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Purification and Characterization of an Extracellular Cholesterol Oxidase of *Bacillus subtilis* Isolated from Tiger Excreta

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Abstract A mesophilic *Bacillus* sp. initially isolated from tiger excreta and later identified as a *Bacillus subtilis* strain was used to produce an extracellular cholesterol oxidase (C_{OX}) in cholesterol-enriched broth. This bacterial isolate was studied for the production of C_{OX} by manipulation of various physicochemical parameters. The extracellular C_{OX} was successfully purified from the cell-free culture broth of *B. subtilis* by successive salting out with ammonium sulfate, dialysis, and riboflavin-affinity chromatography. The purified C_{OX} was characterized for its molecular mass/structure and stability. The enzyme possessed some interesting properties such as high native *M*r (105 kDa), multimeric (pentamer of ~21 kDa protein) nature, organic solvent compatibility, and a half-life of ~2 h at 37 °C. The bacterial C_{OX} exhibited ~22 % higher activity in potassium phosphate buffer (pH 7.5) in the presence of a nonionic detergent Triton X-100 at 0.05 % (*v*/*v*). The K_m and V_{max} value of C_{OX} of *B. subtilis* C_{OX} were found to be 3.25 mM and 2.17 μmol min ml^{-1}, respectively. The purified C_{OX} showed very little cytotoxicity associated with it.

Keywords *Bacillus subtilis* · Extracellular cholesterol oxidase · Purification · Biochemical characterization

Introduction

Cholesterol oxidase (C_{OX}) was first isolated and characterized from *Rhodococcus erythropolis* by Turfitt [32]. In 1947, Turfitt revealed that many strains of *Proactinomyces* were capable of up to 35 % cholesterol decomposition. However, later, many bacterial genera, both Gram

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positive and Gram negative, were found to produce C_{OX}, but the *Actinomycetes* come under most prolific group. A fast-growing strain of *Mycobacterium* sp. VKM Ac-1815D was capable of effective oxidizing of sterols (phytosterol, cholesterol, and ergosterol) to androstenedione and other valuable 3-oxo-steroids [10]. To elucidate the role of cholesterol oxidase in sterol catabolism by the strain, the *choD* gene that was involved in the synthesis of membrane-associated cholesterol oxidase in *Escherichia coli* has been cloned and sequenced. The mutant strain transformed sitosterol forming exclusively 3-keto-4-ene steroids with androstenedione as a major product, thus evidencing that C_{OX} knock out did not abrogate sterol A-ring oxidation. Classical (one-variable-at-a-time) and statistical methods (Plackett–Burman and central composite design) were used [1] to optimize growth medium for the production of C_{OX} from *Rhodococcus* sp. NCIM 2891, but the latter approach gave a cell growth rate that was 29-fold higher than the basal medium.

Medium improvement for the production of C_{OX} employing response surface methodology (RSM) was also optimized [37]. An ultrasound-assisted emulsification as a pretreatment for C_{OX} production by submerge fermentation using *Brevibacterium* sp. in a batch system was studied. They concluded that improved or higher concentration of cholesterol, Tween 80, and increased treatment time resulted in higher production of C_{OX}, which was 83.57 % greater than the control medium [37]. A C_{OX} from *Brevibacterium* sp. M201008 was not stable as free enzyme form; however, when it was covalently immobilized onto functionalized Sepharose particles activated with *N*-ethyl-*N*′-3-dimethylaminopropyl carbodiimide, the immobilized enzyme only lost its 35.8 % activity [5]. Production of extracellular C_{OX} in liquid fermentation conditions by *Monascus* X-1 has been reported [8]. An extracellular C_{OX} producing *B. licheniformis* was isolated from buffalo milk, domestic waste, water sludge, and fermented fish sample by enrichment technique [2]. A recent study on C_{OX} provided the evidence that C_{OX}-B (C_{OX}-Bordetella), isolated from the *Bordetella* species, oxidizes membrane cholesterol to 4-cholesten-3-one and H_2O_2 resulting in a decrease of cholesterol content and an increase of ROS levels causing cell apoptosis by inactivation of the Akt and ERK1/2 pathway besides activation of caspase-3 [19]. A C_{OX} from the *Streptomyces* species for serum assay has been reported to be superior to those from other microorganisms due to lower cost of production and longer shelf life [22]. Ca-alginate immobilized cells of *Streptomyces* sp. studied for the production of C_{OX} [24] suggested that immobilized cells could be used for three consecutive fermentation cycles for C_{OX} production in higher quantities as compared with free cells. In the present study, we have successfully isolated an extracellular cholesterol oxidase producing *Bacillus subtilis* strain from the excreta sample of a carnivore/tiger. The enzyme was purified by an efficient riboflavin-affinity chromatography and was biochemically characterized.

Materials and Methods

Chemicals

$NaNO_3$, K_2HPO_4, KCl, $MgSO_4{\cdot}7H_2O$, $FeSO4{\cdot}7H_2O$, $(NH_4)_2SO_4$, and Celite 545 (S.D. Fine Chem Ltd., Hyderabad, India); $MgCl_2$, cholic acid, sodium borohydried, sodium taurocholate, cholesterol and yeast extract (Himedia Laboratory, Ltd., Mumbai, India); sucrose, KCl, KNO_3, isopropanol, *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine (Temed), ammonium persulfate, 2-mercaptoethanol, hydrochloric acid, and bromophenol blue (Merck Ltd., Mumbai, India); sodium dodecyl sulfate (SDS), acrylamide, bisacrylamide (N,N′-methylenebisacrylamide),

glycerol, glycine, Tris (2-hydroxymethyl-2-methyl-1, 3-propanediol), cholesterol oxidase, and horseradish peroxidase (Sigma Chemicals Co., USA) were procured from various commercial suppliers. All chemicals were of analytical grade and were used as received.

Collection of Samples for Isolation of C_{OX}-Producing Bacterial Strains

A total of 80 soil/water/fecal (dog and tiger) samples for the isolation of cholesterol-degrading microorganisms were collected from different regions of Himachal Pradesh, India.

Screening of Extracellular C_{OX}-Producing Microbes

Each of the colony-forming units (cfus) obtained on cholesterol-enriched (CE) agar-based medium was assigned a separate code number (S, soil sample; T, tiger fecal sample; D, dog fecal sample; and W, water sample). The CE agar Petri plates were incubated at 37±1 °C for about 24 h, and bacterial colonies that appeared were replica plated by toothpick onto the Petri plates containing isolation medium. The replica plates were incubated at 37±1 °C for 24 h to select the C_{OX}-producing strains. Filter papers dipped in 0.5 % (*w*/*v*) cholesterol, 1.7 % 4-aminoantipyrine, 6 % phenol, and 3000 units ml^{-1} HRP in 100 mM potassium phosphate buffer (KPB), pH 7.2±0.1, were placed on colonies grown on agar medium followed by incubation at 37±1 °C. The C_{OX} activity of the selected colonies was indicated by the development of a red color around the C_{OX}-producing colony due to the formation of quinoneimine dye as an end product. Out of the 35 isolates showing red-colored colonies during primary isolation, 8 cfus presenting strong red color (relatively higher extracellular C_{OX} synthesis) were selected for secondary screening. These isolates were grown at 37±1 °C in 50 ml CE broth under continuous shaking (120 rpm) for 24 h. The bacterial cells were sediment by centrifugation, and cell-free culture broth was assayed for extracellular activity of C_{OX}. The bacterial strain showing the maximum extracellular C_{OX} activity was selected for further studies.

16S rDNA Sequence Determination and Phylogenetic Analysis

Chromosomal DNA isolation, PCR amplification, and 16S rDNA sequencing of bacterial isolate T3 were done. Database searches were conducted with the BLAST algorithm provided by the National Center for Biotechnology Information. The 16S rDNA sequence determined for bacterial strain T3 was aligned with the GenBank database using multiple sequence alignment software CLUSTAL W. A phylogenetic tree was constructed. The phylogenetic tree presented a close similarity of bacterial strain T3 to *Bacillus* sp. (biochemically identified as *Bacillus subtilis* by MTCC, Sector 39-A, Chandigarh, India).

Cholesterol Oxidase Activity Assay

A previously reported colorimetric method [21] was used for the assay of extracellular C_{OX} in the cell-free broth or approximately diluted commercial grade C_{OX} using cholesterol as a substrate. One unit (U) of cholesterol oxidase was defined as the amount of enzyme capable of converting 1.0 μmol of cholesterol to 4-cholesten-3-one per minute at pH 7.5±0.1 and at a temperature of 37±1 °C. The protein

concentrations in the production broth and cell-free broth/fractions were determined by a standard method [3].

Purification of *B. subtilis* C_{OX} by Riboflavin–Sephadex G-25 Affinity Column

C_{OX} is a FAD-dependent enzyme, thus riboflavin was covalently linked to amino-Sephadex G-25 through a spacer (cyanuric chloride) according to a previously described method [17]. The riboflavin–Sephadex G-25 matrix was packed in a glass column using glass-wool plug at the base. The dialyzate sample was loaded on the riboflavin–Sephadex column followed by addition of a 20-mM sodium phosphate buffer (pH 7.5). The column was developed with the 20-mM sodium phosphate buffer (pH 7.5), and 20 fractions of 3 ml each were collected. Each of these fractions was checked for A_{280} and cholesterol oxidase activity.

Determination of Molecular Mass [*Mr*] of Purified C_{OX}

The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (12 %) and native-PAGE (8 %) were performed to determine the purity, native molecular mass, and the subunits molecular mass of affinity column-purified *B. subtilis* C_{OX} [15].

Characterization of Purified C_{OX}

The riboflavin-affinity-purified C_{OX} of *B. subtilis* was further characterized by studying the effect of various physiochemical factors in in vitro studies. All the reactions were performed in duplicates under static conditions in a final reaction volume of 3.0 ml at 37 °C in monopotassium phosphate buffer (50 mM, pH 7.2) for 5 min.

Effect of Temperature on Purified C_{OX}

To evaluate the effect of reaction temperature on C_{OX} activity, cholesterol oxidase (100 μl) was incubated at selected temperatures (30, 35, 37, 40, 45, and 50±1 °C) for 5 min in a potassium monophosphate buffer. The C_{OX} activity was assayed at A_{500}, thereafter.

Effect of Buffer pH on Purified C_{OX}

To evaluate the effect of pH of potassium monophosphate, cholesterol oxidase (100 μl) was incubated at selected temperatures 37±1 °C for 5 min in a potassium monophosphate buffer (50 mM) set to varying pH viz. 6, 6.5, 7, 7.5, 8, and 8.5±0.1. The C_{OX} activity was assayed at A_{500}, thereafter.

Effect of Various Buffers on Purified C_{OX}

To study the effect of type of buffer, 50 mM potassium monophosphate buffer, sodium citrate buffer, glycine-NaOH, or Tris–HCl buffer were used with pH set to 8.5. The C_{OX} (100 μl) was incubated at 37±1 °C for 5 min in each of the above-stated buffers, and C_{OX} activity was assayed at thereafter.

Effect of Molarity of Potassium Phosphate Buffer on Purified C_{OX}

The effect of molarity of potassium phosphate buffer on purified C_{OX} was studied by incubating the reaction cocktail to different molarities (viz. 25, 50, 75, and 100 mM) of potassium monophosphate buffer optimized at temperature 37±1 °C for 140 min, and enzyme activity was checked after regular interval of 20 min.

Thermostability of Purified C_{OX}

To examine the effect of temperature on stability of the enzyme, purified C_{OX} was incubated at optimized temperature (37±1 °C) for 140 min, and enzyme activity was checked after regular interval of 20 min.

Effect of Selected Detergents on Purified C_{OX} Activity

In order to check the effects of selected detergents both ionic as well as nonionic on the activity of purified C_{OX}, if any, the selected detergent (Tween 20, Tween 40, Tween 60, Tween 80, and Triton-X 100) 0.05 % (*v*/*v*) was included in the reaction mixture followed by incubation at 37±1 °C for 5 min, and C_{OX} activity was recorded at A_{500} thereafter.

Effect of Time of Incubation on Assay of C_{OX} Activity

The effect of incubation/reaction time on enzyme activity was optimized by incubating the purified C_{OX} in the reaction cocktail at 37±1 °C for varying periods of time followed by assay of C_{OX}.

Kinetic Behavior of Purified C_{OX} of B. subtilis

Substrate specificity and effect of concentration of cholesterol on the reaction rate of C_{OX} of *B. subtilis* were studied to determine V_{max} and K_m of the purified C_{OX} toward cholesterol (substrate) with the help of a Lineweaver–Burk plot. The concentration of the cholesterol was varied in the reaction cocktail while using 100 μl of purified C_{OX}. The reaction was permitted at 37±1 °C followed by assay of enzyme activity. The rate of reaction (V_{max}) was determined by performing the reaction at 37±1 °C for varying periods of time in the presence of 0.1 % (*w*/*v*) cholesterol.

In Vitro Cytotoxic Assay of the Purified C_{OX}

To examine the toxic effect of *B. subtilis* C_{OX} by exposing Hep2C mammalian cells in vitro to varying amounts of purified C_{OX}, a cytotoxic assay was performed using MTT [23]. The purple-colored formazan product entrapped in the cells was extracted with 100 μl of DMSO. After keeping the tissue culture plate for 5 min at room temperature in dark, the color was read at A_{570}, and the percent viability of the cells was determined.

Results and Discussion

Among 35 bacterial isolated colonies, only 8 isolates (D13, D14, T1, T2, T3, T4, S10, and S11) showed considerable extracellular C_{OX} activities (Table 1). Over 276 bacteria and 132 *Actinomycetes* strains have been known to produce C_{OX} activity [20]. Some of these bacteria secrete intracellular enzyme, and many of them have the ability to produce extracellular form of C_{OX} [20]. Out of the above mentioned eight bacterial isolates, only one isolate T3 was selected for further studies due to its relatively high extracellular C_{OX} producing activity (0.214 U ml^{-1}) in comparison to the other seven bacterial isolates (Table 1; Fig. 1). A strain of *Rhodococcus* sp. isolated from soil expressed C_{OX} in both extracellular and membrane-bound forms. It was reported that a high amount of C_{OX} enzyme produced by *Rhodococcus* is extracellular form, and only a low amount of C_{OX} is membrane-bound or intracellular type [14]. Many microorganisms such as *Nocardia rhodocorus*, *Arthrobacter simplex*, *Pseudomonas* spp., *Rhodococcus* spp., Coryneform bacterium, *Actinomyces lavendulae*, *Streptomyces hygroscopicus*, *Brevibacterium* and a few fungal species have been reported to produce C_{OX} [35]. It is interesting to notice that a *Bacillus* sp. SFF34 produced two different extracellular C_{OX} enzymes [27]. *Bacillus cereus* from soil of agriculture waste using a cholesterol-Tween-80 medium was used to isolate C_{OX} [11]. Thus, it seems that some of the *Bacillus* spp. do produce extracellular C_{OX} in the natural environment or in an appropriate growth medium.

The hyper extracellular C_{OX}-producing bacterial strain was selected due to the formation of intense red color around the cfu(s). The characterization of this bacterial isolate T3 was done on the basis of biochemical and 16S rRNA molecular typing at the species level. The bacterial isolate T3 was biochemically identified as *Bacillus subtilis*.

Purification of *B. subtilis* Cholesterol Oxidase

The cell-free broth when saturated with varying concentrations of ammonium sulfate resulted in a maximal C_{OX} activity in the pellet obtained using a 60 % (*w*/*v*) concentration of ammonium sulfate (32.5 U ml^{-1} activity, specific activity 22.1 U mg^{-1} protein). The reconstituted protein precipitates after dialysis showed a C_{OX} activity of 20.8 U ml^{-1} (~10 ml; protein 153.0 mg ml^{-1}, specific activity 0.135 U/mg protein) that indicated an approximate ~28.4-fold concentration of enzyme (Table 2). The purification of dialyzed

Table 1 Screening of selected bacterial isolates for extracellular C_{OX} activity

S. No.	Code	Red-colored colonies	C_{OX} (U ml^{-1})±SD
1	D13	+	0.091±0.001
2	D14	+	0.085±0.002
3	T1	+	0.178±0.001
4	T2	+	0.184±0.001
5	T3	+	0.214±0.002
6	T4	+	0.154±0.002
7	S10	+	0.089±0.001
8	S11	+	0.094±0.001

+ C_{OX}-producing colonies, *D* dog excreta, *T* tiger excreta, *S* soil sample

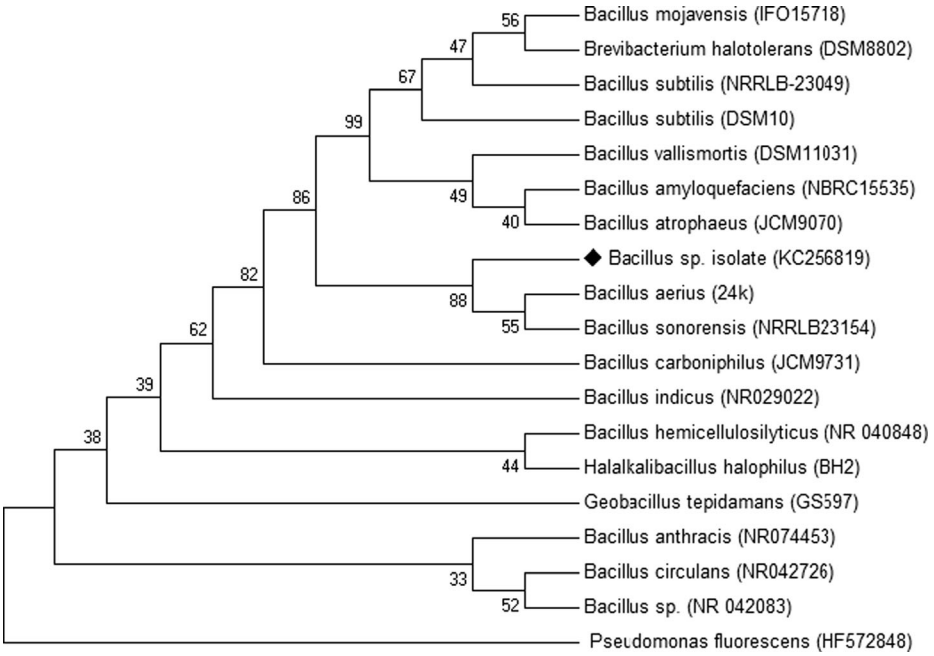

Fig. 1 Phylogenetic dendrogram of the *Bacillus* sp. strain (KC256819) based on 16S rDNA sequence. *Numbers in parenthesis* are accession numbers of published sequences. Bootstrap values were based on 1000 replicates

C_{OX} on a riboflavin–Sepharose G-25 affinity column resulted in a single peak (Fig. 2). The pooled fraction represented an activity of 14.9 U ml^{-1} with a specific activity of 0.135 U mg^{-1} of protein. The overall purification steps indicated a final yield of ~6.2 % with 28.4-fold purification of extracellular C_{OX} of *B. subtilis*. The riboflavin-affinity column-purified C_{OX} fraction was further evaluated for its electrophoretic homogeneity on gel electrophoresis. Several C_{OX} purified from various bacterial species have been found to possess *M*r in the range of 52 to 61 kDa. A small C_{OX} with 31 kDa of *M*r was reported from *Brevibacterium sterolicum* [33].

The analysis of bacterial C_{OX} under reducing and denaturing SDS-PAGE revealed that the purified C_{OX} possessed a single band of *Mr* 21.5 kDa (Fig. 3a). The characteristics of the electrophoretic pattern revealed that *B. subtilis* C_{OX} was a homogeneous protein. A single protein band of 105 kDa was observed in native-PAGE (12 %; Fig. 3b). This protein band was carefully cut and macerated into small pieces to colorimetrically assay the associated C_{OX} activity (0.087 U ml^{-1}). The substrate (cholesterol) hydrolysis study showed that most of the enzymatic activity was retained by the protein in non-reducing native gel as the 105 kDa

Table 2 Summary of steps involved in the purification of C_{OX} of *B. subtilis*

Purification step	Total C_{OX} activity (U)	Protein (mg)	Specific activity (U mg^{-1} protein)	Fold purification	Yield (%)
Crude C_{OX}	241.0	4845.0	0.049	1.0	100.0
Dialyzate	20.8	153.0	0.135	2.7	8.6
Riboflavin affinity chromatography	14.9	10.7	1.39	28.4	6.2

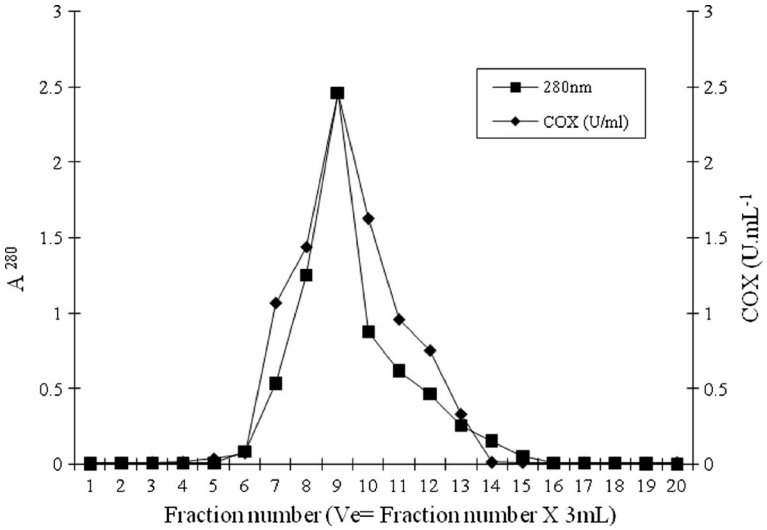

Fig. 2 Riboflavin-affinity column chromatography for purification of *B. subtilis* C_{OX}

protein-containing gel when incubated in 50 mM phosphate buffer (pH 7.2) containing HRP (dissolved in reaction cocktail) yielded brown color. Two novel extracellular cholesterol oxidases designated CO1 and CO2, from *Bacillus* sp. SFF34, were purified 5.6 and 5.9-fold giving *M*r values of 36 and 37 kDa [27]. The molecular weight of purified C_{OX} from *Bacillus cereus* was estimated at 55 kDa [11].

Characteristics of Purified C_{OX} of *B. subtilis*

The purified C_{OX} exhibited a maximum enzyme activity at 37±1 °C (0.73 U ml^{-1}) at pH 7.2 of a 50-mM potassium monophosphate buffer (Fig. 4). However, a decline in enzyme activity of up to 41 % was observed when reaction temperature was increased to 45±1 °C in comparison to optimum temperature, i.e., 37±1 °C. Similarly, relatively lower C_{OX} activities were obtained at 40–50±1 °C. Two novel extracellular cholesterol oxidases designated CO1 and CO2 were previously reported from a *Bacillus* sp. [27]. The optimum temperature for their C_{OX} activity was 60±1 °C (CO1) and 40±1 °C (CO2). The immobilized and free cells produced maximum C_{OX} from *Streptomyces* sp. in the culture incubated at 37 and 30 °C, respectively [24].

Optimization of Different Buffers for the Assay of C_{OX} Activity

Out of the four buffers tested to perform enzyme assay using cholesterol as a substrate, the maximum activity of C_{OX} (Table 3) was recorded in 50 mM potassium monophosphate buffer (pH 7.5) at 37±1 °C. In the other buffer systems, more than 50 % decline in the original activity of purified C_{OX} of *B. subtilis* was noticed. Thus, 50 mM potassium monophosphate buffer remained the best one for in vitro assay of C_{OX} activity. In most of the cases, phosphate buffer was used to optimize C_{OX} activity. To determine some properties of the C_{OX} from a *Brevibacterium* strain isolated from buffalo's milk, phosphate buffer (100 mM) had been used [28].

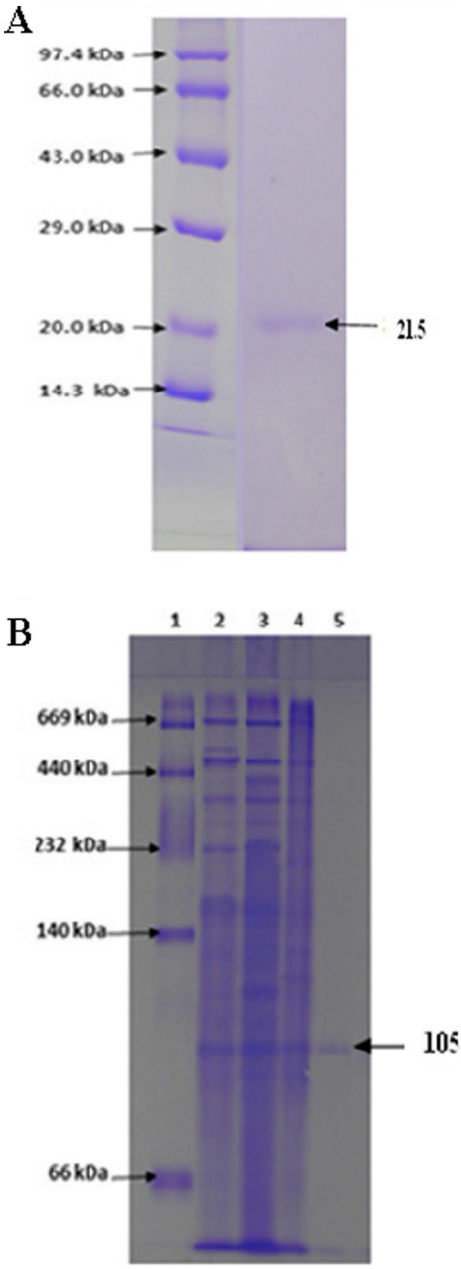

Fig. 3 **a** Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (stacking T4% and resolving gel T12%) of purified cholesterol oxidase of *B. subtilis*. Commercially procured protein reference markers were used to determine the *M*r of the purified enzyme. *Left lane*, standard protein markers; *right lane*, purified C_{OX} of *B. subtilis*. **b** Native-polyacrylamide gel electrophoresis (T8%) of *Bacillus subtilis*. *Lane 1*, reference protein markers; *lane 2*, crude enzyme; *lanes 3 and 4*, dialyzate; *lane 5*, riboflavin-affinity purified fraction indicating presence of ~105 kDa. Commercially procured protein reference markers were used to determine the *M*r of the purified enzyme

Effect of pH of Phosphate Buffer on Activity of Purified C_{OX}

The true ionogenic state of enzyme is essential for its optimal activity. The optimal activity of purified C_{OX} of *B. subtilis* was observed at pH 7.5±0.1 of 50 mM potassium monophosphate (0.739 U ml^{-1}; Fig. 5) at 37±1 °C. The pH influences the structure of proteins/enzymes, and hence governs their catalytic activity. Many of

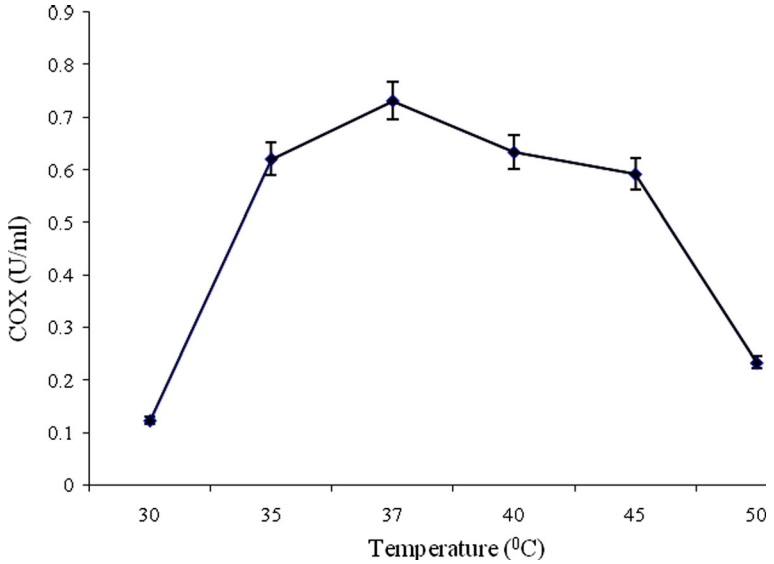

Fig. 4 Effect of incubation temperature on activity of purified C_{OX} of *B. subtilis*

the previously reported bacteria such as *Brevibacterium sterolicum* [34], *Streptomyces violascencs* [31], *Arthrobacter simplex* [18], *Brevibacterium sterolicum*, *Nocardia rhodochrous* [4], and *Pseudomonas* sp. [16] possessed optimal C_{OX} activity at pH 7.0–7.5. A few other bacteria that included *Pseudomonas* sp. strain ST200 [6], *Bacillus* sp. [27], and *Schizophyllum commune* [7] produced an optimal amount of C_{OX} at pH 4.0 to 6.8. However, a pH 8.0 was optimal for extracellular C_{OX} production by *Rhodococcus equi* [12]. An optimum pH for the C_{OX} activity was found to exist between 7.0 and 8.0 as noticed for enzymes from *Actinomyces lavendulae mycelium* [25], *Corynebacterium cholesterolicum* [29], *Streptoverticillium cholesterolicum* [9], *Rhodococcus equi* no. 23 [36], and from *Streptomyces violascens* [31].

Effect of Molarity of Potassium Monophosphate Buffer on Purified C_{OX}

After selection of an appropriate assay buffer for C_{OX} activity, effect of molarity of the selected potassium monophosphate buffer on the purified C_{OX} was studied (Table 4). The recorded data indicated that 50 mM concentration of potassium monophosphate buffers (pH 7.5) remained the best one among the tested molar concentrations of the buffer (0.745 U ml^{-1}).

Table 3 Effect of buffer system on purified C_{OX} activity of *B. subtilis*

Buffer system (50 mM)	Activity (U ml^{-1})±SD	Relative activity (%)
Potassium monophosphate buffer	0.742±0.001	100.0
Tris–HCl buffer	0.329±0.001	43.6
Glycine-NaOH buffer	0.325±0.002	43.1
Sodium phosphate buffer	0.355±0.002	47.1

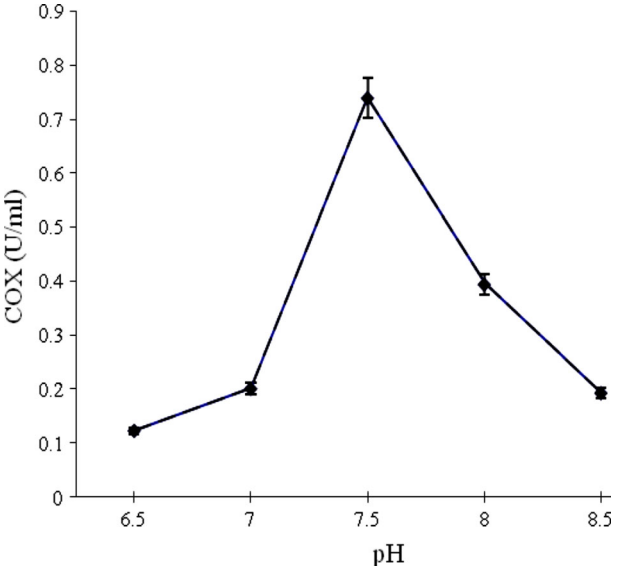

Fig. 5 Effect of pH on purified C_{OX} activity

Effect of Selected Detergents on Purified C_{OX} Activity

Exposure of purified *B. subtilis* C_{OX} to low concentration 0.05 % (*v*/*v*) of each of the selected detergents such as Tween 20 (0.241 U ml^{-1}), Tween 40 (0.431 U ml^{-1}), Tween 60 (0.643 U ml^{-1}), and Tween 80 (0.765 U ml^{-1}), except Triton X-100 (1.02 U ml^{-1}), caused a decline in the enzyme activity (Fig. 6). Thus, the addition of Triton X-100 at 0.05 % (*v*/*v*) in the potassium monophosphate buffer (pH 7.5) was considered optimum for in vitro assay of C_{OX} in the subsequent experiments. At 0.5 % Triton X-100, all cholesterol oxidases showed high stability, while in the presence of sodium cholate, only the C_{OX} from *Proteobacterium* Y-134, *Chromo bacterium* DS-1, and *Pseudomonas* were stable [26]. In 1988, Cees Veeger's group published a very important work regarding effect of the solvent/detergent on the reaction catalyzed by C_{OX} [13].

Thermostability of Purified C_{OX} of *B. subtilis*

In order to check the thermostability of the purified *B. subtilis* C_{OX} preparation, the enzyme solution (0.999 U ml^{-1}) was incubated at 37±1 °C for 160 min in a water bath. The observed residual activities obtained at periodic intervals indicated approximately 20 % decline (0.890 U ml^{-1}) in its original activity after 20 min of incubation at 37±1 °C (Fig. 7). The half-life ($T_{1/2}$) of the purified C_{OX} of *B. subtilis* was found to be ~120 min (0.47 U ml^{-1}) at 37

Table 4 Effect of buffer molarity on purified C_{OX} activity of *B. subtilis*

Phosphate buffer molarity (mM)	C_{OX} (U ml^{-1})±SD	Relative activity (%)
25	0.342±0.001	45.2
50	**0.745**±0.001	**100.0**
75	0.425±0.002	56.3
100	0.155±0.002	20.1

The bold entities indicated highest observed values

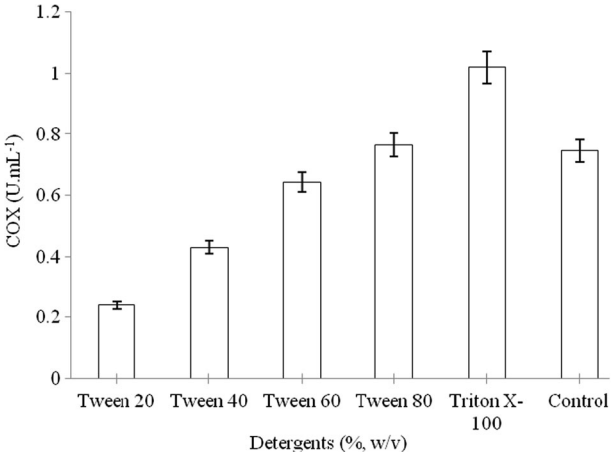

Fig. 6 Effect of detergents on purified C_{OX} activity of *B. subtilis*

±1 °C. Two novel extracellular cholesterol oxidases designated CO1 and CO2 were previously reported from a *Bacillus* sp. [27]. The optimum temperature for their C_{OX} activity was 60± 1 °C (CO1) and 40±1 °C (CO2), and the optimum pH was 6.25 (CO1) and 6 (CO2), respectively, over a 30-min reaction time.

Kinetic Behavior of Purified C_{OX} of *B. subtilis*

The K_m and V_{max} of the purified cholesterol oxidase of *B. subtilis* was determined by a Lineweaver–Burk plot (considering 1/[V] versus 1/[S]). The concentration range of the substrate (cholesterol) used was 0.016, 0.033, 0.05, 0.066, 0.083, 0.1, 0.11, 0.13, 0.15, and 0.16 mM prepared in 50 mM potassium monophosphate buffer (pH 7.5) containing 0.05 % (*v*/*v*) Triton

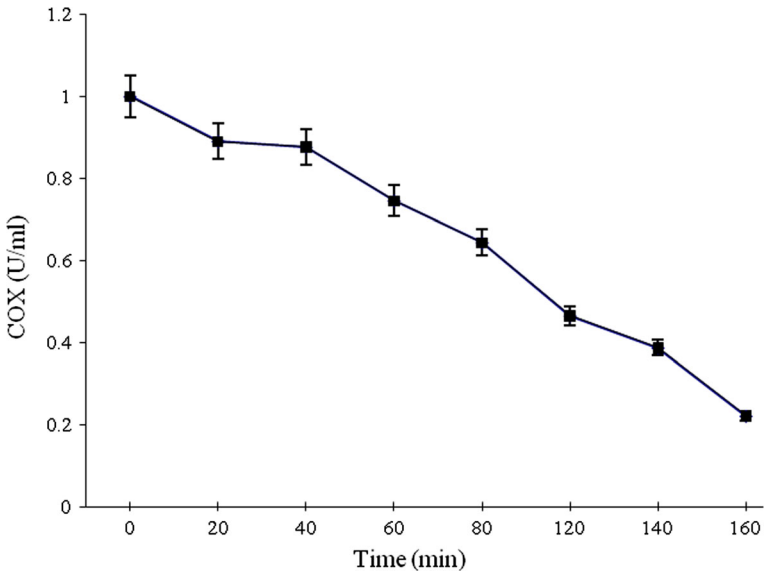

Fig. 7 Thermostability of purified *B. subtilis* C_{OX} at 37±1 °C

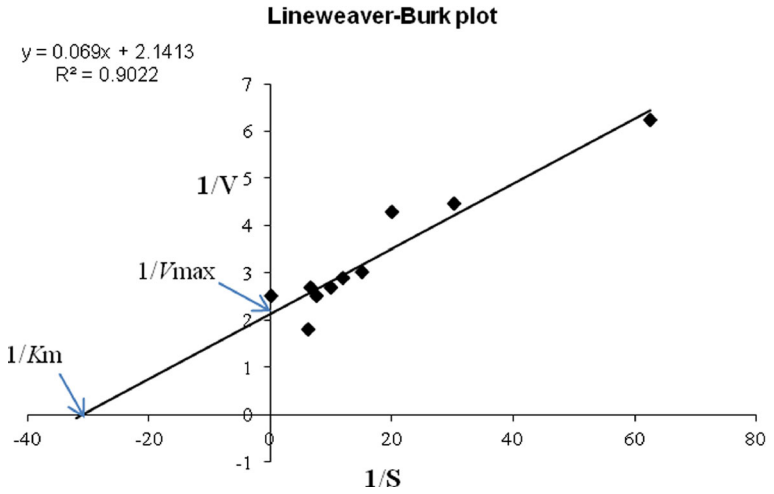

Fig. 8 Kinetic behavior of purified C_{OX} of *B. subtilis*

X-100. The K_m and V_{max} value of the C_{OX} was found to be 3.25 mM and 2.17 μmol min ml^{-1}, respectively (Fig. 8). The K_m and V_{max} of *Brevibacterium*, *Streptomyces*, *Pseudomonas fluorescens*, and *Cellulomonas* cholesterol oxidase were recorded from the previous literature [30]. *Brevibacterium* gave the highest K_m value of 230.3×10^{-4} M, followed by *Streptomyces* 2.17×10^{-4} M, *Cellulomonas* 0.84×10^{-4} M and *Pseudomonas* 0.61×10^{-4} M. In the present study, the K_m and V_{max} value of 2.17 μM and 3.25 mM min^{-1} ml^{-1}, respectively, were recorded for purified C_{OX} of *B. subtilis* using cholesterol as a substrate.

In Vitro Cytotoxicity Assay of Purified C_{OX} of *B. subtilis*

When the mammalian cells of Hep2C cell line grown in DMEM supplemented with FCS (5 %, *v*/*v*) were exposed to varying amounts of purified C_{OX} of *B. subtilis* in vitro had little cytotoxicity when tested at the 0.24 to 1.4-U ml^{-1} concentration in the growth medium at 37±1 °C (Table 5). Interestingly, the addition of the bacterial C_{OX} at 1.4-U ml^{-1} concentrations in the growth medium slightly promoted the cell viability, while the bacterial C_{OX} at 1.9 U ml^{-1} in the growth medium exerted a cytotoxic effect on the cell growth as reflected by the ~10.6 % decline in the cell viability of the mammalian cells recorded in the MTT assay.

Table 5 Cytotoxicity of purified C_{OX} of *B. subtilis* toward Hep2C mammalian cells

C_{OX} in microliters [U/ml]	0	20 [0.24]	40 [0.48]	60 [0.72]	80 [0.96]	100 [1.10]	120 [1.40]	150 [1.90]
	0.140	0.142	0.142	0.142	0.142	0.148	0.157	0.128
	0.138	0.141	0.133	0.133	0.133	0.139	0.161	0.121
	0.147	0.145	0.149	0.149	0.149	0.143	0.156	0.129
Mean±SD	0.141± 0.003	0.142± 0.002	0.141± 0.003	0.141± 0.002	0.141± 0.003	0.143± 0.001	0.158± 0.001	0.126± 0.002
Effect on cell viability (%)	Little effect						↑12.1	↓10.6

Conclusion

The present study provided a cholesterol oxidase producing mesophilic *Bacillus subtilis* strain isolated from tiger fecal sample that produced an appreciable amount of extracellular enzyme in the culture broth. The enzyme possessed some interesting properties like high *M*r and multimeric enzyme as an organic solvent-compatible/tolerant biocatalytic nature as well as anti-cholesterolemic therapeutic efficacy with an adequate half-life of ~2 h at 37 °C. The purified enzyme had little cytotoxicity associated with it.

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Conflict of Interest The authors have no conflict of interest at their place of work.

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