene
FF	Fast flow
FMN	Flavin mononucleotide
FPLC	Fast performance liquid chromatography
FTIR	Fourier Transform Infrared
GMC	Glucose-methanol-choline

HCEO	6β-hydroperoxycholest-4-en-3-ne
HPLC	High performance liquid chromatography
HQ	8-Hydroxyquinoline
HRP	Horse radish peroxidase
ID	Identity
kDa	Kilo dalton
LBS	Sodium lauryl benzenesulfonate
NAD	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NCIM	National Collection of Industrial Micro-organisms
PDB	Protein data bank
PEG	Polyethylene glycol
PHMS	2-Pyridylhydroxymethane sulfate
PPB	Potassium phosphate buffer
PVA	Polyvinyl alcohol
RSM	Response surface methodology
RT	Room temperature
SCC	Side-chain-cleaving
SDRs	Short-chain alcohol dehydrogenase/reductase
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
Tris	Tris(hydroxymethyl)aminomethane
U	Unit
UDP	Uridine diphosphate
UV	Ultra violet

LIST OF SYMBOL

g	Relative centrifugal force
K_m	Michaelis-Menten constant
K_{cat}	Turnover number
K_{cat}/K_m	Catalytic efficieny
R^2	Regression coefficient
$t_{1/2}$	Half- life
ε	Extinction coefficient
λ	Wavelength
P	Partition coefficient
Y	Response
β_o	Model intercept
β_i	Linear coefficient
β_{ii}	Quadratic coefficient
β_{ij}	Interaction coefficient
x_i	Level of the independent variable
k_d	Thermo-deactivation constant
E_{ad}	Deactivation energy
A	Pre-exponential factor
R	Gas constant
h	Plank constant
k_B	Boltzmann constant
ΔH	Enthalpy
ΔG	Free energy
ΔS	Entropy
D	Decimal reduction time

Chapter 1

Introduction

A wide spectrum of microorganisms degrades cholesterol for growth and survival. The first step of this degradation is catalyzed primarily by either 3β-hydroxysteroid dehydrogenase or cholesterol oxidase. The 3β-hydroxysteroid dehydrogenase (EC 1.1.1.145) catalyses the conversion of 3β-hydroxy-5-ene-steroids to 3-oxo-4-ene-steroids through consecutive oxidation and isomerization reactions using NAD or NADP as cofactors. Studies on the application potential of 3β-hydroxysteroid dehydrogenase have been done to a limited extent and among them the determination of cholesterol in clinical samples are prominent (Kishi et al. 2002). The need of supplementing the cofactor in the reaction medium is the prime reason that impedes the progress on the application of the dehydrogenase enzyme for developing techno-economically viable device or process. Although there is a continuous effort to regenerate the co-factor in the reaction system to sustain the catalytic activity of the enzyme, efficient cofactor regenerating system for application of dehydrogenase enzymes in general, is yet to develop properly. Conversely, the cholesterol oxidase (EC 1.1.3.6) is a flavoenzyme consisting of avidly bound flavin co-factor in the redox center of the enzyme. During the oxidation process the cholesterol oxidase does not require supplementation of any external cofactor in the reaction medium (Goswami et al. 2013). This makes the enzyme cholesterol oxidase an attractive biocatalyst for investigating its application potentials in pharmaceutical, environmental and agricultural fields (Smith and Brooks 1976; Watanabe et al. 1986; Purcell et al. 1993). Notably, the clinical application of cholesterol oxidase for detection of cholesterol in human blood serum has already been established.

A ketosteroid, namely, cholestenone (4-cholesten-3-one) is formed from the cholesterol oxidase catalysed oxidation of the substrate cholesterol. Cholestenone has high demand in the pharmaceutical industries since it is a known precursor for the synthesis of hormones and many intermediate steroidal compounds used to produce anabolic drugs and contraceptive (Chaudhari et al. 2010). This ketosteroid also has anti-obesity effect thus depicting its potential as food additive or medicine for preventing obesity related diseases (Suzuki 1993). The production of cholestenone from the chemical procedures is normally not preferred due to the fact that the action of chemical oxidizing agents on the hydroxysteroid is vigorous and uncontrollable, as a result the chemical procedures produce relatively small yields of the desired ketosteroid and even these small amounts were also contaminated with large amounts of undesirable oxidation products (Schmidt and Hughes 1944). Cholestenone is generally produced through biotransformation route using microbial cells as catalysts (Liu et al. 1996). However, the downstream processing for the isolation of the pure product from the culture broth of microbial cell-based biocatalyzed process is a difficult task as the target product is frequently contaminated by other metabolic products and nutrient substrates. Application of sole enzyme based process for the production of cholestenone is not yet properly developed and the reason may be attributed to the low process economy due to high enzymes cost and poor stability of the enzymes. One of the ways to mitigate the above problem for enzyme based process in general is to use immobilized enzyme for biotransformation reaction considering its distinct advantages over the free form such as, easy handling in the downstream processing and reusability of the enzyme which finally contribute to the process economy. Biocompatibility of the immobilisation matrix for stability of the enzyme has also received increasing attention due to its obvious support to the reusability of the enzyme that eventually contributes to the economy of the enzyme based process.

A variety of microorganisms have been reported to produce cholesterol oxidase, among which *Rhodococcus* species have drawn our attention for their high production capability of the enzyme. The molecular characteristics of the cholesterol oxidase from *Rhodococcus* strains have been explored to some extent, while the physico-chemical properties of the enzyme which are relevant to the bioprocess development are not yet adequately explored. It has been found that cholesterol oxidases from *Rhodococcus* are highly stable and active in broad pH range (Wang et al. 2008), the production and characterization of cholesterol oxidases from *Rhodococcus* sp. and its uses for the synthesis of different ketosteroid production have not been adequately studied. Considering the above facts and challenges, the following objectives has been addressed in this thesis: (1). Production of cholesterol oxidase from *Rhodococcus* sp. NCIM 2891. (2) Isolation, purification and characterization of the cholesterol oxidase from *Rhodococcus* sp. NCIM 2891. (3). Immobilization of cholesterol oxidase from *Rhodococcus* sp. NCIM 2891 in a suitable support material. (4). Application of the immobilized cholesterol oxidase for cholestenone production.

The contents of this thesis entitled as **"**Studies on Production, Characterization, and Application Potential of Cholesterol Oxidase from a *Rhodococcus* Sp. NCIM 2891**"** have been divided into five chapters.

Chapter 1. Introduction

The current **Chapter 1** provides a brief introduction defining the origin of the work that comprises the motivation for pursuing the work, challenges involved and objectives undertaken to address the challenges.

Chapter 2. Review of Literature

The chapter summarizes the progress of the work on the subject as a whole, focusing the gaps and important findings on the area. Current knowledge on the cholesterol oxidase enzymes involved in the oxidation of steroids into corresponding ketosteroids and different keto derivatives from different sources is also presented here. Also, the different techniques used for cholesterol oxidase immobilization and its application are discussed here.

Chapter 3. Production of Cholesterol Oxidase from *Rhodococcus* sp. NCIM 2891

This chapter describes the optimum production conditions of the enzyme, different production methods and its pros and cons. It also describes the optimum concentration of different medium constituents that supports high the enzyme production yield.

Chapter 4. Isolation, Purification and Characterization of the Cholesterol Oxidase from *Rhodococcus* sp. NCIM 2891

This chapter describes the isolation and purification of cholesterol oxidase from *Rhodococcus* Sp. NCIM 2891. It also describes the novel physical and chemical characteristics of the enzyme revealed from the investigation.

Chapter 5. Immobilization and Application of the Cholesterol Oxidase from *Rhodococcus* sp. NCIM 2891

This chapter describes the immobilization of the cholesterol oxidase on a suitable biocompatible support matrix for biotransformation application of the enzyme to produce cholestenone.

Following Chapter 5, overall conclusions of the research work highlighting the significant findings and scope of future work is included, which is followed by a Bibliography section.

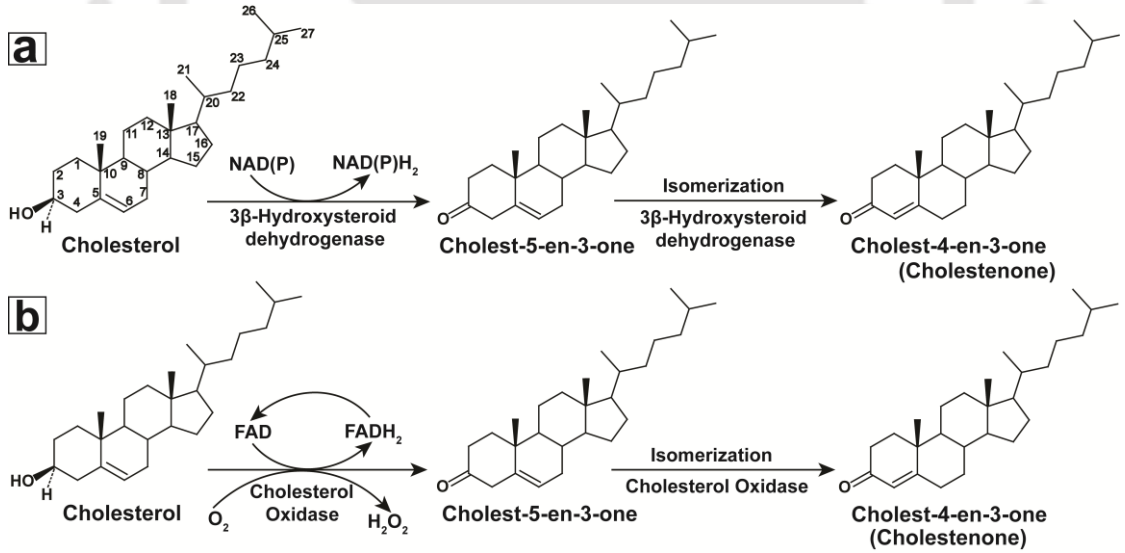
Chapter 2

Review of Literature

The microbial degradation of cholesterol was first reported by Söhngen (1913) and the researcher attributed *Mycobacterium* sp. as the causative microbial agent for the degradation. Later, Tak (1942) had demonstrated chemical evidence on the degradation of cholesterol by *Mycobacterium* sp. Subsequently, Turfit (1944b) had identified cholestenone as the microbial degradation product of cholesterol in the culture medium of *Proactinomyces erythropolis* (later known as *Rhodococcus erythropolis*). After a long gap, the work of Arima et al. (1969) established that cholesterol could be oxidized by various types of microorganisms such as, actinomycetes, molds, and yeasts. Since then, a large number of microorganisms capable of oxidizing cholesterol has been reported and among which *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Gordonia*, *Microbacterium*, *Mycobacterium*, *Nocardia*, *Streptomyces*, and *Streptoverticillium*, *Bordetella*, *Burkholderia*, *Chromobacterium*, *Chryseobacterium*, *Enterobacter*, *Proteobacterium Pseudomonas* and *Schizophyllum* are well documented (Goswami et al. 2013). The microbial degradation of cholesterol is known to occur both in aerobic and anaerobic conditions. Taylor and colleagues (1981) demonstrated that some bacteria can also degrade cholesterol under denitrifying conditions. In a related study, groups of scientists (Groh et al. 1993; Freier et al. 1994) reported that cholesterol is also converted into coprostanol by many intestinal fermenting bacteria. Under the anaerobic conditions the double bond of the cholesterol is reduced, yielding coprostanol (Li et al. 1995; Harder and Probian 1997). However, under aerobic conditions, the microbial degradation of cholesterol in general, involves two processes: sterol side-chain elimination and steroid ring opening (Van der Geize and Dijkhuizen 2004). The

order of these two processes *in vivo* is unknown and may vary among microorganisms (Rosłoniec et al. 2009). The bacteria completely catabolize the A-ring to CO_2 while incorporating side chain carbons into their lipid pools (Pandey and Sassetti 2008). Furthermore, the bacteria completely catabolize the core ring system into pyruvate and succinate (Ismail and Chiang 2011; García et al. 2012). The complete aerobic mineralization of cholesterol was reported by various genera of bacteria, such as *Arthrobacter*, *Corynebacterium*, *Mycobacterium*, *Nocardia*, *Pseudomonas*, and *Rhodococcus* (Ismail and Chiang 2011).

Stadtman et al. (1954) first reported the activity of enzyme cholesterol oxidase that catalyzes the aerobic oxidation of cholesterol in the culture of *Mycobacterium cholesterolicum*. Since then, the enzyme has been isolated and purified by many researchers from various microorganisms (Doukyu 2009).



Scheme 2.1 Mechanism of cholesterol oxidation by (a) 3β-Hydroxysteroid dehydrogenase and (b) Cholesterol oxidase.

However, it was revealed latter that in nature, the oxidation of cholesterol is also catalyzed by an NAD or NADP based dehydrogenase enzyme, known as 3β-hydroxysteroid

dehydrogenase (Ismail et al. 2011). The mechanism of catalysis is different for both the enzymes as shown in the scheme 2.1. Considering the focus of our work on cholesterol oxidase we are presenting here only a brief description on the 3β-hydroxysteroid dehydrogenase followed by the literature review on the enzyme cholesterol oxidase.

2.1 3β-Hydroxysteroid dehydrogenase

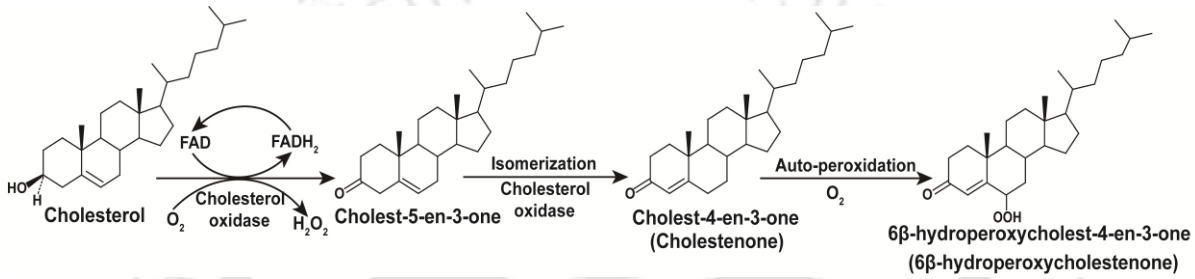
The 3β-hydroxysteroid dehydrogenase (EC 1.1.1.145) catalyses the conversion of 3β-hydroxy-5-ene-steroids to 3-oxo-4-ene-steroids through consecutive oxidation and isomerization reactions using NAD or NADP as cofactors (Scheme 2.1a). The NAD(P)-dependent hydroxysteroid dehydrogenase are distributed in microorganisms: *Clostridium innocuum* (Edenharder et al. 1989), *Peptostreptococcus roductus* (Edenharder et al. 1989), *Pseudomonas testosteroni* (Sqire et al. 1964), *Nocardia* sp., (Akiba 1983), *Mycobacterium tuberculosis* (Yang et al. 2007), *Mycobacterium smegmatis* (Uhía et al. 2011a); plants: *Digitalis lanata* (Seidel et al. 1990; Finsterbusch et al. 1999) and animals (Morel et al. 1997). In Human, the 3β-hydroxysteroid dehydrogenase catalyzes the transformation of pregnenolone to progesterone, 17α-hydroxypregnenolone to 17α-hydroxyprogesterone, dehydroepiandrosterone to 4-androstenedione, and 5-androstene-3β,17β-diol to testosterone and is therefore an essential step in the biosynthesis of all classes of hormonal steroids, namely progesterone, mineralocorticoids, glucocorticoids, androgens, and estrogens (Lachance et al. 1990). The 3β-hydroxysteroid dehydrogenase isoenzymes have been characterized in human and several other vertebrate species: human types I and II; macaque; bovine; rat types I, II, III, and IV; mouse types I, II, III, IV, V and VI; hamster types 1, II and III; and rainbow trout (Morel et al. 1997). Hydroxysteroid dehydrogenases belong to two different protein families. They are either members of the superfamily of the short-chain

alcohol dehydrogenase/reductase family (SDRs) such as, 3β-hydroxysteroid dehydrogenase/ketosteroid isomerase, 11β-hydroxysteroid dehydrogenase, and 17β-hydroxysteroid dehydrogenase, and the aldo-keto reductases (AKRs) super family such as 3α-hydroxysteroid dehydrogenase and 20α-hydroxysteroid dehydrogenase (Malekinejad et al. 2006). Gene database analysis (Baker and Blasco 1992) revealed that the bacterial hydroxysteroid dehydrogenases, mammalian 3β-hydroxysteroid dehydrogenases, plant dihydroflavonol reductases, bacterial UDP-galactose-4-epimerases and viral 3β-hydroxysteroid dehydrogenase all comes under same protein super family. The catalytic property of the 3β-hydroxysteroid dehydrogenase enzyme has been used for many purposes such as, for the determination of cholesterol in serum (Kayamori et al. 1999; Kishi et al. 2002) and also for the development of cholesterol biosensors (Ishige et al. 2009; Wallace-Davis et al. 2011; Pei and Young 2013).

2.2 Cholesterol oxidase

Cholesterol oxidase (cholesterol: oxygen oxidoreductase, EC 1.1.3.6) catalyzes the oxidation of cholesterol to the corresponding ketones using molecular oxygen as a co-substrate as shown in Scheme 2.1b. Unlike 3β-hydroxysteroid dehydrogenase, cholesterol oxidase does not require supplementation of any external cofactor during the oxidation process. This makes the cholesterol oxidase a high potential biocatalyst than the 3β-hydroxysteroid dehydrogenase for several applications. Although, there are many reports on the oxidation of cholesterol by various types of microorganisms, the in-depth study on the oxidative conversion is limited and reported mostly from the prokaryotic microorganisms. In general, cholesterol oxidase catalyses the oxidation and isomerization of cholesterol to cholestenone with the concomitant production of hydrogen peroxide (Smith and Brooks 1974). However, Molnar and group first

reported a bacterial enzyme that oxidizes cholesterol to 6β-hydroperoxycholest-4-en-3-ne (HCEO) but not the CEO catalyzed by most cholesterol oxidases. This HCEO is formed by the oxygenation in the sixth position on the cholesterol nucleus by the cholesterol oxidase catalysis (Molnar et al. 1993). Since then, two distinctive types of bacterial cholesterol oxidases have been documented: one forms CEO and the other forms HCEO as the major product (MacLachlan et al. 2000; Doukyu 2009; Kreit and Sampson 2009) as shown in the scheme 2.2.



Scheme 2.2 Mechanism of 6 β-hydroperoxycholestenone production from cholesterol by cholesterol oxidase catalyzed reaction

The cholesterol oxidases are further classified into two types, type I and type II on the basis of the nature of the FAD linkages to the enzyme protein molecules (Fig. 2.1). The type I and II involve non-covalent and covalent linkage of the FAD cofactors to the protein molecules, respectively (Croteau and Vrielink 1996; Sampson and Vrielink 2003). The type I enzyme belongs to the GMC (glucose/methanol/choline) oxidoreductase family, while the type II enzyme belongs to the VAO (vanillyl alcohol oxidase) family (Sampson and Vrielink 2003). Type I enzymes have been identified mostly in actinomycetes such as, *Streptomyces* sp. SA-COO, *Rhodococcus equi*, *Mycobacterium tuberculosis*, *Corynebacterium urealyticum* (Vrielink and Ghisla 2009). The type I enzymes showed a consensus sequence GlyXGlyXXGly, for FAD binding in the N-terminal region (Dym and Eisenberg 2001).

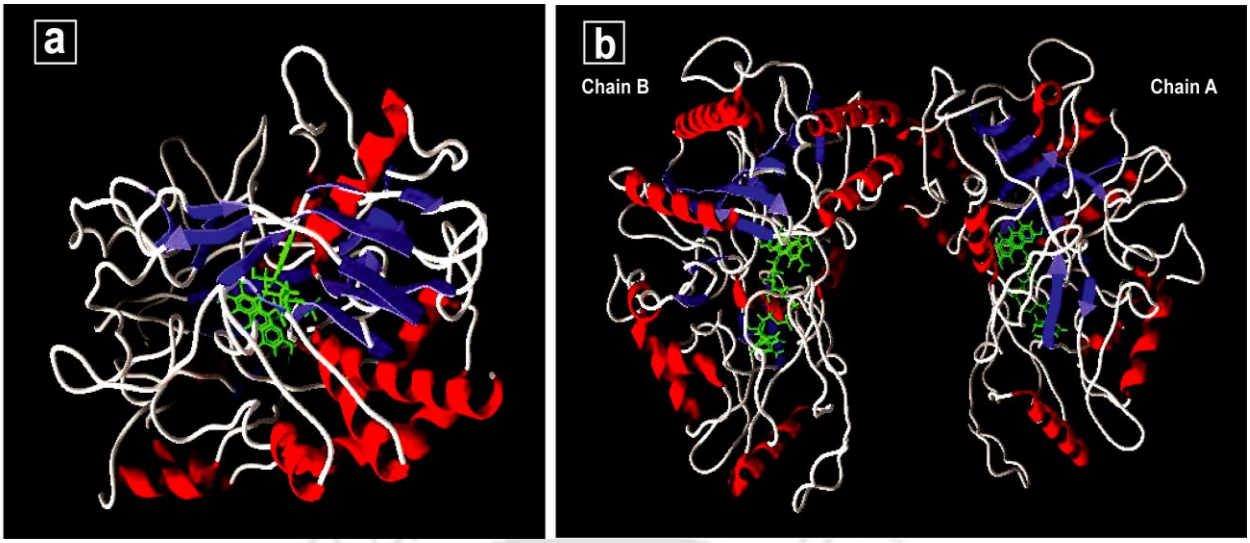


Fig. 2.1 Secondary structure view of (a) type I cholesterol oxidase (PDB ID: 1B4V) from *Streptomyces* (b) type II cholesterol oxidase (PDB ID: 1I19) from *B. sterolicum*. α-helices, β-sheets and loops are depicted in red coil ribbon, blue sheets ribbon and white color ribbon, respectively. The FAD cofactor is shown in green color. The structures are created using Molegro Virtual Docker (Thomsen and Christensen 2006).

The analysis of type I from *Streptomyces* sp. revealed that His447 and Glu361 residues are involved in the activity for oxidation and isomerization and these residues are conserved in many of them (Yue et al. 1999) (Fig. 2.2). The phylogenetic tree analysis showed that the type I enzymes can be further divided into two groups: type I-1 and I-2. Unlike the type I-1 enzymes, the type I-2 enzymes lack a signal sequence suggesting its cytoplasmic origin (Doukyu 2009). Type II enzymes have been reported from *Brevibacterium sterolicum*, *Burkholderia cepacia*, *Chromobacterium* sp. DS-1, *Rhodococcus erythropolis*, *Rhodococcus* sp. PTCC 1633 (Vrielink and Ghisla 2009; Ghasemian et al. 2009). X-ray crystallographic (Coulombe et al. 2001) structure of *B. sterolicum* enzyme (type II) suggested covalent attachment of FAD to an active-site histidine via the C8 α group of the flavin isoalloxazine ring.

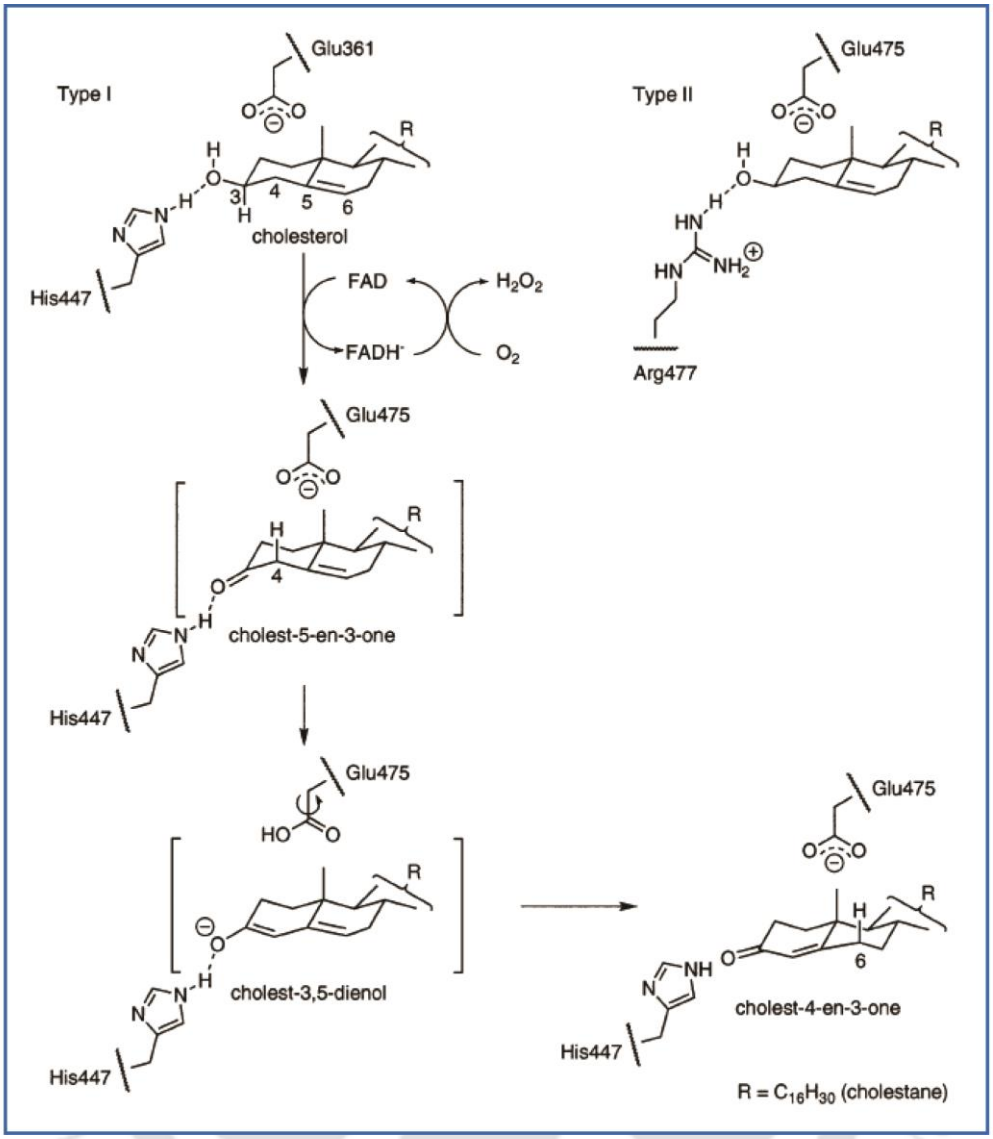


Fig. 2.2 Reaction mechanisms on type I and type II cholesterol oxidase catalyses. (Adopted from Sampson and Vrielink 2003).

This covalent interaction is correlated to redox potential and 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 type II enzymes (Fig. 2.2). Both classes of cholesterol oxidases share the same catalytic activity but show significant differences in their redox and kinetic properties. In general, they exhibit a broad range of

steroid specificities and can oxidize a number of hydroxysterols, but the presence of a 3β-hydroxyl group is an important requirement for their activity (Pollegioni et al. 1999). No mammalian homolog of cholesterol oxidases has been reported till now. In Human, in aerobic condition the cholesterol is degraded into pregnenolone. It is first and rate-limiting step in the synthesis of all steroid hormones. This degradation involves three steps: 20-hydroxylation, 22-hydroxylation, and cleavage of the C_{20}-C_{22} bond to produce pregnenolone and isocaproic acid. These three steps are mediated by a single mitochondrial cytochrome called as P450scc [cholesterol, reduced-adrenal-ferrodoxin: oxygen oxidoreductase (side-chain-cleaving), EC 1.14.15.67] (Chung et al. 1986).

2.2.1 Production of cholesterol oxidase

Various sources of microbial cholesterol oxidases, its production condition and theirs yield are summarized in Table 2.1. This family of enzymes produced by the microorganism in three forms: extracellular, cell-bound/cell-linked and intracellular. Most cholesterol oxidases produced extracellularly. The *Mycobacterium*, *Nocardia*, γ-*Proteobacterium*, *Rhodococcus* sp. produce cell-bound enzymes (Smith et al. 1993; Minuth et al. 1995; Isobe et al. 2003; Kreit et al. 1994). However, cholesterol oxidases from the *Mycobacterium*, *Rhodococcus* sp., *Streptomyces* (Stadtman et al. 1954; Wilmanska et al. 1995; Zajaczkowska and Sedlaczek 1988; Varma and Nene 2003) are also produced intracellularly. Furthermore, some microorganisms such as *Nocardia*, *Mycobacterium*, *Brevibacterium* and *Rhodococcus* produce both extracellular and cell-bound forms of cholesterol oxidases (Richmond 1973; Chipley et al. 1975; Salva et al. 1999; Johnson and Somkuti 1991; Sojo et al. 1997; Elalami et al. 1999; Yazdi et al. 2001; Sojo et al. 2002; Lashkarian et al. 2010).

Table 2.1 Production and localization of cholesterol oxidase enzyme from different sources.

Source organism	Production level	Production condition	Localization	Enzyme activity	References
Arthrobacter simplex (a) USA18 (b) US3011	Fermentor	-	Extracellular	(a) 0.13 U/mg (b) 0.24 U/mg	Liu et al. 1988
Arthrobacter sp. IM 79	-	-	Extracellular	-	Wilmanska and Sedlaczek 1988
Bacillus sp.	Flasks	pH, 7.0; Temp, 30 °C; Time, 36 h; rpm, 150	Extracellular	1.40 U/mg	Rhee et al. 2002
Bacillus subtilis SFF34	Flasks	pH, 7.0; Temp, 30 °C; Time, 24 h; rpm, 150	Extracellular	3.14 U/ml	Kim et al. 2002
Bordetella sp.	Flasks	pH, 7.0; Temp, 37 °C; Time, 24 h; rpm, 260	Extracellular	1.70 U/ml	Lin et al. 2010
Brevibacterium sterolicum	Fermentor	pH, 7.3; Temp, 30°C; Time, 24 h; rpm, 300; Aeration, 15 liters air/min.	Extracellular	0.014 U/mg	Uwajima et al. 1973
Brevibacterium sp.	Flasks	pH, 7.0; Temp, 37°C; Time, 7 days; rpm, 150	Extracellular, cell-bound	1.93 U/ml	Salva et al. 1999
Chromobacterium sp. DS-1	-	Temp, 30°C; Time, 16 h	Extracellular	0.78 U/mg	Doukyu et al. 2008
Chryseobacterium gleum	Flasks	pH, 8.0; Temp, 30°C; Time, 24 h; rpm, 120	Extracellular	167 U/mg	Chaudhari et al. 2010
Corynebacterium cholesterolicum	-	-	-	-	Shirokane et al. 1977
Enterobacter sp.	-	pH, 7.0; Temp, 30°C; Time, 48 h; rpm, 200	Extracellular	0.59 U/mg	Ye et al. 2008

Cont…

Source organism	Production level	Production condition	Localization	Enzyme activity	References
Gordonia cholesterolivorans	Flasks	-	Intracellular	-	Drzyzga et al. 2011
Gordonia neofelifaecis	Flasks	pH, 7.0; Temp, 30°C; Time, 24 h; rpm, 200	Extracellular	0.21 U/ml	Liu et al. 2011
Microbacterium sp.	Flasks	pH, 7.0; Temp, 30°C; Time, 72 h; rpm, 150	Extracellular	1.02 U/ml	Parekh and Desai 2013
Mycobacterium cholesterolicum	-	pH, 7.0-7.5; Temp, 26-32°C; Time, 24-30 h	Intracellular	-	Stadtman et al. 1954
Mycobacterium sp. ATCC 19652	Flasks	-	Extracellular, cell-bound	-	Chipley et al. 1975
Mycobacterium NRRL B-3683, DP	Flasks	pH, 7.0-7.2; Temp, 30 °C; rpm, 150-200	cell-bound	-	Smith et al. 1993
Mycohacterium vaccae, fortuitum, phlei, smegmatis,	Flasks	pH, 7.0; Temp, 28 °C; Time, 7 days; rpm, 120	Intracellular, cell-bound	-	Wilmanska et al. 1995
Nocardia sp.	Flasks	pH, 7.0; Temp, 29 °C; Time, 24 h	Extracellular, cell-bound	-	Richmond 1973
Nocardia rhodocrous	Fermentor	-	-	-	Buckland et al. 1976
Nocardia rhodocrous	Fermentor	pH, 6.9; Temp, 30 °C; rpm, 450, 550; pO_2 ,12 %	Cell-bound	0.34 U/ml	Minuth et al. 1995
γ-*proteobacterium* Y-134	Flasks	pH, 8.0; Temp, 30 °C; Time, 12 days; rpm, 120	Cell-bound	0.42 U/mg	Isobe et al. 2003
Pseudomonas sp. COX629	Flasks	pH, 7.2; Temp, 30 °C; Time, 24 h	Extracellular	0.012 U/mg	Lee et al. 1989
Pseudomonas sp. ST-200	-	Temp, 30 °C; Time, 17 h	Extracellular	0.42 U/mg	Doukyu and Aono 1998

Cont…

Source organism	Production level	Production condition	Localization	Enzyme activity	References
Rhodococcus sp. IM 58	-	-	Intracellular, cell-bound	-	Zajaczkowska and Sedlaczek 1988
Rhodococcus equi No. 23	Flasks	pH, 7.0; Temp, 37 °C; Time, 40 h	Extracellular	0.37 U/mg	Watanabe et al. 1989
Rhodococcus equi ATCC33706	-	-	Extracellular, cell-bound	-	Johnson and Somkuti 1991
Rhodococcus equi ATCC 33701	Flasks	Temp, 37 °C; Time, 72 h; rpm, 100	Extracellular	1400 U/mg	Machang'u and Prescott 1991
Rhodococcus erythropolis ATCC 25544	Flasks	Temp, 29 °C; rpm, 160	Extracellular, cell-bound	22.4 U/ml	Sojo et al. 1997
Rhodococcus equi No. 23	Flasks	pH, 7.0; Temp, 37 °C; Time, 72 h; rpm, 150	Extracellular	0.24 U/ml	Lee et al. 1998
Rhodococcus equi No. 23	Fermentor	Aeration, 5.0 l/min; First phase: pH, 6.5; Temp, 39 °C; Time, 0-24 h; rpm, 200; Second phase: pH, 7.5; Temp, 37 °C; Time, 25-30 h; rpm, 300	Extracellular	0.34 U/ml	Chou et al. 1999
Rhodococcus sp. GK_1	Flasks	-	Extracellular	-	Kreit et al. 1992
Rhodococcus sp. GK_1	Flasks	pH, 7.0; Temp, 28 °C	Cell-bound	0.41 U/ml	Kreit et al. 1994

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Source organism	Production level	Production condition	Localization	Enzyme activity	References
Rhodococcus sp. GK $_1$	Flasks	pH, 7.0; Temp, 28 °C	Extracellular, cell-bound	0.75 U/ml	Elalami et al. 1999
Rhodococcus sp.	Flasks	pH, 7.0; Temp, 30 °C; Time, 24 h; rpm, 150	Extracellular, cell-bound	0.24 U/ml	Yazdi et al. 2001
Rhodococcus erythropolis ATCC 25544	Fermentor	Aeration, 2.6 v/v/min; pH, 6.75; Temp, 29 °C; Time, 70 h	Extracellular, cell-bound	360 U/ml	Sojo et al. 2002
Rhodococcus equi No. 23 (a) Free cells (b) Immobilised cells	Flasks	(a) pH, 6.2; Temp, 37 °C;Time,48 h; rpm, 150 (b) -do-	Extracellular	(a) 0.13 U/ml (b) 0.18 U/ml	Chang and Chou 2002
Rhodococcus equi No. 23 (a) Free cells (b) Immobilised cells	Flasks	(a) pH, 7.0;Temp,30°C; Time, 48 h; rpm, 250 (b) -do-	Extracellular	(a) 0.84 U/ml (b) 0.94 U/ml	Chang et al. 2005
Rhodococcus sp. R $_{14-2}$	Flasks	pH, 7.4; Temp, 33 °C; Time, 60 h; rpm, 150	Extracellular	1.5 U/ml	Wang et al. 2008
Rhodococcus sp.	Flasks	pH, 7.0; Temp, 30 °C	Extracellular, cell-bound	-	Lashkarian et al. 2010
Schizophyllum commune	-	-	-	-	Fukuyama and Miyake 1979
Streptomyces violascens	Flasks	Temp, 27-28 °C; Time, 96 h	Extracellular	-	Fukuda et al. 1973
Streptomyces violascens	-	-	Extracellular	-	Tomioka et al. 1976
Streptomyces violascens	-	-	Extracellular	0.47 mg/ml	Kamei et al. 1978

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Source organism	Production level	Production condition	Localization	Enzyme activity	References
Streptoverticilliu m cholesterolicum	Flasks	Temp, 27 °C; Time, 96 h	Extracellular	0.19 U/mg	Inouye et al. 1982
Streptomyces sp.	-	-	Extracellular	13.0 U/ml	Lartillot and Kedziora 1990
Streptomyces fradiae	Flasks	pH, 7.2; Temp, 34 °C; Time, 96 h; rpm, 150	Extracellular	0.20 U/ml	Yazdi et al. 2001
Streptomyces polychromogenes IFO 13072	-	pH, 7.3; Temp, 28 °C; rpm, 150	-	-	Kim et al. 2002
Streptomyces lavendulae NCIM 2421	Flasks	pH, 7.0; Temp, 30 °C; Time, 72 h; rpm, 200	Extracellular, intracellular	2.0 U/ml	Varma and Nene 2003
Streptomyces parvus	Flasks	pH, 7.2; Temp, 30 °C; Time, 72 h; rpm, 150	Extracellular	1.07 U/ml	Praveen et al. 2011
Streptomyces fradiae (a) Wild (b) Mutant	Flasks	(a) pH, 7.0; Temp, 30 °C; Time, 6 days; rpm, 40-120 (b) pH, 7-8; Temp, 30-35 °C; Time, 6 days; rpm, 40-150	Extracellular	(a) 0.29 U/mg (b) 0.20 U/mg	Ouf et al. 2012
Streptomyces lavendulae NCIM 2499	Flasks	pH, 7.5; Temp, 30 °C; Time, 72 h; rpm, 180	Extracellular	2.21U/ml	Chauhan et al. 2009
Streptomyces badius	Flasks	pH, 6.5; Temp, 30 °C; Time, 48 h	Extracellular	2.38 U/ml	Moradpour et al. 2013
Streptomyces sp.	Flasks	pH, 7.2; Temp, 30 °C; Time, 96 h; rpm, 180	Extracellular	6.20 U/ml	Niwas et al. 2013

There is an increasing demand on cholesterol oxidase for its various industrial and clinical applications. The natural yield of cholesterol oxidase in the culture broth of microorganisms is very low and not sufficient for commercial applications. The yields could be increased by optimizing the media components in basic/basal medium. The enhancement of enzyme yields can be achieved either by conventional or statistical approach. The conventional 'one-variable-at-a-time' approach of optimization is often tedious and time-consuming but is also often incapable of reaching the true optimum, especially due to it's failing to identify the interactions between factors (Kennedy et al. 1994). However, these hurdles could be substantially overcome by adapting statistical approach for optimizing the parameters (Lee et al. 1997; Banker et al. 2009; Kumar and Satyanarayana 2007; Farid et al. 2013). The statistical approaches undertake a rational study by using suitable statistical designs that are adequately experimental to decrease the number of experiments. The statistical approach can be either executed directly through response surface methodology (RSM) or Plackett-Burman factorial design followed by RSM (Lee et al. 1997, 1998, 1999; Moradpour et al. 2013). Plackett-Burman design (Plackett and Burman 1946) is a widely used statistical technique for screening and selection of most significant culture variables, while the response surface methodology (Box and Wilson 1951) provides important information regarding the optimum level of each variable along with its interactions with other variables and their effects on product yield.

2.2.2 Physico-chemical characteristics of cholesterol oxidase

Cholesterol oxidases from several microorganisms have been purified and characterized. Various properties of microbial cholesterol oxidases (viz. molecular weight, pH and temperature optima, stability, and inhibition characteristics) are summarized in Table 2.2. The molecular weights of the cholesterol oxidases have been reported in the range of 47-60

Table 2.2 Physico-chemical properties of cholesterol oxidase enzymes from different sources.

Source organism	Mol.weight (kDa)	*pI*	K_m (µM) for cholesterol	pH optima	pH stability	Temp. Optima (°C)	Temp. stability	Inhibitors	Activators	References
Arthrobacter simplex USA18	57	-	28.40	7.5	pH 6-10 (30 °C, 2 h)	50	-	*p*-chloromercuric benzoate, Hg^{2+}	-	Liu et al. 1988
Arthrobacter sp. IM 79	63	-	-	7.5-8.5	-	40-50	-	-	-	Wilmanska and Sedlaczek 1988
Arthrobacter simplex F2	55	8.5	-	-	-	-	-	-	-	Chen et al. 2006
Bacillus sp. (a) CO1	36	-	6.76	6.25	-	60	60 ° C, 1 h (90 %)	-	-	Rhee et al. 2002
(b) CO2	37		4.50	6.0		40	-do-			
Bordetella sp.	55	-	556	7.0	pH 4-10, 80 % (4 °C, 2 h)	37	50 ° C, 2 h (80 %)	Hg^{2+}, Ag^{+}	Cu^{2+}	Lin et al. 2010
Brevibacterium sterolicum	32.5	8.9	-	7.5	pH 4-10 (37 °C, 30 min)	-	50 ° C, 30 min (100 %)	*p*-chloromercuric benzoate, Hg^{2+}, Ag^{+}	-	Uwajima et al. 1974
Brevibacterium sterolicum	55	4.9-5.1	140	-	-	-	-	-	-	Gadda et al. 1997

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Source organism	Mol.weight (kDa)	*pI*	K_m (µM) for cholesterol	pH optima	pH stability	Temp. Optima (°C)	Temp. stability	Inhibitors	Activators	References
Brevibacterium sterolicum (a) COX I	54.9	8.9	1100	7.0	-	-		*p*-chloromercuric benzoate, Hg^{2+}, Ag^{+}	-	Fujishiro et al. 2002
(b) COX II	46.5	4.7	30	6.5	pH 5-8, ~100 % (50 °C, 1 h)	55			-	
Brevibacterium sp.	-	-	-	7.5	-	53	45 ° C, 30 min (80 %)	-do-	-	Salva et al. 1999
Brevibacterium sp.	-	8.5	-	7.5	pH 6-8, (37 °C)	60	-	Hg^{2+}, Ag^{+}	-	Wang et al. 2007
Chromobacterium sp. DS-1	58		26	7.0	pH 3-11, >80 % (30 °C, 1 h)	65	85 ° C, 30 min (80 %)	Zn^{2+}	-	Doukyu et al. 2008
Corynebacterium cholesterolicum	57	8.7	-	7.0-7.5	pH 5-9 (30 °C, 1 h)	40-42	50 ° C, 30 min (96 %)	Hg^{2+}, Ag^{+}	-	Shirokane et al. 1977
Enterobacter sp.	58	-	120	7.0	-	25	60 ° C, 30 min (30 %)	Hg^{2+}, Fe^{3+}, Ag^{+}	Cu^{2+}	Ye et al. 2008
Nocardia sp.	-	-	14	7.0	-	-	-	Hg^{2+}	-	Richmond 1973
Nocardia rhodocrous	-	-	24	7.0	-	30	-	Hg^{2+}	-	Cheetham et al. 1982

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Source organism	Mol.weight (kDa)	*pI*	K_m (µM) for cholesterol	pH optima	pH stability	Temp. Optima (°C)	Temp. stability	Inhibitors	Activators	References
γ-*Proteobacterium* Y-134 (a) CHO-A	115	4.3	65	6.5	pH 5-8, >60 % (60 °C, 1h)	50	60 ° C, 1 h (90 %)	*HQ, *PHMS, *o*-Phenanthroline	Mn^{2+}, Mg^{2+},Ni^{2+}	Isobe et al. 2003
(b) CHO-U	58	7.0	26	6.0	pH 5-8.5, >80 % (60 °C, 1h)	-do-	70 ° C, 1 h (60 %)	*HQ, *PHMS, *o*-Phenanthroline, Co^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}	-	Isobe et al. 2003
Pseudomonas sp. COX629	56	-	200	7.0	pH 5-8, (60 °C, 1 min)	-	70 ° C, 30 min (85 %)	Hg^{2+}, Zn^{2+} ,Fe^{2+}	Mn^{2+}	Lee et al. 1989
Pseudomonas sp. ST-200	60	-	4.04	7.0	pH 5.0-8.5	60	60 ° C, 30 min (73 %)	-	-	Doukyu and Aono 1998
Rhodococcus erythropolis ATCC 25544	55	-	31	-	-	-	-	-	-	Sojo et al. 1997
Rhodococcus sp. GK $_1$	40	-	20	7.0-7.5	-	30	-	-	-	Kreit et al. 1992
Rhodococcus equi No. 23	56	9.1	900	7.8	-	47	50 ° C, 10 min (100 %)	Hg^{2+}, Ag^{+} , N-bromosuccinimide, *p*-chloromercuric benzoate	-	Watanabe et al. 1989

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Source organism	Mol.weight (kDa)	*pI*	K_m (µM) for cholesterol	pH optima	pH stability	Temp. Optima (°C)	Temp. stability	Inhibitors	Activators	References
Rhodococcus sp. GK_1	80	-	19	6.0-8.2	-	-	-	-	-	Kreit et al. 1994
Rhodococcus sp. GK_1	59	8.9	-	-	-	30	60 ° C, 20 min (10 %)	Hg^{2+}, Zn^{2+}, 2-[(ethylmercurio)-thio]benzoic acid	-	Elalami et al. 1999
Rhodococcus sp. PTCC 1633	55	-	15	7.5	pH 5-9, ~100 % (4 °C, 24 h)	40	40 ° C, 24 h (65 %)	-	-	Yazdi et al. 2001
Rhodococcus sp. R_{14-2}	60	8.5	55	7.0	pH 4-10, 100 % (4 °C, 2 h)	50	50 ° C, 30 min (100 %)	Hg^{2+}, Cu^{2+} Ag^{+}, Zn^{2+} , Fe^{3+} , *p*-chloromercuric benzoate, fenpropimorph	-	Wang et al. 2008
Schizophyllum commune	53	-	330	5.0	-	-	-	-	-	Fukuyama and Miyake 1979
Streptoverticillium cholesterolicum	56	-	400	7.0-7.5	pH 4-12.5, 100 % (25 °C, 1 h)	-	-	Hg^{2+}, Cu^{2+}, Fe^{3+}, Ag^{+} N-bromosuccinimide, iodine, *FDB	-	Inouye et al. 1982

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Source organism	Mol.weight (kDa)	*pI*	K_m (µM) for cholesterol	pH optima	pH stability	Temp. Optima (°C)	Temp. stability	Inhibitors	Activators	References
Streptomyces violascens	-	-	450, 670	7.5	-	50	-	Fe^{2+}, Fe^{3+}, Ag^{+}, KCN, NaN_3, N-bromosuccinimide, iodine	-	Tomioka et al. 1976
Streptomyces violascens	61	-	-	-	-	-	-	-	-	Kamei et al. 1978
Streptomyces sp.	35	-	-	7.0	-	-	-	-	-	Lartillot and Kedziora 1990
Streptomyces hygroscopicus	53	4.4-4.5	200	-	-	-	-	-	-	Gadda et al. 1997
Streptomyces fradiae	60	-	70.60	7.0	pH 4-10, 100 % (4 °C, 4 h)	70	70 ° C, 1 h (10 %)	-	-	Yazdi et al. 2001
Streptomyces polychromogenes IFO 13072	52	-	2500	7.0	pH 6-7, (25 °C)	37	-	Hg^{2+}, Fe^{2+}, dithiothreitol, mercaptoethanol, isonicotinic acid	-	Kim et al. 2002
Streptomyces parvus	55	-	20	7.2	pH 6-10, 40 % (50 °C, 30 min)	50	55 ° C, 30 min (68 %)	Hg^{2+}, Pb^{2+} Ag^{2+}, Zn^{2+}	Mn^{2+}	Praveen et al. 2011
Streptomyces sp.	62	-	101.30	7.0	pH 6-8, 80% (4 °C, 24 h)	37	30 - 40° C, 120 min (90 %)	Hg^{2+}, Ba^{2+}	Cu^{2+}	Niwas et al. 2013

* PHMS = 2-Pyridylhydroxymethane sulfate; HQ = 8-Hydroxyquinoline; FDB = 1-fluoro-2,4-dinitrobenzene

kDa. Microbial cholesterol oxidases generally have neutral pH optima and possess stability over a wide range. The enzymes have temperature optima in the range 40-70 °C (Doukyu 2009).

2.2.2.1 Substrate specificity and kinetic parameters of cholesterol oxidase

The length and structure of the 17-side chain on the steroid ring D often affect the oxidation rate. Next to cholesterol, most enzymes oxidized β-cholestanol at a high rate. The double bond between the positions of the 5 and 6 do not seems to be very important for this enzyme activity. The sterols with the short side chain to be oxidized at a low rate (Doukyu 2009). Generally the pregnenolone was oxidized by most enzymes. However, the cholesterol oxidase obtained from γ-*Proteobacterium* was unable to oxidized pregnenolone (Isobe et al. 2003). The protein genetic engineering is the best approach in modifying the thermostability, catalytic activity and substrate specificity of the protein. Toyoma et al. (2002) succeeded in the alteration of substrate specificity of *Streptomyces* cholesterol oxidase by site directed mutagenesis. The catalytic efficiencies (k_{cat}/K_m) of the S379T mutant for cholesterol and pregnenolone were 1.8- and 6.0-folds higher than those of the wild type, respectively. Doukyu et al. (2009) determined the K_m and V_{max} values of cholesterol oxidases from various bacterial origins. The K_m values were 26.2, 18.4, 18.8, 76.8, 183 and 315 μM from *Chromobacterium* sp. DS-1, *Nocardia* sp., *N. erythropolis*, *B. cepacia*, *P. fluorescens* and *Streptomyces* sp., respectively. Thus, the K_m values of *Nocardia* sp. were relatively lower than those of other enzymes. The V_{max} value of the *Chromobacterium* sp. DS-1 enzyme were the highest among the enzymes tested.

2.2.2.2 Effect of pH and temperature on the cholesterol oxidase activity and stability

The cholesterol oxidase obtained from *Rhodococcus* sp. R_{14-2} showed maximum activity at pH 7.0. The purified enzyme retained its full activity at pH range 4-10 for 2 h at 4 °C, but at higher or lower pH values the enzyme activity was decreased significantly (Wang et al. 2008). The cholesterol oxidase from *Bordetella* sp. exhibited optimum activity at a pH value around 7.0 and retained more than 80 % of the initial activity in the pH range of 4.0-10.0 at 4 °C for 48 h and was more stable at pH 5.0 (Lin et al. 2010). The cholesterol oxidase from *Streptomyces parvus* was most active at pH 7.2 and was stable from pH 4.0 to 11. A loss of 40 % activity occurred at pH 11 at 50 °C for 30 min (Praveen et al. 2011). The cholesterol oxidase from *Streptomyces* sp. was most active at pH 7.0 and retained 80 % activity at pH 6-8.0 for 24 h, but at higher or lower pH, the enzyme activity was decreased significantly and at pH 10, only 10.16 % enzyme was found to be active (Niwas et al. 2013).

The optimum temperature of cholesterol oxidase activity from *Rhodococcus* sp. GKI was found to be around 30 °C. At temperatures 45 °C, the enzyme retained its 92 % relative activity to the optimal activity. At the temperatures 20, 30 and 40 °C enzyme was stable for 2 h while, the half-life of cholesterol oxidase activity at 50 °C was around 70 min (Elalami et al. 1999). Cholesterol oxidase obtained from *Rhodococcus* sp. R_{14-2} retained its maximum activity for 30 min at 50 °C but at temperatures higher than 60 °C, enzyme rapidly lost its activity and at 70 °C was completely inactivated within 1 h (Wang et al. 2008). Cholesterol oxidase from *Chromobacterium* sp. strain DS-1 is highly thermo-stable in comparison to the cholesterol oxidases obtained from various bacterial sources such as *Streptomyces* sp., *Cellulomonas* sp., *Nocardia* sp., *Nocardia erythropolis*, *Pseudomonas fluorescens*, and *B. cepacia* ST-200 (Doukyu et al. 2008). All of these commercial enzymes lost most of their activity after incubation for 30 min at 60-80 °C (Doukyu and Aono 2001; Doukyu et al. 2008). The CD spectra analysis of recombinant DS-1 enzyme and *B. cepacia* ST-200 enzyme at various temperatures showed that in both enzymes, the transitions of the β-sheet to other

secondary structures were observed at higher temperatures. In particular, the loss of the β-sheet of the *B. cepacia* ST-200 enzyme was more distinctive than that of the *Chromobacterium* sp. strain DS-1 enzyme. Since the thermal stability of the *B. cepacia* ST-200 enzyme was remarkably lower than that of the *Chromobacterium* sp. strain DS-1 enzyme, it was suggested that the thermal stability of these enzymes might depend on the structural stability of the β-sheet at high temperatures (Doukyu et al. 2009). Nishiya et al. (1998) improved the thermal stability of *Streptomyces* cholesterol oxidase by random mutagenesis. The half-life of a multiple mutant (S103T, V121A and R135H) showing the highest stability was 52.2 min at 60 °C, while that of the wild type was 7.8 min. The *Chromobacterium* sp. strain DS-1 enzyme retained more than 90 % of its original activity after incubation for 240 min at 60 °C (Doukyu et al. 2008). Therefore, the thermal stability of the *Chromobacterium* sp. strain DS-1 enzyme is much higher than that of the multiple mutants from the *Streptomyces* enzyme. Praveen et al. (2011) reported that cholesterol oxidase from *Streptomyces parvus* was stable from 4 °C to 65 °C. 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.

2.2.2.3 Effect of detergents and solvents on the cholesterol oxidase activity

The solubility of cholesterol in water is very low. It is often solubilized with detergents as well as organic solvents. However, detergents and organic solvents often influence the cholesterol oxidase activity (Shirokane et al. 1977; Cheetham et al. 1982; Pollegioni et al. 1999) and often inactivate cholesterol oxidase enzymes (Isobe et al. 2003; Doukyu and Aono 2001; Doukyu et al. 2008, 2009). Cholesterol oxidase has been used for the optical resolution of 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, a cholesterol oxidase with high activity and stability in the presence of a wide range of detergents and organic solvents would be useful for these applications. Isobe et al. (2003) reported a detergent-tolerant cholesterol oxidase from γ-*Proteobacterium* Y-134. The Y-134 enzyme retained more than 80 % of its original activity in 0.5 % Triton X-405 and sodium cholate after incubation for 1 h at 60 °C. In this experimental condition, commercially available enzymes from *Nocardia*, *Brevibacterium*, and *Streptomyces* lost most of their activities. Doukyu and Aono (2001) examined the stability of cholesterol oxidase activity from the *Burkholderia cepacia* with the 0.1 % or 1 % detergent at 30 °C for 1 h together with commercially available enzymes of *Pseudomonas* sp., *Nocardia erythropolis*, *Streptomyces* sp. SA-COO and *Brevibacterium* sp. None of the cholesterol oxidases tested was markedly inhibited by the addition of Triton X-100, Tween 20, sodium cholate and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS). However, the commercial cholesterol oxidases from *Brevibacterium* sp. and *Streptomyces* sp. SA-COO were partially inactivated by the addition of 1 % sarcosyl. Furthermore, the all of the commercial cholesterol oxidases were completely inactivated by the addition of 0.1 % SDS, whereas the recombinant enzyme from the *Burkholderia cepacia* retained about 60 % activity, even in the presence of 1 % SDS. Doukyu et al. (2008) examined the stability of the *Chromobacterium* sp. strain DS-1 enzyme in the presence of 0.5 % detergents at various temperatures together with commercially available enzymes of *Streptomyces* sp., *Cellulomonas* sp., *Nocardia* sp., *N. erythropolis*, and *P. fluorescens*. All cholesterol oxidases tested were stable in Tween 20, Triton X-100, Triton X-405, sodium cholate, and sodium polyoxyethylene alkyl ether sulfate (Emal 20 CM) after incubation for 1 h at 30 °C. Commercially available enzymes were completely inactivated by the addition of ionic

detergents such as sodium dodecyl sulfate (SDS) or sodium lauryl benzenesulfonate (LBS) after incubation for 1 h at 30 °C. By contrast, the *Chromobacterium* sp. strain DS-1 enzyme was relatively tolerant to SDS and LBS. In addition, the treatment with sodium dodecyl sarcosinate or Emal 20 CM completely inactivated all of the enzymes except the *Chromobacterium* sp. strain DS-1 enzyme after 1 h at 60 °C. The *Chromobacterium* sp. strain DS-1 enzyme was relatively tolerant to these detergents even at 60 °C. Praveen et al. (2011) showed that cholesterol oxidase obtained from *Streptomyces parvus* exhibit good stability in the presence of a wide range of detergents (0.5 %). 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 %), cholesterol oxidase 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. Niwas et al. (2013) has reported that cholesterol oxidase from *Streptomyces* sp. shown remarkable stability in the presence of different detergents (0.5 %) such as Tween-80, Triton X-100, Triton X-114 and sodium cholate and organic solvents. The enzyme activity was found to be highest in Triton X-100. However, presence of only 0.1 % SDS fully inactivated the enzyme.

Laane et al. (1987) had reported that organic solvents with log P_{ow} (logarithm of partition coefficient) values less than 2.0 inactivate most enzymes through structural denaturation. Pollegioni et al. (1999) examined the stability of cholesterol oxidases from *Streptomyces hygroscopicus* and *B. sterolicum* in the presence of various concentrations of isopropanol. The activity of the *B. sterolicum* enzyme is rapidly inactivated, whereas the *S. hygroscopicus* enzyme retained 70 % of the initial activity after 5 h in the presence of 30 % 2-propanol at 25 °C. Doukyu and Aono (2001) reported that the recombinant cholesterol oxidase from the *Burkholderia cepacia* was inactivated by acetone after incubation for 12 h at 37 °C. However, the enzyme retained about 70 % activity after 3 h in the presence of acetone.

The recombinant enzyme was partially inactivated by isopropanol or butanol and was scarcely inactivated by the addition of ethyl acetate, chloroform, benzene, toluene, *p*-xylene, diphenylmethane or cyclooctane. All of the commercial cholesterol oxidases were completely inactivated by the addition of acetone or isopropanol. The treatment with ethyl acetate and butanol completely inactivated the enzymes of *Brevibacterium* sp. and *Streptomyces* sp. SA-COO and partially inactivated the enzymes of *Pseudomonas* sp. and *Nocardia erythropolis*. The recombinant enzyme was relatively tolerant to these solvents. None of the cholesterol oxidases tested was inactivated markedly by the solvents with log P_{ow} values above 4.1. Doukyu et al. (2008) examined the stability of various cholesterol oxidases in the presence of organic solvents. Commercially available cholesterol oxidases, including *Streptomyces* sp., *Cellulomonas* sp., *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 24 h incubation at 37 °C. By contrast, *Chromobacterium* sp. strain DS-1 and *B. cepacia* ST-200 enzymes were stable in the presence of all solvents except for acetone. Lin et al. (2010) reported that cholesterol oxidase from *Bordetella* sp. mostly inactivated by methanol, ethanol, and acetone. However, the enzyme was stable in the presence of ethyl acetate, butanol, chloroform, benzene, xylene, or cyclohexane. This enzyme was stable even in the presence of dimethylsulfoxide, which has a low log P_{ow} value. Praveen et al. (2011) reported that cholesterol oxidase from *Streptomyces parvus* showed moderate stability (50-75 %) towards different organic solvents (10 %) such as methanol, ethanol, ethylacetate, isopopanol and dimethylsulfoxide except acetone, benzene, and chloroform. The increase in concentrations of isopropanol up to 5-10 % (v/v) showed an increase in cholesterol oxidase activity while the activity was decreased with further increase in the solvent concentration. Niwas et al. (2013) has reported that cholesterol oxidase from

Streptomyces sp. retained more than 80 % activity with organic solvents such as methanol, ethanol, isopropanol, dimethylsulfoxide and benzene except acetone and chloroform.

2.2.2.4 Effect of chemicals and metal ions on the cholesterol oxidase activity

The cholesterol oxidase activity reduced with the thiol reducing agents such as glutathione reduced, dithiothreitol and β-mercaptoethanol (Uwajima et al. 1974; Kim et al. 2002; Praveen et al. 2011; Niwas et al. 2013). Chelating agents such as EDTA, *o*-phenanthroline, and 8-hydroxyquinoline, did not show a significant inhibitory effect on the enzyme activity (Tomioka et al. 1976; Inouye et al. 1982; Isobe et al. 2003; Doukyu et al. 2008). However, the enzyme activity was completely inhibited by N-bromosuccinimide and iodine (Tomioka et al. 1976; Inouye et al. 1982). Metal ions generally did not exert any remarkable effect on the enzyme activity. However, in many cases cholesterol oxidase activity is markedly inhibited by SH inhibitors such as, Hg^{2+}, or Ag^{+} (Table 2.2). By contrast, Ag^{+} scarcely influenced the activity of the enzyme from *Chromobacterium* sp. strain DS-1 (Doukyu et al. 2008). $FeCl_3$ and $FeSO_4$ remarkably inhibited the activity of the enzyme from *S. violascens* (Tomioka et al. 1976). However, $FeCl_3$ partially inhibited the enzyme activity from the *Enterobacter* sp. (Ye et al. 2008). $CuSO_4$ scarcely inhibited the activity of the enzyme from *Streptoverticillium cholesterolicum* and *Rhodococcus equi* No. 23 (Inouye et al. 1982; Watanabe et al. 1989). However, the $CuSO_4$ substantially activated the enzyme from *Enterobacter* sp. and *Bordetella* sp. (Ye et al. 2008; Lin et al. 2010). The addition of *p*-chloromercuric benzoate partially reduced the activity of the enzymes from *B. sterolicum* (Uwajima et al. 1974), *A. simplex* (Liu et al. 1988) and *Rhodococcus equi* No. 23 (Watanabe et al. 1989), where as *p*-chloromercuric benzoate and fenpropimorph drastically inhibited the enzyme activity from *Rhodococcus* sp. (Wang et al. 2008). The activities of the enzymes

from *Pseudomonas* sp. COX629, γ-*Proteobacterium* and *Streptomyces parvus* were partially activated by the addition of Mn^{2+} (Lee et al. 1989; Isobe et al. 2003; Praveen et al. 2011).

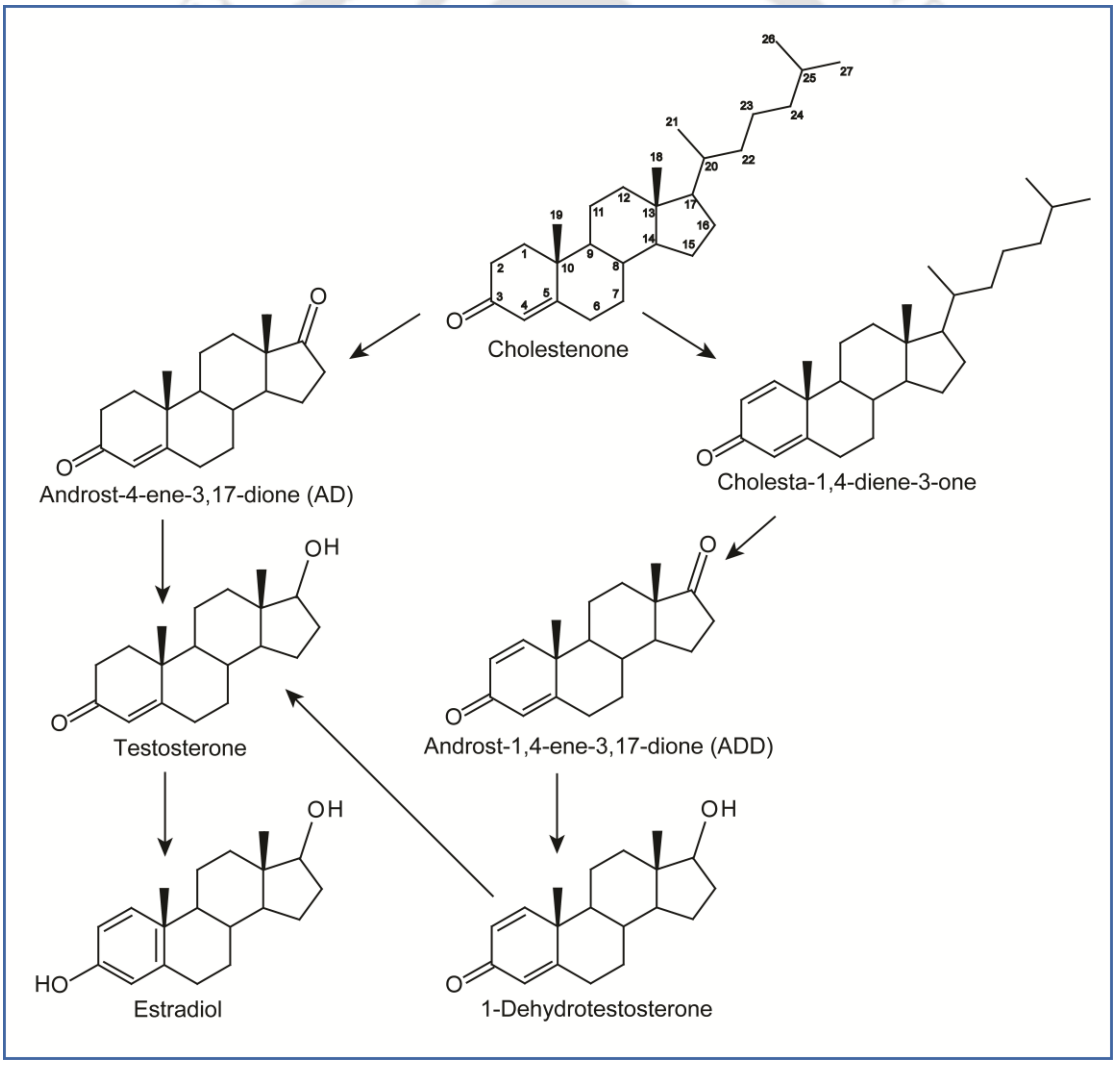
2.3 Immobilization of cholesterol oxidase

Cholesterol oxidase has been immobilized in several support materials such as, glass beads (Suman and Pundir 2003), cellulose acetate and poly (2-hydroxyethyl methacrylate-co-glycidyl methacrylate) membranes (Wang et al. 2004; Akgöl et al. 2002), polypyrrole and polyaniline films (Singh et al. 2004; Wang and Mu 1999; Singh et al. 2006), gold nanoparticle and multiwalled carbon nanotube based nanocomposites (Eguílaz et al. 2011; Saxena et al. 2011), mesoporous materials (Murai and Kato 2011), poly(acrylamide-co-acrylic acid)/ polyethyleneimine and polyvinyl alcohol (PVA) hydrogels (Akkaya et al. 2009; Marques et al. 2012), chitosan (Yapar et al. 2009), sepharose particles (Chen et al. 2013) and silk mat (Saxena and Goswami 2010). However, more focus has recently been laid on the use of biocompatible, biodegradable and low cost materials such as, chitosan to immobilize enzyme for industrial applications (Yapar et al. 2009; Krajewska 2004; Aranaz et al. 2009). The immobilization of enzymes on support materials may be executed by different methods such as adsorption, covalent cross-linking, encapsulation and entrapment. However, the success of an immobilization method largely depends on the nature of the support material and the reaction environment being used (Brady and Jordaan 2009; Sassolas et al. 2012).

2.4 Production of cholestenone

Cholestenone is a derivative of the cholestane, which is a saturated steroid with 27-carbon atom. It serves as the basis of precursor for many organic molecules. It differs from the cholesterol in having double bond at C_5-C_4 position and a keto group at C_3 position.

Cholestenone has many pharmaceutical applications as shown in the scheme 2.3. This has been used as a precursor for the synthesis of hormones and many intermediate steroidal compounds such as androst-4-ene-3,17-dione (AD) and androsta-1,4-diene-3,17-dione (ADD), which are eventually used to produce anabolic drugs and contraceptive (Lee et al. 1993; Sugano et al. 1995; Chaudhari et al. 2010). This ketosteroid also has anti-obesity effect thus depicting its potential as food additive or medicine for preventing obesity related diseases (Suzuki 1993). Cholestenone has been produced by chemically as well as biologically.



Scheme 2.3 The schematic diagram on the conversion of cholestenone to pharmaceutically important products.

2.4.1 Chemical methods

Cholestenone could be produced from cholesterol by means of chemical procedures. Oppenauer (1941) had synthesized cholestenone using cholesterol along with acetone, benzene and aluminum *tert*-butoxide (catalyst) by heating the reaction mixture in an oil bath maintained at 75-85 °C. Eastham and Teranishi (1955) also produced cholestenone from cholesterol by using toluene, cyclohexanone and aluminum isopropoxide (catalyst). The cholestenone production from the chemical procedures is generally not preferred due to the fact that the action of oxidizing agents on the hydroxysteroid is vigorous and uncontrollable. Due to this, the chemical procedures produce relatively small yields of the desired ketosteroid and even these small amounts were also contaminated with large amounts of undesirable oxidation products (Schmidt and Hughes 1944).

2.4.2 Biological methods

2.4.2.1 Whole cell based catalysis

Cholestenone is generally produced through biotransformation route using microbial cells as catalysts. Buckland et al. (1975) reported the formation of 7 g/h cholestenone using 100 g of *Nocardia* cells in 200 ml of carbon tetrachloride containing 16 % (w/v) cholesterol, at 20 °C, while Liu et al. (1996) reported that 94.6 % of added cholesterol could be converted to cholestenone by the 6 g wet cells of *Arthrobacter* in 300 ml aqueous/carbon tetrachloride two-phase system containing 10 % (w/v) cholesterol at 30 °C.

2.4.2.2 Enzyme based catalysis

Myamato and Toyoda (1994) reported the cholestenone production from cholesterol by using the cholesterol oxidase of *Rhodococcus* as catalyst. Chen-feng et al. (2001) reported

the biotransformation of cholesterol in aqueous/*n*-octane two phase systems by cholesterol oxidase from *Brevibacterium* sp. Nearly 92 % cholesterol conversion was achieved using 16 U of cholesterol oxidase and 40 g/l of cholesterol under the oxygen flow rate of 40 l/min and shaking rate of 300 rpm at 40 °C in 40 min. Marques et al. (2012) reported 36 M of cholestenone production in 300 h with immobilized cholesterol oxidase and catalase. The production was carried out within Y-shaped microchannel reactors in an aqueous/organic two-phase system. Substrate was delivered from the organic phase to aqueous phase containing cholesterol oxidase and the product formed partitions back to the organic phase. The aqueous phase was then forced through a plug-flow reactor, containing immobilized catalase which degrade the hydrogen peroxide formed as a by-product during cholesterol oxidation, to avoid cholesterol oxidase deactivation.

Chapter 3

Production of Cholesterol Oxidase from *Rhodococcus* sp. NCIM 2891

3.1 Overview

Cholesterol oxidase (EC 1.1.3.6) is widely used in clinical settings to detect cholesterol (MacLachlan et al. 2000) and steroids (Ahmad et al. 1992) in biological samples. Cholesterol oxidase also has potential applications for the biotransformation of sterols, optical resolution of of allylic alcohols, production of steroid hormones precursors, biosynthesis of antifungal antibiotics etc (Doukyu 2009). Cholesterol oxidase is a flavoenzyme that catalyses the oxidation and isomerization of cholesterol to cholest-4-en-3-one (Kreit and Sampson 2009). The enzyme is mainly generated in bacteria, although there are a few reports of cholesterol oxidase production in fungi as well (Fukuyama and Miyake 1979). This family of enzymes occurs in both the membrane-bound and secreted forms (Inouye et al. 1982; Kreit et al. 1994; Sojo et al. 1997). Cholesterol oxidase enzymes from different microbial sources have been purified and characterized (Doukyu 2009); however, reports on cholesterol oxidase production methods are limited (Aihara et al. 1986; Kreit et al. 1994; Lee et al. 1998; Lee et al. 1999; Yazdi et al. 2001; Chang et al. 2005; Chauhan et al. 2009) even though the applications of cholesterol oxidase have been rapidly expanding.

Cholesterol oxidase produced from *Rhodococcus* strains has gained importance due to its high stability and activity over a wide range of pH values (Wang et al. 2008) and its high yields (Aihara et al. 1986; Watanabe et al. 1989). *Rhodococcus* species can produce both secreted and cell-bound forms of cholesterol oxidase (Watanabe et al. 1989; Kreit et al. 1992; Kreit et al. 1994; Sojo et al. 1997). We first studied the effect of different nutritional factors

on the production of total cholesterol oxidase by a *Rhodococcus* sp. NCIM 2891 strain, following the classical 'one-variable-at-a-time' method. The medium components were then analysed for their significant effects on enzyme production using a statistical Plackett-Burman factorial design (Plackett and Burman 1946). Further, we used a full-factorial central composite design (CCD) for response surface methodology (RSM) to derive important information on the optimal level of each variable, along with its interactions with other variables and its effects on product yield. A detailed account on the findings is presented here.

3.2 Experimental approaches

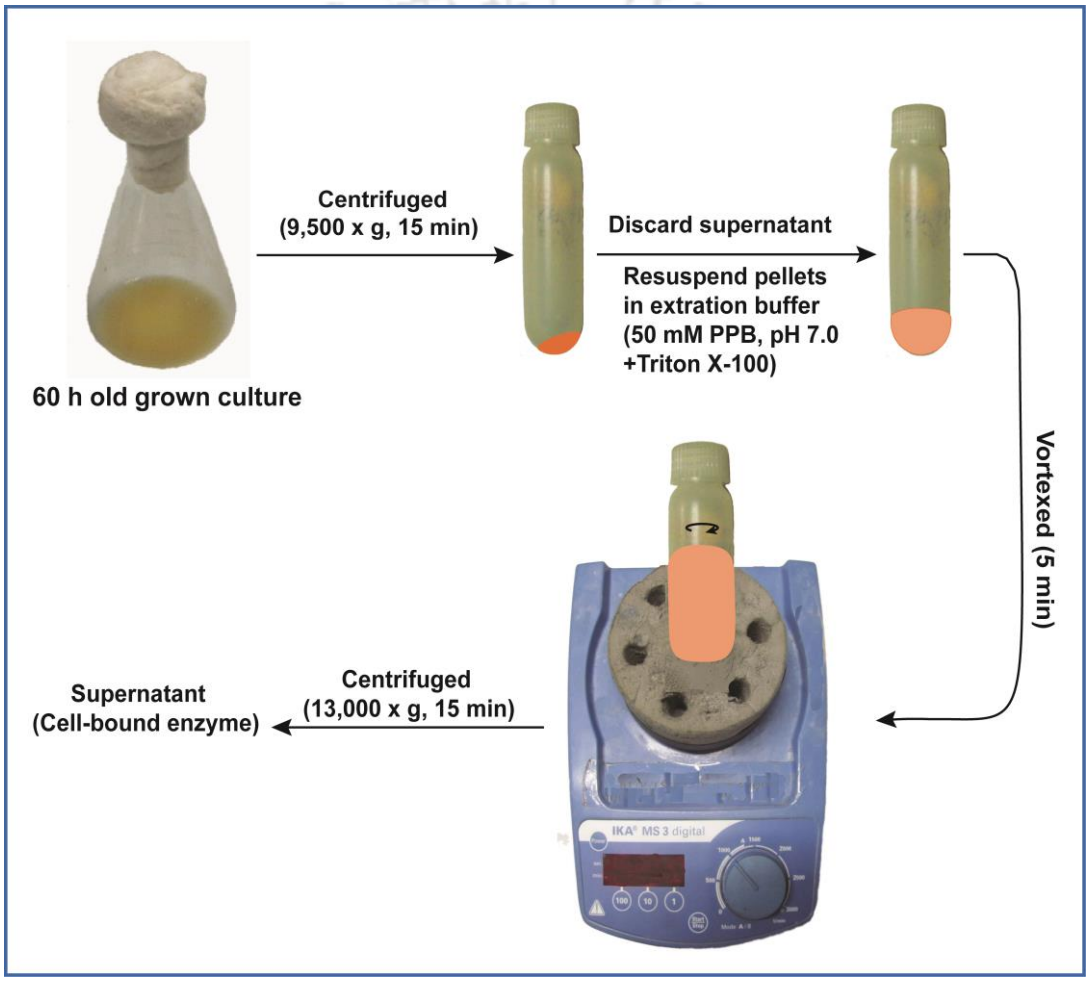
3.2.1 Microorganism and culture conditions

Rhodococcus sp. NCIM 2891 was obtained from the National Collection of Industrial Micro-organisms (NCIM) (National Chemical Laboratory, Pune, India). This strain was cultured in the modified culture medium (basal medium) based on (Yazdi et al. 2001), which contained the following additives (in g/l): $(NH_4)_2HPO_4$, 2.0; yeast extract, 5.0; K_2HPO_4, 0.25; $FeSO_4 \cdot 7H2O$, 0.1; NaCl, 1.0; cholesterol, 2.0; and Tween 80, 2.0 (v/v). The pH was adjusted to 7.0. The active seed culture (~ 1×10^9 bacterial cells) was added to 50 ml of sterilized medium in 250 ml Erlenmeyer flasks and incubated at 30 °C on a rotary shaker (200 rpm). To measure the bacterial cell count, 1 ml samples were withdrawn from the flask at 12 h growth intervals and diluted by 10-fold with phosphate buffered saline which contained the following (in g/l): NaCl, 8.0; KCl, 0.2; Na_2HPO_4, 1.44 and KH_2PO_4, 0.24; pH 7.4. Nutrient agar plates were uniformly streaked with 100 µl of the diluted broth sample using a cell spreader and were incubated for a maximum of 6 days at 30° C. The colonies formed on each plate were

then counted to estimate the colony forming unit (CFU) per ml of the original sample (CFU/ml).

3.2.2 Isolation and assay of enzyme

After 60 h of cultivation, bacterial cultures were centrifuged at 9,500×*g* for 15 min at 4° C and the collected supernatant fluid was used as the source of the extracellular enzyme.



Scheme 3.2.1 Schematic representation of isolation of cell-bound cholesterol oxidase from *Rhodococcus* sp. NCIM 2891.

The cell pellets were re-suspended in 5 ml of 50 mM potassium phosphate buffer pH 7.0, containing 1 % Triton X-100 (extraction buffer) and mixed vigorously for 5 min with a

vortex mixture (IKA MS 3 digital). The cell-homogenate was then centrifuged (13,000×*g* for 15 min) to collect the cell-free supernatant, which was used for measuring cell-bound cholesterol oxidase (Scheme 3.2.1). Cholesterol oxidase activity was determined by cholesterol oxidase and horseradish peroxidase (HRP)-coupled assay method. The method involves monitoring H_2O_2, formed in the reaction by measuring 2,2′-azino-bis [3-ethylbenzothiazoline-6-sulphonic acid] diammonium salt (ABTS) radical at $\lambda_{405\ nm}$ (ε_{405nm} = 18400/M/cm) at 35° C (Werner et al. 1970). The enzyme was assayed by kinetic assay method in a final volume of 1 ml containing 50 mM potassium phosphate buffer (pH 7.0); HRP, 1080 U/ml (5 µl); ABTS, 14 mg/ml (50 µl); cholesterol 3 mM (50 µl). A clear solution of 20 mM cholesterol was prepared in 10 ml capped glass bottle vial by dissolving powdered cholesterol in 0.5 ml Triton X-100 (100 %) by heating at ~165 °C for 5 min and then made up the volume to 5 ml with distilled water and stored as stock solution at 4 °C. Every time before the experiment, the stock solution was heated to ~165 °C to get a clear solution and then a suitable volume of it was diluted with water containing 1 % Triton X-100 to get a final cholesterol concentration of 3 mM for using as ready stock solution. The amount of enzyme activity (U/ml) was calculated using the Eq. 3.1.

$$\text{Enzyme activity (U/ml)} = \frac{\Delta A_{405nm/min} \times \text{Volume of reaction mixture (ml)}}{36.8 \times \text{Volume of enzyme used (ml)}} \qquad (3.1)$$

where 36.8 is the millimolar extinction coefficient for two molecules of oxidised ABTS because the reduction of one molecule of H_2O_2 requires two molecules of ABTS. One enzyme unit was defined as the amount of enzyme that forms 1 µmol hydrogen peroxide/min at 35° C.

3.2.3 Optimization of medium components by the experimental designs

The culture medium was optimized using the Plackett-Burman design and CCD. Each factor was examined at two levels: -1 and +1 for low and high levels, respectively. A centre point was run to evaluate the linear and curvature effects of the variables. The Plackett-Burman experimental design is based on a first-order polynomial model as shown in Eq. 3.2.

$$Y = \beta_{\circ} + \sum \beta_i x_i \qquad (3.2)$$

where Y is the response (productivity), β_o is the model intercept, β_i is the linear coefficient and x_i is the level of the independent variable. This model was used to screen and evaluate the medium components influencing cholesterol oxidase production. Seven assigned variables were screened in twelve experimental runs with an additional two runs made at their centre points as shown in Table 3.6.1. From regression analysis of the variables, factors with greater than 95 % significance ($P < 0.05$) were considered to have a significant effect on cholesterol oxidase production and were further optimized by RSM.

A CCD with five settings for each factor was used to evaluate the quadratic effects and two-way interactions among these variables. A full factorial CCD was used to optimize the key variables for cholesterol oxidase production in shaking cultures. The variables used were cholesterol, $(NH_4)_2HPO_4$, and yeast extract each at five coded levels ($-\alpha$,-1, 0, +1, $+\alpha$) as shown in Table 3.6.2. The relation between the coded forms and the actual value of the variables are described in Eq. 3.3.

$$x_i = \frac{X_i - X_0}{\Delta X_i},\ i = 1, 2, 3.....,k \qquad (3.3)$$

where x_i is a coded value and X_i is the actual value of variable, X_0 is the actual value of the same variable at the centre point, and ΔX_i is the step change of the variable.

These three factors each with five coded levels consisting of 20 experimental runs that included six replicates at the centre point with α = 1.682 to measure the entire range of variable combinations as shown in Table 3.6.3. For predicting the optimal point, a second-order polynomial model (Eq. 3.4) was used to correlate the relationship between the variables and the response.

$$Y = \beta_{\circ} + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i} \sum_{j} \beta_{ij} X_i X_j \qquad (3.4)$$

where Y is the predicted response, k is the number of factor variables, β_o is the model constant, β_i is the linear coefficient, β_{ii} is the quadratic coefficient, and β_{ij} is the interaction coefficient.

Statistical analysis was performed using MINITAB 15, which was used to evaluate the analysis of variance (ANOVA) to determine the significance of each term in the fitted equations and to estimate the goodness of fit in each case. The fit of the model was checked by the coefficient of determination R^2. As the values of R^2 approach 1, the model better explains the variability between the experimental and predicted values. To validate the conditions optimized with CCD, we used the newly optimized medium to study cholesterol oxidase production.

3.3 Results and discussion

3.3.1 Optimization of cholesterol oxidase production using classical methods

The effects of pH, temperature, cultivation time, and different types of carbon and nitrogen sources on cholesterol oxidase production by *Rhodococcus* sp. NCIM 2891 were studied in the basal medium using the ‘one-variable-at-a-time’ method. The optimal

conditions for cholesterol oxidase production in the basal medium were 30° C, pH 7.0 and 60 h cultivation time. Enzyme production under these optimal conditions was 0.335 U/ml. Cell-bound cholesterol oxidase contributed half of the total enzymatic activity in the basal medium. To know the effect of different carbon sources for cholesterol oxidase production, steroidal and non-steroidal (glucose and *n*-hexanoate) substrates were studied. All of the tested carbon substrates produced cholesterol oxidase; however, only the steroidal substrates (β-sitosterol, cholesterol and stigmasterol) produced a significant increase in cholesterol oxidase levels. Out of the tested sterols, cholesterol produced the most cholesterol oxidase (Fig.3.5.1a). We also tested the effects of the combinations of inorganic and organic nitrogen sources on cell growth and cholesterol oxidase production. The combination of $(NH_4)_2HPO_4$ and yeast extract supported the highest enzyme production (0.353 U/ml) (Fig. 3.5.1b). When grown in the control medium containing 0.2 % cholesterol, cholesterol oxidase activity reached 0.346 U/ml. Increasing the cholesterol content from 0.2 % to 0.35 % also increased the cholesterol oxidase activity by 24.15 %, to 0.433 U/ml. Cholesterol oxidase production reached saturation at 0.4 % cholesterol (Fig. 3.5.2). Adding $(NH_4)_2HPO_4$ to the medium stimulated the production of cholesterol oxidase (Fig. 3.5.2). Maximum cholesterol oxidase activity (0.397 U/ml) was observed for media containing $(NH_4)_2HPO_4$ concentrations of 0.25 %. However, at higher concentrations of $(NH_4)_2HPO_4$ the enzyme production declined. The effects of adding yeast extract in medium on cholesterol oxidase activity were also tested. Yeast extract first caused a noticeable effect at 0.1 % concentration and reached a maximum activity of 0.483 U/ml at 0.9 %. Further increases in yeast extract concentrations did not improve cholesterol oxidase production (Fig. 3.5.2). Optimal concentrations of $(NH_4)_2HPO_4$, yeast extract and cholesterol in our classically optimized medium were 2.5, 9.0 and 3.5 g/l,

respectively. Under these optimal conditions the enzyme production was 0.75 U/ml, with cell-bound cholesterol oxidase accounting for 65.71 % of the total enzyme activity.

3.3.2 Evaluation of significant components by the Plackett-Burman design

Cholesterol oxidase activity varied widely (0.157±0.01 to 1.248±0.02 U/ml) in the experiments (Table 3.6.1), which indicates the potential for optimizing cholesterol oxidase production. The analysis of regression coefficients, *t*-values and *P*-values for these seven variables is shown in Table 3.6.4. Both the *t*-value and the *P*-value (< 0.05) were used to confirm the significance of the parameters studied. Cholesterol was significant to a 99.4 % confidence level, while $(NH_4)_2HPO_4$ and yeast extract had significance levels of 99.9 % and 98.9 %, respectively. Based on *P*-values, cholesterol, $(NH_4)_2HPO_4$, and yeast extract were chosen for further optimization. Because NaCl, K_2HPO_4, Tween 80 and $FeSO_4 \cdot 7H_2O$ did not have significant effects on cholesterol oxidase production, they were added in the cultivation medium at their median values for subsequent experiments.

3.3.3 Optimization of medium components by central composite design

The significant variables from the Plackett-Burman factorial design were further examined by CCD. The fitting of the second order polynomial model was verified by the coefficient of determination, R^2. The model shows a high determination coefficient (R^2 = 99.82), which explains almost 100 % of the variability in the response. The regression coefficients were calculated, and the regression equation obtained is shown in the Eq. 3.5.

$$\begin{aligned} Y_{COX\ activity} &= 1.73869 + 0.40542X_1 + 0.18352X_2 + 0.50360X_3 \\ &\quad - 0.20465X_1^2 - 0.22551X_2^2 - 0.22957X_3^2 \\ &\quad + 0.37375X_1X_2 + 0.17550X_1X_3 + 0.03450X_2X_3 \end{aligned} \qquad (3.5)$$

where Y, X_1, X_2, and X_3 represent the response, and the amounts of cholesterol, $(NH_4)_2HPO_4$, and yeast extract, respectively. The F-value of model is 612.62 ($p < 0.05$), which indicates that the model is a good fit and explains most of the observed response variation. All of the model terms X_1, X_2, X_3, X_1^2, X_2^2 X_3^2 $X_1 X_2$, $X_1 X_3$ and $X_2 X_3$ exhibited confidence levels above 95 % ($P < 0.05$), which indicates that our chosen variables (cholesterol, $(NH_4)_2HPO_4$, yeast extract, and their interaction effects) were significant in our model equation for both linear and quadratic fits as shown in Table 3.6.5. The regression equation is graphically represented by the contour plots to visualise the effect of the component concentration on the expected response (Fig 3.5.3). It is evident that the interactions of cholesterol with yeast extract (Fig. 3.5.3a) cholesterol and $(NH_4)_2HPO_4$ (Fig. 3.5.3b) are highly significant, while the interaction between yeast extract and $(NH_4)_2HPO_4$ (Fig. 3.5.3c) is only moderately significant.

3.3.4 Experimental validation of the medium composition optimized by central composite design

The CCD model predicted that maximum cholesterol oxidase activity of 3.36 U/ml would occur when the medium contained cholesterol, $(NH_4)_2HPO_4$, and yeast extract at the concentrations of 8.95 g/l, 10.57 g/l and 26.75 g/l, respectively identified from our optimization studies and keeping the other components at their centre point concentrations. The model was experimentally validated by RSM for the statistically optimized medium. Cholesterol oxidase production in the statistically optimized medium is shown in Fig. 3.5.4a. The cholesterol oxidase production reached a maximum of 3.25 U/ml at nearly 60 h of cultivation, which agrees with the predicted value. Cell-bound cholesterol oxidase accounted for 90.68 % of the total enzyme activity in the statistically optimized broth. By comparing cell-bound and extracellular cholesterol oxidase production, we showed that the optimized

media increased cell-bound cholesterol oxidase levels by 40.52 %, while only marginally increasing the extracellular cholesterol oxidase by 15.15 % from basal medium levels.

The growth rate and enzyme production rate within the exponential phases (36-60 h) of growth were calculated from the slope of the lines shown in Fig. 3.5.4b. The growth rates (CFU/ml/h) in statistically optimized, classically optimized and basal medium were 3 x 10^7, 3 x 10^7 and 2 x 10^7, respectively, while the corresponding enzyme production rates (U/ml/h) were 0.087, 0.011 and 0.002. Thus, the ratio (x10^{-10}) of enzyme production rate to growth rate in statistically optimized, classically optimized and basal medium are 29, 3.6 and 1, respectively. These results imply that the ratio of the enzyme production rate to the growth rate in statistically optimized medium is 8.05-fold greater than the classically optimized medium, and 29-fold higher than the basal medium. The results demonstrated that the production of the cell-bound cholesterol oxidase though linked with growth of the cells mass the level of its production (viz. activity per unit cell mass) could be increased by adjusting the critical nutrient components identified through this investigation at the statistically determined ratio. The cell mass obtained here by using the growth stimulating nutrients, namely yeast extract and $(NH_4)_2HPO_4$, supports the accumulation of the cholesterol oxidase that is induced by the cholesterol substrate in the cell wall.

The literature on the optimization of cholesterol oxidase production by microorganisms using statistical method is few. Various compounds such as cholesterol and yeast extract (Lee et al. 1998; Yazdi et al. 2001), glycerol, malt extract and soyabean meal (Chauhan et al. 2009), potato starch, yeast extract, malt extract and peptone (Varma and Nene 2003) are reported as substrates for the enhanced production of cholesterol oxidase. The cholesterol oxidase production obtained by us (3.25 U/ml) following the statistical method is

comparable and even improved than many reported values (Lee et al. 1998; Yazdi et al. 2001; Chauhan et al. 2009; Varma and Nene 2003).

3.4 Conclusions

This work demonstrated that statistical methods were effective in determining the optimized concentration of medium components for cholesterol oxidase production than the classical method. Statistically optimized medium can drastically increase cholesterol oxidase production in *Rhodococcus* sp. NCIM 2891 compared to production in the classically optimized medium. Enhanced cholesterol oxidase production was largely due to an increase in cell-bound cholesterol oxidase. The enzyme production rate far exceeded the organism's intrinsic growth, thus implying that production of the cell-bound cholesterol oxidase though linked with cell growth, the level of its production in terms of activity per unit cell mass could be increased by using critical nutrient components at statistically determined ratios. The high production of cell-bound cholesterol oxidase by *Rhodococcus* sp. NCIM 2891 in a simple medium under suitable environmental conditions is therefore a potential source of cholesterol oxidase for industrial applications.

3.5 Figures

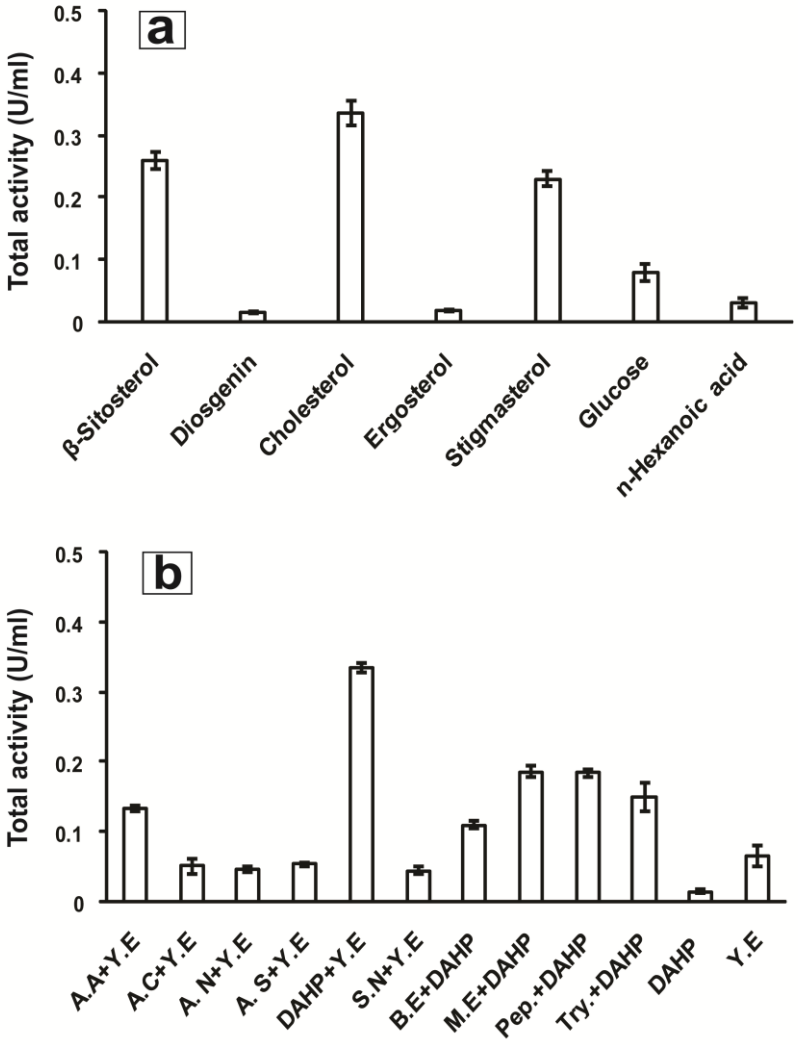


Fig. 3.5.1 Effects of (**a**) carbon sources (0.2 % w/v) and (**b**) inorganic (0.2 % w/v) and organic (0.5 % w/v) nitrogen sources on cholesterol oxidase production by *Rhodococcus* sp. NCIM 2891. A.A = CH_3COONH_4, A.C = NH_4Cl, A.N = NH_4NO_3, A.S = $(NH_4)_2SO_4$, S.N = $NaNO_3$, DAHP = $(NH_4)_2HPO_4$, B.E = Beef extract, M.E = Malt extract, Y.E = Yeast extract, Pep = Peptone, Try = Tryptone

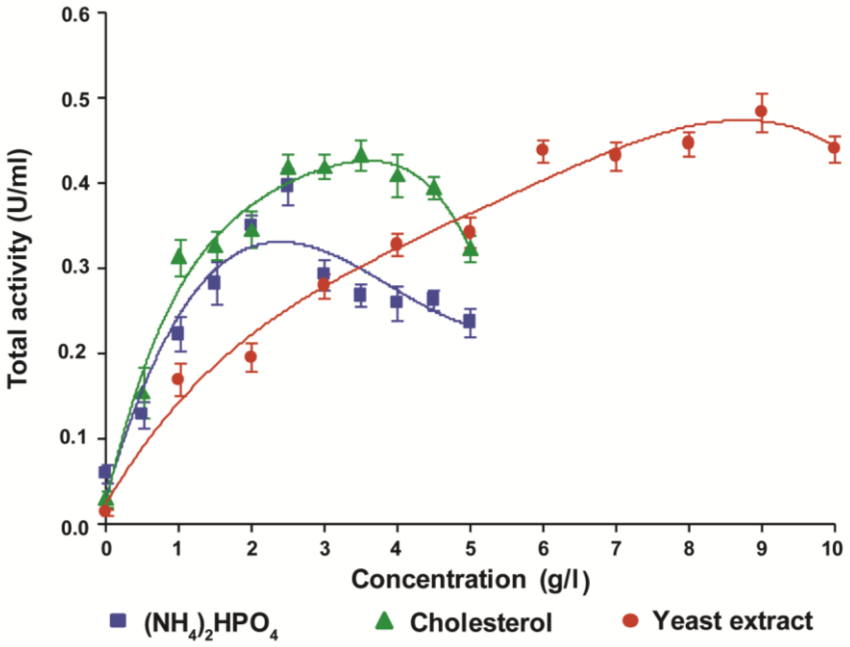


Fig. 3.5.2 Effect of different concentrations of $(NH_4)_2HPO_4$,, cholesterol and yeast extract on cholesterol oxidase production by *Rhodococcus* sp. NCIM 2891.

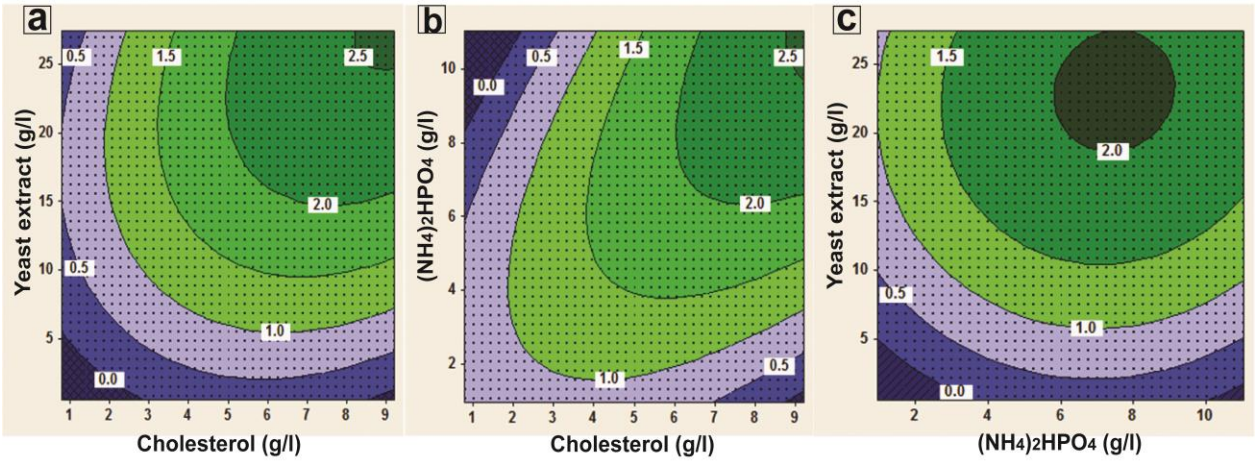


Fig. 3.5.3 Contour plots showing the effect of (**a**) yeast extract and cholesterol, (**b**) $(NH_4)_2HPO_4$ and cholesterol and (**c**) yeast extract and $(NH_4)_2HPO_4$ on cholesterol oxidase production by *Rhodococcus* sp. NCIM 2891.

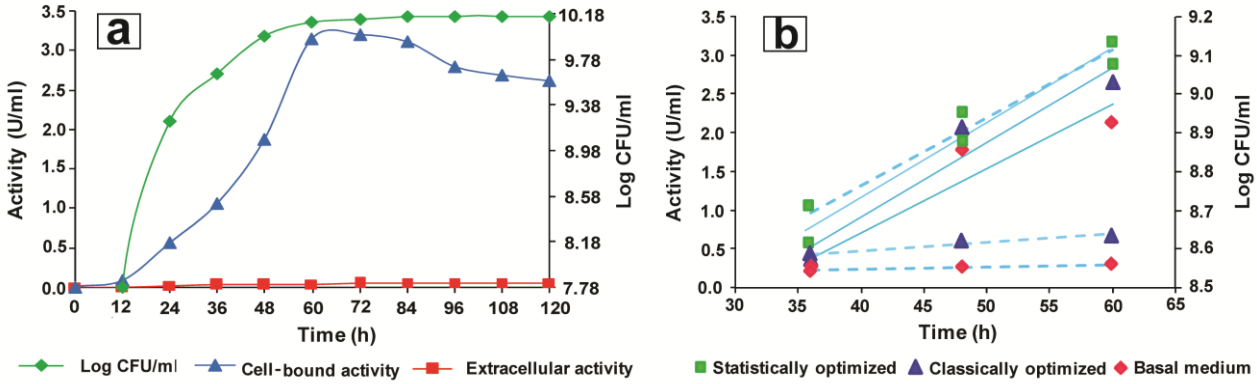


Fig. 3.5.4 (**a**) Production profile of cholesterol oxidase by *Rhodococcus sp.* NCIM 2891 in statistically optimized medium. (**b**) Comparison of growth (bold line) and enzyme production (dotted line) in statistically optimized, classically optimized and basal medium.

3.6 Tables

Table 3.6.1 Plackett-Burman design for seven variables, with the observed results for cholesterol oxidase activity.

Run order	Cholesterol (g/l)	$(NH_4)_2HPO_4$ (g/l)	Yeast extract (g/l)	NaCl (g/l)	K_2HPO_4 (g/l)	Tween 80 (g/l)	$FeSO_4 \cdot 7H_2O$ (g/l)	ChOx[a] (U/ml)
1	5.00	0.50	18.00	0.50	0.10	0.50	0.70	0.767±0.03
2	5.00	5.50	6.00	7.50	0.10	0.50	0.10	0.952±0.02
3	2.00	5.50	18.00	0.50	0.90	0.50	0.10	0.935±0.04
4	5.00	0.50	18.00	7.50	0.10	4.50	0.10	0.437±0.01
5	5.00	5.50	6.00	7.50	0.90	0.50	0.70	0.742±0.02
6	5.00	5.50	18.00	0.50	0.90	4.50	0.10	1.248±0.02
7	2.00	5.50	18.00	7.50	0.10	4.50	0.70	0.609±0.01
8	2.00	0.50	18.00	7.50	0.90	0.50	0.70	0.327±0.02
9	2.00	0.50	6.00	7.50	0.90	4.50	0.10	0.157±0.01
10	5.00	0.50	6.00	0.50	0.90	4.50	0.70	0.276±0.03
11	2.00	5.50	6.00	0.50	0.10	4.50	0.70	0.378±0.01
12	2.00	0.50	6.00	0.50	0.10	0.50	0.10	0.319±0.01
13	3.50	3.00	12.00	4.00	0.50	2.50	0.40	0.535±0.03
14	3.50	3.00	12.00	4.00	0.50	2.50	0.40	0.523±0.02

[a] Values are mean ± SD (n = 3); ChOx (cholesterol oxidase)

Table 3.6.2 Central composite design levels.

Factor (g/l)	Factor code	Levels				
		-α	-1	0	+1	+α
Cholesterol	X_1	0.79	2.50	5.00	7.50	9.20
$(NH_4)_2HPO_4$	X_2	0.96	3.00	6.00	9.00	11.04
Yeast extract	X_3	0.55	6.00	14.00	22.00	27.45

Table 3.6.3 Full factorial CCD design of three variables and the response of cholesterol oxidase activity.

Run order	Cholesterol (g/l)	$(NH_4)_2HPO_4$ (g/l)	Yeast extract (g/l)	ChOx[a] (U/ml)
1	2.50	3.00	6.00	0.557±0.05
2	7.50	3.00	6.00	0.312±0.02
3	2.50	9.00	6.00	0.145±0.01
4	7.50	9.00	6.00	1.301±0.02
5	2.50	3.00	22.00	1.221±0.02
6	7.50	3.00	22.00	1.584±0.01
7	2.50	9.00	22.00	0.853±0.03
8	7.50	9.00	22.00	2.805±0.02
9	0.79	6.00	14.00	0.447±0.05
10	9.20	6.00	14.00	1.821±0.04
11	5.00	0.95	14.00	0.755±0.04
12	5.00	11.05	14.00	1.395±0.03
13	5.00	6.00	0.55	0.252±0.04
14	5.00	6.00	27.45	1.875±0.06
15	5.00	6.00	14.00	1.745±0.02
16	5.00	6.00	14.00	1.729±0.01
17	5.00	6.00	14.00	1.742±0.01
18	5.00	6.00	14.00	1.737±0.02
19	5.00	6.00	14.00	1.725±0.03
20	5.00	6.00	14.00	1.763±0.02

[a] Values are mean ± SD (n = 3); ChOx (cholesterol oxidase)

Table 3.6.4 Statistical analysis of the Plackett-Burman design showing the coefficient values, *t*-values and *P*-values for each variable for cholesterol oxidase activity.

Variable	Cholesterol oxidase activity (U/ml)			
	Coefficient	*t*-value	*P*-value	Confidence levels (%)
Intercept	0.59558	18.97	0.000	100.00
Cholesterol (X_1)	0.14142	4.50	0.006[b]	99.40
$(NH_4)_2HPO_4$ (X_2)	0.21508	6.85	0.001[b]	99.90
Yeast extract (X_3)	0.12492	3.98	0.011[b]	98.90
NaCl (X_4)	-0.05825	-1.86	0.123	87.70
K_2HPO_4 (X_5)	0.01858	0.59	0.580	42.00
Tween 80 (X_6)	-0.07808	-2.49	0.055	94.50
$FeSO_4 \cdot 7H_2O$ (X_7)	-0.07908	-2.52	0.053	94.70

[b] Indicates ≥ 95% confidence

Table 3.6.5 Model coefficient estimated by multiple linear regressions for cholesterol oxidase activity.

Model term	Parameter estimate	Standard error	Computed *t* -value	*P*- value
Intercept	1.73869	0.01674	103.847	<0.0001
X_1	0.40542	0.01111	36.497	<0.0001
X_2	0.18352	0.01111	16.521	<0.0001
X_3	0.50360	0.01111	45.334	<0.0001
X_1^2	-0.20465	0.01081	-18.925	<0.0001
X_2^2	-0.22551	0.01081	-20.853	<0.0001
X_3^2	-0.22957	0.01081	-21.229	<0.0001
$X_1 X_2$	0.37375	0.01451	25.751	<0.0001
$X_1 X_3$	0.17550	0.01451	12.092	<0.0001
$X_2 X_3$	0.03450	0.01451	2.377	0.0390

Where, X_1 = Cholesterol, X_2 = $(NH_4)_2HPO_4$, X_3 = Yeast extract

Chapter 4

Isolation, Purification and Characterization of Cell-bound Cholesterol Oxidase from *Rhodococcus* sp. NCIM 2891

4.1 Overview

A variety of microorganisms produce cholesterol oxidase, among which *Rhodococcus* species have attracted wide attention for their high production capability of the enzyme (Doukyu 2009; Pollegioni et al. 2009). Both secreted and cell-bound forms of the enzyme are produced by many *Rhodococcus* strains (Sojo et al. 1997; Aihara et al. 1986; Watanabe et al. 1989). The molecular characteristics of the cholesterol oxidase from *Rhodococcus* strains have been explored to some extent (Johnson et al. 1991; Machang'u et al. 1991; Elalami et al. 1999; Sojo et al. 2002; Wang et al. 2008; Yazdi et al. 2008), while the physico-chemical properties of the enzyme which are relevant to the bioprocess development are yet to be adequately elucidated. Here, we report some critical physico-chemical characteristics of the cholesterol oxidase from the *Rhodococcus* sp. NCIM 2891.

4.2 Experimental approaches

4.2.1 Isolation of cell-bound cholesterol oxidase from bacterial culture

The culture conditions of *Rhodococcus* sp. NCIM 2891 and the enzyme production were based on our previous work (Ahmad and Goswami 2013). Briefly, the medium contained the following (in g/l): cholesterol, 3.5; $(NH_4)_2HPO_4$, 9.0; yeast extract, 22.0; K_2HPO_4, 0.25; $FeSO_4 \cdot 7H_2O$, 0.1; NaCl, 1.0; Tween 80, 2.0 (v/v), pH 7.0. The active seed culture (~ 1×10^9 bacterial cells) was added to 50 ml of sterilized medium in 250 ml

Erlenmeyer flasks and incubated at 30 °C on a rotary shaker (200 rpm). The cultures (50 ml) harvested after 60 h of cultivation were subjected to 9,500×*g* for 15 min at 4 °C. The cell-pellets (~ 0.2 g, wet weight) were suspended in 3 ml of 50 mM potassium phosphate buffer (PPB) (pH 7.0), containing different concentrations of Triton X-100 (extraction buffer) and vortexed (MS 3 digital, Germany) for six min as shown in the scheme 3.2.1. The cell homogenates were then subjected to 13,000×*g* for 15 min to collect the cell-free supernatant containing cell-bound enzymes.

4.2.2 Enzyme assay and protein measurement

A clear solution of 20 mM cholesterol was prepared in a 10 ml capped glass bottle vial by dissolving powdered cholesterol in 0.5 ml Triton X-100 (100 %) by heating at ~165 °C for 5 min and then made up the volume to 5 ml with distilled water and stored as stock solution at 4 °C. Every time before the experiment, the stock solution was heated to ~165 °C to get a clear solution and then a suitable volume of it was diluted with water containing 1 % Triton X-100 to get a final cholesterol concentration of 3 mM for using as ready stock solution. For measuring the enzyme activity a total 50 µl of the ready stock solution was diluted with 50 mM PPB (pH 7.0) to 1 ml final reaction volume containing 0.15 mM cholesterol. The reaction was initiated by adding 50 µl of free enzyme solution at 25 °C for 5 min. The reaction with the free enzyme was terminated by suspending the eppendorf tubes containing the reaction mixture in a boiling water bath for 4 min and then cooled to room temperature of 25 °C (RT) before taking the reading. Cholesterol oxidase activity was measured in a spectrophotometer (Cary 100, Agilent Technologies) by taking absorbance of cholestenone formed in the reaction mixture at $\lambda_{240\ nm}$ ($\varepsilon_{240\ nm}$ = 14,000/M/cm) (Kreit et al. 1992). One unit of enzyme activity was defined as the amount of enzyme that forms 1 µmol

cholestenone/min. The kinetic parameters, K_m and k_{cat}, of enzyme were determined by measuring the enzyme activity as described above at different substrate concentration (12-240 µM). The values of kinetic parameters were discerned from the Lineweaver-Burk plot.

For substrate specificity study, the enzyme activity was determined by cholesterol oxidase and horseradish peroxidase (HRP)-coupled assay method. The method involves monitoring H_2O_2, formed in the reaction by measuring 2,2′-azino-bis [3-ethylbenzothiazoline-6-sulphonic acid] diammonium salt (ABTS) radical at $\lambda_{405\ nm}$ ($\varepsilon_{405\ nm}$ = 18400/M/cm) at RT (Werner et al. 1970). The enzyme was assayed by kinetic assay method in a final volume of 1 ml of 50 mM PPB (pH 7.0) containing 50 µl cholesterol oxidase , 5 U HRP, 0.7 mg of ABTS and 0.15 mM sterols. The solutions of different sterols were prepared in the same manner as described elsewhere for the cholesterol.

The protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard. For estimation of protein, the samples were sufficiently diluted to keep the Triton-X100 less than 0.1 %. All experiments were conducted in triplicate and average values have been reported.

4.2.3 Enzyme purification and SDS-PAGE analysis

The cell free supernatant containing cell-bound enzyme was first centrifuged at 13,000×*g* for 15 min to remove the particulate matters. A total 20 ml of clear enzyme solution in a dialysis tube was dialyzed against the 20 mM Tris-HCl buffer, pH 8.0 (1000 ml x 3) overnight at 4 ºC. All the dialyzed enzyme solutions were then subjected to 13,000×*g* for 15 min to discard the insoluble particulate matter before loading into column. A weak anion exchanger column containing Hitrap DEAE-sepharose FF (1.6×2.5 cm, 90 µm) connected to FPLC AKTA system (GE Healthcare) was equilibrated with 20 mM Tris-HCl buffer, pH 8.0.

The enzyme sample was loaded into the pre-equilibrated column at a flow rate of 1 ml/min. The same buffer containing sodium chloride gradient (0-0.5 M) was used for elution of proteins with a flow rate of 3 ml/min and monitored the fractions at $\lambda_{280\ nm}$ by UV detector. The tubes with protein peak containing enzyme activity were pooled, dialysed against 50 mM PPB, pH 7.0, and then concentrated by using PEG 6000 (6 %, w/v) and stored at 4 °C for further use. SDS-PAGE was carried out according to Laemmli (1970) at RT under reducing conditions using stacking and separating gel at 5 % and 12 %, respectively. Coomassie brilliant blue staining was performed (Candiano et al. 2004) to visualize the sample and standard (10-250 kDa, New England Biolab) protein bands in the gel.

4.2.4 Effect of pH, temperature and chemicals on cholesterol oxidase activity and stability

The optimum pH of cholesterol oxidase was determined by analyzing its activity at various pH levels (3.5-11.0). The buffer systems used were sodium acetate buffer (pH 3.5-5.5), PPB (pH 6.0-7.5), Tris-HCl buffer (pH 8.0-9.5), glycine-NaOH buffer (pH 10.0-12.0) each at a concentration of 50 mM. The optimum temperature of enzyme was investigated in the range of 25-65 °C in 50 mM PPB at pH 7.0. Prior to the enzyme assays, substrate solution was also equilibrated at the corresponding temperature. For pH stability, the enzyme was incubated in different pH buffers (5.0-10.0) up to 96 h at 4 °C and residual enzyme activities were determined. The temperature stability was studied by incubating the enzyme at different temperatures *viz.* 40.0, 50.0 and 60.0 °C in water bath for different time periods and residual activities were measured.

The effect of different organic solvents at different concentrations *viz.* 5.0, 10.0 and 15.0 % (v/v) on the activity of enzyme was determined by incubating individually in a reaction mixture for 30 min at RT and then the residual enzyme activities were measured. To

determine the effects of chemicals and metal ions on the enzyme activity, free enzyme was incubated individually with reducing agents (10 mM), inhibitors and metal ions (0.1 mM) at the corresponding final concentrations shown in the brackets at RT for 10 min in the reaction mixture and residual enzyme activities were determined.

The storage stabilities of enzyme were studied at optimum pH value (7.0) at different temperatures in PPB (50 mM). The experiment was conducted by storing the enzyme (8 ml) in three sterile small glass bottle vials separately at RT, 4 °C and –20 °C, respectively. The activities of the incubated enzymes were measured periodically after each week. The half-life ($t_{1/2}$) of the enzyme is calculated by using Eq. (4.1). The decimal reduction time (D), which is defined as the time required for one $\log_{10}$ reduction (90 % reduction) in the enzyme activity, was calculated by using the Eq. (4.2).

$$t_{½} = \frac{0.693}{k_d} \quad (4.1)$$

$$D = \frac{2.303}{k_d} \quad (4.2)$$

4.2.5 Thermo-deactivation studies and estimation of thermodynamic parameters

Thermo-deactivation assays were carried out by preheating the 2 ml enzyme solution (0.54 mg protein/ml) at different temperatures at pH 7.0. A total 50 µl of enzyme samples were collected for different time intervals ranging from 1.5 min to 90.0 min for 55, 60, 65 °C while, 1.5 min to 7.5 min for 70 °C and then cooled to RT. After cooling, the residual activities were determined as described in the section 4.2.2. The thermo-deactivation constant (k_d) and the deactivation energy (E_{ad}) of the purified enzyme was calculated by using the equation 4.3 and 4.4, respectively.

$$\ln(v) = -k_d\, t \tag{4.3}$$

$$\ln(k_d) = \ln(A) - \frac{E_{ad}}{R}\left(\frac{1}{T}\right) \tag{4.4}$$

where, t is the time, k_d is the first-order thermo-deactivation constant since the enzyme activity decreases with time, E_{ad} is the energy required to denature the protein, A is the pre-exponential factor or frequency factor and R is the gas constant (8.314 J/mol/K). The value of k_d is obtained by the slope of the plot of natural logarithm of residual activity, ln (v), versus time, t; while, the value of E_{ad} was estimated from the slope of the plot of ln (k_d) versus /T (K). The enthalpy (ΔH), free energy (ΔG) and entropy (ΔS) were calculated by using the following equations 4.5, 4.6 and 4.7, respectively.

$$\Delta H = E_{ad} - RT \tag{4.5}$$

$$\Delta G = -RT \ln\left(\frac{k_d\, h}{k_B\, T}\right) \tag{4.6}$$

$$\Delta S = \frac{\Delta H - \Delta G}{T} \tag{4.7}$$

where, T is the corresponding absolute temperature (K), h is the Plank constant (11.04×10^{-36} J min), k_B is the Boltzmann constant (1.38×10^{-23} J/K).

4.3 Results and discussion

4.3.1 Isolation and purification of cell-bound cholesterol oxidase

The total yield of enzyme activity in a pooled extract obtained by extracting the bacterial cells twice with the extracting buffer containing 1.0 % Triton X-100 was considered 100 %. Considering this as reference, the total yield of enzyme obtained by extracting with each of the extraction buffers containing 0.2, 0.4, and 0.5 % of Triton X-100 were 85, 97 and 98 %, respectively (Table 4.6.1). Although, the buffer containing 1.0 % Triton X-100 could extract nearly the entire cell-bound enzyme in its first extraction (97 %), we used only 0.4 % Triton X-100 for the extraction considering the fact that the high concentration of Triton X-100 may impedes the downstream processing of the enzyme. Since the extraction efficiencies with the both 0.4 % and 0.5 % Triton X-100 were found to be nearly similar, the former concentration was used for extracting the cell-bound enzyme for further studies. The isolated cholesterol oxidase was purified 3-fold by DEAE sepharose column in a single step and the yield of purification was 88 % (Table 4.6.2). The bound enzyme was started eluting from the column when the concentration of the elution buffer reached 75 % (Fig. 4.5.1). The binding of the cholesterol oxidase to the anion exchange column at the pH 8.0 indicates that the isoelectric point of the enzyme is < 8.0. The purified enzyme solution appeared creamy yellow indicating typical nature of flavoprotein. The solution exhibited three absorption maxima at wavelengths 280, 370, and 450 nm (Fig. 4.5.1). The purified enzyme gave a single band in SDS-PAGE gel with a molecular mass of ~ 55 kDa (Fig. 4.5.2), which is closer to the molecular masses of 55 kDa (Sojo et al. 1997; Yazdi et al. 2008), 59 kDa (Elalami et al. 1999) and 60 kDa (Machang'u et al. 1991; Wang et al. 2008) reported from other *Rhodococcu*s sp.

4.3.2 Substrate specificity and kinetic parameters of cholesterol oxidase

The substrate specificity study was performed with cholesterol oxidase and horseradish peroxidase (HRP)-coupled assay method at $\lambda_{405\ nm}$ to obviate the probable inclusion of error due to the varying molar absorptivities of the carbonyl products may formed from different sterol substrates at $\lambda_{240\ nm}$. The enzyme was active on 3β-hydroxysteroids such as cholesterol, β-sitosterol, stigmasterol and ergosterol, whereas it was inactive on diosgenin, cholesteryl oleate and cholestenone (Table 4.6.3). The enzyme displayed low reactivity with 3β-hydroxysteroids containing unsaturated side chain attached to position 17, such as stigmasterol and ergosterol. The cholesterol oxidase was inactive towards A-ring modified sterols. The length and structure of the 17-side chain on the steroid ring D affect the oxidation rate of sterols. The alkyl chain in sterol molecules appeared to be necessary for the adequate oxidation of the 3β-hydroxy sterol (Pollegioni et al. 2009; Wang et al. 2008). The K_m and k_{cat} values for the cholesterol substrate for the cholesterol oxidase were 151 µM and ~ 1295/min, respectively. The K_m values reported from other *Rhodococcus* sp. cholesterol oxidases for the same substrate were 55 µM (Wang et al. 2008).

4.3.3 Effect of pH and temperature on the cholesterol oxidase activity and stability

The enzyme maximal activities were observed in a broad pH range (Fig. 4.5.3a). The highest activity of enzyme was 20±0.14 U at optimum pH value. The optimum pH range of the enzyme was (6.5-8.0). The optimum temperature for the activity of the enzyme was 45 °C (Fig. 4.5.3b). The optimum pH and temperature ranges of cholesterol oxidase reported from different sources were 5.0-8.5 and 30-70 °C, respectively (Doukyu 2009; Elalami et al. 1999; Wang et al. 2008; Praveen et al. 2011).

The enzyme was stable at pH 7.0 and 8.0 till 96 h of incubation at 4 °C (Fig. 4.5.4a). The $t_{1/2}$ (day) of the enzyme at pH, 5 (4.0), 6 (8.0), 9 (3.0) and 10 (2.5) are shown in the

parentheses. The stabilities of enzyme were diminished steadily with increasing temperature within the studied temperature range of 40-60 °C (Fig. 4.5.4b). For enzyme the K_d at 40, 50 and 60 °C were 2.40×10^{-2}, 6.50×10^{-2}, and 4.51×10^{-1} /h, respectively and the corresponding $t_{1/2}$ (h) were 29, 11 and 1.50, respectively. The enzyme retained their entire activity for 6 weeks when stored at 4 °C and at –20 °C (Fig. 4.5.5). The $t_{1/2}$ of cholesterol oxidase at RT were ~ 6 weeks and it retained 100 % activity for ~ 1week. The storage life of cholesterol oxidase enzyme from *S. lavendulae* NCIM 2421 was 5 days at RT (Varma et al. 2003). However, the storage life of cholesterol oxidase enzyme from *S. parvus* was 6 months at 4 °C (Praveen et al. 2011). The high stability of the *Rhodococcus* sp. NCIM 2891 cholesterol oxidase at RT made it suitable for the biotransformation studies.

4.3.4 Effect of solvents and chemicals on cholesterol oxidase activity

The activity of cholesterol oxidase in all the tested organic solvents except for acetone, propanol and 2-propanol, was only moderately decreased at the solvent concentration of 5 % (v/v) (Table 4.6.4). The activity of cholesterol oxidase in 5 % acetone were reduced to zero, while in 5 % propanol or 2-propanol the activity of the enzyme was increased. When treated with dimethylsulfoxide (DMSO), which is known as an environmentally friendly solvent, the cholesterol oxidase isolated by us retained nearly 91, 82 and 78 % enzyme activities at the corresponding solvent concentration of 5, 10 and 15 %, respectively. Activity of cholesterol oxidase obtained from *P. fluorescens* and *Bordetella* sp. was drastically decreased in the presence of methanol and ethanol (Doukyu et al. 2008; Lin et al. 2010). Similarly, the activity of cholesterol oxidases from different sources such as, from *P. fluorescens, Nocardia* sp., *Cellulomonas* sp. and *Streptomyces* sp., were considerably decreased in the presence of dimethylsulfoxide (Doukyu et al. 2008). Synthesis of steroid

hormones and biotransformation of a number of 3β-hydroxysteroids are generally carried out in presence of organic solvents (Doukyu et al. 1996; Guo et al. 2003) to solubilise the hydrophobic steroidal substrates. There is a growing mandate on the use of non-chlorinated and environmentally safe organic solvent such as DMSO for biotransformation and bioprocess studies. Therefore, the cholesterol oxidase produced by the *Rhodococcus* sp. NCIM 2891 showed good promise for the biotransformation applications of steroidal compounds.

Treatment of the enzyme with thiol reducing agents such as, glutathione reduced, dithiothreitol and β-mercaptoethanol reduced the enzyme activity, suggesting that the disulfide linkage is essential for the activity of enzyme (Table 4.6.5). Sodium azide and ethylenediamine tetraacetic acid (EDTA) also marginally activated the enzyme. It suggests that the enzyme does not have any metal ion as cofactor. Fe^{3+}, Co^{2+}, Cu^{2+}, Mn^{2+} did not exert any remarkable effect on the enzyme activity. However, the addition of Fe^{2+}, Pb^{2+} and Hg^{2+} reduced the activity to 71 %, 61 % and 12 %, respectively; while addition of Ag^{+}, Ca^{2+}, Mg^{2+}, Ni^{2+} and Zn^{2+} at similar concentration (0.1 mM) activated the enzyme by 9 %, 9 %, 15 %, 14 % and 16 %, respectively. Contrary to the findings, the activity of cholesterol oxidase from *Rhodococcus* sp. $R_{14\text{-}2}$ and *Streptomyces* sp. were reported to be decreased by Ag^{+} and Zn^{2+} (Wang et al. 2008; Praveen et al. 2011).

4.3.5 Thermo-deactivation studies of cholesterol oxidase

The thermal-deactivation studies of free cholesterol oxidase indicated the first order deactivation kinetics in the temperature range of 55-70 °C when fitted by linear regression (Fig. 4.5.6a) suggesting its irreversible inactivation at higher temperatures. The calculated value of deactivation energy (E_{ad}) is 228.39 kJ/mol, which agrees with the energy required for

the stable conformational changes of the catalytically active form of enzyme into catalytically inactive form (Klibanov 1983; Rao et al. 2009). The thermal inactivation curve was calculated according to Lopez and Burgos (1995). The thermal inactivation curve traversing one log cycle (*z*) expresses the temperature dependence of thermal inactivation, and it was obtained from the plot of log *D* against temperature as the number of degrees required for the thermal inactivation curve to traverse one log cycle. The *z* value was found to be approximately 9.5 °C (Fig. 4.5.6b). Such low *z* value suggests that the enzyme is more sensitive towards the increase in temperature than the duration of heat treatment (Tayefi-Nasrabadi and Asadpour 2008). The results of the deactivation kinetics and thermodynamic parameters of cholesterol oxidase calculated at different temperature viz 55, 60, 65 and 70 °C were shown in Table 4.6.6. The enthalpy (ΔH) shows practically no dependence on temperature (there is no change in enzyme heat capacity) whereas the free energy (ΔG) decreases with the increasing temperature and the effect was more prominent at 70 °C which is also revealed from the sudden increase in entropy (ΔS) at 70 °C. This high temperature greatly facilitates the rapid destruction of the enzyme structure from ordered into disordered states (Cobos and Estrada 2003). The $t_{1/2}$ of the cholesterol oxidase enzyme at 70 °C is quite less (5.13 min). K_{d70} is 45 times more than the $K_{d55,}$ while K_{d65} and K_{d60} are 6 times and 3 times higher than the K_{d55}. These values also suggest that the cholesterol oxidase is unstable at high temperature (70 °C).

4.4 Conclusion

The cell-bound cholesterol oxidase isolated from the *Rhodococcus* sp. NCIM 2891 was found to be active in a wide pH range with the physiological pH at centre of the range, stable at RT for several weeks and in many organic solvents commonly being used in

biotransformation studies. The enzyme was identified as monomeric flavoprotein. Cholesterol was found to be the best substrate among all the steroids studied for the enzyme. The enzyme does not have any metal ions as a cofactor for its activity as its activity was not inhibited by EDTA. The enzyme was more sensitive towards the increase in temperature than the duration of heat treatment.

4.5 Figure

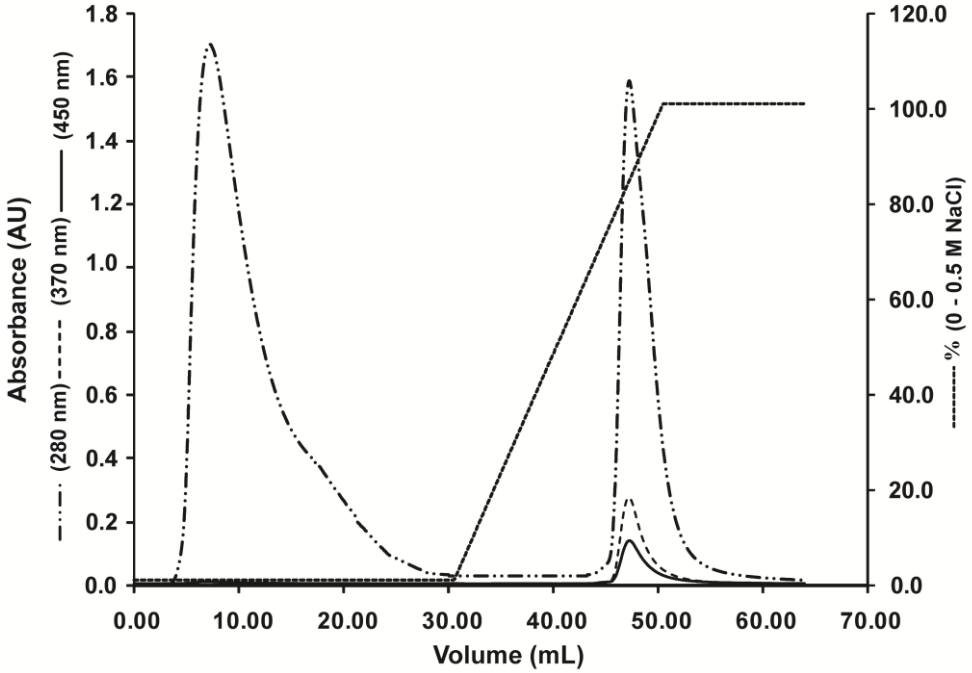


Fig. 4.5.1 Anion exchange chromatogram of cholesterol oxidase. The crude sample (5 ml) was absorbed on to a pre-equilibrated Hitrap DEAE sepharose FF column (1.6×2.5 cm, 90 μm) at a flow rate of 1 ml/min. The protein was eluted with (0-0.5M) NaCl in linear gradient at flow rate of 3 ml/min. The eluted fractions were monitored at λ_{280nm} for protein and at λ_{370nm} and $\lambda_{450\ nm}$ for flavin.

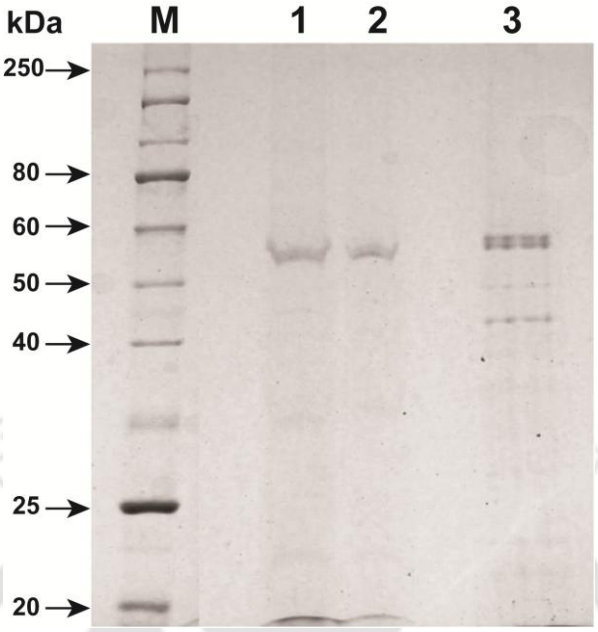


Fig. 4.5.2 SDS-PAGE of cholesterol oxidase protein from *Rhodococcus* sp. NCIM 2891. The protein samples under reducing conditions were subjected to 95 ºC for 10 min and then electrophoresed on 12 % SDS-polyacrylamide gel. The gel was stained with Coomassie G-250. Lane M: marker, Lane 1and 2: purified cholesterol oxidase protein, Lane 3: crude samples. 10 µg proteins were loaded in each lane (1, 2 and 3).

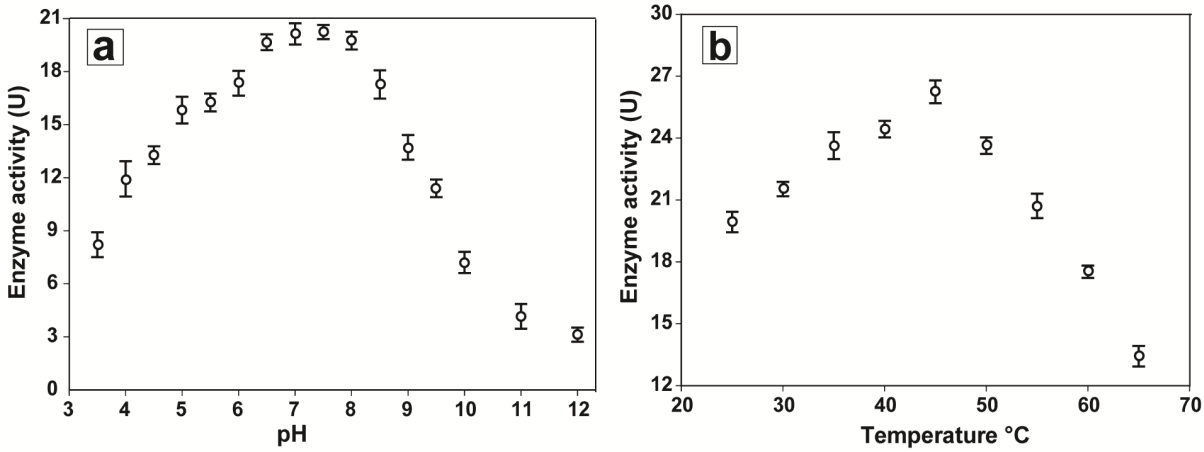


Fig. 4.5.3 (a) pH and **(b)** temperature optima of cholesterol oxidase isolated from *Rhodococcus* sp. NCIM 2891. The buffer systems used for pH optima study were sodium acetate buffer (pH 3.5-5.5), PPB (pH 6.0-7.5), tris buffer (pH 8.0-9.5), glycine-NaOH buffer (pH 10.0-12.0) each at a concentration of 50 mM. For optimum pH, the enzyme activity was assayed for 5 min at 25 °C, while for the optimum temperature the enzyme activity was assayed for 5 min at pH 7.0. Each datum point represents the mean of triplicate values, with the range indicated with error bars.

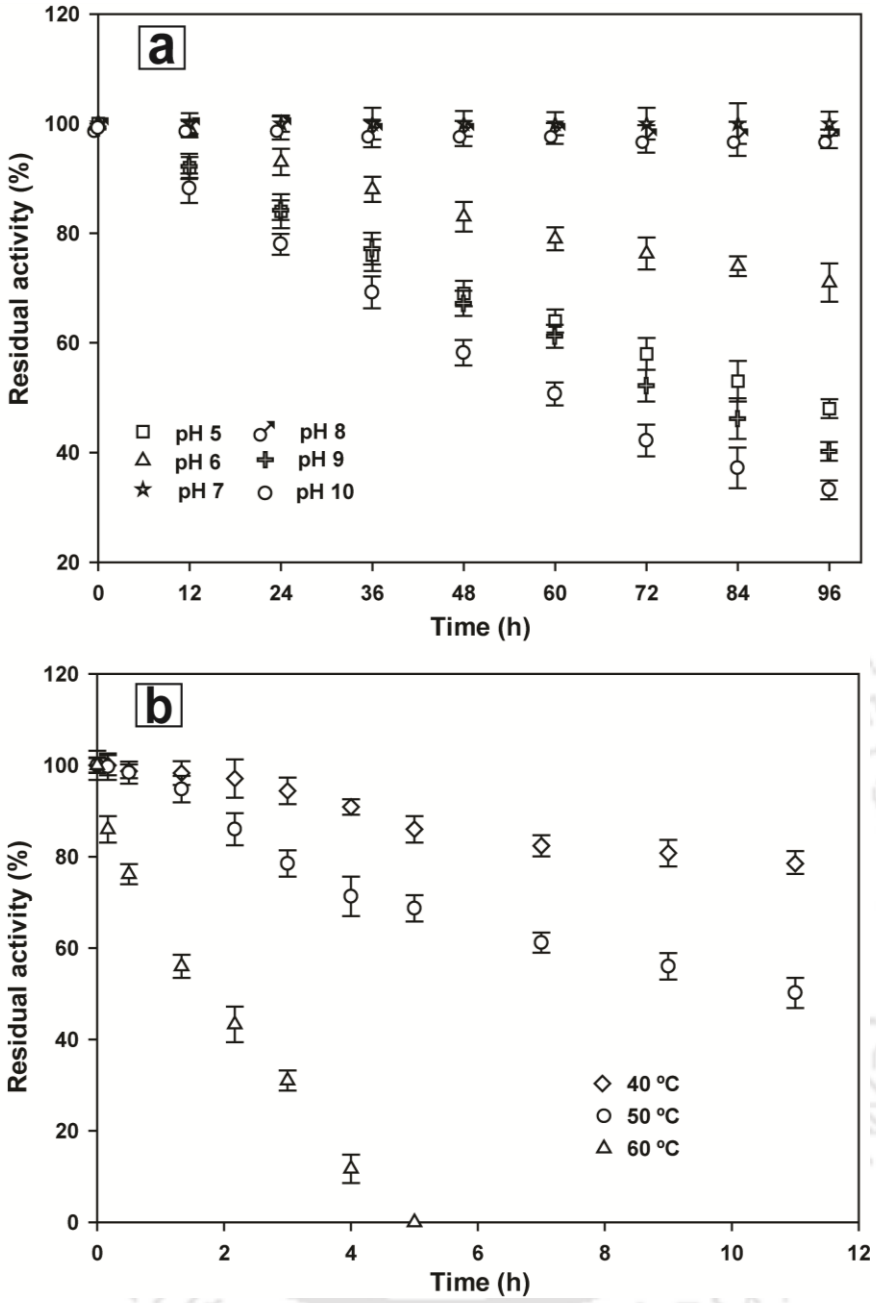


Fig. 4.5.4 (a) pH and **(b)** temperature stability of cholesterol oxidase isolated from the *Rhodococcus* sp. NCIM 2891. The enzyme was incubated in different pH values (5-10) for 96 h at 4 °C and the residual activities were measured. 100 % residual activity of cholesterol oxidase corresponds to 20 U. Each datum point represents the mean of triplicate values, with the range indicated with error bars.

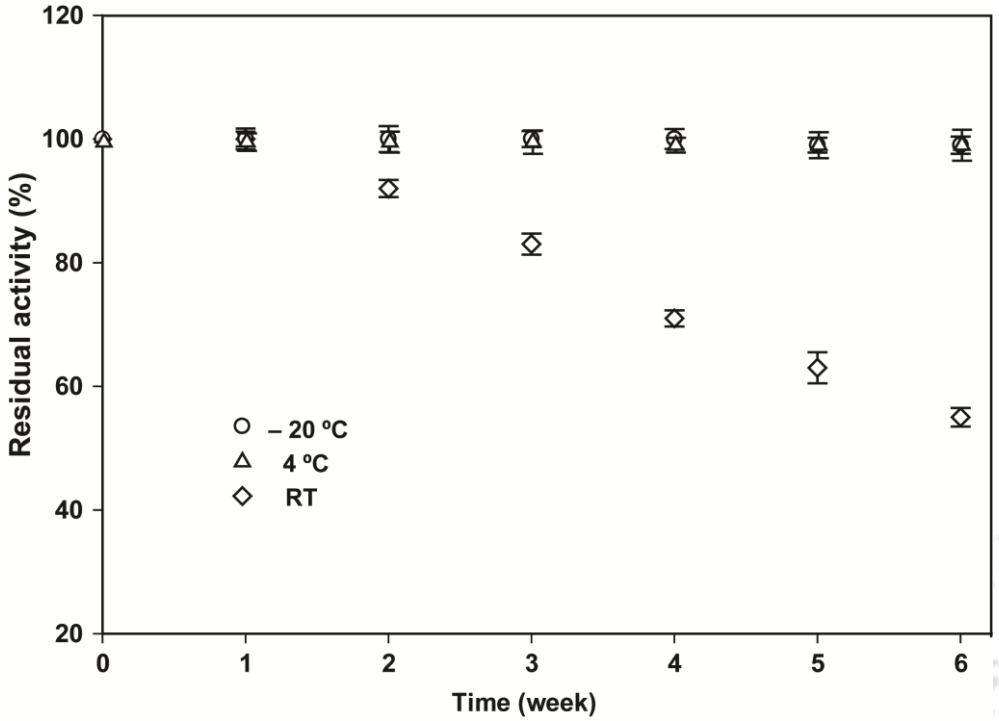


Fig. 4.5.5 Storage stability of the cholesterol oxidase at different incubation temperatures. A total of 8 ml enzyme solution (1225 U/mg) in 50 mM PPB, pH 7.0 was kept in small sterile glass bottle vials and incubated at the specified temperature. The enzyme activity was assayed for 5 min at pH 7.0 and 25 °C. Each datum point represents the mean of triplicate values, with the range indicated with error bars.

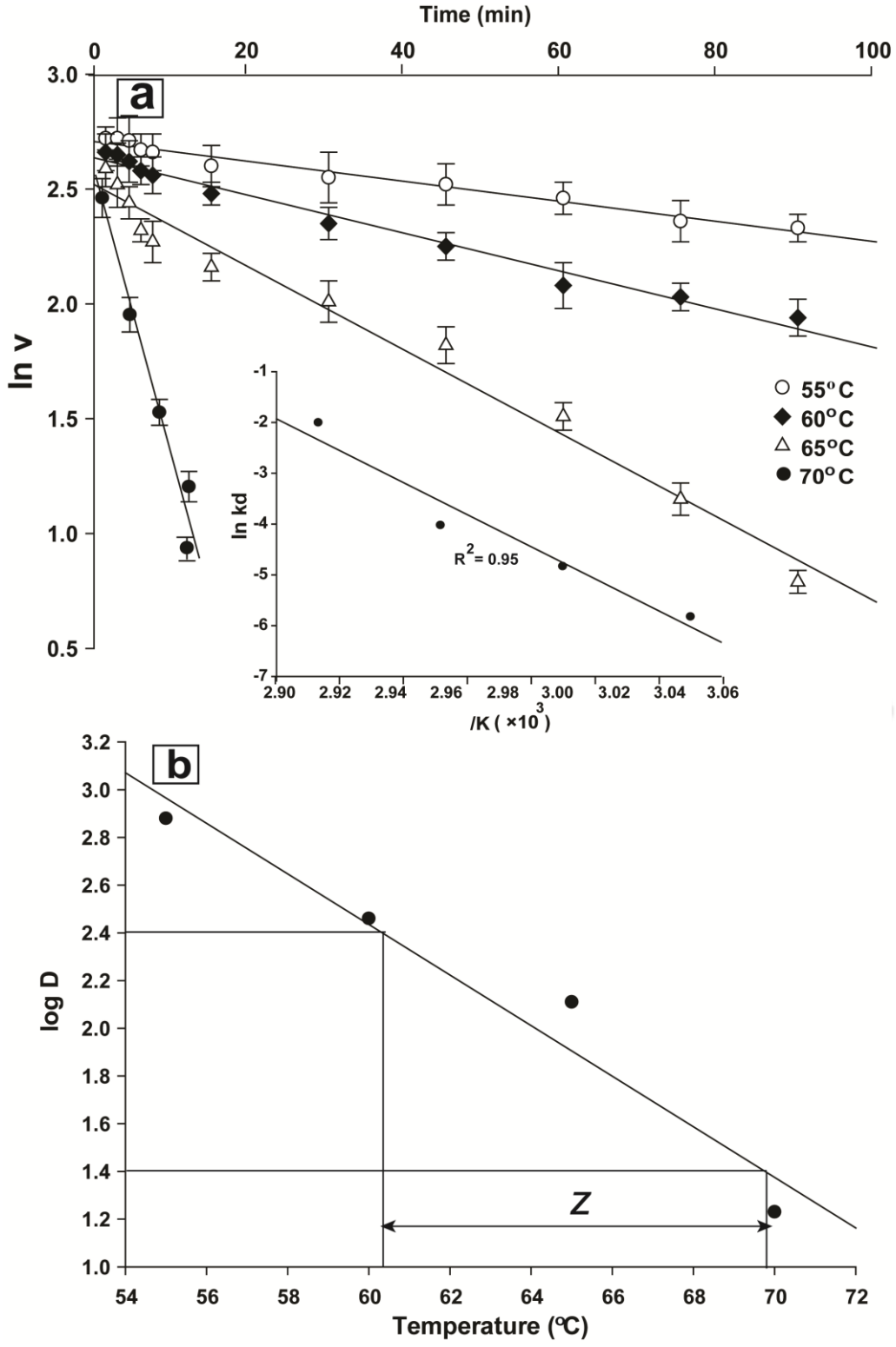


Fig. 4.5.6 (a) Temperature dependence of the thermo-deactivation constant (inset, ln kd *vs* temperature) and **(b)** Temperature dependence of the decimal reduction of purified cholesterol oxidase enzyme.

4.6 Tables

Table 4.6.1 Isolation of the cell-bound cholesterol oxidase by extraction buffers with different concentration of Triton X-100.

Triton X-100 (%)	Activity (U) [a]		Total activity (%)
	1st extraction [b]	2nd extraction [c]	
0.2	486±27	150±2.5	85
0.4	650±30	75±2.1	97
0.5	665±25	67±1.9	98
1.0	725±21	22±1.3	100

[a] The activity was measured with cholesterol under standard conditions (pH 7.0 and 25 °C).

[b] The cell-bound enzyme in supernatant was obtained by vigorously vortexing (6 min) the cell-pellets (from 50 ml bacterial culture) with extraction buffer and then separated the cell-pellets from the resulted cell homogenate at 9,500×*g* for 15 min at 4 °C.

[c] Cell-bound enzyme in supernatant was obtained by vigorously vortexing (6 min) the cell-pellets of 1st extraction with extraction buffer and the separated the cell-pellets from the cell homogenate at 9,500×*g* for 15 min at 4 °C.

Each datum point represents the mean of triplicate values ± standard deviation.

Table 4.6.2 Purification of the cell-bound cholesterol oxidase from *Rhodococcus* sp. NCIM 2891.

Purification step	Total protein (mg)	Total activity [b] (U)	Specific activity [b] (U/mg)	Purification (fold)	Yield (%)
Cell-pellet supernatant [a]	49.82±0.65	20,130±115	404±35.0	-	100
DEAE-Sepharose	14.65±0.34	17,808±90	1215±44.6	3	88

[a] The cell-pellet supernatant was obtained by vigorously vortexing (6 min) the cell-pellets (from 1.6 l bacterial culture) with 0.4 % Triton X-100 and the cell homogenate was then subjected to 13,000×*g* for 15 min. [b] The activity was measured with cholesterol under standard conditions (pH 7.0 and 25 °C). Each datum point represents the mean of triplicate values ± standard deviation.

Table 4.6.3 Substrate specificity of cholesterol oxidase from *Rhodococcus* sp. NCIM 2891.

Substrate	Relative activity (%)
Cholesterol	100
β-Sitosterol	53±1.8
Stigmasterol	23±1.2
Ergosterol	18±0.9
Diosgenin	0.0
Cholesteryl oleate	0.0
Cholestenone	0.0

The substrate specificity of the cholesterol oxidase was determined by cholesterol oxidase and horseradish peroxidase (HRP)-coupled assay method. The method involves measuring ABTS radical at $\lambda_{405\ nm}$. The cholesterol oxidase activity was measured under standard conditions (pH 7.0 and 25 °C). 100 % relative activity of the enzyme corresponds to 22 U with cholesterol. Each datum point represents the mean of triplicate values ± standard deviation.

Table 4.6.4 Stability of cholesterol oxidase in different organic solvents.

Organic solvent	Relative activity at different solvent concentrations (v/v)		
	5%	10%	15%
Control	100.00[a]	100.00[a]	100.00[a]
Methanol	79.50±2	55.14±2.3	35.36±0.8
Ethanol	92.42±0.8	70.76±1.3	65.00±1.4
Propanol	101.50±1.4	70.50±0.9	51.62±2.2
2-Propanol	115.62±1.7	91.27±1.5	87.11±1.8
Dimethylsulfoxide	91.23±2.0	82.00±2.0	78.35±0.9
Acetonitrile	56.63±0.9	41.72±1.3	23.75±1.8
2-methyl-2-propanol	86.46±1.3	82.92±2.4	74.00±2.7
Acetone	0.00	0.00	0.00

The cholesterol oxidase activity was determined by incubating 20 µg proteins in the 50 mM PPB at pH 7.0 with each of the organic solvents at their final concentration in reaction mixture for 30 min at RT and residual activities were measured. [a]100 % relative activity corresponds to 20 U. Each datum point represents the mean of triplicate values ± standard deviation.

Table 4.6.5 Effect of chemicals and metal ions on cholesterol oxidase activity.

Chemical and metal ion	Relative activity (%)
Control	100
Glutathione reduced	35±0.4
Dithiothreitol	23±0.7
β-Mercaptoethanol	59±1.3
EDTA	109±0.9
NaN_3	110±1.6
Ag^+ ($AgNO_3$)	109±3.0
Ca^{2+} ($CaCl_2$)	109±1.5
Mg^{2+} ($MgCl_2$)	115±1.1
Co^{2+} ($CoCl_2$)	105±2.3
Hg^{2+} ($HgCl_2$)	12±0.8
Fe^{3+} ($FeCl_3$)	83±1.4
Fe^{2+} ($FeSO_4$)	71±1.8
Cu^{2+} ($CuSO_4$)	93±2.7
Mn^{2+} ($MnSO_4$)	99±1.0
Ni^{2+} ($NiSO_4$)	114±2.3
Zn^{2+} ($ZnSO_4$)	116±1.7
Pb^{2+} [$Pb(NO_3)_2$]	61±1.3

The cholesterol oxidase activity was determined by incubating enzyme in the 50 mM PPB (pH 7.0) containing one of the chemicals (reducing agents, 10 mM; inhibitors, 0.1 mM; metal ions, 0.1 mM; final concentration) at RT for 10 min then the residual activity was measured by addition of cholesterol. 100 % relative activity corresponds to 20 U. Each datum point represents the mean of triplicate values ± standard deviation.

Table 4.6.6 Deactivation kinetics and thermodynamic parameters of cholesterol oxidase.

Temperature (ºC)	Deactivation kinetic and thermodynamic parameters					
	K_d (/min)	$t_{1/2}$ (min)	D (min)	ΔG (kJ/mol)	ΔH (kJ/mol)	ΔS (J/mol/K)
55	3.00×10^{-3}	231.00	767.67	107.63	225.66	359.84
60	8.00×10^{-3}	86.63	287.87	106.59	225.62	357.45
65	1.80×10^{-2}	38.50	127.94	105.92	225.57	353.98
70	1.35×10^{-1}	5.13	17.06	101.80	225.53	360.73

Thermo-deactivation studies were carried out by preheating 2 ml enzyme solution in 50 mM PPB, pH 7.0 containing 0.54 mg/ml protein at different temperatures. A total 50 µl enzyme samples were collected for different time intervals ranging from 1.5 min to 90.0 min for 55, 60, 65 °C and 1.5 min to 7.5 min for 70 °C and cooled to RT. The residual activity was measured with cholesterol at 25 °C and pH 7.0.

Chapter 5

Immobilization and Application of Cholesterol Oxidase from *Rhodococcus* sp. NCIM 2891

5.1 Overview

The ketosteroid, cholestenone has been used as a precursor for the synthesis of hormones and many intermediate steroidal compounds such as androst-4-ene-3,17-dione (AD) and androsta-1,4-diene-3,17-dione (ADD), which are eventually used to produce anabolic drugs and contraceptive (Lee et al. 1993; Sugano et al. 1995; Chaudhari et al. 2010). This ketosteroid also has anti-obesity effect thus depicting its potential as food additive or medicine for preventing obesity related diseases (Suzuki 1993). Cholestenone is generally produced from cholesterol through biotransformation route using microbial cells as catalysts (Buckland et al. 1975; Liu et al. 1996) with limited report where enzyme also has been used as catalyst (Marques et al. 2012). Here, we report biotransformation application of cholesterol oxidase from the *Rhodococcus* sp. NCIM 2891 for the production of cholestenone using the substrate cholesterol.

Enzymes are usually applied in immobilized form for biotransformation reaction considering its distinct advantages over the free form such as, easy handling in the downstream processing and reusability of the enzyme which finally contribute to the process economy (Brena et al. 2006). Cholesterol oxidase has been immobilized in several support materials (Suman and Pundir 2003; Wang et al. 2004; Akgöl et al. 2002; Singh et al. 2004; Wang and Mu 1999; Singh et al. 2006; Eguílaz et al. 2011; Saxena et al. 2011; Murai and Kato 2011; Akkaya et al. 2009; Marques et al. 2012; Yapar et al. 2009; Chen et al. 2013;

Saxena and Goswami 2010). However, more focus has recently been laid on the use of biocompatible, biodegradable and low cost materials such as, chitosan to immobilize enzyme for industrial applications (Yapar et al. 2009; Krajewska 2004; Aranaz et al. 2009). The success of an immobilization method largely depends on the nature of the support material and the reaction environment being used (Brady and Jordaan 2009; Sassolas et al. 2012). We employed here chitosan beads for immobilizing the cholesterol oxidase for the biotransformation study. The immobilization of the enzyme on the surface of the chitosan beads was performed by well-established glutaraldehyde based cross-linking reaction considering the fact that the chemical immobilization prevents enzyme leaching from the support medium during prolong biotransformation operation. The surface immobilized enzymes are though susceptible to shear stress, it surmounts the substrate diffusion problems frequently encountered with the matrix encapsulated enzyme (Brena et al. 2006).

5.2 Experimental approaches

5.2.1 Enzyme assay and protein measurement of cholesterol oxidase immobilized beads

A clear solution of 20 mM cholesterol was prepared in a 10 ml capped glass bottle vial by dissolving powdered cholesterol in 0.5 ml Triton X-100 (100 %) by heating at ~165 °C for 5 min and then made up the volume to 5 ml with distilled water and stored as stock solution at 4 °C. Every time before the experiment, the stock solution was heated to ~165 °C to get a clear solution and then a suitable volume of it was diluted with water containing 1 % Triton X-100 to get a final cholesterol concentration of 3 mM for using as ready stock solution. For measuring the enzyme activity a total 50 µl of the ready stock solution was diluted with 50 mM PPB (pH 7.0) to 1 ml final reaction volume containing 0.15 mM

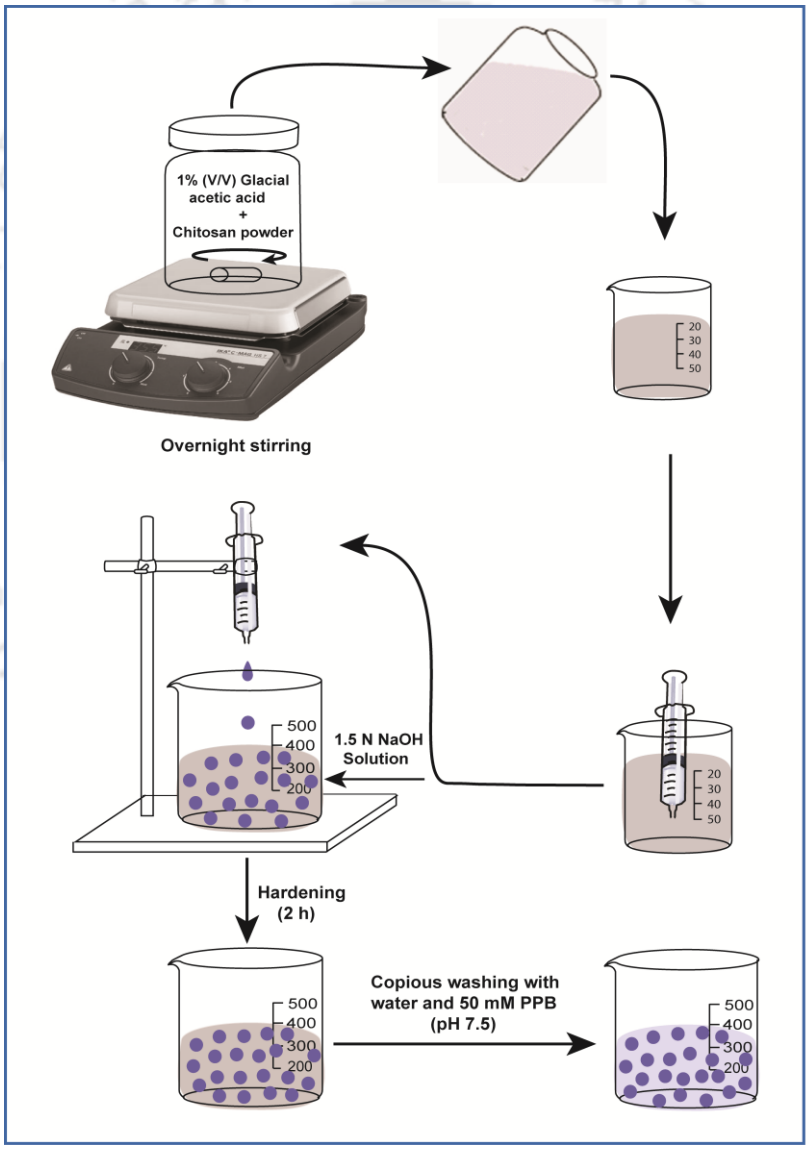
cholesterol. The reaction was initiated with six of cholesterol oxidase immobilized beads at 25 °C for 5 min. The reaction with the cholesterol oxidase immobilized beads was terminated by removing the beads from the reaction mixture immediately after the reaction time before taking the reading at RT. Cholesterol oxidase activity of immobilized enzyme was measured in a spectrophotometer (Cary 100, Agilent Technologies) by taking absorbance of cholestenone formed in the reaction mixture at $\lambda_{240\ nm}$ ($\varepsilon_{240\ nm}$ = 14,000/M/cm) (Kreit et al. 1992). One unit of enzyme was defined as the amount of enzyme that forms 1 μmol cholestenone/min. The kinetic parameters, K_m and k_{cat}, of free and immobilized enzymes were determined by measuring the enzyme activity as described above at different substrate concentration (12-240 μM). The values of kinetic parameters were discerned from the Lineweaver-Burk plot.

The protein content was determined by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as standard. For estimation of protein, the samples were sufficiently diluted to keep the Triton-X100 less than 0.1 %. The amount of protein immobilized on chitosan beads was determined by subtracting the residual free protein content left in solution, which also includes wash out protein content from the beads, from the total protein used for the immobilization. The protein content leached out from the chitosan beads during the biotransformation study was measured in the supernatant obtained by separating the immobilized beads from the reaction mixture at the end of each cycle. All experiments were conducted in triplicate and average values have been reported.

5.2.2 Preparation and characterization of cholesterol oxidase immobilized chitosan beads

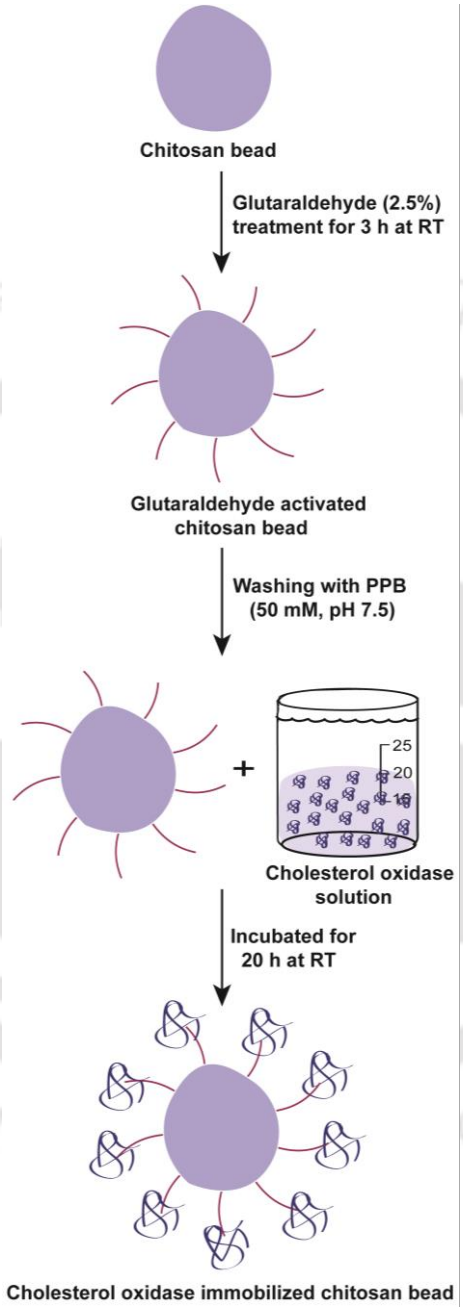
Chitosan powder was dissolved in 1.0 % (v/v) glacial acetic acid to prepare stock solutions in the range of 0.5-2.5 % (w/v). The stock solution of chitosan from a 5 ml syringe

was dispensed drop by drop into a 1.5 N NaOH solution under static conditions for the formation of chitosan beads, which were then allowed to stand for hardening in the solution for 2 h as shown in the scheme 5.2.1. After that the beads were washed with deionized water (0.078 µS/cm) followed by PPB (50 mM, pH 7.5) and stored in the same buffer at 4 °C. The beads were activated with a 2.5 % (v/v) glutaraldehyde for 3 h at RT and then washed extensively with PPB (50 mM, pH 7.5) to remove unreacted glutaraldehyde and stored in the same buffer at 4 °C.



Scheme 5.2.1 Schematic diagram on the preparation of chitosan beads.

For immobilization of cholesterol oxidase, a total 56 of glutaraldehyde activated beads were suitably submerged in 3 ml enzyme (1200 U) solution and incubated for 20 h at RT as shown in the scheme 5.2.2.



Scheme 5.2.2 Schematic diagram of immobilization of cholesterol oxidase on chitosan bead.

The beads were then washed with PPB (pH 7.5) until no enzyme activity was detected in the wash out buffer solution. The activation of the bead with glutaraldehyde and immobilization of the enzyme on the beads were examined by Fourier Transformed Infrared (FTIR) (Nicolet iS10, Thermo Scientific). The surface morphology of the enzyme immobilized beads at different stages of its preparation was analyzed by Scanning Electron Microscopy (SEM) (LEO 1430 VP, 10.00 kV) and Energy Dispersive X-ray (EDX).

5.2.3 Effect of pH, temperature and chemicals on cholesterol oxidase activity

The optimum pH of the immobilized enzyme was determined by analyzing its activity at various pH levels (3.5-11.0). The buffer systems used were sodium acetate buffer (pH 3.5-5.5), PPB (pH 6.0-7.5), Tris-HCl buffer (pH 8.0-9.5), glycine-NaOH buffer (pH 10.0-12.0) each at a concentration of 50 mM. The optimum temperatures for immobilized enzymes were investigated in the range of 25-65 °C in 50 mM PPB at pH 7.5. Prior to the enzyme assays, substrate solution was also equilibrated at the corresponding temperature. For pH stability, the immobilized enzyme was incubated in different pH buffers (5.0-10.0) up to 96 h at 4 °C and residual enzyme activities were determined. The temperature stability was studied by incubating the immobilized enzymes at different temperatures *viz*. 40.0, 50.0 and 60.0 °C in water bath for different time periods and residual activities were measured.

The effect of different organic solvents at their different concentrations *viz*. 5.0, 10.0 and 15.0 % (v/v) on the activity of immobilized enzyme was determined by incubating individually in a reaction mixture for 30 min at RT and then the residual enzyme activities were measured.

5.2.4 Storage stability study of cholesterol oxidase

The storage stability of immobilized enzyme was studied at the optimum pH (7.5) at different temperatures in PPB (50 mM). The immobilized cholesterol oxidase using 56 beads was investigated only at 4 °C and RT. The activities of the incubated enzymes were measured periodically after each week.

The half-life ($t_{1/2}$) of the enzyme is calculated by using Eq. (4.1). The decimal reduction time (D), which is defined as the time required for one $\log_{10}$ reduction (90 % reduction) in the enzyme activity, was calculated by using the Eq. (4.2).

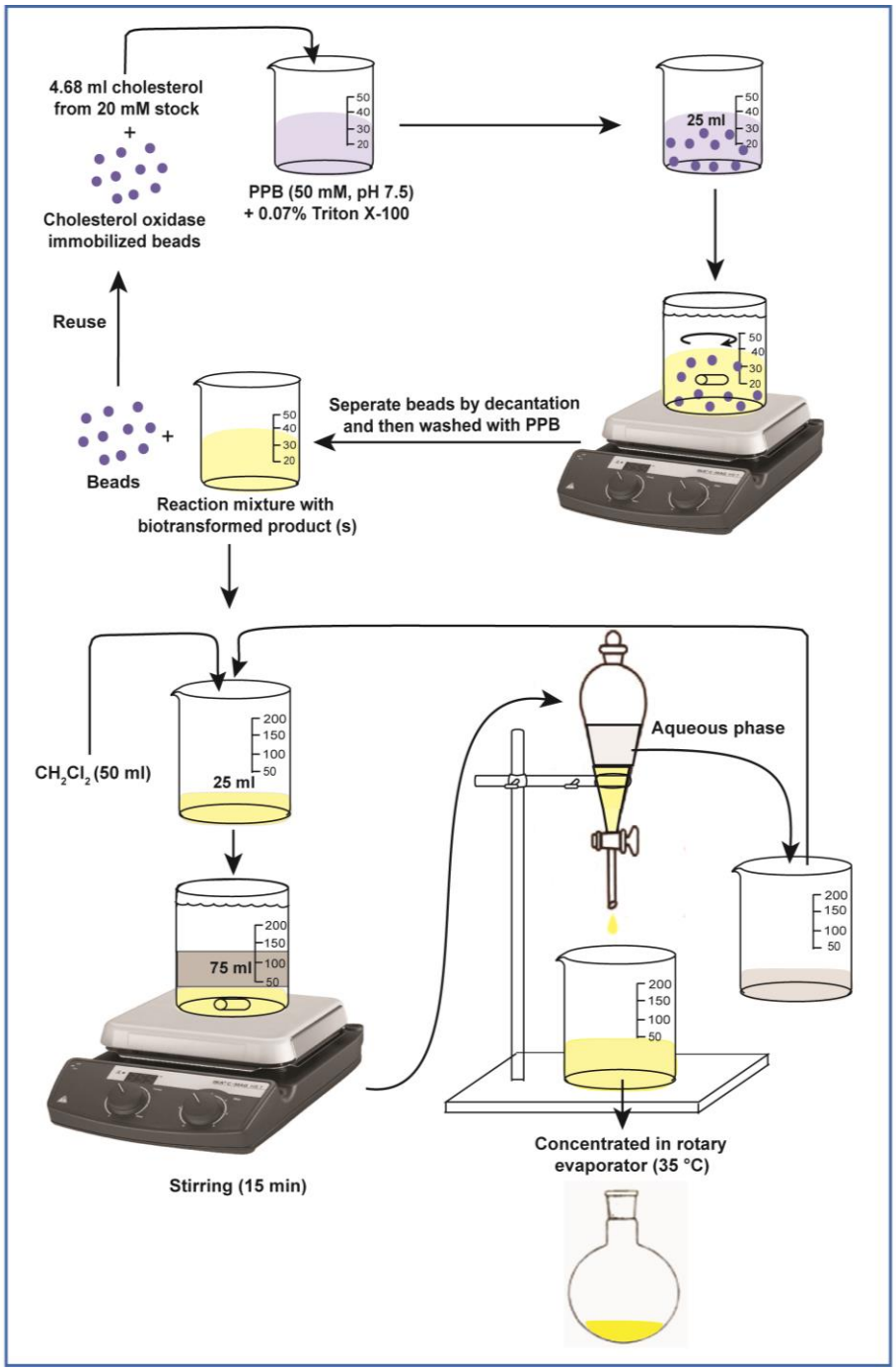
5.2.5 Biotransformation studies

The biotransformation reaction of cholesterol to cholestenone was studied by using 10 cholesterol oxidase immobilized beads (3.50 U) in 25 ml of 50 mM PPB (pH 7.5) containing 3.75 mM cholesterol in a 50 ml beaker covered with aluminium foil. The reaction was carried out for 11 h by stirring at 260 rpm with micro spin magnetic bar (3x10 mm size) at RT as shown in the scheme 5.2.3. The cholesterol solution for biotransformation was prepared as mentioned in the section 2.2. The cholestenone yield was determined by measuring the absorbance at $\lambda_{240\ nm}$ at 25 °C using $\varepsilon_{240\ nm}$ = 14,000/M/cm (Kreit et al. 1992).

To study the operational stability, the enzyme immobilized beads were separated from the reaction mixture following each cycle of operation and then washed three times with PPB (50 mM, pH 7.5) to remove any residual substrate present on the beads. The beads were again reintroduced into fresh reaction medium to obtain the residual enzyme activities.

5.2.6 Analysis of biotransformation products

The reaction mixture was extracted with two volumes of dichloromethane in a separating funnel.



Scheme 5.2.3 Schematic diagram of biotransformation of cholesterol and extraction of biotransformed product.

The dichloromethane phase was recovered, and the aqueous layer was re-extracted with two volume of dichloromethane as shown in the scheme 5.2.3. The extracts were pooled, dehydrated with anhydrous sodium sulphate, concentrated in a rotary evaporator (Equitron Roteva, India) at 35 °C and then filtered with syringe membrane filter of pore size 0.22 µm (Genetix Biotech Asia) prior to analysis. The extract was analyzed by HPLC equipped with a Shodex, C18-4E column (25 cm × 4.6 mm; 5 µm) connected to 1220 infinity LC system (Agilent Technologies) with 20 µl injection loop using methanol as mobile phase at a flow rate of 1 ml/min. The product was detected by UV detector ($\lambda_{240\ nm}$) at RT. The standard stock solution of cholestenone (98 %, Sigma-Aldrich) (500 µg/ml) in methanol was used for identification and quantification of the product based on retention time and peak area in the chromatogram.

5.3 Results and discussion

5.3.1 Immobilization of cholesterol oxidase on chitosan beads

Spherical chitosan beads with uniform shape of ~3 mm diameter were prepared with 2 % chitosan solution (Fig. 5.5.1a). At the chitosan concentration less than 2 %, the beads obtained were fragile, while at higher concentrations beads were difficult to prepare due to high viscous nature of the chitosan solution. The beads activated with 2.5 % glutaraldehyde were used for the enzyme immobilization as beyond this concentration (3 %) of glutaraldehyde the generated beads were brittle in nature. The normal and activated beads were analysed by FTIR spectroscopy (Fig. 5.5.2).The prominent peaks at 1572/cm represents the C=N bond indicating the formation of imine bonds due to cross-linking reaction of free amino groups of chitosan with the aldehydic groups of glutaraldehyde (Ray et al. 2010)

which is absent in the normal chitosan beads. The peak at 1654/cm confirms the presence of carbonyl group which is present in both the normal and activated chitosan beads. However, the peak is more prominent in the activated chitosan bead due to additional free aldehyde groups from glutaraldehyde. These free aldehyde groups of glutaraldehyde react with $–NH_2$ present in the enzyme and form the covalent bond leading to the immobilization of the cholesterol oxidase protein. Activation of chitosan beads with 2.5 % glutaraldehyde for 3 h and incubation of activated chitosan beads for 20 h with 1200 U enzyme leads to 83±1.33 % enzyme immobilization. The enzyme immobilization was confirmed by SEM and EDX analysis of the beads. SEM images were captured to understand the difference in the surface morphology among normal, activated and cholesterol oxidase immobilized beads. The surface morphology of the normal (Fig. 5.5.1b) and the glutaraldehyde activated beads (Fig. 5.5.1c) were smooth and nearly same. This indicates that glutaraldehyde (molecular mass ~ 100 Da) immobilization did not significantly alter the surface morphology of the normal beads. The smooth bead surfaces however, were transformed to rough morphology when cholesterol oxidase proteins were immobilized on them (Fig. 5.5.1d). The EDX analysis of different bead surfaces showed that the nitrogen % on the immobilized beads significantly increased depicting the formation of protein layer on the beads surface (Table 5.6.1).

5.3.2 Kinetic parameters of immobilized cholesterol oxidase

The K_m and k_{cat} value of the immobilized enzyme for the cholesterol substrate were 266.27 µM and ~ 668/min, respectively. The increase in the K_m value for the immobilized cholesterol oxidase has been previously reported for the immobilization matrices, namely, poly (2-hydroxyethyl methacrylate-co-glycidyl methacrylate) membranes (Akgöl et al. 2002),

poly(acrylamide-co-acrylic acid)/polyethyleneimine (Akkaya et al. 2009) and silk mat (Saxena and Goswami 2010).

5.3.3 Effect of pH and temperature on the cholesterol oxidase activity and stability

The enzyme maximal activities were observed in a broad pH range (Fig. 5.5.3a). The highest activity of the immobilized enzyme was 2.11±0.02 U at the pH value of 7.5. The activity of the enzyme after immobilization was decreased. The decrease in enzyme activity of the immobilized enzyme may be due to partial distortions in the enzyme structure triggered by the enzyme-support attachments. The pH optima of the immobilized enzyme (7.0-8.5) showed a marginal forward shift from the free enzyme (6.5-8.0). The reason may be attributed to the change in charge on the support material caused by the buffer system which in turn influenced the amino acids-support material interaction (Gawande et al. 1998). The optimum temperature for the activity of the immobilized enzyme was 50 °C (Fig. 5.5.3b). The increase in optimum temperature for immobilized enzyme may be ascribed to the increase in enzyme rigidity upon its covalent immobilization on the support matrix (Ortega et al. 2009). Akgöl et al. (2002), showed that the optimum pH of the free and immobilized cholesterol oxidase remain same, while the optimum temperature for the poly(hydroxyethyl methacrylate-co-glycidyl methacrylate) reactive membrane immobilized enzyme was increased by 5 °C from the free enzyme. However, Akkaya et al. (2009), reported the optimum pH values for functional polymeric support immobilized and free enzyme were 7.5 and 7.0, respectively.

The immobilized enzyme was stable at pH 7.0 and 8.0 till 96 h of incubation at 4 °C (Fig. 5.5.4a). The $t_{1/2}$ (day) of the immobilized enzyme at pH, 5 (2.41), 6 (4.81), 9 (9.62) and 10 (5.76) are shown in the parentheses. Thus the immobilized enzyme was less stable in

acidic pH than the basic pH conditions as compared to the free enzyme. This may be due to lower stability of the chitosan beads in acidic pH than the basic pH. The stability of the immobilized enzyme was diminished steadily with increasing temperature within the studied temperature range of 40-60 °C (Fig. 5.5.4b). For immobilized enzyme the K_d at 40, 50 and 60 °C were 2.10×10^{-2}, 5.70×10^{-2} and 4.38×10^{-1} /h, respectively and the corresponding $t_{1/2}$ (h) were 33, 12.16 and 2, respectively. Thus, the results infer that the immobilized enzyme is more thermostable than the free enzyme. The immobilized enzyme retained their entire activity for 6 weeks when stored at 4 °C (Fig. 5.5.5). The stability of immobilized enzyme was not performed at –20 °C due to brittleness of the beads at this temperature. The $t_{1/2}$ of the immobilized enzyme at RT was ~ 7 weeks and it retained 100 % activity ~ 1week.

5.3.4 Effect of solvents on cholesterol oxidase activity

The activities of immobilized enzyme in all the tested organic solvents except for acetone, propanol and 2-propanol, were only moderately decreased at the solvent concentration of 5 % (v/v) (Table 5.6.2). The activities of the enzyme in 5 % acetone were reduced to zero, while in 5 % propanol or 2-propanol the activity of the immobilized enzyme was increased. When treated with dimethylsulfoxide (DMSO), which is known as an environmentally friendly solvent, the cholesterol oxidase isolated by us retained nearly 91, 82 and 78 % enzyme activities at the corresponding solvent concentration of 5, 10 and 15 %, respectively. Synthesis of steroid hormones and biotransformation of a number of 3β-hydroxysteroids are generally carried out in presence of organic solvents (Doukyu et al. 1996; Guo et al. 2003) to solubilise the hydrophobic steroidal substrates. There is a growing mandate on the use of non-chlorinated and environmentally safe organic solvent such as DMSO for biotransformation and bioprocess studies. Therefore, the cholesterol oxidase

produced by the *Rhodococcus* sp. NCIM 2891 showed good promise for the biotransformation applications of steroidal compounds.

5.3.5 Biotransformation of cholesterol

The biotransformation was performed at RT, which was closer to the ambient temperature to reduce the enzyme denaturation and the energy cost in the process by excluding the high energy consuming thermostat based equipment in the experiment. The biotransformation of cholesterol to cholestenone was increased with time and reached to a nearly stationery state after 9 h of the reaction (Fig. 5.5.6). The biotransformation rate (dx/dt) was 5.75±0.32 µM/min in the initial nine hours of reaction. A total 3.31±0.11 mM cholestenone was produced out of 3.75 mM cholesterol, which is equivalent to ~ 88 % milli molar cholesterol biotransformation. HPLC analysis of the biotransformed product of cholesterol showed single major peak with the similar retention time of the standard cholestenone (Fig. 5.5.7) thus, validating cholestenone as the sole ketosteroid product of the biotransformation reaction. The amount of cholestenone obtained from the HPLC analysis was ~ 31 mg which is equivalent to ~ 86 % degree of biotransformation. Thus, the value obtained by HPLC method is closer to the one obtained by UV-spectroscopoic based method (~ 88 %) as mentioned above.

The information on the production of cholestenone is limited and among which the whole cell-biocatalysts based biotransformation route is widely reported. Buckland et al. (1975) reported the formation of 7 g/h cholestenone using 100 g of *Nocardia* cells in 200 ml of carbon tetrachloride containing 16 % (w/v) cholesterol, at 20 °C, while Liu et al. (1996) reported that 94.6 % of added cholesterol could be converted to cholestenone by the 6 g wet cells of *Arthrobacter* in 300 ml aqueous/carbon tetrachloride two-phase system containing 10

% (w/v) cholesterol at 30 °C. Marques et al. (2012) reported a conversion yield of 67 % of cholesterol (1g/l) within a microchannel reactor at a fluid flow rate of 14 µl/min in an enzyme based biotransformation process. Considering the results obtained through the present investigation it may be inferred that there is a scope of increasing the product yield by increasing the quantity of enzyme immobilized beads and cholesterol concentration in the reaction medium. Moreover, the pure product obtained through the present study may reduce the downstream processing cost of this enzyme catalyzed based process as compared to the whole-cell based biotransformation process, where the target product is frequently contaminated by other metabolic products and nutrient substrates. The results thus validated the prospect of the present cholesterol oxidase immobilized chitosan bead based biotransformation of cholesterol without using any chlorinated organic solvent and subsequent separation of the pure cholestenone product with least downstream processing steps.

5.3.6 Operational stability of immobilized cholesterol oxidase

Operational stability of immobilized enzyme is one of the major factors that grossly influence the process economy in the large scale production of compound through biotransformation route. In the present study, the enzyme activity decreased from 100 % to ~ 80 % in the second cycle of biotransformation by reusing the same cholesterol oxidase immobilized beads. However, in the subsequent cycles, the loss of enzyme activity was comparatively reduced (Fig. 5.5.8). The reason is attributed to the loss of loosely associated enzyme from the fresh beads during the first cycle of operation. There was no loss of protein from the beads in the subsequent cycles; however decrease in specific activity of the immobilized enzyme was observed after each cycle of operation and the reason is attributed

to the denaturation of the enzyme caused by shear stress. The decrease in specific activity of the immobilized enzyme with the reaction cycle number however, followed a non-linear behavior (Fig. 5.5.8). The comparative assessment on the operational stability of the enzyme from *Rhodococcus* sp. NCIM 2891 with other cholesterol oxidases (Akgöl et al. 2002; Akkaya et al. 2009; Saxena and Goswami 2010) is difficult due to varying reaction environment and immobilizing support matrices across different studies. However, the retention of ~ 67 % activity of the immobilized enzyme even after twelve successive cycles of operation observed in the present case is encouraging and showed prospect for up-scaling the production of cholestenone.

5.4 Conclusion

The enzyme, covalently immobilized on chitosan beads showed higher pH and temperature stability than its free form. The immobilized cholesterol oxidase could catalyze the formation of cholestenone as the sole ketosteroid product in the reaction mixture from the corresponding cholesterol substrate at RT without using any hazardous organic solvent. Further, the immobilized enzyme was stable in many organic solvents commonly being used in biotransformation studies. The immobilized enzyme showed high operational stability in many successive batches of operations. The proposed biotransformation method using chitosan bead immobilized cholesterol oxidase from the *Rhodococcus* sp. has therefore demonstrated great potential for the production of pharmaceutically important cholestenone in pure form in an environmental friendly reaction environment.

5.5 Figure

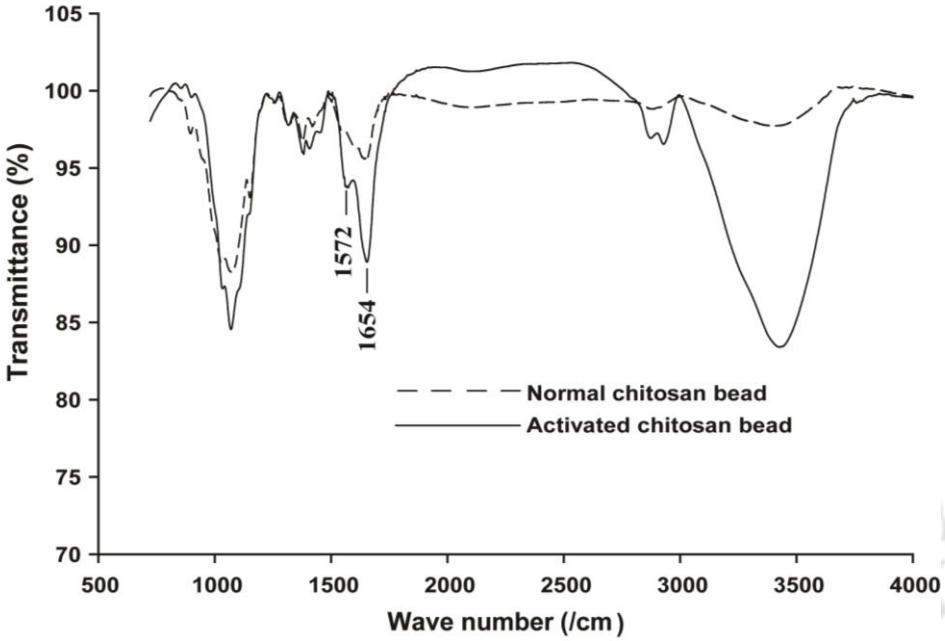


Fig. 5.5.1 Comparison of FTIR spectra of normal chitosan beads (dotted lines) and glutaraldehyde-activated beads (solid lines) for the confirmation of activation of glutaraldehyde treated beads.

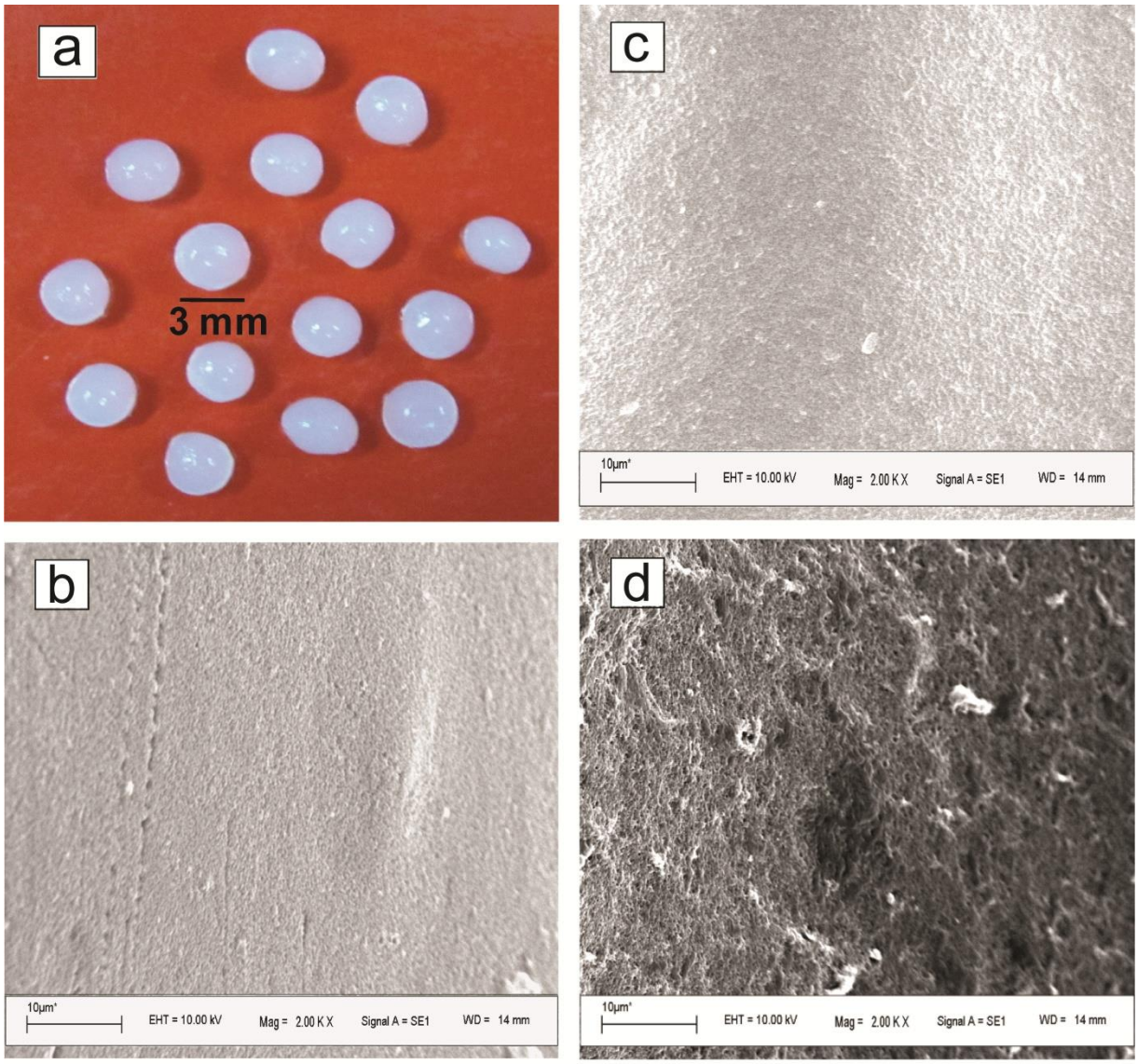


Fig. 5.5.2 (a) Normal image of chitosan beads captured by digital camera (Canon power shot, SX 120, Japan). SEM images of surface of **(b)** normal chitosan bead **(c)** glutaraldehyde activated chitosan bead and (d) cholesterol oxidase immobilized chitosan beads.

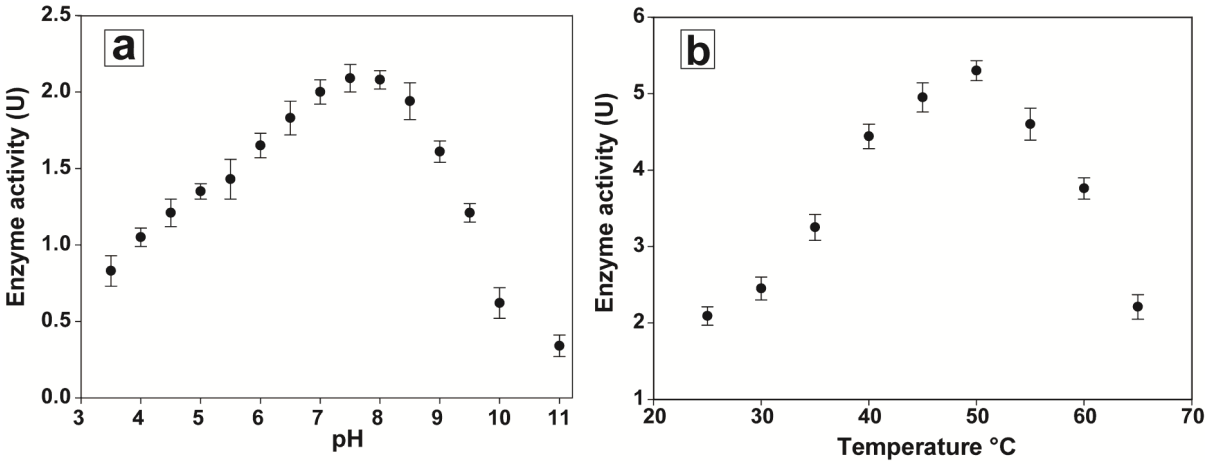


Fig. 5.5.3 (a) pH and **(b)** temperature optima of the immobilized cholesterol oxidase from *Rhodococcus* sp. NCIM 2891. The buffer systems used for pH optima study were sodium acetate buffer (pH 3.5-5.5), PPB (pH 6.0-7.5), tris buffer (pH 8.0-9.5), glycine-NaOH buffer (pH 10.0-12.0) each at a concentration of 50 mM. For optimum pH, the enzyme activity was assayed for 5 min at 25 °C, while for the optimum temperature the enzyme activity was assayed for 5 min at pH 7.5. Each datum point represents the mean of triplicate values, with the range indicated with error bars.

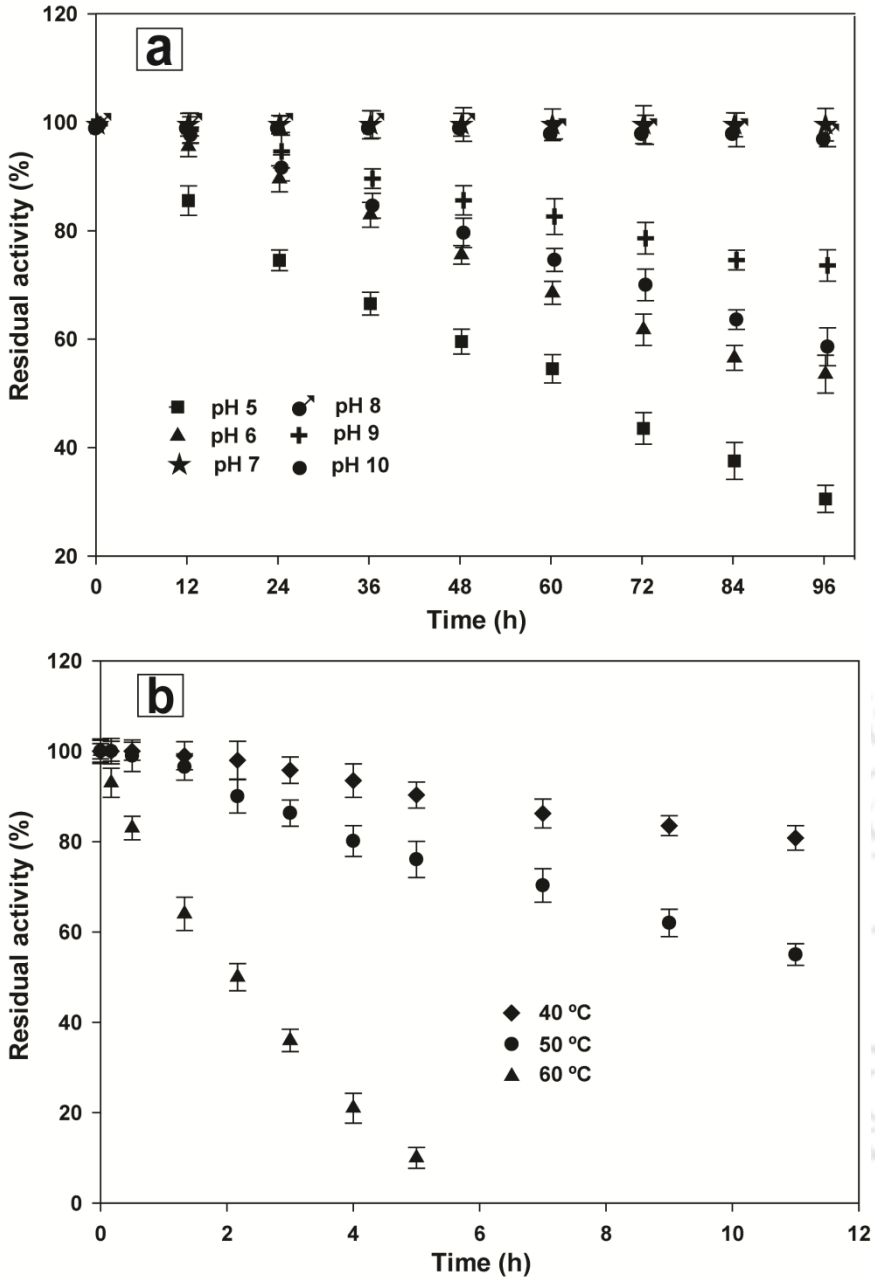


Fig. 5.5.4 (a) pH and **(b)** temperature stability of cholesterol oxidase from the *Rhodococcus* sp. NCIM 2891. The enzyme was incubated in different pH values (5-10) for 96 h at 4 °C and residual activities was measured. 100 % residual activity of immobilized cholesterol oxidase corresponds to 2.11U. Each datum point represents the mean of triplicate values, with the range indicated with error bars.

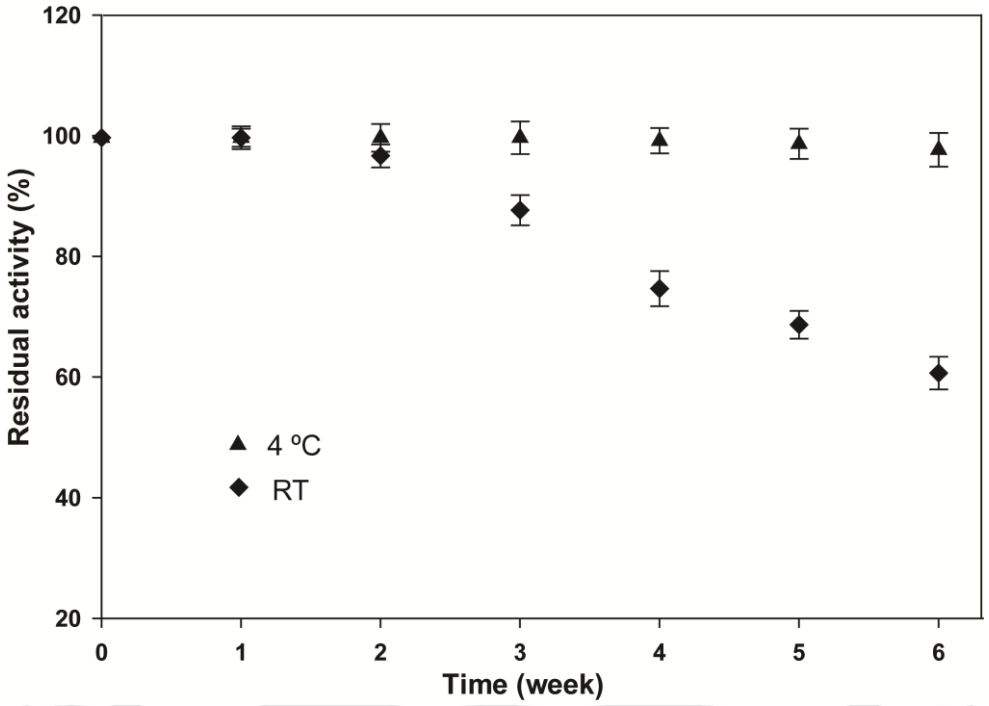


Fig. 5.5.5 Storage stability of immobilized cholesterol oxidase at different temperatures. The immobilized enzyme beads (56) were kept in a beaker in the PPB (50 mM, pH 7.5). The enzyme activity was assayed for 5 min at pH 7.0 and 25 °C. Each datum point represents the mean of triplicate values, with the range indicated with error bars.

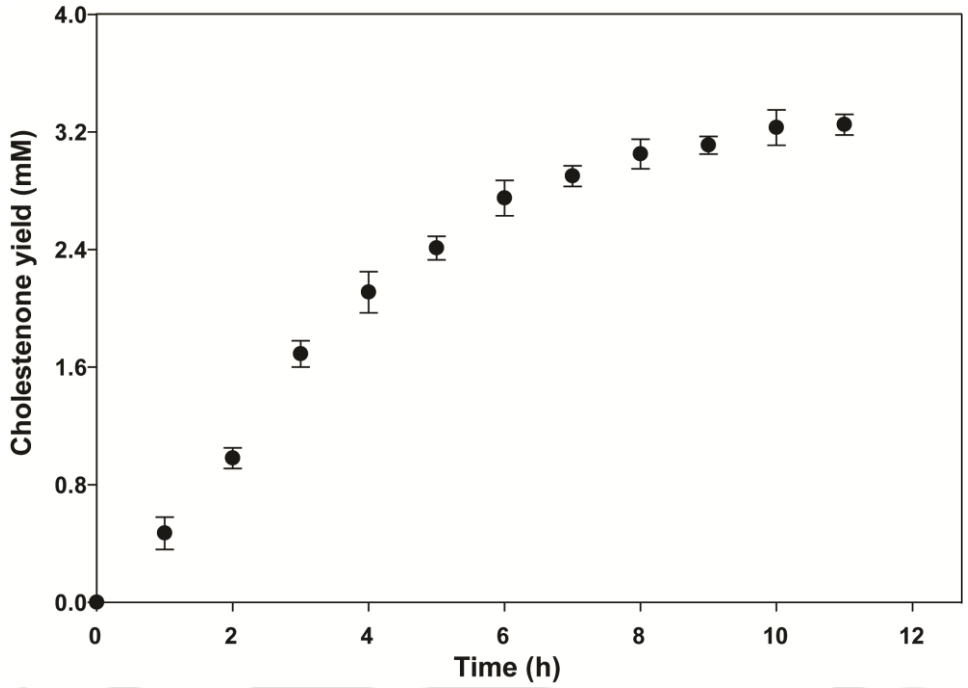


Fig. 5.5.6 Cholestenone yield *vs*. reaction time during biotransformation of cholesterol with cholesterol oxidase immobilized chitosan beads. Each datum point represents the mean of triplicate values, with the range indicated with error bars.

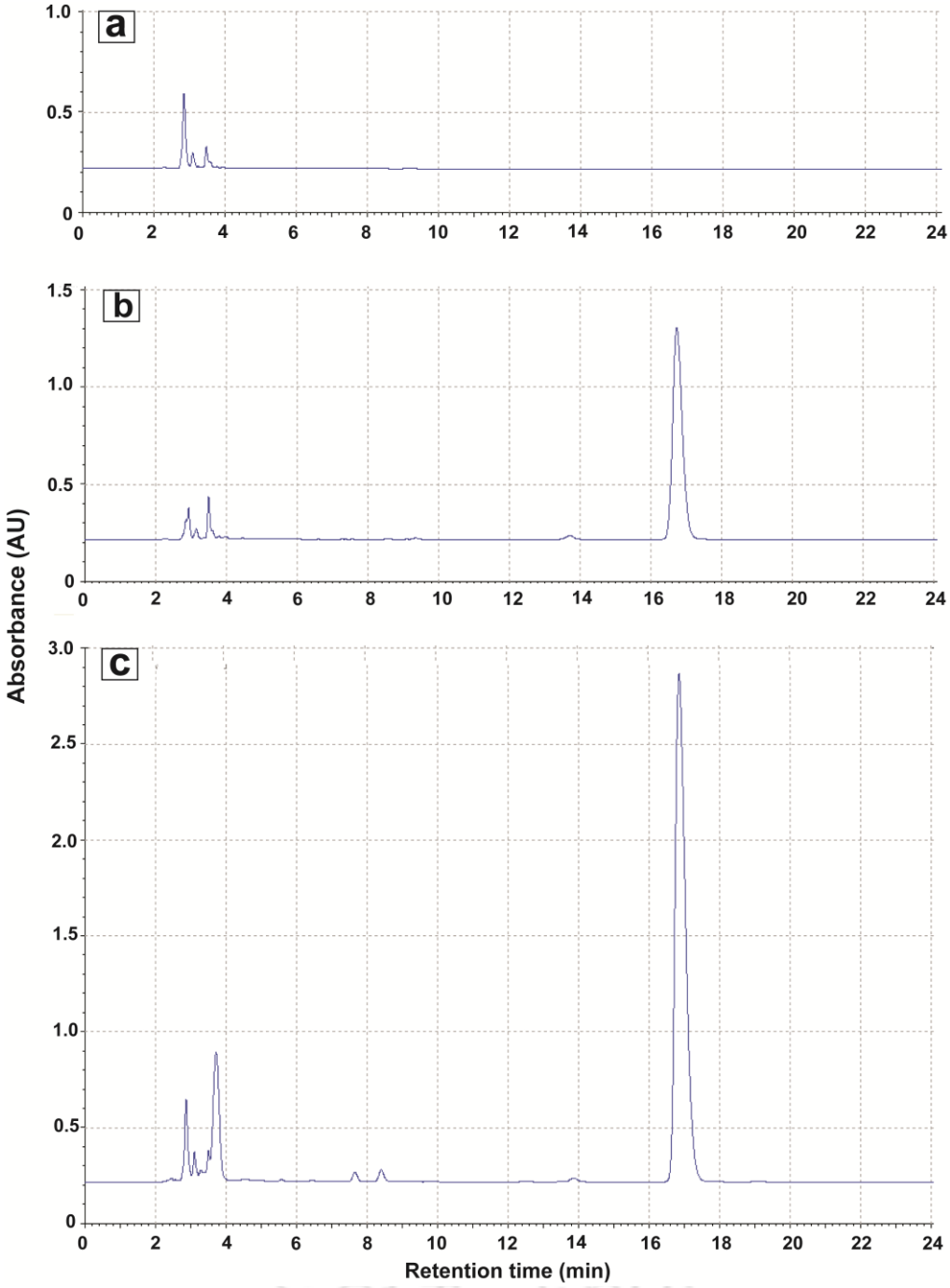


Fig. 5.5.7 HPLC chromatogram of **(a)** Control (membrane filtered methanol) **(b)** Standard solution of cholestenone (500µg/ml in methanol) **(c)** Biotransformation reaction mixture of cholesterol. Chromatographic conditions: Shodex, C18-4E column; methanol as a mobile phase at a flow rate of 1 ml/min; detection at $\lambda_{240\ nm}$.

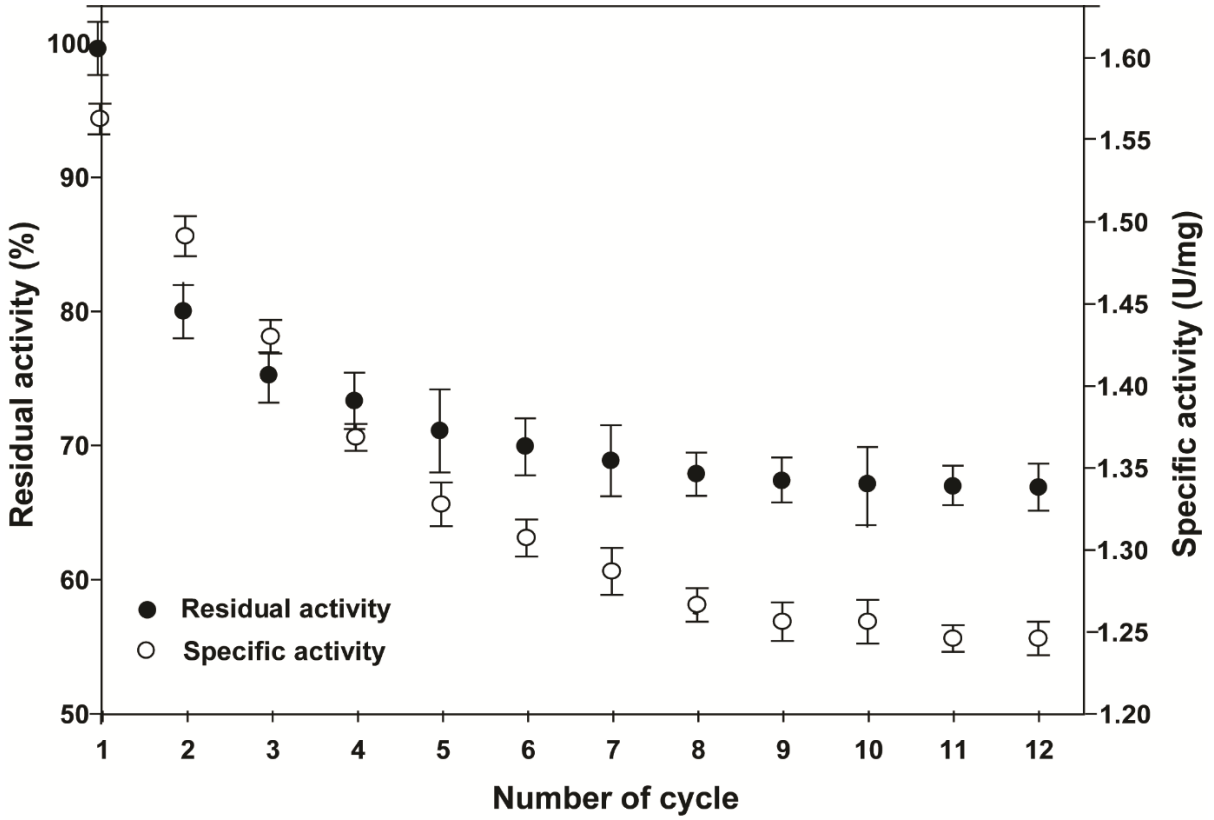


Fig. 5.5.8 Residual activities and specific activities *vs.* no. of reaction cycle during biotransformation of cholesterol with the immobilized cholesterol oxidase. The duration of each cycle was 9 h and no. of cholesterol oxidase immobilized beads was 10. The reaction was performed at 25 °C using 3.75 mM cholesterol as the substrate. For reusability study the enzyme immobilized beads collected after each cycle were washed and kept at RT to use in the next cycle. Each datum point represents the mean of triplicate values, with the range indicated with error bars.

5.6 Tables

Table 5.6.1 EDX analysis of chitosan bead surface.

Element	Atom (%)		
	A	B	C
C	45.80	42.61	28.42
N	22.22	22.23	36.17
O	31.98	35.16	35.41

A: Normal chitosan bead; B: Glutaraldehyde activated chitosan bead; C: Cholesterol oxidase immobilized chitosan bead.

Table 5.6.2 Stability of immobilized cholesterol oxidase in different organic solvents.

Organic solvent	Relative activity of immobilized cholesterol oxidase at different concentrations (v/v)		
	5%	10%	15%
Control	100.00[a]	100.00[a]	100.00[a]
Methanol	81.23±1.8	56.50±3	38.00±0.9
Ethanol	91.75±1.3	73.12±1.3	68.43±1.7
Propanol	103.25±0.7	72.00±0.8	53.47±1.5
2-Propanol	113.24±2.1	90.34±1.9	90.35±1.3
Dimethylsulfoxide	93.13±1.7	84.75±1.4	80.18±1.6
Acetonitrile	54.85±1.9	43.25±1.7	21.89±2.3
2-methyl-2-propanol	85.32±1.5	81.65±1.2	75.92±1.9
Acetone	0.00	0.00	0.00

The cholesterol oxidase activity was determined by incubating six cholesterol oxidase immobilized beads containing 88 µg proteins in the 50 mM PPB at pH 7.5 with each of the organic solvents at their final concentration in reaction mixture for 30 min at RT and residual activities were measured. [a]100 % relative activity corresponds to 2.11 U. Each datum point represents the mean of triplicate values ± standard deviation.

Conclusions and Scope for Future Work

Conclusions

The present study was carried out to find the optimum medium composition for higher cholesterol oxidase production, its characterization and application for cholestenone production. The optimum pH and temperature for cholesterol oxidase production from *Rhodococcus* sp. NCIM 2891 in the basal medium were 7.0 and 30 °C, respectively. The maximum enzyme production takes place with 60 h cultivation time. Cholesterol, $(NH_4)_2HPO_4$, and yeast extract were most significantly influencing medium components. The production of cholesterol oxidase was increased by 9.7 fold in statistically optimized medium as compared to basal (un-optimized) medium and 2.21 fold increase as compared to classically optimized medium. The production of the cell-bound cholesterol oxidase though induced by cholesterol, the level of its production was also linked with growth of the cells mass. The molecular weight of cholesterol oxidase determined by SDS-PAGE was ~ 55 kDa. Among all steroids, cholesterol was the best substrate for the cholesterol oxidase activity. The enzyme activity was not decreased by EDTA, which indicates that the enzyme does not have any metal ions as a cofactor. The optimum pH range of the cholesterol oxidase was 6.5-8.0 while the optimum temperature was 45 ºC. The *z* value was found to be approximately 9.5 °C which indicates that the enzyme was more sensitive towards the increase in temperature than the duration of heat treatment. The chitosan beads obtained were spherical and uniform shape with the diameter of 3 mm. The percentage immobilization of cholesterol oxidase was 83 %. The optimum pH range and temperature of the immobilized enzyme were 7.0-8.5 and 50 ºC, respectively. The immobilized enzyme was more thermostable than the free enzyme. The $t_{1/2}$ of immobilized enzyme at RT was ~ 7 weeks and it retained 100 % activity ~ 1 week. The biotransformation rate (dx/dt) was 5.75±0.32 µM/min in the initial nine hours of reaction. A

total 3.31±0.11 mM cholestenone was produced out of 3.75 mM cholesterol, which is equivalent to ~ 88 % milli molar cholesterol biotransformation. The immobilized enzyme retained ~ 67 % activity even after twelve successive cycles of operation. Cholestenone was the main ketosteroid product of the biotransformation reaction. The optimum medium composition for higher cholesterol oxidase production was successfully identified. The enzyme was physico-chemically characterized and successfully immobilized on chitosan beads for cholestenone production.

Scope for Future Work

Cloning and expression of the cholesterol oxidase and characterization of the functionally active enzyme protein by x-ray crystallographic study expected to furnish important information regarding the structural features, catalytic sites and catalytic mechanism of the enzyme. Further, the upscale production of cholestenone following the immobilization techniques adapted here using a suitably designed bioreactor needs to be investigated. Finally, the techno-economic feasibility studies on the production of the pharmaceutically important ketosteroid (cholestenone) required to be investigated for their potential industrial application.

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List of Publications

Publication in Referred Journals

1. **Seraj Ahmad**, Pranab Goswami (2014). Application of chitosan beads immobilized *Rhodococcus* sp. NCIM 2891 cholesterol oxidase for cholestenone production. Process Biochemistry 49:2149-2157.
2. **Seraj Ahmad**, Pranab Goswami (2013). Enhanced production of cell-bound cholesterol oxidase from *Rhodococcus* sp. NCIM 2891 by the statistical method. Annals of Microbiology 63:199-205.
3. Urmila Saxena, Madhuri Das, **Seraj Ahmad**, Lepakshi Barbora, Mala Borthakur, Anil Verma, Utpal Bora, Pranab Goswami (2011). Multiwalled carbon nanotube-based enzyme electrode for total cholesterol estimation in human serum. Journal of Experimental Nanoscience 6 (1) 84-95.

Abstract Published in Conferences

1. **Seraj Ahmad**, Pranab Goswami (2011). Statistical evaluation of medium components by experimental design for enhancing the cholesterol oxidase production from *Rhodococcus* sp. World Congress on Biotechnology, Hyderabad International Convention Centre (HICC), Hyderabad, India, P 72, 21-23 March.
2. Urmila Saxena, Madhuri Das, **Seraj Ahmad**, Pranab Goswami (2009). An amperometric cholesterol biosensor based on multi-walled carbon nanotube-nafion-cholesterol oxidase-cholesterol esterase nanobiocomposite. International Conference on Advance Nanomaterial and Nanotechnology (ICANN), IITG, Guwahati, Abstr. No. E-134, 9-11 December.

3. **Seraj Ahmad**, Pranab Goswami (2009). Production and partial characterization of cholesterol oxidase from *Rhodococcus sp*. 50th Annual conference, Association of Microbiologists of India, AMI 2009, NCL, Pune, P 278, 15-18 December.