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Purification and Characterization of the Enzyme Cholesterol Oxidase from a New Isolate of *Streptomyces* sp.

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Abstract An extracellular cholesterol oxidase (cho) enzyme was isolated from the *Streptomyces parvus*, a new source and purified 18-fold by ion exchange and gel filtration chromatography. Specific activity of the purified enzyme was found to be 20 U/mg with a 55 kDa molecular mass. The enzyme was stable at pH 7.2 and 50 °C. The enzyme activity was inhibited in the presence of Pb^{2+}, Ag^{2+}, Hg^{2+}, and Zn^{2+} and enhanced in the presence of Mn^{2+}. The enzyme activity was inhibited by the thiol-reducing reagents (DTT, β-mercaptoethanol), suggesting that disulfide linkage is essential for the enzyme activity. The enzyme activity was found to be maximum in the presence of Triton X-100 and X-114 detergents whereas sodium dodecyl sulfate fully inactivated the enzyme. The enzyme showed moderate stability towards all organic solvents except acetone, benzene, chloroform and the activity increased in the presence of isopropanol and ethanol. The K_m value for the oxidation of cholesterol by this enzyme was 0.02 mM.

Keywords Cholesterol oxidase · *Streptomyces parvus* · Ion exchange · Gel filtration chromatography · Isopropanol · β-mercaptoethanol

Introduction

Cholesterol oxidase (EC 1.1.3.6) is a flavin adenine dinucleotide-dependent enzyme that catalyzes the oxidation of cholesterol (cholest-5-en-3β-ol) using oxygen as an electron acceptor to form cholest-4-en-3-one and hydrogen peroxide (H_2O_2) [1]. With only the exception of glucose oxidase, cholesterol oxidase (cho) is the most widely used enzyme in clinical laboratories. Cho is used to determine cholesterol in food and blood serum by coupling of the enzyme with peroxidase. A high blood cholesterol level is considered to be related to cardiovascular disease. Since the monitoring of high-density lipoprotein (HDL) cholesterol and low-density lipoprotein (LDL) cholesterol in serum is important for the

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diagnosis of hyperlipemia or atherosclerotic diseases, several methods for the separation of HDL or LDL cholesterol with various detergents have been devised [2]. Therefore, cho with high stability in the presence of a wide range of detergents is expected to improve the differential assay method for HDL and LDL cholesterol in serum.

Cho is also used for the production of precursors for the chemical synthesis of steroid hormones [3], degradation of dietary cholesterol in foods [4], and for the production of an insecticidal protein against boll weevil larvae [5]. In *Streptomyces natalensis*, cho (encoded by the pimE gene) plays a main role in the biosynthesis of the polyene macrolide pimaricin [6]. Cho have been purified and characterized from a variety of microorganisms including *Arthrobacter* sp, *Brevibacterium* sp, *Corynebacterium* sp, *Pseudomonas* sp, *Rhodococcus* sp, *Schizophyllum* sp, and *Streptomyces* sp. [7]. Cho from *Streptomyces* species has been reported to be superior to those from other microorganisms on account of lower cost of production, stability and longer shelf life [8]. *Streptomyces* sp. strain SA-COO (Toyobo Co. Ltd., Tsuruga, Japan) is used for the commercial production of cho.

In the present study, we have described the taxonomic characterization of a novel cho-producing bacterial strain, *Streptomyces parvus* which showed markedly high cho activity in culture broth. Purification and characterization of the extracellular cho have also been attempted.

Materials and Methods

Chemicals

Diethylaminoethyl (DEAE) cellulose and Sephadex G-100 were purchased from Pharmacia, Uppsala, Sweden. Cholesterol, horseradish peroxidase, 4-aminoantipyrine, and Triton X-100 were obtained from Sigma Chemical Co., St. Louis, Mo, USA. Molecular weight standard proteins and Mini Protean cell was from Bio-Rad Laboratories, Richmond, CA, USA. All other chemicals were from commercial sources and were of analytical grade.

Microorganism and Culture Conditions

A small amount of the soil sample, collected from effluent sediment of a steel plant in Barabanki (Uttar Pradesh, India), was suspended in sterile 0.8% NaCl solution and a portion of the suspension was spread on cholesterol enrichment medium containing (g L^{-1}) glucose, 20; yeast extract, 10; KH_2PO_4,0.05; KNO_3, 1; $MgSO_4$, 0.5; $FeSO_4$, 0.01; NaCl, 0.5; cholesterol, 1 (dissolved in 0.3% Triton X-100); agar 20 and incubated at 30 °C for 7 days. Colonies that formed halos were isolated by repeated single-colony isolation on the same medium. Single colonies were streaked on cholesterol oxidase indicator plates [9]. The plates were incubated at 30 °C for 2–4 days. Colonies that formed brown pigments around them were isolated as cho-producing cultures and maintained on cholesterol enrichment medium slants at 4 °C.

A strain showing high cho production, selected for further studies, was cultivated as seed in medium containing (g L^{-1}): 1.0 K_2HPO_4, 0.5 $(NH_4)_2HPO_4$, 0.5 $MgSO_4$, 3.0 NaCl, 15.0 soybean meal and 15.0 glucose, pH 7.2 at 30 °C for 48 h with shaking (200 rpm) and used to inoculate the production medium (200 ml in 1 L shaker flask), identical to the seed medium. Cultivation was carried out at 30 °C for 72 h with shaking (150 rpm). All experiments were done in triplicate. In a separate experiment, cultivation was carried out in the same medium supplemented with 0.3% cholesterol under similar conditions.

Taxonomic Studies of the Production Strain

Various cultural, physiological, and biochemical characteristics of the strain were studied according to the methods described by Shirling [10] and Bergey's mannual [11]. Cell morphology was determined by scanning electron microscope (SEM) following cell growth on ISP-2 agar medium according to the method described by Castillo [12].

For 16S rRNA homology studies, the producer strain was grown on ISP-2 agar. Isolation of genomic DNA, polymerase chain reaction (PCR) amplification, and the sequencing of PCR-amplified product was carried out according to the methods described earlier [13]. Closely related homolog were identified by comparing partial 16S rRNA sequence with the sequences deposited in database at National Center for Biotechnology Information (NCBI) web server (http://www.ncbi.nlm.nih.gov), using the standard nucleotide–nucleotide BLAST program [14]. The phylogenetic tree was constructed using the neighbor-joining method [15] with the program of *Mega 4* (http://www.kumarlab.net/publications).

Purification of the Enzyme

All the purification procedures were carried out at 4–10 °C.

Step 1 Preparation of crude enzyme solution

The culture (1 L) was centrifuged at 8,000×*g* for 20 min. Proteins in the supernatant fluid were precipitated with $(NH_4)_2SO_4$ (70% saturation) overnight. The precipitate was recovered by centrifugation (10,000×*g*, 20 min), dissolved in 50 ml of 10 mM Tris–HCl (pH 7.2) and dialyzed twice against the same buffer.

Step 2 Anion exchange chromatography

The enzyme solution (100 ml) was loaded onto a column (10×50 cm) of DEAE cellulose DE52 which had been equilibrated and washed with 10 mM Tris–HCl (pH 7.2) buffer. Two buffers, buffer A (Tris–HCl buffer, pH 7.0, 10 mM) and buffer B (buffer A+0.3 M NaCl) were used along with 0.3% Triton X-100 for eluting the protein. A linear gradient of 0–100% buffer B was used with a flow rate of 0.5 ml min^{-1}. Twenty milliliter fractions were collected until no cho activity could be detected. The absorbance at 380 and 460 nm was monitored as an indicator of the presence of a flavoprotein during the purification steps. The cho-positive fractions were pooled and lyophilized.

Step 3 Gel filtration

The enzyme solution (80 ml) was loaded on a Sephadex G-100 column (10× 50 cm) that was equilibrated and eluted with a buffer consisting of 10 mM Tris –HCl (pH 7.2), 30 mM NaCl and 5 mM sodium cholate at a flow rate of 1.5 ml min^{-1}. Fractions were collected in 10 ml aliquots. In the absence of sodium cholate, the cho eluted over a wide range of fractions and was not separated from impurities. Cho-positive fractions were pooled and lyophilized and used as purified enzyme.

Analytical Methods

Enzyme Assay and Protein Determination The protein concentrations were determined according to the method of Lowry et al. [16]. Cho activity was measured by the method of Kinya et al. [17]. The assay of the enzyme was based on the formation of the quinoneimine

dye. The rate of hydrogen peroxide formation was determined by measuring the color developed at 500 nm. The reaction mixture consisted of 3 μmol of cholesterol in 1.0 ml of 1% Triton X-100, 300 μmol of phosphate buffer (pH 7.0), 0.1 ml of enzyme solution, 1.2 μmol 4-aminoantipyrine, 21 μmol of phenol, and 20 U of horseradish peroxidase in a final volume of 3 ml. Reaction was performed at 37 °C for 10 min and terminated by heating at 100 °C for 3 min. One unit of enzyme was defined as the amount of enzyme that formed 1 μmol of H_2O_2 per minute at 37 °C.

Identification of the Cholesterol Oxidation Product The conversion of cholesterol to 4-cholesten-3-one was confirmed by HPLC on a reverse phase C_{18} analytical column and UV detection at 205 nm with isopropanol: methanol (10:90) as eluting solvent at a flow rate of 0.7 ml min^{-1}.

SDS-PAGE

Purified enzyme was dissolved in a solution containing 2% (*w*/*v*) sodium dodecyl sulfate (SDS), 4% (*v*/*v*) β-mercaptoethanol, 20% (*v*/*v*) glycerol, and 10 mM Tris–HCl (pH 7.2) and heated in a boiling water bath for 5 min. SDS-polyacrylamide gel electrophoresis (PAGE) of purified enzyme [18] was carried out at 25 mA at 25 °C in a Mini Protean cell with 12% resolving gel.

Table 1 Physiological characteristics of *S. parvus*

Characteristics	C-46	Characteristics	C-46
Urease	–	Decarboxylation of	
Amylases	++ +	Na citrate	+
Tyrosinase	–	Pyruvate	+
Protease	+ +	Na tartrate	–
Gelatinase	–	Utilization of	
Chitinase	+++	L-Arabinose	+ ++
Phenylalanine deaminse	–	D-Dextrose	4 +
Catalase activity	+	Fructose	+++
Nitrate reductase	–	D+ galactose	+ + +
H_2S production	+	Meso Inositol	+ + +
Melanin production			
ISP-6	+	D-Raffinose	+ + +
ISP-7	–	Sucrose	+ +
ISP-2	–	L-Rhamnose	+ +
Antibiotic resistence (30 μg/disk)		NaCl tolerance	
R	R	1%	+ +
S	R	3	+
Cf	R	5	–
M	S	7	–

4+=heavy growth, 2+=moderate growth

R rifampicin, *S* streptomycin, *Cf* ciprofloxacin, *M* methicillin, *C-46 S. parvus*

Physico–Chemical Characterization of the Enzyme

Enzyme Stability Thermal stability of cho was examined by incubating the enzyme in sodium phosphate (10 mM, pH 7.0) buffer at various temperatures for 30 min. Enzyme

Table 2 Cultural characteristics of *S. parvus*

Media		Cultural characteristics
Nitrate agar	Gr	+++, White and rough colonies
	RC	White
	S	None
	DP	Light yellow
Urea agar base	Gr	+, White, small and round colonies
	RC	Pink
	S	++, White
	DP	None
Simmons citrate agar	Gr	+
	RC	Blue
	S	None
	DP	Blue
ISP-2 agar	Gr	++, Medium, round and intruded into the agar medium
	RC	Yellow
	S	+++, Yellow
	DP	Bright yellow
ISP-3 (oatmeal agar)	Gr	+++, Gray, medium sized round colonies
	RC	Dirty gray
	S	++, Dirty gray
	DP	Dirty gray
ISP-4 (inorganic salt starch agar)	Gr	+++, Yellow, small and round
	RC	Cream yellow
	S	+, Cream
	DP	Light yellow
ISP-5 (glycerol asparagine agar)	Gr	++, Cream, small and round colonies
	RC	Cream
	S	+, White
	DP	Bright yellow
ISP-6	Gr	+, Cream, small and round colonies
	RC	Light brown
	S	Scanty, cream
	DP	Light brown
ISP-7 (tyrosine agar)	Gr	++, Gray
	RC	White
	S	++, Gray
	DP	None

Gr mycelial growth, *RC* reverse side color of the slant, *S* sporulation, *DP* diffusible pigment
+++ = heavy growth, ++ = moderate growth, + = poor growth, – = no growth

stability, as a function of pH, was studied by taking 10 ml aliquots of the supernatant of the centrifuged broth in test tubes and adjusting the pH in the range of 4–12 using 1 N NaOH or HCl. The tubes were kept standing at 50 °C for 30 min. Stability in organic solvent and detergents was measured by incubating 500 μl crude enzyme solution (100 μg ml^{-1} protein) with 500 μl of different organic solvents/detergents at 50 °C for 30 min and residual activities were calculated.

Shelf life of the enzyme at pH 7.2 was studied by storing the enzyme in two sterile tubes; one was kept at room temperature while the other was kept at 4 °C. Cho activities were determined at regular intervals. To determine the effect of metal ions on the cho activity, enzyme activity was measured at 50 °C in the presence of various metal ions at a concentration of 10 mM.

Substrate Specificity Substrate specificity was investigated by comparing the rates of oxidation of cholesterol analogs with that of cholesterol at the same molarities.

Determination of K_m and V_{max} Values The K_m and V_{max} values were estimated from Michaelis–Menten plot of data obtained with the assay solution containing 0–0.2 mM cholesterol.

Results and Discussion

Strain Characterization

Total 20 cho-producing strains were isolated. Final screening for cho-producing bacteria was done on cho indicator agar plates. For further studies, a high cho-producing culture designated as *S. parvus* was selected. SEM images showed the presence of straight spore chains with 15–20 smooth and yellow colored spores (0.8×0.3 μm) per chain (SEM image not shown). Bright yellow colonies were powdery and circular with wavy margin and anchored in the agar. Other physiological and cultural characteristics have been summarized in Tables 1 and 2.

Sequence Analysis

Out of 1,352 16S rRNA nucleotides of *S. parvus*, 1,349 were found to be identical with those of *S. parvus* strain 13647 J (EU741140.1, score 2,486), indicating that the sequence similarity of the 16S rRNA of the two bacteria was 99%. Phylogenetic tree (Fig. 1) revealed that *S. parvus* 16S rRNA gene sequence evolved from a common ancestor in a

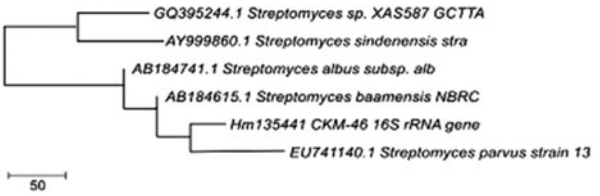

Fig. 1 Phylogenetic tree of *S. parvus* (phylogenetic tree was inferred by neighbour-joining method. The software package *MEGA 4* was used for analysis)

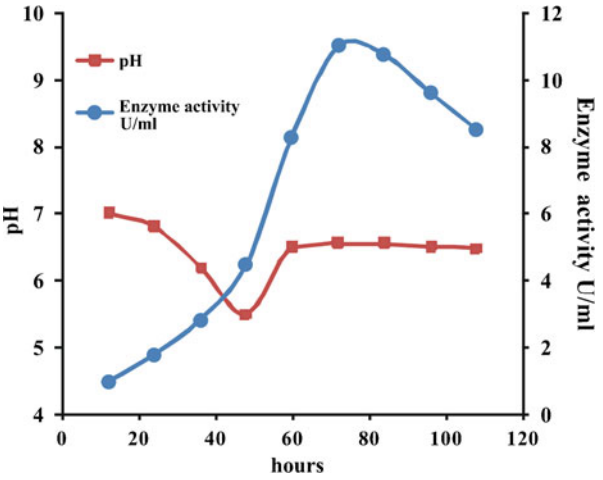

Fig. 2 Cho production and pH variation during growth of *S. parvus*

clade with 24 other *Streptomyces* strains with the score values of 2486 and 99% similarity. The culture was identified as *S. parvus* strain 1,3647 J. Nearly complete (1,352 bp) 16S rDNA sequence has been deposited in the NCBI GenBank database under accession number HM135441.

Purification of Extracellular Cholesterol Oxidase

Maximum enzyme production was observed at 72 h (Fig. 2). pH was observed as 5.5 to decrease up to 40 h and increased sharply after 48 h and remained almost constant for rest of the fermentation period.

The enzyme purification steps followed elution profiles from a DEAE cellulose and Sephadex G-100 column chromatography are shown in (Figs. 3, 4) and summarizes in Table 3. Major enzyme activity was found in fraction no. 8, 9, 10, and 11, eluted from DEAE cellulose column. In this step, the efficacy of Triton X-100 was 2.5 times more than the amount of cho recovered by NaCl alone. In the next step (gel filtration), cho activity was found in fraction no. 5, 6, and 7. Cho was purified 18-fold with an overall yield of 22.54% from culture broth. The purified enzyme had a specific

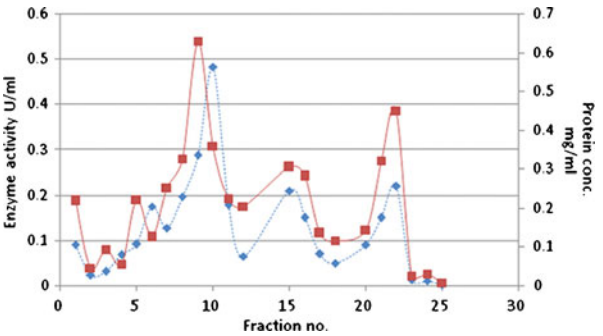

Fig. 3 Elution profile of cholesterol oxidase from DEAE celluose DE-52

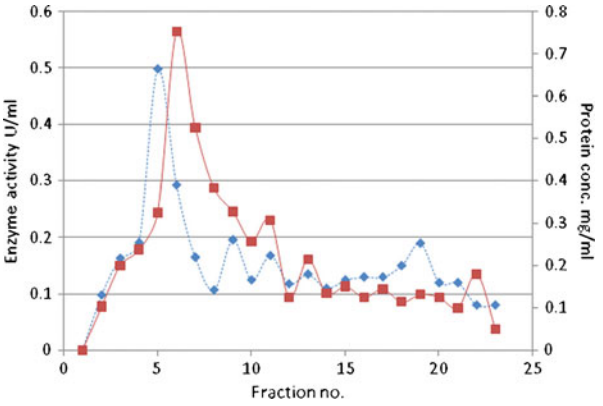

Fig. 4 Elution profile of cholesterol oxidase from Sephadex G-100 column chromatography

activity of 20 U mg^{-1} of purified protein. The purified enzyme preparation gave a single band by SDS-PAGE and the molecular mass was estimated to be 55 kDa (Fig. 5).

Physicochemical Properties and Characterization of Cholesterol Oxidase

The purified enzyme solution was cream yellow and like a typical flavoprotein, exhibited three absorption maxima at 280, 380, and 460 nm (Fig. 6). As cholesterol is poorly soluble in water, it was emulsified with Triton X-100 (0.3%). The enzyme 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 (Fig. 7).

The enzyme was stable from 4 °C to 65 °C temperature. The optimum temperature at pH 7.2 was 50 °C (Fig. 7). The enzyme retained about 68%, 66%, and 46% of its activity after incubation for 30 min at 55 °C, 60 °C, and 65 °C, respectively. However, the enzyme lost almost all (86%) activity after 30 min at 75 °C. No detectable change in activity occurred when the enzyme was stored in buffered Triton X-100 (0.3%) for 6 months at 4 °C. Among the cho from *Streptomyces* sp., thermal stability of *S. parvus* (temperature optimum at 50 °C) was second highest after *Streptomyces fradiae* (temperature optimum at 70 °C) [19]. Reported pH optima for most of bacterial cho range between 7.0 and 7.5 [9, 20].

Ca^{2+}, Cu^{2+}, Mg^{2+}, Ni^{2+}, and Fe^{2+} did not exert any remarkable effect on the enzyme activity. However, the addition of Pb^{2+}, Ag^{2+}, Hg^{2+}, and Zn^{2+} reduced the activity to 65%,

Table 3 Purification steps of the enzyme

Purification step	Protein (mg)	Activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Culture supernatant	950	1,065	1.12	100	1
$(NH_4)_2SO_4$	187	795	4.25	74.65	3.79
DEAE cellulose	41.6	520	12.5	48.83	11.16
Sephadex G-100	12	240	20	22.54	17.86

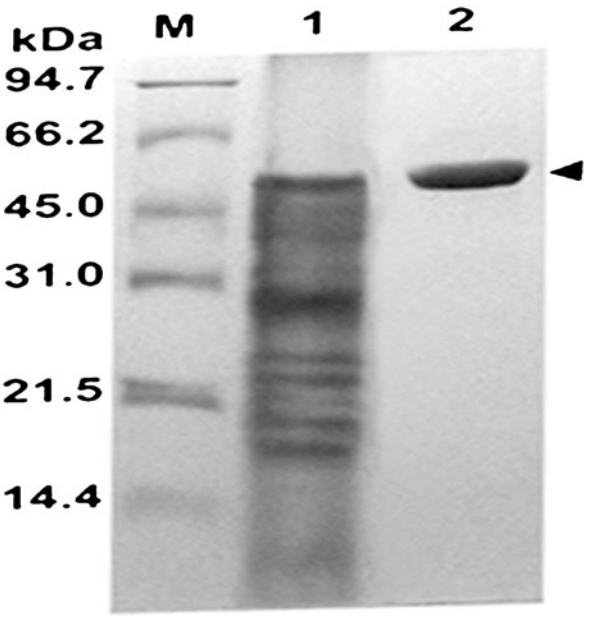

Fig. 5 SDS-PAGE of cholesterol oxidase, *1* crude ppt. obtained with 70% (NH4)2 SO4, *2* purified enzyme from Sephadex G-100 column, *M* molecular size markers

55%, 50%, and 74%, respectively. Both thiol-reducing reagents, DTT and β-mercaptoethanol, decreased the enzyme activity, suggesting that disulfide linkage is essential for the enzyme activity. Addition of Mn^{2+} activated the enzyme by 30% Table 4. Inhibition by Ag^{2+} and Hg^{2+} were completely prevented by the addition of glutathione (10 mM) in the assay mixture [21]. This suggested that –SH group may be involved in the catalytic activity of cho from *S. parvus* Cho from *Pseudomonas* sp. COX629 and γ-*Proteobaceterium* were also found to be activated by Mn^{2+} [22, 23]. The enzyme showed good stability in the presence of a wide range of detergents. The enzyme activity was highest in Triton X-100 and Triton X-114 at 50 °C Table 5. At a low concentration of Triton X-100 (0.03–0.5%), cho activity increased whereas at higher detergent concentrations the opposite effect occurred. Presence of only 0.1% SDS fully inactivated the enzyme where as 100% activity was observed in presence of sodium cholate.

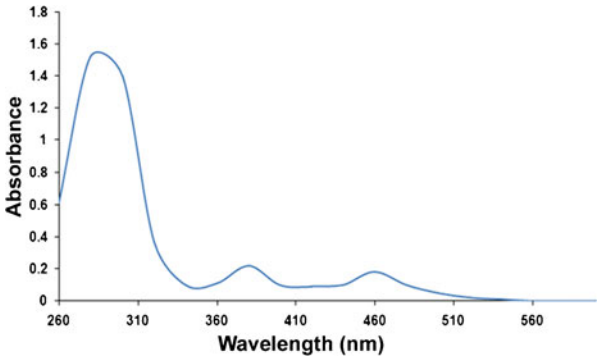

Fig. 6 UV spectra of cho in phosphate buffer

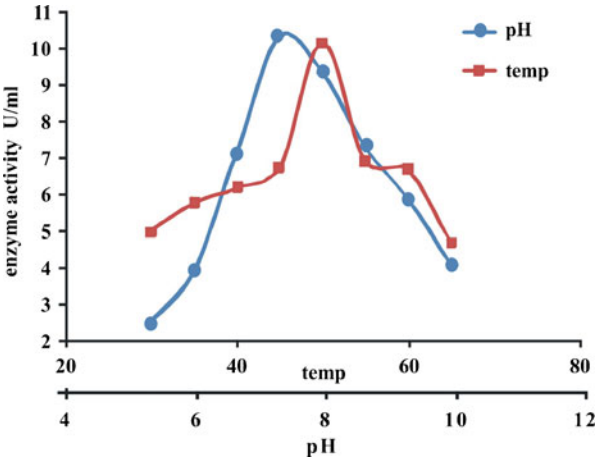

Fig. 7 Effect of temperature and pH on enzyme activity

Cho stability was studied in the presence of various solvents. The enzyme showed moderate stability (50–75%) towards all the organic solvents tested except acetone, benzene, and chloroform. The increase in concentrations of isopropanol up to 5–10% (*v*/*v*) showed an increase in cho activity and decreased at higher solvent concentration. Synthesis of steroid hormones and bioconversion of a number of 3β-hydroxysteroids is carried out in presence of organic solvents [24]. Cho is an enzyme that interacts with membranes and micelles and acts on hydrophobic substrates whose solubility is increased by using detergents and solvents.

Substrate Specificity

The enzyme oxidized most 3β-hydroxysteroids at high rates (Table 6). The enzyme was not reactive for 3α-hydroxysteroids such as epicholesterol. β-Cholestanol and β-sitosterol were rapidly oxidized (more than 50%). Pregnenolone, dehydroepiandros-

Table 4 Effect of divalent metal ions on the enzyme

Divalent metal ions	Relative activity (%)
Control	100
Ca^{2+}	98
Cu^{2+}	96
Mg^{2+}	100
Ni^{2+}	99
Fe^{2+}	98
Pb^{2+}	65
Ag^{2+}	55
Hg^{2+}	50
Zn^{2+}	74
Mn^{2+}	130

Table 5 Enzyme stability in detergents and organic solvents

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

terone, and epiandrosterone were oxidized at very low rates. The enzyme displayed low reactivity with 3β-hydroxysteroids having short side chain attached to position 17, such as pregnenolone, dehydroepiandrosterone, and epiandrosterone. Kinetic behaviors of enzyme were examined under assay conditions except for changing the substrate concentration. The K_m value for cholesterol was determined to be 0.02 mM by the method of Lineweaver and Burk [25] (Fig. 8), showing error bars with standard error. *S. parvus* cho showed higher affinity for cholesterol as compared to other *Streptomyces* cho which showed K_m values in the range of 0.07–0.2 mM [19, 26].

Table 6 Substrate specificity of the enzyme

Substrate (0.5 mM)	Relative activity (%)
Cholesterol (cholest-5-en-3 β-ol)	100
β-Cholestanol (5α-cholestan-5-en-3 β-ol)	100
β-Sitosterol (sitost-5-en-3 β-ol)	88
β-Stigmasterol (sigmast-5-en-3 β-ol)	68
Pregnenolone (3β-hydroxypregn-5-en-20-one)	42
Dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one)	30
Epiandrosterone (5α-androstan-3-ol-17-one)	15
Ergosterol (ergosta-5,7,22-trien-3 β-ol)	18
Epicholesterol (cholest-5-en-3 α-ol)	0

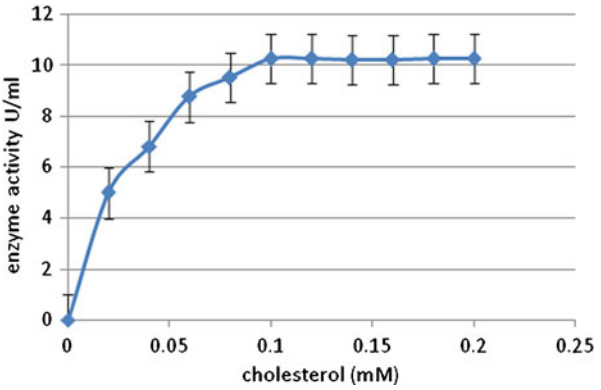

Fig. 8 Substrate saturation curve of the enzyme showing standard errors

Identification of the Cholesterol Oxidation Product

The extracellular cho of the culture oxidized cholesterol to yield 4-cholesten-3-one as one major product. HPLC analysis of the reaction mixture showed a peak of cholesterol at 11.73 and 4-cholesten-3-one at 13.20 min of retention time (Fig. 9).

Conclusion

It is notable that the new strain, characterized as *S. parvus*, isolated and studied has not been reported earlier to produce cho. Cho production with *S. parvus* was found to be only extracellular. Heat-stable cho with long shelf life and tolerance for detergents and organic solvents are the characteristic features that make the strain *S. parvus* a potential source of cho to be used for clinical and other research purposes. This strain

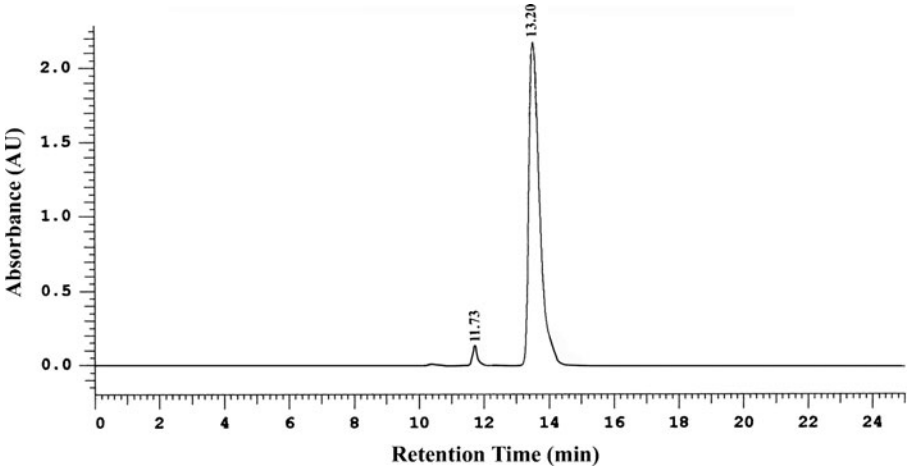

Fig. 9 HPLC chromatogram of the reaction mixture showing the peaks of cholestreol and 4-cholesten-3-one at 11.73 and 13.20 min retention times, respectively

may also be useful for metabolic engineering to construct cho-producing recombinant strains.

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