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PURIFICATION AND CHARACTERIZATION OF CHOLESTEROL OXIDASE FROM NOVEL NATIVE ISOLATE *BACILLUS SUBTILIS* YS01 ISOLATED FROM MEAT SAMPLE

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ABSTRACT

Cholesterol oxidase (COX) has various clinical and industrial applications. Recently, microbial cholesterol oxidase has received a great attention for its wide usage in medicine. In the present study, cholesterol oxidase was purified and characterized from *Bacillus subtilis* YS01 (GenBank accession number: AB924083) isolated from meat sample. The extracellular cholesterol oxidase produced by *Bacillus subtilis* YS01 was purified by ammonium sulphate precipitation, dialysis, DEAE-cellulose ion-exchange chromatography and Sephadex G-100 gel filtration. The enzyme was purified 19.69-fold and with specific activity of 99.7 U/mg of protein. The purified cholesterol oxidase appeared as a single protein band in SDS-PAGE and with a molecular weight of approximately 56 kDa. The optimum temperature for the activity was 40°C and the optimum pH was 7.2. The maximum Cholesterol oxidase activity was obtained at 1% concentration of cholesterol. At lower concentrations and higher concentration of substrate showed a decrease in Cholesterol oxidase activity.

Keywords: Cholesterol oxidase (COX), *Bacillus subtilis* YS01, SDS-PAGE. Meat sample

INTRODUCTION

Cholesterol oxidase (COX, EC 1.1.3.6) is a bacterial enzyme that has proven to be very useful in biotechnological applications related to the conversion of cholesterol and to the disruption of cholesterol-containing membranes.COX is widely used in clinical diagnosis and determining lipid disorders (Vrielink A and Ghisla S., 2009). COX also exhibits an insecticidal effect against several species of lepidopteron cotton insects which occurs during membrane fusion, since the curvature, including tobacco budworm (Heliothis virescens), corn earworm (*Helicoverpa zea*), and pink bollworm (*Pectinophora gossypiella*).COX is used as an insecticide (Kreit J & Sampson NS., 2009) and also plays a role in lysis of macrophages and leukocytes (J. Navas et al. 2001). As described in the two accompanying reviews in this miniseries (Hamed et al. 2010; Kim et al. 2002), COX catalyzes the oxidation of the C_3-OH group of cholesterol (and other sterols) to give the corresponding D5-3-ketone (Cholest-5-en-3-one) and its isomerization to D4-3-ketone (Cholest-4-en-3-one).Since the first report on cholesterol oxidation in microorganism(Turfit.,1949),the same reaction has been reported in different organisms such as Mycobacterium spas (stadtmen et al. 1954), Nocardia restricus (Richmond. 1973).Morever,the enzyme has been purified and partially characterized from several microorganisms(uwajima et al . 1973;Tomiokaetal. 1976;kameietal. 1978).This Present study was taken up with the objective for the purification of cholesterol oxidas from the meat sample by using *Bacillus subtilis* YS01 and screening for factors affecting the cholesterol oxidase production such as incubation temperature, pH was studied and optimized.

MATERIALS AND METHODS

Micro- organism and substrates

Collection of sample:

Meat sample was collected from Local market, Hyderabad, A.P, India. The samples were collected in sterilized bottles for isolation of cholesterol oxidase producing bacteria.

Isolation of bacteria

In order to isolate bacteria, 1 g of meat sample was suspended in 100 ml of distilled water. The suspension was shaken strongly for 30 min. A volume of 100 µl of supernatant was inoculated in medium containing cholesterol as the sole carbon source as described by Hamed et al (2010).

Larger colonies were sub cultured again in the same media.

Maintenance of pure cultures

The identified colonies were sub cultured on nutrient agar slants and preserved at 4ºC. Sub-culturing was performed at one month interval.

Identification of bacterial strain

Bacterial culture is identified based on the molecular methods. Genomic DNA was isolated from the bacterial culture, The DNA was used in PCR to amplify the 16S region using 16S Forward and 16S Reverse primers described in the literature. The ~1500 bp amplicon was gel eluted and subjected to sequencing. The sequencing results were assembled and compared with NCBI database.

Production of cholesterol oxidase

The media for the production of cholesterol oxides was prepared according to Kim et al. 2001. The composition of the media was as follows (%w/v) (g/1000 ml): Cholesterol 1, Glucose 20, Yeast extracts 5, NH_4NO_3 2, K_2HPO_4 0.2, $MgSO_4.7H_2O$ 0.3. The pH of the media was adjusted to 7 and sterilized as mentioned above. Bacterial strain was grown at 30^{o}C for 36 h with shaking at 150 rpm as described by Watanabe et al. 1989.

Crude enzyme extraction

The culture supernatant was obtained by centrifugation of the culture broth at 25 000 × *g* for 30 min and the obtained supernatant used as a crude enzyme for enzyme assay and for further experiments.

Determination of protein content

Protein content was determined by the method of Bradford (1976) using a Bio-Rad dye reagent concentrates and bovine serum albumin as the standard.

Enzyme assay

The crude enzyme was subjected to cholesterol oxidase assay, which was carried out using the method of Richmond (1973). The reaction mixture was composed of 3 ml of 0.1 M sodium phosphate buffer (pH 7.0)-0.05% Triton X-100, 0.05 ml of 6 mM cholesterol solution in isopropanol and 0.05 ml of the crude enzyme solution. The enzyme reaction was carried out at 30^{o}C for 1 min in a 10 mm light path cuvette, suitable for use in a thermostat double-beam spectrophotometer. After incubation, the increase in absorbance resulting from the oxidation of cholesterol was measured at 240 NM. The enzyme unit of cholesterol oxidase was defined as the amount of enzyme oxidizing 1 µmole of cholesterol to 4-cholesten-3-one per min at 30^{o}C.

Enzyme purification

The organism was grown for 36 hours as described previously. The supernatant was fractionated by precipitation with ammonium sulfate. All subsequent steps were carried out at 4°C. The protein was resuspended in 0.1 M sodium phosphate buffer (pH 7.0) and dialyzed against the same buffer overnight at 4^0C. The dialyzed sample was then loaded with a pre-equilibrated DEAE-cellulose column chromatography (2.6 x 20 cm), and washed with the 0.1 M sodium phosphate buffer (pH 7.0). The proteins were eluted with a stepwise gradient of NaCl (0 - 1.0 M) at a flow rate of 48 ml/h. Fractions of 2.5 ml were collected and the absorbance was read at 280 nm in a spectrophotometer. The fractions with cholesterol oxidase activity were combined, dialyzed against the phosphate buffer (100 mM, pH 7.0), and concentration by lyophilization. The concentrated sample was again applied to column of Sephadex G-100 (1.5 × 24 cm) (Sigma-Aldrich, St Louis, MO) equilibrated with phosphate buffer, pH 7.0. The column was eluted at a flow rate of 60 mL/h with a 1:1 volume gradient from 0.1M to 1M NaCl in the same buffer. The elution fraction (2.5 mL) were collected and assayed for cholesterol oxidases activity and those fractions which shown high activity was collected and used for SDS-PAGE analysis.

SDS- PAGE and molecular weight determination

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed under non reducing conditions (Laemmli, 1970). Electrophoresis was performed at 50V in stacking gel (4%) and for resolving gel (12%) at 100V. The gels were stained with coomassie brilliant blue staining (coomassie 0.25%, methanol 15%, acetic acid 7.5%) for 2hrs and destained overnight with the stain solution excluding the dye. The molecular weight of protease was determined with the help of protein markers.

Characterization of Purified Enzyme

Factors affecting activity

The following factors were studied to obtain the optimal condition for the activity of purified Cholesterol oxidase. These factors include incubation period, incubation temperature, pH and concentration of substrate.

Effect of Temperature on enzyme activity

This experiment was carried out in order to determine the effect of different incubation temperatures of the reaction mixture on the purified Cholesterol oxidase enzyme activity. This was performed by incubating the

enzyme reaction mixtures at different temperatures viz: 10, 20, 30, 35, 40, 50 and 55^0C respectively. The assay of Cholesterol oxidase activity was performed as previously mentioned.

Effect of pH on enzyme activity

This experiment was performed in order to investigate the effect of different pH values on the purified Cholesterol oxidase enzyme activities. The purified enzyme reaction mixture was incubated at different pH values via: 5.8, 6.0, 6.2, 6.4, 6.6, 6.8, 7.0, 7.2, 7.4, 7.6, 7.8 and 8.0 using 0.2M phosphate buffer. The enzyme activities in each case were determined.

Effect of Substrate concentration on enzyme activity

This experiment was undertaken to investigate the effect of different substrate concentrations on the activity of the purified Cholesterol oxidase. Carboxymethyl Cellulose was applied at the concentrations viz. (W/V, %) 0.25, 0.5, 1.0, 2.0, 5.0 and 10.0 respectively. Enzyme activities were determined.

RESULTS AND DISCUSSION

Isolation and screening of cholesterol oxides producing bacteria

Out 6 pure cultures isolated, one was found to be fast growing and largest colony. This colony was sub cultured on nutrient agar slants and preserved at 4ºC. Sub-culturing was performed at twenty days to one month interval. (Fig.1 and 2). This colony identified by molecular methods.

Purification of cholesterol oxidase

The cholesterol oxidase produced *by Bacillus subtilis* YS01 was concentrated by ammonium sulfate (70%) precipitation and purified consecutively by ion-exchange chromatography and gel filtration. The results of the purification procedure are summarized in Table 1. The precipitate was purified by a DEAE-cellulose column, and eluted at 0.1-0.3M NaCl in the buffer (Fig. 3). Following DEAE-cellulose column, the enzyme was purified 10.45-fold with a recovery of 10.46% and specific activity of 49.15 U/mg of protein. The concentrated-active fractions were further purified by a Sephadex G-100 column chromatography. The elution profiles of protein and cholesterol oxidase activity are shown in Fig. 4. After the final purification step, the enzyme was purified 19.69-fold with a recovery of 5.09% and specific activity of 102.7 U/mg of protein.

Molecular weight of purified protein

The purified cholesterol oxidase appeared as a single protein band in SDS-PAGE and with

a molecular weight of approximately 56 kDa (Fig. 5). Yanliang et al (2010) reported the molecular weight of cholesterol oxidase as 55KDa.

Characterization of Purified Enzyme

Effect of temperature

The results showed that the activity of Cholesterol oxidase was increased up to 30^0C which represented the optimum temperature (Relative activity was higher for Cholesterol oxidase). Beyond 40^0C the activity decreased with the increase of the temperature. However, the Cholesterol oxidase was showing a responsible activity within the temperature range of 10-50^0C.

Effect of pH

The results revealed that the optimum pH for the activity of Cholesterol oxidase was attained at pH7.2.Beyond pH 7.2, the activity decreased with the increase of the pH. However, the Cholesterol oxidase was showing a responsible activity within the pH values ranged from 6.8 to 7.6.

Effect of substrate concentration

The results showed the maximum Cholesterol oxidase activity was obtained at 1% concentration of cholesterol. At lower concentrations, e.g. 0.25 and 0.5% (W/V) and higher concentration of substrate (more than 1%) showed a dramatic decrease in Cholesterol oxidase activity. The activity of Cholesterol oxidase attained their maximum activities, beyond which no increase of enzyme activity was recorded.

Fig.1 *Bacillus subtilis* YS01 on a nutrient agar plate

Fig.2 *Bacillus subtilis* YS01 on nutrient agar slants

Table 1: Purification steps of cholesterol oxidase from *Bacillus subtilis* YS01

Step	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Yield (%)
Crude enzyme	84.7	381.1	4.5	1.0	100
Ammonium sulfate	24.64	268.56	10.9	3.43	29.1
DEAE-cellulose	8.1	398.11	49.15	10.45	10.46
Sephadex G-100	4.3	428.71	99.7	19.69	5.09

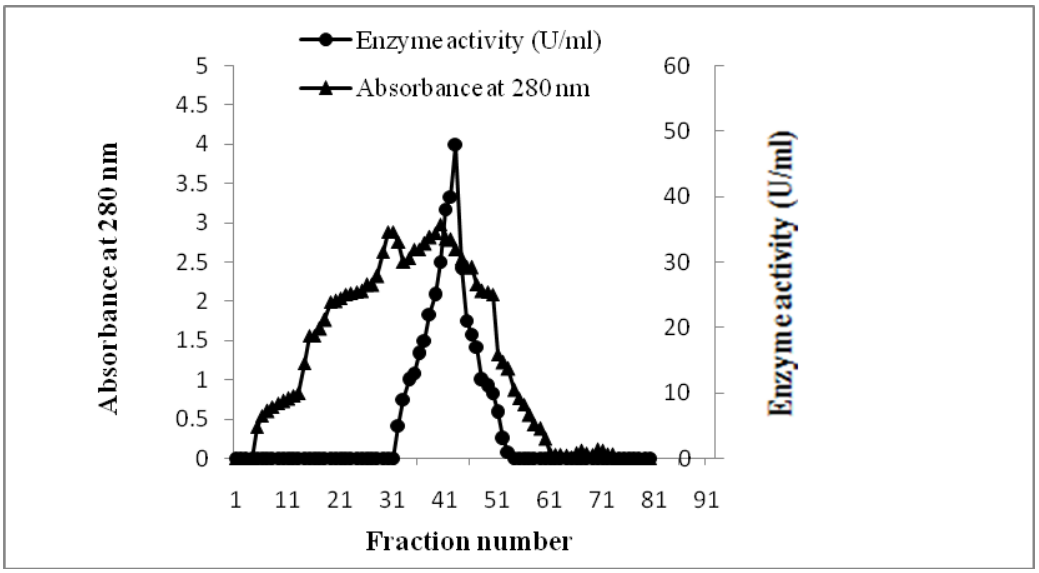

Fig. 3: Chromatogram of the cholesterol oxidase from *Bacillus subtilis* YS01 on DEAE-cellulose (2.6 × 20 cm). The column was eluted with NaCl (0-1.0 M) at a flow rate of 24 ml/h. Fractions of 2.5 ml were collected.

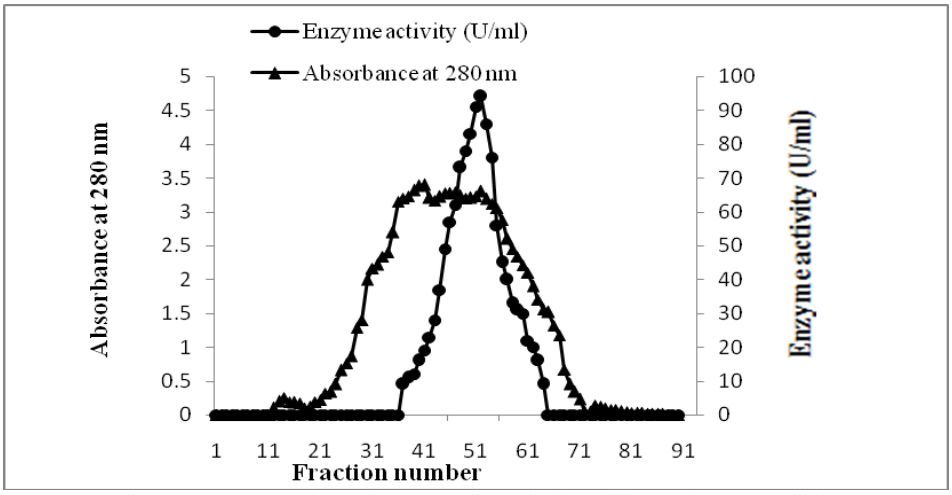

Fig. 4: Chromatogram of the cholesterol oxidase from *Bacillus subtilis* YS01 on a Sephadex G-100 column (1.6 × 36 cm). The column was eluted with a 50 mM sodium acetate buffer (pH 5.0) at a flow rate of 15 ml/h. Fractions of 3 ml were collected

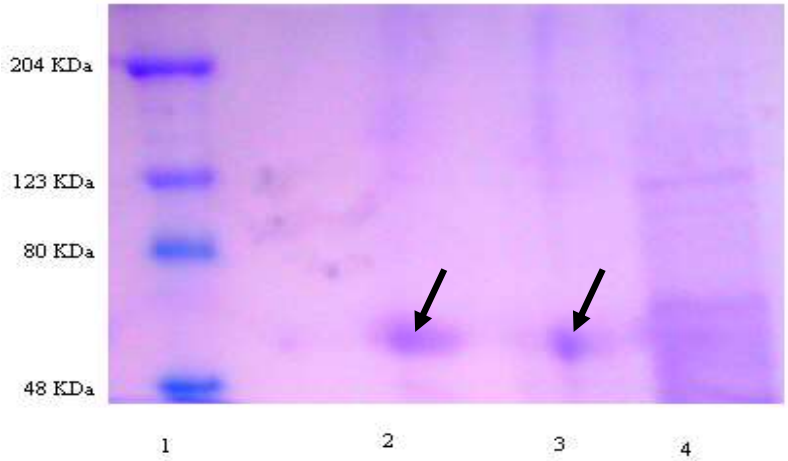

Fig.5: SDS PAGE of purified enzyme Lane 1: Bio-Rad prestained protein marker, Lane 2 and 3: purified cholesterol oxides, Lane 4: crude enzyme

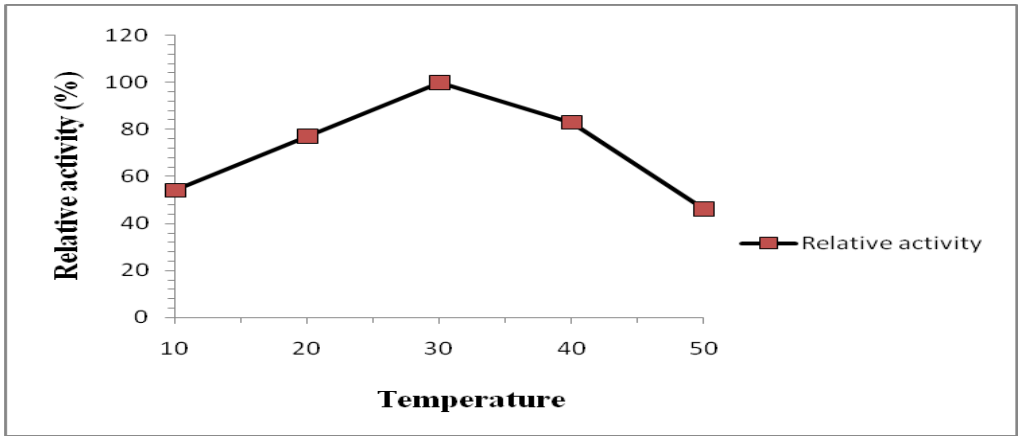

Fig.6: Effect of temperature on cholesterol oxidase activity

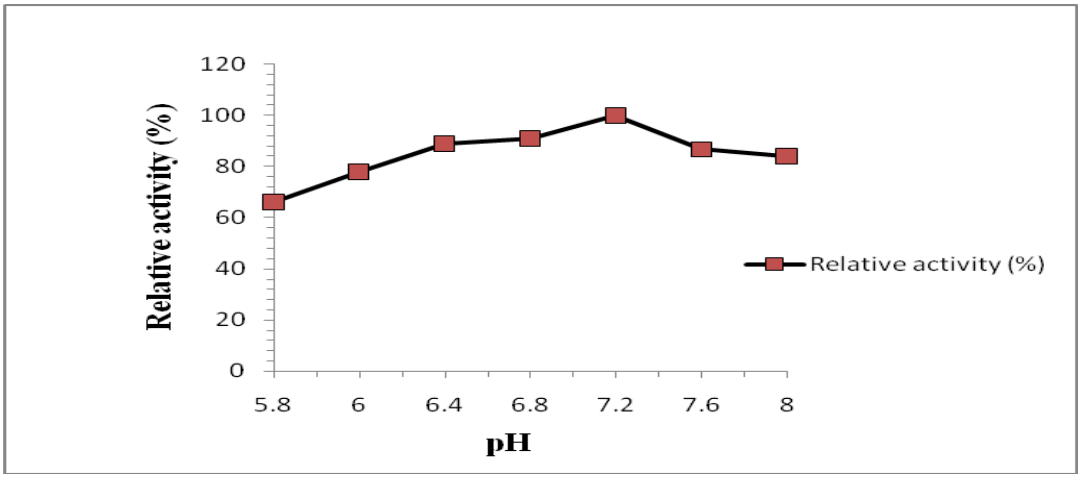

Fig.7: Effect of pH on cholesterol oxidase activity

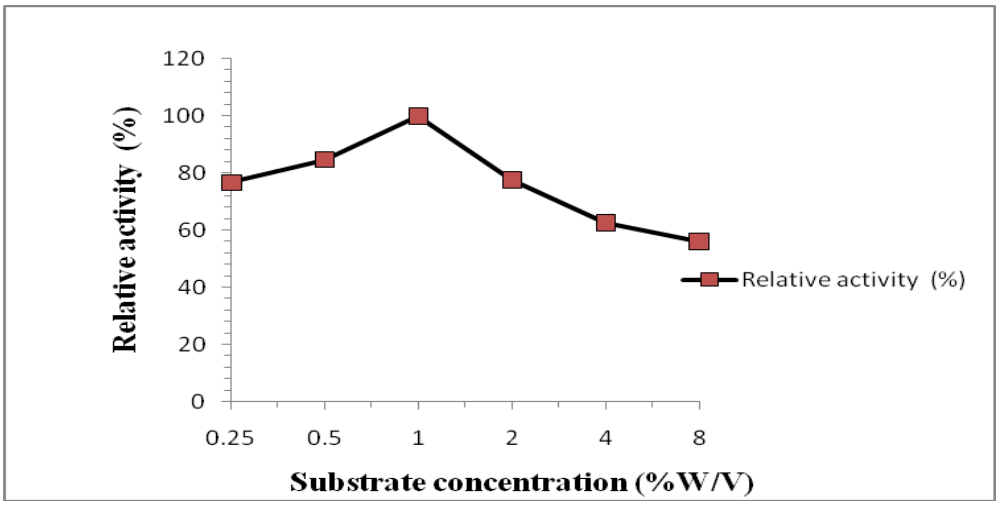

Fig.8: Effect of substrate concentration on Enzyme activity

CONCLUSION

In the present study, cholesterol oxidase from bacteria was isolated from meat sample and identified as Bacillus subtilis YS01 based on molecular identifications. The extracellular cholesterol oxidase from *Bacillus subtilis* YS01 was purified by ammonium sulphate precipitation, dialysis, DEAE-cellulose ion-exchange chromatography and sephadex G-100 gel filtration. The enzyme was purified 19.69-fold and with specific activity of 99.7 U/mg of protein. The purified cholesterol oxidase appeared as a single protein band in SDS-PAGE and with a molecular weight of approximately 56 kDa. The optimum temperature for the activity was 40^0C and the optimum pH was 7.2. The maximum Cholesterol oxidase activity was obtained at 1% concentration of cholesterol. At lower concentrations and higher concentration of

substrate showed a decrease in Cholesterol oxidase activity.

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Conflict of interest The authors hereby declare no conflict of Interest.

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