6.1. Overview

Chox is an enzyme of high commercial value due to its extensive industrial applications. Chox has widespread applications in the clinical, pharmaceuticals, food, and agricultural industries which have substantially increased the demand for this enzyme. The food industry and clinical pathology encompass the central role of Chox as a diagnostic enzyme where it is used to determine the cholesterol concentration in food and blood serum by coupling Chox with horseradish peroxidase (Allain et al. 1974; Lolekha and Jantaveesirirat 1992). Chox is also used for the degradation of dietary cholesterol in foods in the food industry. The pharmaceutical industry makes use of Chox in the production of precursors for the chemical synthesis of steroid hormone (MacLachlan et al. 2000; Watanabe et al. 1986). In the field of agriculture it is used as a biological control agent (Corbin et al. 2001; Martin et al. 2007; Doukyu et al. 2008).

Chox is exclusively a microbial enzyme; no other homologue of Chox has been reported till date. There are pieces of the literature that suggest that there is a wide array of microorganisms (including bacteria, actinomycetes, fungi) producing Chox with specific properties (Buchland, 1975; Petrova et al., 1981; Liu et al., 1988; Shirshova et al., 1992; Noriyaku and Rikizo, 1998; Tabatabei et al, 2001).

It has been established from the previously available literature that the Chox produced and purified from different microbial sources exhibits many diverse physicochemical properties (Lee et al., 1989; Yazdi et al., 2000; Yazdi et al., 2008; Lin et al., 2010; EI-Shora et al., 2011; Yehia et al., 2015; Rodrigues F and Palani P., 2016;). The study of physicochemical properties of the Chox obtained from different sources may be of enough industrial and academic interest and can be commercially exploited for clinical and pharmaceutical purposes or the food industry.

All the above-mentioned industrial applications make use of Chox in the purified form. The knowledge of the biochemical characteristics of the enzyme is essential for an understanding of their structure and mechanisms of action and thermo-stability analysis. The purification of the Chox produced from *S. olivaceus MTCC 6820* using PEG-ammonium sulfate-water ATPS was covered in Chapter 5. In the present chapter, the physicochemical properties of the partially purified Chox including pH optima, temperature optima, the effect of substrate concentration, and the effect of various metal ions and some inhibitors on enzyme activity have been studied. The stability of purified Chox under various conditions viz., pH stability, thermo-stability, and in the presence of different organic solvents and detergents were studied.

6.2. Experimental

6.2.1. Chemicals used

Cholesterol (for enzyme assay) was purchased from Sigma Aldrich. Sodium acetate, potassium phosphate, Tris-HCl, sodium dodecyl sulfate (SDS) were purchased from Sisco Research Laboratories, Mumbai, India. Magnesium sulfate, manganous sulfate, copper sulfate, calcium chloride, zinc sulfate, barium sulfate, and urea were purchased from Sd Fine-Chem Limited, Mumbai, India. Methanol, ethanol, acetone, ethyl acetate, tertiary butanol, isopropanol were obtained from Merck, Germany. Benzene, p-xylene, toluene, dichloromethane, acetonitrile, tween-20, tween-80, and triton X-100 were purchased from Loba Cheme. All the chemicals and media components used were of analytical grade.

6.2.2. Preparation of purified Cholesterol oxidase sample

From the partitioning studies carried out in Chapter-5, PEG 4000 [10.63% (w/w)]-ammonium sulfate [14.5% (w/w)]-water was found to be the most suitable system

component for partitioning of Chox, therefore the same ATPS composition was used for further studies. The supernatant obtained by centrifugation of the fermented broth at 12,000 rpm (4ºC) for 20 min was collected as crude Chox and it was concentrated by rotary vacuum evaporator (Vaiometra, RE 2000A, Shanghai Ya Rong, China) to remove the excess of water in the broth. The concentrated crude Chox was homogeneously mixed with the PEG 4000-ammonium sulfate mixture (with the above-mentioned composition) on a magnetic stirrer for at least 45 min and allowed to stand for a maximum of 1 h leading to complete phase separation. The top and bottom phases were separated carefully using a Pasteur pipette. The Chox activity (U/ml) of the top phase was analyzed for the study of the physicochemical properties of the chox.

6.2.3. Enzyme Assay

Chox activity was estimated by the method of Allain et al. (Allain et al., 1974; MacLachlan et al., 2000). 50 µl of 0.6% (w/v) cholesterol (dissolved in dimethyl formamide containing 5% (v/v) Triton X-100) was added to 1 ml of reaction mixture containing 1.5 mM 4-aminoantipyrine, 5 mM phenol, 10 U/ml horseradish peroxidase and sodium phosphate buffer (20 mM, pH 8.0) and pre-incubated at 30ºC for 5 min. 100 µl of partitioned enzyme sample (top phase) was taken and added to the pre-incubated reaction mixture to start the reaction and the incubation continued at 30ºC for 10 min. The reaction was terminated by placing the samples in a boiling water bath for 2 min and then immediately placed in an ice bath for 2 min for color development. Absorbance was recorded at 500 nm by discontinuous spectrophotometric method. Blank was prepared by taking the ATPS (top phase) samples with no enzyme loaded on it. The enzyme activity in the top phase was measured against the enzyme blank of the same top phase. Since maximum Chox was recovered from the top phase of ATPS (mentioned in Chapter-5),

therefore, in further studies including the study of physicochemical properties of the Chox, only the top phase enzyme activity was measured.

One unit of Chox activity was defined as the formation of 1 µmol of hydrogen peroxide (0.5 µmol of quinoneimine dye) per minute at 30ºC and pH 8.0.

6.2.4. Protein Determination

Protein concentration was determined by Bradford's method using Coomassie Brilliant Blue G-250 dye. Blank was prepared by taking ATPS (top/bottom phase) samples containing no crude Chox. The standard curve of BSA was prepared in the concentration range 0.01 to 0.1 mg/ml and the absorbance was recorded at 595nm (Bradford, 1976).

6.2.5. Studies of the physicochemical properties of partitioned Cholesterol oxidase

Various properties of the purified Chox were studied including pH optima, temperature optima, pH stability, thermo-stability, the effect of various metal ions, and some inhibitors. The stability of purified Chox in the presence of various organic solvents and detergents was studied.

6.2.5.1. Effect of pH on the activity of Cholesterol oxidase

The effect of different pH (4 - 10) on the activity of purified Chox was studied using the following buffers (0.1 M): sodium acetate buffer (pH 4 - 6), potassium phosphate buffer (pH 6.5 - 8.5), and Tris-HCl buffer (pH 9 - 10). The reaction was carried out at 30°C for 10 min.

6.2.5.2. Effect of temperature on the activity of Cholesterol oxidase

The effect of temperature on the activity of Chox was studied by incubating the reaction mixture containing Chox at different temperatures (15 - 80°C) using cholesterol as a substrate in 0.1 M phosphate buffer, pH 8.0.

6.2.5.3. Effect of substrate concentration

The effect of the increase in substrate concentration on the reaction rate of purified Chox was studied using cholesterol as a substrate at pH 8.0, 30°C. 70µl of partitioned Chox was used and the cholesterol concentration was varied in the range of 0.1 - 1 mM. The rate of change in initial reaction velocity (Vo) was measured at every 30 seconds for each substrate concentration [S] and K_m was determined by the Michaelis-Menten curve. The maximum reaction rate (Vmax) was determined by Lineweaver–Burk plot between 1/[S] versus 1/V.

6.2.6 Stability Studies

Most enzymes lost their structure and function when exposed to conditions such as extreme pH, temperature, inhibitors, organic solvents, and detergents. Therefore, it is necessary to examine the stability of partitioned Chox under the conditions described below for various applications.

6.2.6.1. pH stability

The stability of purified Chox was examined by incubating Chox with buffers (0.1 M) of different pH (4 - 12) for 24 h at room temperature. 100 µl aliquot of samples from each pH variant were assayed for residual Chox activity at pH 8.0, 30°C for 10 min.

6.2.6.2. Thermo-stability

Thermo-stability studies of purified Chox were performed by incubating the purified Chox (100 µl) with phosphate buffer (0.1 M), pH 8.0 for 30 min in a water bath at different temperatures (15 - 90°C). 100 µl aliquot of samples from each temperature variable was assayed for residual Chox activity with cholesterol as substrate at pH 8.0, 30°C for 10 min.

6.2.6.3. Effect of organic solvents and detergents

Different organic solvents are used for solubilizing the cholesterol in the fermentation medium, for solubilizing cholesterol (used as a substrate) in Chox enzyme assay, for the elution of Chox in DEAE IEX chromatography, and in biopharmaceuticals for the production of steroids and other derivatives. It is therefore necessary to examine the stability of purified Chox in the presence of various organic solvents and detergents. Stability studies of purified Chox were evaluated in the presence of dimethyl sulphoxide (DMSO), methanol, ethanol, acetone, ethyl acetate, tertiary butanol, benzene, p-xylene, toluene, dichloromethane, acetonitrile, and isopropanol. 500 µl of purified Chox (14.9 IU) was added to each organic solvent [0.5 ml, 10% (v/v)] in eppendorf tubes. This mixture was incubated for 24 h with continuous shaking at 30°C. An aliquot (100 µl) of the sample was examined for enzyme activity under specified conditions (at pH 8.0, 30°C, reaction time 10 min). Blank was prepared separately taking into consideration each organic solvent by the addition of inactivated enzyme.

Often detergents are also used as solubilizers for steroidal compounds like cholesterol and many others. The stability of purified Chox in the presence of various ionic and non-ionic detergents was examined. The effect on the stability of purified Chox in the presence of non-ionic detergents including Tween 20, Tween 80, and Triton X - 100 as well as ionic detergents including Extran (commercial lab detergent) and sodium dodecyl sulfate (SDS) were studied. The purified Chox was incubated with different detergent solutions [1% (v/v)] in eppendorf tubes at room temperature for 24 h. An aliquot (100 µl) of the sample was examined for residual Chox activity at pH 8.0, 30°C for 10 min.

6.2.6.4. Effect of denaturant and chelating agent

The effect of denaturing agent (urea) on purified Chox was examined by incubating Chox with urea (0.1 ml, 50 mM) at room temperature for 2 hours. An aliquot (100 µl) of the sample was assayed for residual Chox activity at pH 8.0, 30°C for 10 min.

To assess the effect of chelating agent i.e., ethylene diamine tetra ammonium salt (EDTA) on purified Chox was done by incubating Chox (0.1 ml) with EDTA (50 mM) for 2 h at room temperature. An aliquot (100 µl) of the sample was withdrawn and assayed for residual Chox activity at pH 8.0, 30°C for 10 min.

6.2.6.5. Effect of metal ions on Chox activity

The effect of various metal ions Mg^{2+}, Mn^{2+}, Cu^{2+}, K^{+}, Na^{+}, Ca^{2+}, $Zn^{2+,}$ and Ba^{2+} on Chox activity was studied by incubating 0.1 ml, 100 mM metal salt solution with purified Chox for 24 h at room temperature. Relative activity (%) was measured by assaying Chox activity of an aliquot (100 µl) withdrawn.

6.3 Results and Discussion

6.3.1. Physicochemical properties of partitioned Cholesterol oxidase

Some preliminary studies for the determination of physicochemical properties of the partitioned Chox (covered in Chapter 5) were carried out including the study of pH and temperature optima, and the effect of substrate concentration on reaction velocity. The stability studies of the purified Chox included pH stability and thermo-stability, stability in the presence of organic solvents, detergents, metal ions, chelating agents, and denaturants, which have been discussed section-wise.

6.3.1.1. pH optima

The pH optima of the purified Chox were examined by performing Chox assay at different pH (4-10) using three different types of buffer and cholesterol as a substrate at

30°C for 10 min. The maximum activity of Chox was obtained at optimum pH 7.0; **Fig 6.1**. Although a significant amount of activity was also noticed at pH 6.5 and 7.5, it indicates that Chox possesses a considerable amount of activity between pH 6-8. The enzyme is most active at neutral pH range while slightly acidic pH below 6 and slightly alkaline pH above 7.5 may significantly reduce the Chox activity. However, the optimum pH for the crude extract of Chox was found to be 8.0 in Chapter 3 which was slightly different from the optimum pH obtained for purified Chox. This result points towards the presence of metal salts and other interfering substances in the fermented broth of Chox, which affect the optimum pH of the purified Chox.

6.3.1.2. Temperature optima

The effect of temperature on the activity of Chox was analyzed in the temperature range of 15 - 80°C, as shown in **Fig 6.2**. It was found that the enzyme activity was very low at lower temperatures 15°C and 20°C, started increasing at 25°C and reached its optimum at 35°C and steeply reduced after 37°C. The maximum activity of Chox was obtained at optimum temperature 35°C. However, the temperatures 25-37°C were suitable for Chox activity assay. Enzymes show a reduction in catalytic activity beyond the optimal temperature. This may be due to the changes in the secondary and tertiary structure of the protein occurring as a result of the disturbances caused due to the intramolecular interactions (hydrogen bonding and dipole-dipole interaction) between the polar groups as well as the hydrophobic interactions among the non-polar groups within the protein structure.

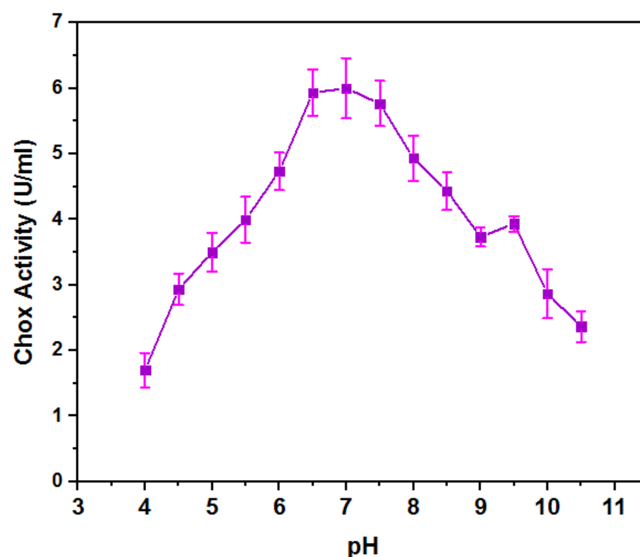

Fig. 6.1 Effect of pH on activity of purified cholesterol oxidase

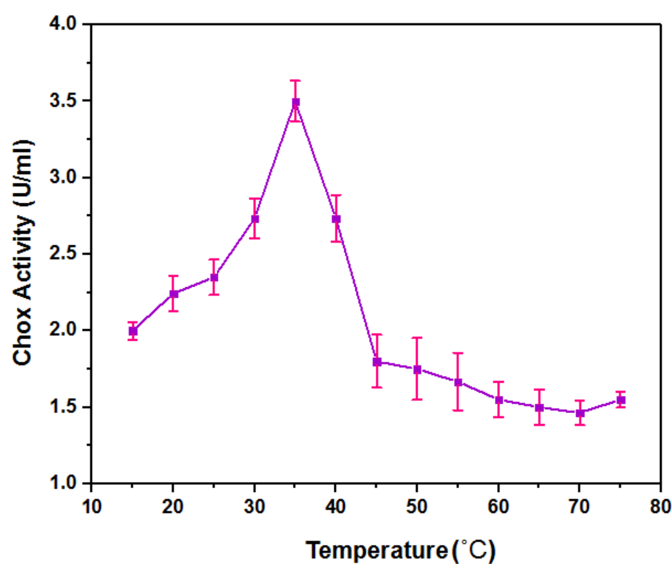

Fig. 6.2 Effect of temperature on activity of purified ChOx

6.3.1.3. Effect of substrate concentration on the reaction velocity

The effect of substrate concentration on Chox produced by *S. olivaceus* MTCC 6820 was studied using different concentrations of cholesterol (0.1 – 1 mM). The effect of increasing substrate concentration on reaction velocity of the purified Chox obeyed the Michaelis - Menten kinetics. The maximum activity of Chox was obtained at 0.7 mM

concentration of cholesterol as shown in **Fig 6.3 (A)**. At higher substrate concentrations, the enzyme activity was found to reach a state of saturation and a rectangular hyperbolic curve was obtained for reaction velocity [V] through the origin against substrate concentration [S] as shown in **Fig 6.3 (A).** The steady-state kinetics of enzyme-catalyzed reactions predicts a hyperbolic relationship between the steady-state velocity (V) and substrate concentration [S], expressed in terms of Michaelis - Menten equation; **eq. (6.11)**

$$V = \frac{Vmax[S]}{Km+[S]} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (6.11)$$

Where V_{max} is maximum velocity and K_m is Michaelis constant. Michaelis constant is defined as the degree of affinity of the enzyme towards the substrate. Michaelis-Menten plot could not be used to determine the accurate value of V_{max} and K_m because the asymptotes cannot be approached closely enough (Eisenthal and Cornish-Bowden, 1974). For this purpose, the Lineweaver Burk plot (1/V versus 1/[S]) was plotted to determine the kinetic parameters considering the following equation, **eq. (6.12)**:

$$\frac{1}{V} = \frac{1}{Vmax} + \frac{Km}{Vmax[S]} \quad \ldots\ldots\ldots\ldots\ldots\ldots (6.12)$$

The K_m and V_{max} values of purified Chox from *S. olivaceus* MTCC 6820 were obtained as 0.36 mM and 1.62 µM/min/mg-protein respectively using Lineweaver-Burk plot, displayed in **Fig 6.6 (B).** The Chox purified from another *Streptomyces sp.* had a different Km value as 101.3 µM (Niwas et al., 2013).

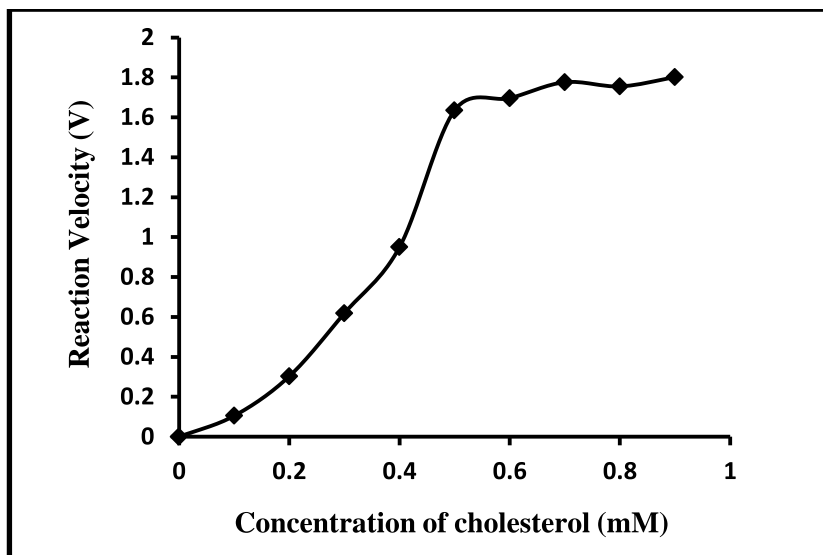

Fig 6.3 (A)

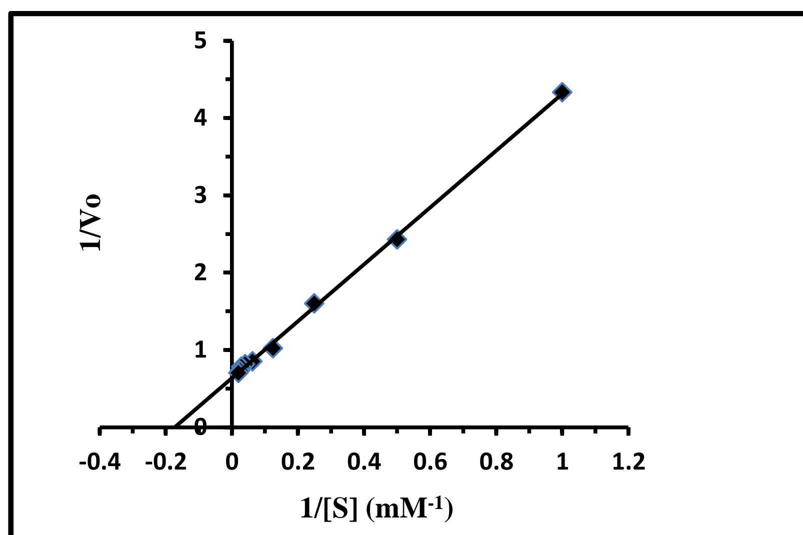

Fig. 6.3 (B)

Fig 6.3 (A-B) Effect of substrate concentration on reaction velocity of Cholesterol oxidase

(A) At the higher substrate concentrations, the activity of Chox undergoes saturation in accordance with the Michaelis-Menten equation. (B) Lineweaver-Burk plot. Km was calculated according to the Michaelis-Menten equation. Km of Chox was found to be 0.36 mM.

The physicochemical properties of Chox purified from *S. olivaceus* MTCC 6820 was compared with the Chox obtained from other microbial sources as tabulated in **Table 6.1**.

Table 6.1 Comparison of physicochemical properties of Cholesterol oxidase purified from different microorganisms

Microorganism	MW (KDa)	pH optima	Temp. optima (^{0}C)	Stability		Km (mM)	References
				pH	Temp.		
Enterobacter cloacae	56.50	7	40	-	-	-	Rodrigues F and Palani P., 2016
Staphylococcus epidermidis	-	8	40	-	-	0.23	EI-Shora et al., 2011
Enterococcus hirae	60	7.8	40	-	-	-	Yehia et al., 2015
Streptomyces fradiae	60	7	70	4-10	50	0.07	Yazdi et al., 2000
Bordetella species	55	7	37	5	50	0.556	Lin et al., 2010
Pseudomonas sp.	56	7	-	4-11	High	0.2	Lee et al., 1989
Rhodococcus sp. PTCC 1633	55	7-7.5	40	-	40	0.015	Yazdi et al., 2008

6.3.2. Stability studies of purified Cholesterol oxidase

The stability of Chox purified from *S. olivaceus* MTCC 6820 was examined under various conditions which included pH stability and thermo-stability, stability in the presence of organic solvents, detergents, metal ions, chelating agents, and denaturants.

6.3.2.1. pH stability

The stability of purified Chox was studied in the pH range of 4 - 12, shown in **Fig 6.4**. The pH stability studies were carried out by incubating the enzyme with the buffers of different pH at room temperature for 24 h. The purified Chox retained 98-100% of residual activity between pH 6 - 8.5 while it retained almost 69 - 71% of its residual activity between pH 9.5 - 12 for 24 h. At pH 5.0, it retained 79% of residual activity. This shows that the Chox was stable over a wide range of pH, which makes it suitable for various applications. With varying pH, the charge on the amino acid present at the active site of the enzyme changes thus changing the electrostatic state of the enzymes, which affects the enzyme-substrate interaction (Kang et al., 1994). However, Chox with pH optima and stability near-neutral pH range was found to be suitable for clinical diagnostic purposes (Lolekha et al., 2004; Srisawasdi et al., 2006). Niwas et al. reported Chox from a new *Streptomyces sp.* which was stable in the pH range of 6 – 8 for 24 h at 4˚C (Niwas et al., 2013).

6.3.2.2.Thermo-stability

The thermo-stability study of purified Chox was carried out in a temperature range of 15°C - 90°C. It is visible in **Fig 6.5** that the enzyme retained 90 - 95% of its residual activity between temperatures 25°C - 50°C after 30 min of incubation at each temperature. Upon incubation of the enzyme for about 30 min at higher temperatures beyond 60°C viz. at temperatures 70°C, 80°C, and 90°C, around 69-79% residual activity was retained. Enzymes exhibit reduced catalytic activity beyond the optimum temperature range, which is due to the loss of protein three-dimensional conformation (tertiary and quaternary structure) causing loss of protein function due to denaturation. The thermal denaturation is slow in Chox purified from *S. olivaceus* MTCC 6820 and it is stable over a wide range of temperatures.

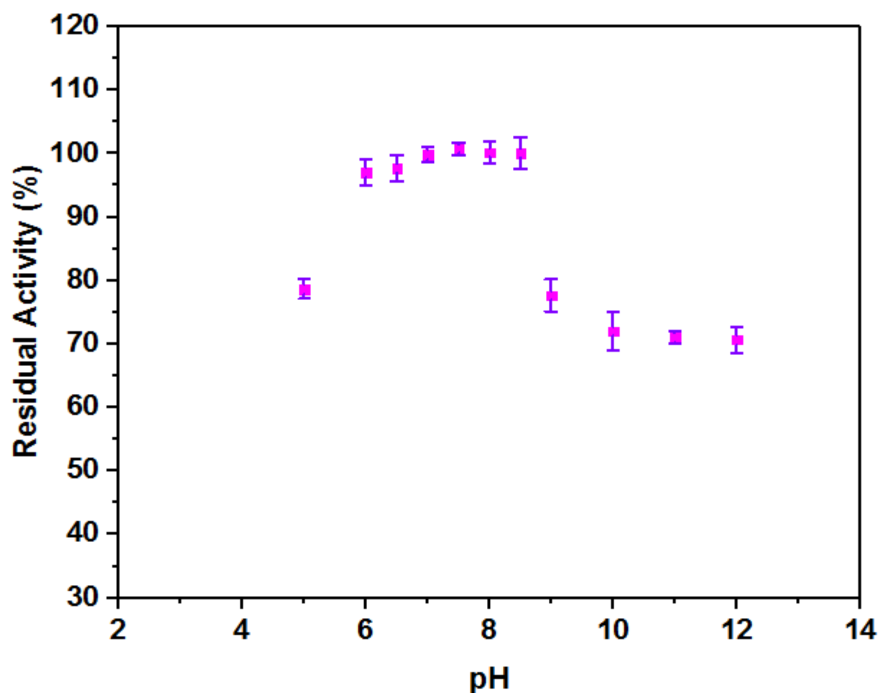

Fig. 6.4 pH stability studies of Cholesterol oxidase

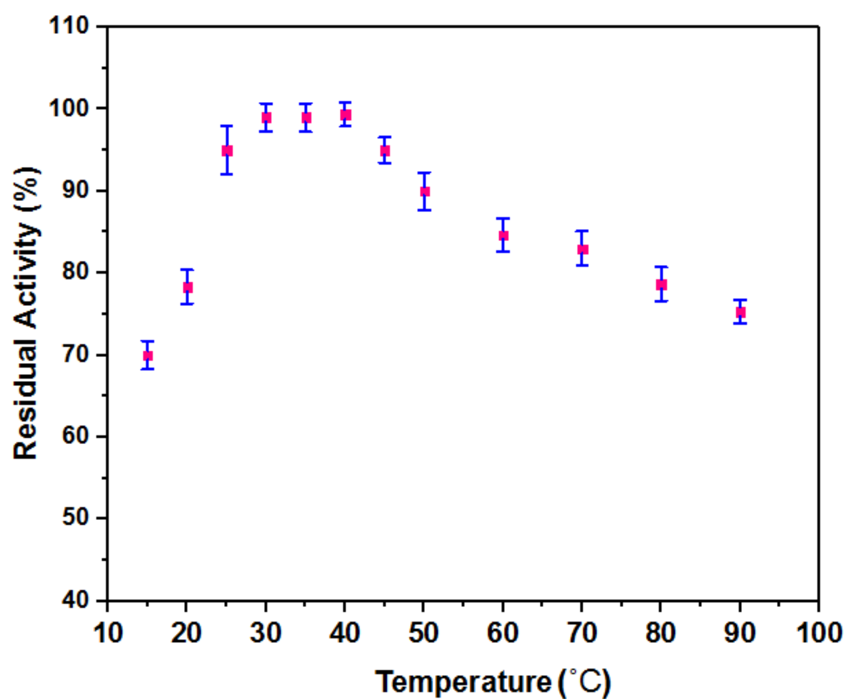

Figure 6.5 Thermostability studies of Cholesterol oxidase

6.3.2.3. Effect of organic solvents

Organic solvents are used as a solubilizing agent for cholesterol and other steroids in the pharmaceutical industry and very often it has been observed that Chox loses its activity in the presence of organic solvents (Doukyu et al., 2008). We examined the stability of purified Chox in the presence of 12 different organic solvents and the results have been tabulated in **Table 6.2**. The relative residual activity was calculated as a percentage of the enzyme activity without organic solvent (control). It was observed that Chox purified from *S.* MTCC 6820 was not only highly stable over a wide range of organic solvents but also was activated with subsequent enhancement of the enzyme activity manifolds.

Relative activity (%) of Chox in the presence of dichloromethane, ethyl acetate, ethanol, isopropanol, methanol, and toluene were obtained as 210.94 ± 0.33, 156.09 ± 0.18, 152.2 ± 0.22, 147.37 ± 0.30, 147.93 ± 0.15and 145.29 ± 0.3 respectively, which were enormously high as compared to the Chox reported from other microorganisms till date. Dimethyl sulphoxide (DMSO) and xylene also activated the purified Chox with relative activity (%) 117.51 and 133.52. These results provide an insight into two strong aspects of the Chox enzyme and the substrate (cholesterol) in the reaction. Firstly, the enzyme remained completely functional with its conformational flexibility uninterrupted in the aqueous mixture of these organic solvents, so that the active site of the enzyme was readily available to bind with the substrate and convert it into the product. And secondly, the solubility of the substrate (cholesterol) comparatively increased in the aqueous-organic co-solvent medium than the sole aqueous medium (as present in Control). Pure cholesterol is a hydrophobic molecule, insoluble in water, which can only be dissolved either with organic solvents or with detergents.

Here, depending upon the miscibility of an organic solvent with water and the relative proportion of the solvent and water in the medium, the mixture constituted three types of organic solvent systems - (i) water + water-miscible organic solvent system (ii) water + water-immiscible organic solvent system and (iii) a nearly anhydrous organic solvent system (Doukyu and Ogino, 2010). In water + water-miscible solvents viz. isopropanol, DMSO, methanol, and ethanol the aqueous rich milieu of the enzyme remained undisturbed, conserving the three-dimensional conformational stability and functionality of the enzyme. Additionally, the improved solubility of cholesterol in these solvent systems increased the substrate availability for the enzyme active site, thereby reducing the mass-transfer limitations leading to rapid reaction rates and yielding the utmost product. In the case of the water + water-immiscible solvents viz. dichloromethane, ethyl acetate, toluene, and p-xylene, a biphasic system is formed consisting of - the aqueous phase containing the dissolved enzyme and the phase of an immiscible organic solvent. Under such conditions, the enzymatic reaction proceeds in the aqueous phase containing enzyme, and the hydrophobic substrate (cholesterol) is located in the organic solvent phase and is later on partitioned into the aqueous phase. The aqueous phase forms a separate layer in contact with the organic solvent phase through the interfacial area. The low rate of mass transfer across the interface is eliminated by intensive stirring, the substrate is thus converted by the enzyme and the product is extracted into the organic phase. In such biphasic systems, the enzyme remains relatively stable because direct contact of the enzyme with organic solvent is minimized resulting in enhancement of the enzyme activity. The relative activity (%) of acetone, tertiary butanol, acetonitrile, and benzene was 41.18 ± 0.23, 47.2 ± 0.24, 28.08 ± 0.27, and 96.82 ± 0.14 respectively, which constituted nearly anhydrous organic solvent system. In the presence of an anhydrous organic solvent system, free soluble enzyme loses

their aqueous rich milieu which is essentially required for maintaining the three-dimensional structure of the protein, to exhibit the enzyme function. At such low water content, the conformational mobility of the enzyme is generally restricted and the protein gets more rigid in this system than in water. Although the cholesterol was soluble in these solvents too, the Chox enzyme activity was greatly reduced due to the loss of protein function. Therefore, despite free soluble Chox, if immobilized Chox is used with these organic solvents, there might be a possibility of getting enhanced enzyme activity because all the organic solvents used were good solubilizers of cholesterol. Besides hydrophobicity and log P value of the solvent, the solvent polarity and denaturation capacity are also some of the factors which decide the stability and catalytic potential of a biocatalyst in an organic medium.

It can be inferred from the results obtained that the Chox from *S. olivaceus* MTCC 6820 is an organic solvent tolerant enzyme, which makes it a potential enzyme for various serum and other clinical samples, biotransformation of 3β-hydroxysteroids, and other biopharmaceutical applications.

Chox obtained from many other microorganisms has also been shown to exhibit organic solvent tolerance. Chox isolated from a *Streptomyces sp.* showed 160, 230 and 115 relative activity (%), was stable in the presence of benzene and DMSO but inactivated in the presence of acetone and chloroform (Niwas et al., 2013). Commercial Chox reported from *Pseudomonas fluorescens, Nocardia erythropolis, Nocardia sp., Cellulomonas sp., and Streptomyces sp.* SA-COO was inactivated with the addition of dimethyl sulfoxide, methanol, ethanol, ethyl acetate, acetone, isopropanol, and butanol (Doukyu et al, 2008).

Table 6.2 Effect of organic solvents on activity of purified Cholesterol oxidase

Organic Solvents	Relative activity (%) ± SD
Control	100 ± 0.25
Di-methyl Sulphoxide	**117.09 ± 0.13**
Methanol	**147.93 ± 0.15**
Ethanol	**152.2 ± 0.22**
Acetone	41.18 ± **0.23**
Ethyl acetate	**156.09 ± 0.18**
Tertiary Butanol	47.2 ± **0.24**
Benzene	96.82 ± **0.14**
p - Xylene	**133.98 ± 0.27**
Toluene	**145.29 ± 0.3**
Di-chloro methane	**210.94 ± 0.33**
Acetonitrile	28.08 ± **0.27**
Isopropanol	**147.37 ± 0.30**

Each organic solvent [0.5 ml, 10% (v/v)] was added with 500 µl of purified Chox (14.9 IU) in eppendorf tubes. The mixture was incubated for 24 h with continuous shaking/stirring at 30°C. An aliquot (100 µl) of the sample was examined for enzyme activity under specified conditions (at pH 8.0, 30°C, reaction time 10 min). Blank was prepared separately taking into consideration each organic solvent by the addition of inactivated enzyme. The relative residual activity was calculated as a percentage of the enzyme activity without organic solvents (control). Mean values and standard deviation for five independent experiments are shown.

6.3.2.4. Effect of detergents

Detergents have also been used as solubilizing cholesterol for the analysis of serum and other clinical samples. The choice of suitable detergent for the dissolution of cholesterol determines the overall enzyme activity and stability of the enzyme. We examined the stability of Chox in the presence of 5 different ionic and non-ionic

detergents, the results have been summarized in **Table 6.3**. 500 μl of purified Chox (14.9 IU) was added to each eppendorf tube containing [0.5 ml, 1% (v/v)] non-ionic detergents viz. Tween-80, Tween-20, and Triton X-100 and ionic detergents viz. Extran and Sodium dodecyl sulfate. The mixture was incubated with shaking for 24 h at 30°C. An aliquot (100 μl) of the sample was examined for enzyme activity under specified conditions (at pH 8.0, 30°C, reaction time 10 min). Blank was prepared separately taking into consideration each detergent by the addition of inactivated enzyme.

The Chox from *S. olivaceus* MTCC 6820 showed remarkable stability in the presence of non-ionic detergents. Relative activity of Chox in the presence of Tween-80, tween-20, and triton X-100 was found to be 178.59 ± 2.38 %, 191.22 ± 1.96 % and 144.61 ± 3.34% respectively. While in the presence of ionic detergents Extran and SDS 56.81 ± 2.46 and 53.74 ± 1.67 % of residual activity was observed respectively. It means the activity of Chox was enhanced in the presence of non-ionic detergents and the Chox not only retained at least 50% of its residual activity but was also stable in both the ionic and non-ionic detergents. These results suggest the application of these Chox in serum cholesterol assay and in the pharmaceutical industry for the bioconversion of cholesterol to 4-Cholestenone.

Cholesterol is a water-insoluble molecule; its solubility increases in the presence of detergents, therefore non-ionic detergents are widely used in various Chox assay procedures. Ionic detergents are supposed to inhibit the activity of Chox. But here the Chox is stable more than 50% in the presence of ionic detergents.

Table 6.3 Effect of detergents on activity of purified Cholesterol oxidase

Detergents 1% (v/v)	Relative activity (%) ± SD
Control	101.76 ± 2.003
Tween-80 (non-ionic)	**178.59 ± 2.38**
Tween-20 (non-ionic)	**191.22 ± 1.96**
Triton X-100 (non-ionic)	**144.61 ± 3.34**
Extran (non-ionic + ionic)	56.81 ± 2.46
Sodium dodecyl sulphate (ionic)	53.74 ± 1.67

500 µl of purified Chox (14.9 IU) was added to each eppendorf tube containing 0.5 ml, 1% (v/v) various detergents. The mixture was incubated with shaking for 24 h at 30°C. An aliquot (100 µl) of the sample was examined for enzyme activity under specified conditions (at pH 8.0, 30°C, reaction time 10 min). Blank was prepared separately taking into consideration each detergent by the addition of inactivated enzyme. The relative residual activity was calculated as a percentage of the enzyme activity without detergent treatment (control). Mean values and standard deviation for five independent experiments are shown.

6.3.2.5. Chelating agent and denaturants

EDTA is a chelating agent and most enzymes lost their activity in the presence of EDTA. While Chox from *S. olivaceus* MTCC 6820 showed 119.34% relative activity up to 2 h in the presence of EDTA. No inhibition of Chox activity was observed in the presence of EDTA. Similar results were found in the case of Chox produced by *γ-Proteobacterium Y-134* by Isobe et al. (Isobe et al., 2003). Urea is a denaturing agent and due to the denaturation of the enzyme, loss of enzyme activity is noticed in many cases. While the partitioned Chox retained 66.03% residual activity up to 2 h.

Control was prepared by incubating the enzyme without EDTA and urea.

6.3.2.6. Effect of metal ions

The effect of various metal ions Mg^{2+}, Mn^{2+}, Cu^{2+}, K^{+}, Na^{+}, Ca^{2+}, $Zn^{2+,}$ and Ba^{2+} on Chox activity was studied, the summarized relative activity of the same has been tabulated in **Table 6.4**. The relative activity for purified Chox in the presence of Zn^{2+} and Ba^{2+} were 167.24 ± 2.64% and 153.34 ± 1.75% respectively. Usually, Ba^{2+} inhibits the enzyme activity, but in the case of Chox, it is behaving as an activator. Na^{+} and K^{+} also seem to be activators of Chox with 121.75 ± 2.31% and 107.32 ± 1.65% relative activity respectively after 24 h of incubation at room temperature. The Chox activity was highly inhibited in the presence of Mn^{2+} as it showed only 2.37 ± 0.77% of relative activity. Cu^{2+} also inhibited Chox activity as it showed 28.37 ± 1.97% relative activity.

Table 6.4 Effect of metal ions on activity of purified Cholesterol oxidase

Metal ions (100mM)	**Relative Activity (%)**
Control	101.48 ± 1.28
Mg^{2+}	**100.19 ± 0.88**
Mn^{2+}	2.37 ± **0.77**
Zn^{2+}	**167.24 ± 2.64**
Ca^{2+}	85.59 ± **2.10**
Cu^{2+}	28.37 ± **1.97**
Na^{+}	**121.75 ± 2.31**
K^{+}	**107.32 ± 1.65**
Ba^{2+}	**153.34 ± 1.75**

* The relative residual activity was calculated as a percentage of the enzyme activity without incubation with metal ions (control). Mean values and standard deviation for five independent experiments are shown.

6.4. CONCLUSION

The pH and temperature optima range for purified Chox were 7.0 and 35˚C which was suitable for its application in serum cholesterol assay. The kinetic constant values K_m and V_{max} for purified Chox were determined as 0.36 mM and 1.62 μM/min/mg protein. An organic solvent and detergent tolerant Chox were purified from *S. olivaceus* MTCC 6820 showing very high stability in the presence of various organic solvents and detergents studied. No significant change in enzyme activity was observed in the presence of chelating agents and chemical denaturants. Apart from these, some metal ions viz. Zn^{2+}, Ba^{2+}, Na^{+} and K^{+} were also found to be the activators of Chox which showed enhanced Chox activity. The relative activity for purified Chox in the presence of Zn^{2+} and Ba^{2+} were 167.24 ± 2.64% and 153.34 ± 1.75% respectively. Usually, Ba^{2+} inhibits the enzyme activity, but in the case of Chox, it was behaving as an activator. Na^{+} and K^{+} also seem to be activators of Chox with 121.75 ± 2.31% and 107.32 ± 1.65% relative activity respectively. The study of the physicochemical properties of Chox purified from *S. olivaceus MTCC 6820* revealed remarkable properties viz. organic solvent and detergent tolerant. Thus, the purified Chox may be used as a potential enzyme for application in various fields like clinical diagnosis, pharmaceutical, and food industries.