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Purification and Characterization of Cholesterol Oxidase from a Novel Source - *Enterobacter cloacae*

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Abstract: *The renewed interest in exploring various natural habitat/environments for discovering novel microbial sources as stable cholesterol oxidase producers is on the incline due to its broad-range of clinical and industrial applications. A search was conducted to isolate cholesterol oxidase producing bacteria from samples collected from different environments. A total of 98 bacterial isolates capable of degrading cholesterol were obtained from various soil samples collected from in and around Chennai. The bacterial strain producing the highest level of cholesterol oxidase was selected, identified as Enterobacter cloacae by molecular studies and used for all further studies. Purification of cholesterol oxidase was carried out by ammonium sulphate precipitation (80% w/v) followed by Q sepharose and DEAE-cellulose chromatographie techniques. The enzyme was purified to 4.63 fold with a recovery of 93.8 mg of purified protein from 2,889 mg of total protein. The purification yield of the enzyme was 14.86% and its specific activity was 21.8 Umg^{-1}. SDS PAGE and MALDI TOF analyses proved that the enzyme was a monomer with a molecular mass of 56.50461. The enzyme exhibited optimum activity at pH 7.0 and was stable up to 40°C for an hour. Metal ions such as, Mn^{+}, K^{+} and Zn^{+} inhibited the enzyme activity by 82, 37.66 and 32.8% respectively while most of the other metal ions showed less than 25% inhibition. Triton X-100 and Tween-80 failed to inhibit its activity, however SDS decreased in its activity by 35.2%. Therefore E.cloacae can be exploited for industrial production of stable cholesterol oxidase.*

Keywords*: cholesterol oxidase, characterization, purification, Enterobacter cloacae*

I. Introduction

Cholesterol oxidase (EC1.1.3.6), or 3β-hydroxysterol oxidase or alcohol deydrogenase/oxidase is a bacterial FAD-containing flavo oxidase which catalyses the initial reaction in cholesterol catabolism. This enzyme is capable of catalyzing two reactions, oxidation and isomerization of cholesterol to give rise to cholest-5-en-3-one and cholest-4-en-3-one respectively [27, 34]. Cholesterol oxidases can be intrinsic membrane bound enzymes located on the outside of the cells, as found in *Nocardia rhodochrous*, *Nocardia erythropolis* and *Mycobacterium* spp., or can be secreted into the growth medium as extracellular enzyme as found in *Streptomyces violascens, Brevibacterium sterolicum, Rhodococcus equi, Streptoverticilium cholesterolicum, Mycobacterium* ATCC 19652 and *Rhodococcus erythropolis* [43]. Most of the isolated strains capable of producing cholesterol oxidase belonged to the genus *Rhodococcus*. These organisms degraded cholesterol with little or no accumulation of steroid intermediates as determined by thin layer chromatography (TLC). In 1984, Ferreira and Tracey [15] compared the numerical taxonomy of cholesterol degrading soil bacteria and they found that the majority of the cholesterol degrading bacteria belonged to the genera *Rhodococcus*, *Mycobacterium* and *Nocardia.* The industrial demand for cholesterol oxidase is met mainly by bacteria. The gene *choE* responsible for cholesterol oxidase has been identified and is homologous to secreted cholesterol oxidase identified from *Streptomyces*. This protein also exhibits significant similarities to putative cholesterol oxidase encoded by *Mycobacterium tuberculosis* and *Mycobacterium leprae*. Many soil bacteria produce cholesterol oxidase either as intracellular or extracellular enzymes; *Rhodococcus erythropolis* has been found to produce both intracellular and extracellular cholesterol oxidase. Lv et al. (2002) [26] have reported an elevated level of cholesterol oxidase (1285 U/L) from a mutant *Brevibacterium* after 36h of cultivation. Later, Wang et al. (2008) [39] discovered higher levels of extracellular cholesterol oxidase (1500 U/L) by *Rhodococcus* sp. R14-2. An elevated level of cholesterol oxidase (~2500 U/L) production has been reported from the non pathogenic *Streptomyces* sp., which has been commercialized. Therefore, the enzyme may be isolated from broth filtrate as an extracellular enzyme or as intracellular membrane bound enzymes located on the outer membrane of the cell. Cholesterol oxidase has gained importance in industrial and clinical fields, thus this investigation was carried out to isolate and identify bacteria capable of hyper producing cholesterol oxidase and to purify and characterize extracellular cholesterol oxidase produced by the selected bacterial species.

II. Materials and Methods

Chemicals

All the analytical grade chemicals used in this study were purchased from Sigma-Aldrich Chemicals Co. (USA). Components for bacteriological media were obtained from Hi-media (India). The organic and inorganic chemicals, and solvents used were of analytical grade, obtained from Qualigens or SD-Fine chemicals (India). Protein molecular weight marker and DNA ladder were purchased from Genei (India). PCR master mix and primers were purchased from Xcleris Genomics (Ahmadabad, India). All the solutions were prepared with double distilled water just before use, unless mentioned otherwise.

Isolation of microorganisms

Various soil samples were collected from vegetable, fish, dairy, poultry, animal farms and markets in and around Chennai. Five grams of each sample was suspended in 100 ml of sterile distilled water, and were spread on a Primary screening agar plate containing cholesterol as the sole carbon source. This medium contained: agar, 15 (g/L); K_2HPO_4 2.5 (g/L); NH_4NO_3 17 (g/L); $MgSO_4.H_2O$ 2.5 (g/L); $FeSO_4$ 0.01 (g/L); NaCl 0.05 (g/L); cholesterol 1.0 (g/L) and tween-80 1.0 (ml/L). The pH of medium was adjusted to 7.0 prior to sterilization. The plates were incubated at 30ºC for 4 days. Halo forming colonies were selected and subcultured on to a secondary screening medium, containing: yeast extract 3.0 (g/L); $(NH_4)_2HPO_4$ 1.0 (g/L); cholesterol 1.5 (g/L); Tween-80 1.0 (ml/L) and agar 15.0 (g/L). Cultures were incubated at 30ºC for 24h [43]. The colonies generated on the secondary medium were used for further identification and differential tests. All the colonies were screened for enzyme production followed by conventional identification of bacteria. Bacterial cultures were centrifuged at 10,000 rpm and the supernatant obtained served as the crude enzyme source and was analyzed for extracellular enzyme activity of cholesterol oxidase.

Cholesterol oxidase indicator plates

Colonies which grew on the secondary medium were selected and grown in a secondary broth. Bacterial cultures were centrifuged at 10,000 rpm and the supernatant obtained served as the crude enzyme source. The supernatant (50µL) obtained were loaded into the wells formed in the indicator plates and incubated at 4ºC for a week. These plates were prepared by adding cholesterol (1.0 g/L), Triton X-100 (1.0 ml/L), o-Dianisidine (0.1 g/L) and Peroxidase (1000 U/L) to 1 liter of LB agar medium [32].

Cholesterol oxidase Assay

Cholesterol oxidase activity was measured at 240 nm by the method of Richmond (1976) [33]. The reaction mixture composed of 3 ml of 0.1 M sodium phosphate buffer (pH 7) with 0.05% Triton X-100, 0.05 ml 6 mM cholesterol in 2-propanol and 0.05 ml culture supernatant. The enzyme reaction was carried out at 30ºC for 30 min followed by the measurement of the increase of absorbance at 240 nm. The molar absorption Δ of 4-cholesten-3- one was 12.2 × 103 l M^{-1} cm^{-1}. Cholesterol oxidase activity was calculated as (ΔA × reaction volume × 0.082)/volume of enzyme used) = ΔA × 5.1 Uml^{-1}. One unit of cholesterol oxidase was defined as the amount of enzyme which oxidizes 1 μ mole of cholesterol to 4-cholesten-3-one per min at 30ºC. Protein estimation was performed by following the method of Bradford (1976) [1].

Identification of cholesterol oxidase positive bacterium

Molecular identification of the bacterial strain FH68 was carried out by amplifying the 16S rRNA gene. The forward primer: AGAGTTTGATCMTGGCTCAG and reverse primer: GYTACCTTGTTACGACTT were used. The product was sequenced using BDT v3.1 Cycle sequencing kit on ABI 3130 Genetic Analyzer. Consensus sequence of 16S rRNA gene was generated from forward and reverse sequence data using aligner software. The 16S rRNA gene sequence was used to carry out BLAST analysis with the NCBI GenBank database. Based on maximum identity score the first ten sequences were selected and aligned using multiple alignment software program ClustalW. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4 software.

Purification of extracellular cholesterol oxidase produced by *Enterobacter cloacae*

E.Cloacae cells were grown in an optimized medium (tallow (0.9% w/v), tween-80 (4.0 g/L), yeast extract (2.0 g/L), $MnSO_4$ (0.01 g/L), $ZnSO_4$ (0.01 g/L), KH_2PO_4 (0.1 g/L), Na_2HPO_4 (0.5 g/L), CaCl (0.01 g/L), NaCl (1.0 g/L), $FeSO_4$ (1.5 g/L) at pH 7.0). The medium was inoculated with 12.5% (v/v) of 48 h old inoculum and incubated for period of 36 h. Cells were harvested and centrifuged at 10000 rpm for 15 min at 4ºC. The supernatant obtained was used for the purification of extracellular enzyme. The supernatant collected as above was subjected to ammonium sulfate (80% w/v) precipitation. The precipitated proteins were dialyzed against sodium phosphate buffer (0.05M; pH 7.0) overnight. Lyophilized proteins were dissolved in Tris-HCl buffer (0.05M; pH 7.0) and the solution was passed through Q-sepharose column (10 x 2.5 cm) with pH 10.0. The column was eluted with Tris-HCl buffer (0.05M; pH 10.0) with a linear gradient of NaCl (0.05 to 0.5). The fractions possessing cholesterol oxidase activity were pooled together, concentrated and dialyzed against sodium phosphate buffer (0.05M; pH 7.0) at 4°C overnight. Lyophilized proteins were dissolved in sodium phosphate buffer (0.05M; pH 8.0) and loaded on to DEAE-cellulose ion exchange column (2.5 by 10 cm) which was equilibrated with sodium phosphate buffer (0.05M; pH 8.0). The bound proteins were eluted with a linear gradient of NaCl (0.05-0.5M) in the same buffer. Protein content and cholesterol oxidase activity were determined at every step of purification and has been listed in table-1. Finally, active cholesterol oxidase fractions were pooled, desalted, lyophilized and used for characterization of the enzyme.

Molecular mass determination

SDS-PAGE was performed according to the protocol described by Laemmli (1970) [19] . The resolving gel and the stacking gels were prepared for to 12.5 and 5% respectively. The purified protein sample was run on SDS-PAGE with a concurrent run of standard protein markers (14 to 95 kDa) obtained from SRL chemicals (India). The gel was carefully removed from the glass plates and was stained using the Imidazole-Zinc method [9]. The molecular mass of the purified protein was determined by comparing the relative mobility value of the unknown protein with known protein molecular weight markers.

Mass Spectrometry analysis of purified cholesterol oxidase.

Mass spectrometry analysis of the purified protein was carried out according to the method of Gundry *et al.*, 2010 [13]. Reduction of disulfide bonds and alkylation of free cysteines, trypsin digestion, and extraction of peptides from trypsin digested protein, desalting of peptides prior to MS analysis and finally MS analysis was carried out. Peptide mass fingerprinting and sequence analysis aided in detecting local as well as global alignments along with the molecular weight of the purified protein. The amino acid sequence obtained from the purified enzyme was compared with other amino acid sequences available in the UniProt database with the aid of computation program, BLAST homology analysis.

Physiochemical properties of cholesterol oxidase.

The optimum pH of the purified enzyme was measured by incubating 2.0 ml (3 U/mL) of purified enzyme with 2.0 ml of buffers with different pH's sodium acetate buffer (0.1 M; pH 4.5–5.5), sodium phosphate buffer (0.1 M; pH 6.0–8.0) and glycine-NaOH buffer (0.1 M; pH 8.5–9.0) at 40°C, without the substrate for 30 min. The residual activity of the enzyme was measured by the assay method. Similarly, the optimum temperature stability of the enzyme was measured by incubating 2.0 ml (3 U/mL) purified enzyme with 2.0 ml buffer (sodium phosphate buffer, 0.1 M; pH 7.0) without a substrate at different temperatures (20–100°C) for 30 min. The effects of 13 metal ions and three detergents on cholesterol oxidase activity were investigated. The enzyme was incubated with metal ions, detergents and a control at a final concentration of 1 mM for 1 h, and the residual enzyme activity was assayed with cholesterol as the substrate.

III. Results and Discussion

In the present investigation, an attempt has been made to explore the possibility of using bacteria for the production of cholesterol oxidase. As a first step, isolation of bacteria was performed with samples collected from different environmental conditions such as poultry farms, dairy farms, fish markets, abattoirs and vegetable gardens. As many as 98 isolates of bacteria from 25 different soil samples were obtained using the primary screening media. These isolates were screened for the production of extracellular cholesterol oxidase both quantitatively and qualitatively. The qualitative detection of cholesterol oxidase producing bacteria was performed by allowing the bacterial cultures to grow on a medium containing cholesterol. The cholesterol oxidase produced by individual bacterium oxidized cholesterol with the concomitant production of H_2O_2. The medium contained horse radish peroxidase and O-dianisidine, which interacted with hydrogen peroxide (H_2O_2) and formed an azo compound which turned the color of the medium to brown. [32]. Based on whether or not halo formation around the colony occurred, cholesterol oxidase positive and negative isolates were segregated. There were 16 cholesterol oxidase positive isolates out of a total number of 98 bacterial isolated in the present study (Table-1). Among the positive isolates, an isolate designated as FH68 formed relatively large halo (25mm; Fig 1) around the colony when compared to other bacterial strains. Several studies have used various methods to confirm the production of cholesterol oxidase by different bacteria [32]. Of the 98 isolates, only 14 isolates exhibited brown halos on the indicator plate (Fig. 1) cholesterol oxidase production by the bacteria was also determined quantitatively and the results are presented in Table-1. In addition to the 14 isolates which were confirmed by qualitative indicator plate for cholesterol oxidase production. Two other isolates namely BT96 and BT98 did not develop brown halos on the indicator plates however when assayed quantitatively showed positive for cholesterol oxidase activity. Out of the 16 positive isolates, a strain designated as FH68 has shown relatively higher cholesterol oxidase specific activity (Table-1). Therefore, this strain was chosen for further studies. The gene

sequence obtained with 16S rRNA gene of strain FH68 was compared with other 16S rRNA gene sequences available in the Gen Bank database of NCBI with the aid of computation program, BLASTN homology analysis. The 16S rRNA gene sequence of the isolate FH68 showed 100% similarity with sequence of *Enterobacter cloacae*. Therefore, the isolate was identified as *Enterobacter cloacae*. The 16S rRNA gene partial sequence has been deposited in the NCBI Gen Bank with the accession number KJ958487.1. A phylogenetic tree was constructed using the 16S rRNA partial gene sequence of FH68 strain and related sequences were retrieved from GenBank database (www.ncbi.nlm.nih.gov) showed the sequences clustered according to the previous taxonomic assignment. The phylogenetic tree was observed within the *Enterobacter* genus clade, possibly due to the high identity shared by the sequences. Hence, the strain FH68 was confirmed as *Enterobacter cloacae.*

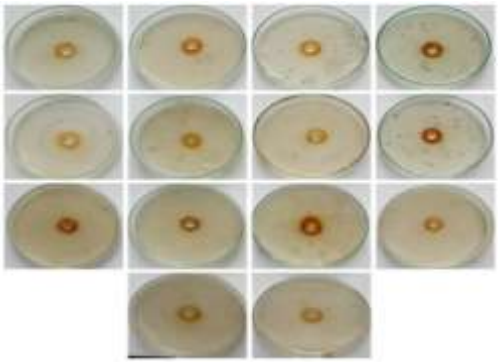

Figure 1: Qualitative detection of cholesterol oxidase activity of different bacterial isolates on indicator plates.

Table 1: Screening and selection of cholesterol oxidase producing bacterial strains

Bacterial strains	Protein	Total cholesterol	Specific activity
ES 2	0.574 ± 0.004	0.55 ± 0.03	0.96 ± 0.05
ES15	0.585 ± 0.004	0.537 ± 0.03	0.8 ± 0.05
KF21	0.415 ± 0.003	0.627 ± 0.01	1.54 ± 0.01
KF22	0.426 ± 0.003	0.707 ± 0.02	1.65 ± 0.06
EW26	0.648 ± 0.001	0.57 ± 0.01	0.87 ± 0.01
EW31	0.724 ± 0.005	0.717 ± 0.02	0.98 ± 0.03
GB36	0.583 ± 0.002	0.827 ± 0.02	1.41 ± 0.03
GB43	0.593 ± 0.005	0.607 ± 0.02	0.99 ± 0.04
BT45	0.564 ± 0.003	0.573 ± 0.02	1.05 ± 0.03
BT65	0.435 ± 0.003	0.513 ± 0.02	1.17 ± 0.03
FH68	**0.564 ± 0.002**	**1.163 ± 0.03**	**2.08 ± 0.04**
VG84	0.495 ± 0.003	0.677 ± 0.02	1.35 ± 0.03
BT88	0.621 ± 0.001	0.607 ± 0.03	1.01 ± 0.04
BT90	0.446 ± 0.003	0.723 ± 0.02	1.58 ± 0.04
BT96	1.473 ± 0.003	0.283 ± 0.03	0.19 ± 0.02
BT98	0.925 ± 0.004	0.327 ± 0.02	0.353 ± 0.03

Each value is the mean of three experiments.

The capacity to degrade cholesterol is widespread among a number of microorganisms that have been explored either as free or immobilized cells [22, 41] or as a source of cholesterol oxidase [5] in steroid biotransformations. A number of investigators have isolated cholesterol oxidase from different sources since its discovery in *Rhodococcus erythropolis* [37].

Many species belonging to the genus *Rhodococcus* possessed the ability to degrade cholesterol [10]. Apart from this genus, extracellular cholesterol oxidase production has been reported from other genera such as *Micrococcus* [17], *Burkholderia* and *Chromobacterium* spp. [7], *Bacillus* [30]*) Nocardia* [35]. *Pseudomonas*.[2, 4, 7, 24], *Schizophyllum* [11], *Streptomyces* ([12, 16, 32, 44] and *Gamma Proteobacterium* Y-134 [14].

The purification of extra cellular cholesterol oxidase was achieved by 80% ammonium sulphate precipitation, Q-Sepharose chromatography and DEAE Cellulose chromatography (Table-2). The purified protein appeared as a single homogenous band with a molecular mass of 56.50 kDa (Fig. 2), as determined by comparing the relative motility of the purified enzyme with standard molecular weight markers. State-of-the-art purification techniques were used to obtain purified extra cellular cholesterol oxidase. The purified protein appeared as a single homogenous band with a molecular mass of 56.50 kDa, which is in agreement to the molecular mass of cholesterol oxidase isolated from most microorganisms, which lie between 31 and 61 kDa [27] The cholesterol oxidase produced by *Pseudomonas* sp. strain CO629 was a monomer with a molecular weight of 56 kDa. On the other hand, the molecular weight of the enzyme from *S. violascens*[38]. *B. sterolicum* [42] and *Schizophyllum commune* [8] have been determined as 61, 31 and 53 kDa respectively. All these enzymes consisted of a single polypeptide chain.

Table 2: Summary of purification steps

Purification steps	Total volume (mL)	Protein (mg/ml)	Total protein (mg)	Total Activity (U/ml)	Specific Activity (U/mg)	Purification (Fold)	% yield (%)
Supernatant	3000	0.963	2889	13800	4.7	100	0
Ammonium sulphate Precipitation	80	10.53	842.4	7704	9.14	1.94	55.82
Q-Sepharose chromatography	52	3.5	182	3172	17.42	3.7	22.98
DEAE Cellulose chromatography	34	2.68	93.8	2052	21.87	4.65	14.86

Each value is the mean of three experiments.

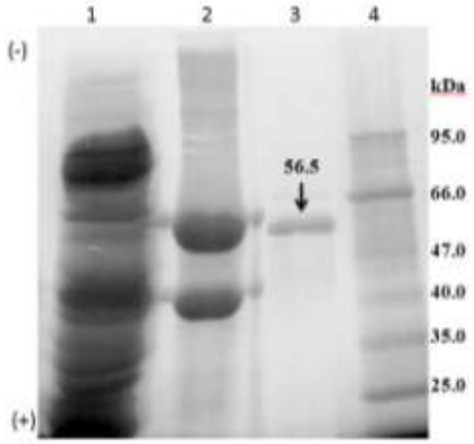

Figure 2: Determination of purity and molecular mass of purified cholesterol oxidase

Lanes: 1-Ammonium sulphate precipitated protein;
2-Protein obtained after Q Sepharose chromatography;
3- Protein obtained after DEAE cellulose chromatography; 4- Standard protein molecular weight markers

The peptide masses from the MALDI TOF spectrum yielded a total number of 42 peptides (Table 3and Fig. 3). The combined molecular weight of the purified enzyme was found to be 56.50461 kDa (Fig. 2 and Table 3). This molecular weight is in agreement with the molecular weight of the purified enzyme obtained from SDS PAGE. The BLAST algorithm detected local as well as global alignments, regions of similarity embedded in otherwise unrelated proteins and based on the similarity provided, the uncharacterized protein was found to possess 70% identity with sequences of cholesterol oxidase obtained from other sources (Table 4). Therefore, the purified enzyme was confirmed as cholesterol oxidase. The molecular mass of cholesterol oxidase isolated from most microorganisms lie between 31 and 61 kDa [27]. The cholesterol oxidase produced by *Pseudomonas* sp. strain CO629 was a monomer with a molecular weight of 56 kDa. On the other hand, the molecular weight of the enzyme from *S. violascens* [38] *B. sterolicum* [42] and *Schizophyllum commune* [8] have been determined as 61, 31 and 53 kDa respectively. All these enzymes consisted of a single polypeptide chain.

Table 3: Peptides obtained from the MALDI-TOF analysis

S.No	Peptide	M.Wt (Da)
1	GSK	290.15901
2	MR	305.1517
3	LAR	358.23284
4	ALTIYR	387.0666
5	TIR	388.24341
6	AFFK	511.2786
7	GGAAPR	527.28157
8	AFWR	578.2965
9	NSIKAG	588.32311
10	AELTGR	645.34457
11	QTKGGR	645.3548
12	AVAVNAAK	742.4324
13	ALDNEGR	773.36676
14	GDFVYDK	842.3796
15	WPTGPFR	859.43405
16	AWDDQAGK	889.39297
17	VNLNWTR	901.4756
18	YYPLVRK	937.53852
19	AGVQVTVLER	1070.60838
20	DQNAPAVNAAR	1125.5509
21	YDLNQNYLR	1197.5779
22	TAFTNDTLPDGGR	1363.63678
23	QLNRYELSCDR	1395.65649
24	YLISVTQLAPEGK	1417.78165
25	ANMIKGQGQGSPSASR	1587.77872

26	SLPSAIAGASNHGNSNGAK	1751.85504
27	QAEATGKTTIYPGHEVK	1828.93189
28	LVLAAGSIGTTELLVSAQAK	1941.11459
29	GAAVGGGSVIFTGVMIQPER	1945.00908
30	AGLGTVFIDSIFNWDVVR	2008.04176
31	NYFDAIFGGGNSYDEMDK	2041.83634
32	MLNLNSMPLDIYNSSPFGHSR	2392.13037
33	GTLPNLNEEIGQGWGSNGDIMTAR	2529.19176
34	YDLFGTQDANANWTAHPLGGAVLDK	2673.28227
35	NIVNLDLDQFGGVMDVTEYPNMTVWR	3025.43134
36	VIGYPGLYVMDGALVPGSTGSVNPALTITALAER	3401.7904
37	IHDRNAGLPVTLENWYVPGVPVNLGIIGSLGMAMDETNR	4218.13534
38	AAGAAALLGAAAGSASSAFP	6294.21361

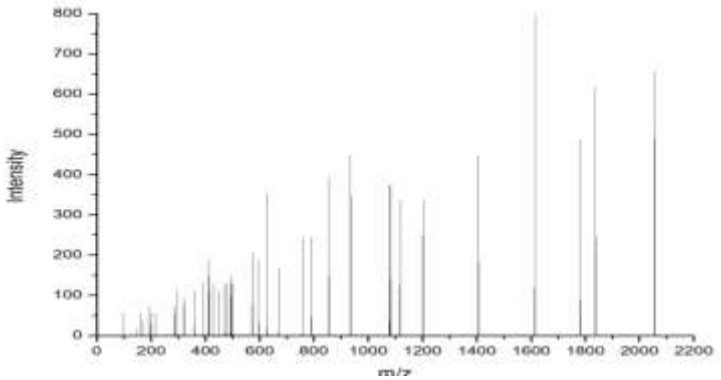

Figure 3: MALDI-TOF MS spectrum obtained from trypsin digested cholesterol oxidase

Table 4: Amino acid sequence alignment of peptides obtained from cholesterol oxidase after trypsin digestion.

Residues	Sequence				
1-50	M**RR**RA**F**FKAA	**GA**AALLGAAA	GSASSAFPAP	HAT**SAL**AAAD	GVYA**APELFQ**
51-100	**P**VDPAPEHSQ	ALV**VGTGFG**A	VAAL**AR**LAR**A**	**G**VQ**VTVLERG**	S**KWPTGPFRT**
101-150	**AFTNDTLPDG**	G**RAFW**RRGGA	**APRNIVN**LDL	DQFG**GVMD**VT	EYP**NMTVWRG**
151-200	**AAVGGGSVIF**	**TGVMIQPERN**	**YFDAIFGGG**N	**SYDEMDKKYY**	**PLVRKMLNLN**
201-250	S**MPLDIYNS**S	**PFGHSRAWDD**	**QAGK**AGL**GTV**	F**IDSIFNWDV**	VR**AELTGRSL**
251-300	**PSAIAGASNH**	**GNSNGAKYDL**	**NQNYL**R**QAEA**	**TG**K**TTIYPGH**	**EV**KTIRQTKG
301-350	GR**YLISV**TQL	A**PEGKQLNRY**	**ELSCDRLVLA**	**AGSIGTTELL**	**VSAQAKGTLP**
351-400	N**LNEEIGQGW**	**GSNGD**IMTAR	ANM**IKG**QG**Q**G	**SPSASRIHDR**	**NAGLPVTLEN**
401-450	**WYVPGVPVNL**	**GIIGSLGMA**M	**DETNRG**D**FVY**	**DKVNL**N**WT**RD	Q**NA**PA**V**NA**AR**
451-500	A**V**AVNAAKAL	TIYRYDLFGT	QDANAN**WTAH**	**PLGAVLDKAL**	**DNEGRVIGY**P
500-534	**GLYVMDGA**LV	**PGSTGSVN**AL	TITA**LAERNS**	**IKAG**	

Physicochemical properties of cholesterol oxidase

The purified cholesterol oxidase enzyme exhibited activity from a pH 4.0 to 9.0 (Fig. 4). However, optimum activity was observed at pH 7.0. The behavior of an enzyme under different micro environment plays a critical role in the application of the enzyme for commercial use. The purified cholesterol oxidase enzyme exhibited activity from a pH 4.0 to 9.0. However, the optimum activity was observed at pH 7.0, which is exactly similar to the observation made by Lashkarian *dococcus* sp. 501. Cholesterol oxidase from *Brevibacterium* sp. has shown maximum activity at pH 7.5 [36] and decreased to ıcubated at pH of 6.0 and 8.7.Cheetham et al.(1982) [3] have observed the optimum pH for cholesterol oxidase activity derive *rdia* and *Rhodococcus* which was found to be at pH 7.5. Moreover, cholesterol oxidase produced by *Rhodococcus* sp. 501 show ı a broad range of pH 3-9 [21].

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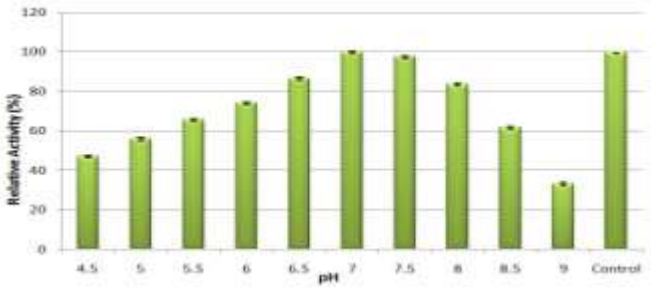

Figure 4: Effect of pH on cholesterol oxidase activity

The activity of cholesterol oxidase did not change when incubated from 10 to 40ºC (Fig. 5). Temperatures beyond 40ºC had reduced the activity of the enzyme, however about 50% of its activity was retained even at 80ºC (Fig. 4) Notably the enzyme did not lose its activity even when incubated at 100ºC. Another interesting observation made with cholesterol oxidase of *Enterobacter cloacae* in the present study is that the enzyme activity did not change when incubated from 10 to 40ºC (Fig. 4). Temperatures beyond 40ºC has reduced the activity of the enzyme, however about 50% of its activity was retained even at 80ºC (Fig. 4) Notably the enzyme did not lose its activity even when incubated at 100ºC. The cholesterol oxidase from *Brevibacterium* sp. had an optimum temperature for enzyme activity at 53°C [36] which is different from the observation made with the same enzyme from *Rhodococcus equi* (47°C) and *Corynebacterium cholesterolicum* (40°C) [28]. The purified enzyme from *Streptomyces violascens*, exhibited an optimum temperature at 50°C and plateaued between 40 and 60°C [43].The optimum temperature of cholesterol oxidase from *Bacillus* sp [25], *Arthrobacter simplex* [3] and *Streptomyces violascens* [40] has been found to range between 50 – 60°C and the enzymes appeared to be thermo-tolerant. Cholesterol oxidase produced by *Rhodococcus* sp. 501 showed marked activity between 20 and 50°C with optimum activity at 35°C [21].

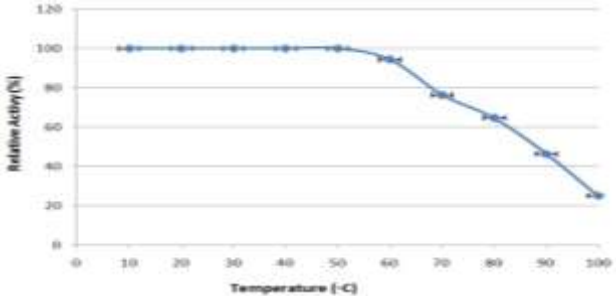

Figure 5: Effect of temperature on cholesterol oxidase activity

Among the 13 metal ions tested in this study (Table- 4), only Mn^+, K^+ and Zn^+ inhibited the enzyme activity by 81.8, 37.66 and 32.8% respectively whereas most of the other metal ions showed less than 25% inhibition. When this enzyme was mixed with nonionic detergents namely Triton X-100, Tween-80 and SDS for 60 min, it retained all of its activity and only SDS showed 35.2 % decrease in its activity. The action of cholesterol oxidase is strongly influenced by the environment such as the concentration of substrate, metal ions and detergent solutions [23].Nagasawa et al. (1969) [31] reported that most metals including $CaCl_2$ were found to inhibit cholesterol oxidase activity strongly. Among the 13 metal ions tested in this study (Table- 5), only Mn^+, K^+ and Zn^+ inhibited the enzyme activity by 81.8, 37.66 and 32.8% respectively whereas most of the other metal ions showed less than 25% inhibition.

Table 5: Effect of metal ions and detergents on enzyme activity

Metal ions and detergents	**Relative Activity (%)**	**Metal ions and detergents**	**Relative Activity (%)**
Control	100	KCl	62.34
$CaCl_2$	85.80	$CuSO_4$	90.1
CuCl	83.10	$AgNO_3$	76.5
MnCl	18.20	$HgCl_2$	71.1
FeCl	89.37	EDTA	79.62
MgCl	87.09	0.1 % SDS	64.81
$ZnSO_4$	67.2	0.1 % Tween-80	101.8
NaCl	84.75	0.1 % Triton X-100	101.23
CoCl	77.16		

Each value is the mean of three experiments.

When this enzyme was mixed with nonionic detergents namely Triton X-100, Tween-80 and SDS for 60 min, it retained all of its activity and only SDS showed 35.2 % decrease in its activity. A detergent-tolerant cholesterol oxidase from *γ-Proteobacterium* Y-134 has been employed for the assay of HDL and LDL cholesterol in serum [14]. When cholesterol oxidase possesses stability in the presence of a wide range of detergents it aids in the improvement of differential assay methods for HDL (High density lipids) and LDL (Low density lipids). Cholesterol oxidase is stable in the presence of organic solvents and it could be used for the optical resolution of non-steroidal compounds and allylic alcohols [6, 14] and the bioconversion of a number of 3β hydroxysteroids in the presence of organic solvents [18, 20, 29]. Organic solvents as well as detergents are also employed to solublize the steroids. Cholesterol oxidase with organic solvent-tolerance would be useful for these applications.

IV. Conclusion

A perusal of literature has clearly shown that the existence of oxidase has not been reported from *Enterobacter cloacae*, which has also been identified as the hyper producer of cholesterol oxidase in the present study. Therefore, this study has provided a novel source for obtaining abundant amount of cholesterol oxidase to meet the needs of the industrial and medicinal fields. These results demonstrate the novelty of the source of cholesterol oxidase. Apart from this, the extracellular enzyme from *Enterobacter cloacae* can be successfully employed in large scale production as its stability and physiochemical properties make it an attractive candidate for industrial and medical applications.

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