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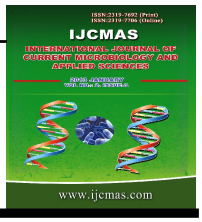

Original Research Article

Bacterial extracellular Cholesterol oxidase and its Pharmaceutical perspectives

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A B S T R A C T

Cholesterol oxidase is an enzyme which catalyzes the oxidation of cholesterol and converts 5-cholesten-3β-ol into 4-cholesten-3-one. Screening and isolation of bacterial strains producing extracellular form of cholesterol oxidase are very important due to its wide spectrum applications. Cholesterol oxidase enzyme has many applications in medicine, agriculture, industry and pharmaceutical sectors. The various samples- buffalo milk, domestic waste water sludge and fermented fish were used for enrichment and isolation. Out of 10 isolates 6 were cholesterol oxidase positive of which one with significantly high enzyme activity was identified as *B. licheniformis.* Optimization of physical parameters for enzyme production such as pH and incubation temperature were determined and found to be 7.0 and 35^0C respectively. Enzyme activity was assayed using o-dianisidin-peroxidase method. Antimicrobial and bioinsecticidal activity of purified enzyme was tested. Also the effect of the enzyme on serum cholesterol was studied. The enzymatic oxidation product of cholesterol in serum sample was characterized by the TLC and found to be 20α-Hydroxycholesterol.

Keywords

Cholesterol oxidase; 5-cholesten-3β-ol; 4-cholesten-3-one; Serum cholesterol; Bioinsecticide.

Introduction

Cholesterol oxidase (CHO) is an enzyme which catalyzes the oxidation of cholesterol and converts 5-cholesten-3β-ol into 4 cholesten-3-ones (Murooka, *et al.*, 1986). CHO enzyme has many applications in agriculture, medicine, industry, and pharmaceutical sectors. For instance, it can be used for production of diagnostic kits to detect blood cholesterol, biological insecticide and precursors for steroid hormones (Bell, *et al.*, 1998) Many bacteria produce this enzyme including members of the genera *Brevibacterium,Arthrobacter, Nocardia, Pseudomonas, Corynebacterium, Rhodococcus, Streptomyces, and Shizophyllum*. This enzyme can be produced by a bacterium in three forms: intracellular, extracellular and membrane bound. Due to the wide spectrum applications of CHO, screening and isolation of bacterial strains producing extracellular form of CHO are of great importance (Yazdi, *et al.*, 2001). Many microorganisms were demonstrated to

produce extracellular CHO including *Rhodococcusequi, Streptomyces sp., Rhodococcuserythropolis, Arthrobacter simplex, Brevibacterium sterolicum, Streptomyces lividans,* and *Shizophylum commune* (Anando *et al*.,2006).

Autoxidation of cholesterol yields toxic products. Cholesterol oxidation products (COP) are similar to cholesterol; contain an additional functional group, such as a hydroxyl, ketone or an epoxide group in the sterol nucleus and/or on the side chain of the molecule. Oxidation of lipids and cholesterol follows the same oxidation patterns such as autoxidation, photoxidation and enzymic oxidation, producing hydroperoxides. It is believed that the hyroperoxides produced from oxidation of unsaturated fatty acids play a significant role to facilitate cholesterol oxidation at Δ-5 double bond, which is more sensitive to oxidation (Lercker *et al*., 2002, Kumari *et al.,* 2006).

The major COP products are 5-cholesten-3β, 7α-diol (7α-HC), 5-cholesten- 3β, 7β-diol (7β-HC), 5-cholesten-3β-ol-7-one (7-KC), 5-cholestan-5α, 6α-epoxy-3β- ol (α-CE), 5-cholestan-5β, 6β-epoxy-3β-ol (β-CE), 5-cholesten-3β,20α-diol (20α-HC), 5-cholesten-3β, 25-diol (25-HC) and 5-cholestane-3β, 5α,6β-triol (CT). These eight COP are usually found in processed foods. COP are present in our diet, especially in foods with high cholesterol contents (egg, whole milk, meat etc). The fresh foods contain low levels of COP and the levels go up during processing and storage (time and condition). Generally, heat, pH, light, oxygen, water activity and the presence of unsaturated fatty acids are the major factors that influence COP formation during food processing or storage (Christie, 2003; Kim *et al.,* 2002). In the present study, *Bacillus licheniformis* was isolated from waste water sludge and fermented fish and its biochemical characters were elucidated. The optimization of temperature and pH conditions for CHO production was also performed. The activity of extracellular CHO enzyme produced by organism was determined using an enzyme assay on cell free supernatant of bacterial culture. The enzymatic oxidation product of cholesterol in serum sample was also characterized by the TLC

Materials and Methods

Collection of sample

The samples gathered for isolation of organisms were wastewater sludge, buffalo milk and fermented fish infusion. In order to isolate microorganisms, 1 ml of each sample was suspended in 10 ml of sterile saline. The suspension was thoroughly vortexed. These samples were then pooled together and enriched in enrichment medium to obtain the all possible organisms capable to produce bacterial cholesterol oxidase.

Isolation of Microorganisms

Composition of enrichment medium (gm/ lit): Cholesterol 1.0; Glucose 20.0; Yeast extract 5.0; NH_4NO_3 2.0; K_2HPO_4 0.2 and $MgSO_4 \cdot 7H_2O$ 0.3 (pH 7.0). The inoculated medium was incubated at room temperature for 7-12 days at 140rmp. After incubation a loop full of enriched sample was streaked on to the plates of same medium but having cholesterol as the only source of carbon and incubated at 30^oC for 2-3 days. The fast growing bacteria generating larger colonies were subcultured.

Identification of Microorganisms

Identification of selected isolated culture showing maximum CHO activity qualitatively as well as quantitatively was performed by morphological, biochemical examination and further confirmed with VITEK-2 system version 05.02.

Screening of organisms by qualitative detection of CHO

Colony staining method

To confirm CHO producing strain, colony staining method was performed on the grown colonies. The filter papers were dipped into the solution containing 0.5% cholesterol; 1.7% 4-aminoantipyrin; 6% phenol and 3000U/l horseradish peroxidase (HRP) in 100 mM potassium buffer phosphate (pH 7.0). Thereafter, soaked disc were located on grown colonies on the plate and incubated at room temperature for 24 h.CHO activity of tested colonies was confirmed by measuring the time required for the development of red color due to the formation of quinoneimine dye. (Lashkarian *et al.*, 2010)

CHO indicator plates

CHO enzyme is able to convert cholesterol into cholest-4-en-3-one and hydrogen peroxide. CHO-producing colonies were selected on suitable indicator plates. These plates were prepared by adding 1.0 g cholesterol, 1.0 g Triton X-100, 0.1 g o-dianisidine and 1000 U/l horseradish peroxidase to 1 litre of LB agar medium. Bacterial colonies were cultured on these plates and incubated at 30^{o}C. Cholesterol penetrates into bacterial cells where it is converted into hydrogen peroxide (H_2O_2) by CHO. Reagents that exist in the medium react with H_2O_2 to form azo compound which turns the colour of medium to intense brown (Lashkarian *et al*., 2010).

Quantitative analysis of CHO

Enzyme activity of the crude CHO and purified CHO extracted from isolates were tested spectrophotometrically using peroxide and o-dianisidine method. Hydrogen peroxide generated during cholesterol oxidation process was measured in this method.

In this reaction, hydrogen peroxide was coupled with o-dianisidine in presence of enzyme peroxidase, which causes oxidation of o-dianisidine. Oxidized o-dianisidine has maximum absorption at 500nm. Increase in the absorbance for reaction mixtures was recorded and calculations for enzyme units were done (Sigma's Quality Control Procedure, 1994).

For enzymatic assay of CHO produced by isolated organisms, following reagents were used.

1. **Reagent A (Buffer):** 50mM Potassium Phosphate Buffer, pH 7.5 at 25°C (Prepared 100 ml in distilled water using KH_2PO_4. Adjusted to pH 7.5 with 1 M KOH)
2. **Reagent B (ODA):** 1% (w/v) o-Dianisidine Solution (Prepared freshly in 5 ml distilled water using o-DianisidineDihydrochloride, Hi-media)
3. **Reagent C (Chol):** 0.5% (w/v) Cholesterol- Hi-media with 10% (v/v) Triton X-100 Solution (Prepared by initially dissolving the Cholesterol in 10 ml of Triton X-100.Heated until the solution clarifies. Then 90 ml of

distilled water was added. Vortexed and stored the solution at 4°C)

4. **Reagent D (POD):** Peroxidase Enzyme Solution (A solution was prepared immediately before use, using Horseradish Peroxidase, Chromus Biotech Ltd. containing 100 units/ml in distilled water i.e. 0.1mg in 3.3ml)
5. **Reagent E** (Enzyme Solution i.e. supernatant of broth)**:** Cholesterol Oxidase Enzyme Solution (Here, we used the supernatant that was obtained by centrifugation of the culture broth at 10000×g for 10 min at 4°C)

A reaction cocktail was prepared by pipetting (in milliliters) the following reagents into a conical flask: Reagent A (Buffer) 40.0ml, Reagent B (ODA) 0.50ml. These reagents were mixed thoroughly by swirling and pH was adjusted to 7.5 at 25°C with 100mMHCl or 100 mM KOH. Then Reagent A was added to make a final volume of 50 ml. The contents were mixed by swirling thoroughly and oxygenated for 10 minutes immediately before use. Further the quantitative estimation of cholesterol was performed as given below:

	Test(ml)	Blank(ml)	Control(ml)
Reaction Cocktail	2.70	2.70	2.70
Reagent C (Chol)	0.10	0.10	0.10
Reagent D (POD)	0.10	0.10	0.10
Mixed by inversion and equilibrated to 25°C. Monitored the A_{500nm} until constant, using a thermostatted spectrophotometer.			
Then added:			
Reagent A (Buffer)	--	0.10	--
Reagent E (Enzyme Solution - i.e.supernatant of culture broth)	0.10	--	--
Supernatant of un-inoculated broth (Control)	--	--	0.1
Immediately mixed by inversion and recorded the increase in A_{500nm} for approximately 5 minutes. Obtained the A_{500nm}/minute using the maximum linear rate for both the Test and Blank.			

Formulae for units/ml CHO and units/mg protein

$$\frac{\text{Units}}{\text{ml}}\text{enzyme} = \frac{\left(\frac{\Delta A_{500nm}}{\min \text{Test}} - \frac{\Delta A_{500nm}}{\min \text{Blank}}\right)}{(7.5)(0.1)} \times (3)\,(df)$$

Where, 3 = volume (in ml) of assay, df = dilution factor, 7.5 = milimolar extinction coefficient of o-Dianisidine, 0.1 = volume (in ml) of enzyme used.

$$\frac{\text{Units}}{\text{mg}}\text{protein} = \frac{\frac{\text{Units}}{\text{ml}}\text{enzyme}}{\text{mg}\frac{\text{protein}}{\text{ml}}\text{enzyme}}$$

To determine units per mg protein by above formula, mg protein present per ml enzyme was determined using Folin-Lowry's method.

Optimization of physical parameters

For preparation of standard inoculum, the isolate *Bacillus licheniformis* was cultured in sterile isolation medium at 30^{o}C on rotary shaker at 150 rpm for 24 h so as to achieve the minimum average viable count of 10^{6} cells/ml of culture broth. For optimization of pH, sterile isolation medium with different pH *viz.*, 6.0,6.5,7.0,7.5 and 8.0 were inoculated with 1 ml of standard inoculum and incubated at 30°C. For optimization of temperature sterile isolation medium, pH 7.0, was incubated at respective temperatures *viz.*, 25,30,35,40 and 45°C. The amount of enzyme produced by the organism at 24, 48 and 72h was determined by the enzymatic assay (Lachlan Mac *et al.*, 2000).

Enzyme Purification

The extracellular enzyme was purified by the organic solvent precipitation using acetone. Precipitation was carried out by using different acetone to broth ratios such as 1:1, 1:2, 1:3 and 1:4. The precipitated enzyme was resuspended in phosphate buffer and stored at 4°C (Ghasemian et al, 2009).

Detection of COP byTLC

In order to know the cholesterol oxidation products (COP) accumulated in the culture medium due to the action of bacterial cells, 50 ml of medium in 250 ml Erlenmeyer flask was inoculated with 24h culture of *B.licheniformis* and incubated for 7 days at 35°C and agitation speed of 150 rpm. After incubation the cells were harvested by centrifugation and equal volume of ethyl acetate was added to the supernatant. The organic phase was recovered and evaporated at 60°C in a rotary evaporator until a white residue was formed.

The methylene chloride was used to dissolve dry residue and analyzed by thin-layer chromatography. Blank culture medium was uninoculated flask, incubated and treated as the above. The reaction medium and procedure used for cholesterol oxidation in this experiment were same as those used to determine the enzyme activity, except tenfold volume of buffer, enzyme and substrate were employed and reactions were arrested by addition of 2 ml of methylene chloride.

The methylene chloride fractions were recovered and analyzed by thin layer chromatography. Reaction blank, where no cholesterol was added to the reaction mixture, was prepared for each time interval and treated in the same way. Thin-layer chromatography was carried out on pre-coated silica gel 60 F254 plate (0.25 mm thick, 20 cm x 20 cm, Merck) at 30°C. Plates were activated at 110°C for 1 hour and spotted with 25 µl of samples. Benzene: ethyl acetate 3:1 (v:v) was used as solvent system and the development was carried out by spraying 95% aq.H_2SO_4, 5% methanol solution followed by heating at 90°C until visualization of the spots (Terezinha *et al.*, 2000).

Applications of purified CHO

The purified enzyme was used to check its effect on serum cholesterol, the antimicrobial and bio insecticidal activity (Noriyuki *et al.*, 2009).

Effect on serum cholesterol: As per the protocol (Table-1) provided in the kit (CHOD-PAP method, Span Diagnostics

Ltd. India) to estimate serum cholesterol, the initial serum cholesterol value was determined. Then the serum was treated separately with 5µl of the purified and diluted (1:1) enzyme samples and kept for incubation at 37°C for 10 min. After completion of incubation period the amount of residual cholesterol was estimated.

Antibacterial activity

Antibacterial activity was tested by well diffusion method. The solidified Muller Hilton agar plates were swaped with the sufficiently turbid (10^8 cells/ml) suspension of the organisms *Escherichia coli, Salmonella typhae, Micrococcus and Bacillus subtilis*. Then the 6 mm wells were dug in the centre of agar plates and 25µl enzyme sample was poured into them and kept at 4^oC for 1h for efficient diffusion. The plates were then incubated at 37°C for 24 h and resulted inhibition zones were measured.

Bio-insecticidal activity

The leaves infected with *Cryptolaemus montrouzieri* were treated with the 1:1 diluted purified CHO enzyme. The enzyme was sprayed on the infected leaf uniformly and observed for 2 h to determine the insect mortality rate (Purcell *et al.,* 1993).

Table. 1 Protocol for testing serum with purified and diluted enzyme sample.

Additions	Quantity in µL				
	CHO		Normal	Standard	Blank
Serum	10	10	10	-	-
Purified CHO sample	5	-	-	-	-
Diluted CHO sample (1:1)	-	5	-	-	-
Incubated for 10 min at 37ºC					
CHOD-PAP Reagent	1000	1000	1000	1000	1000
Std. cholesterol (200mg/dl)	-	-	-	10	-
Incubated for 10 min at 37ºC and absorbance measured at 505 nm					

Result and Discussion

Isolation of microorganisms

The isolation from enriched broth on agar plate gave 10 different isolated colonies. As the medium contained cholesterol as a sole source of carbon, appearance of growth on this medium implicates that the organisms utilize cholesterol. By assuming that they must produce some enzymes to metabolize the cholesterol, among 10 isolates, 6 were screened on the basis of their excellent growth on the screening medium.

Gram staining, endospore staining, motility testing were employed to all screened isolate but only the one most potent isolate IV P II bacillus was subjected to identification.

Identification of microorganisms

From the observations of morphological and biochemical tests employed as per Bergey's Mannual of Systematic Bacteriology 2nd edition, volume 2, the species of the isolate IV P II bacillus was identified to be *Bacillus licheniformis.* This identified organism was further confirmed with VITEK 2 system version 05.02 (Table 2).

Table. 2 VITEK-2 system report for identification of the organism.

Bac-test laboratory Printed Apr. 2. 2012 07:25 IST

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System # — Laboratory Report — Bench:WATER

Bionumber: 1773261715476271
Selected Organism: *Bacillus licheniformis*

Identification information	**Card:** BCL	**Lot Number:** 239213510	**Expires:** Oct 18, 2012 12.00
	Completed: Apr. 2. 2012	**Status:** Final	**Analysis Time:**14.2 h
Selected Organism	93% Probability ***Bacillus licheniformis*** **Bionumber:**1773261715476271 **Confidence:** very good identification		

Biochemical details:

1	BXYL	+	3	LysA	-	4	AspA	-	5	LeuA	+	7	PheA	+	8	ProA	+
9	BGAL	+	10	PyrA	+	11	AGAL	(+)	12	AlaA	+	13	TyrA	+	14	BNAG	-
15	APPA	-	18	CDEX	+	19	dGAL	-	21	GLYG	(-)	22	INO	+	24	MdG	+
25	ELLM	+	26	MdX	-	27	AMAN	-	29	MTE	+	30	GlyA	(+)	31	dMAN	+
32	dMNE	+	34	dMLZ	-	36	NAG	(-)	37	PLE	+	39	IRHA	-	41	BGLU	+
43	BMAN	-	44	PHC	-	45	PVATE	+	46	AGLU	+	47	dTAG	+	48	dTRE	+
50	INU	-	53	dGLU	+	54	dRIB	+	55	PSCNa	-	58	NaCl 6.5%	+	59	KAN	-
60	OLD	+	61	ESC	+	62	TTZ	+	63	POLYB_R	+						

Installed VITEK 2 System Version: 05.02
MIC Interpretation guideline:
Therapeutic Interpretation guideline:
AES Parameter Set Name:
AES Parameter Last Modified:

Screening of organisms by qualitative analysis of CHO

In colony staining method the development of red color was due to formation of quinineimine dye which confirmed the CHO activity. Such colonies were further inoculated on indicator plate, here the medium color changed to intense brown that indicated formation of azo component due to presence of H_2O_2 generated by the reaction of cholesterol oxidase.

Quantitative Analysis of CHO and Enzyme Purification

The enzyme was purified by solvent extraction at acetone to broth ratio of 1:1. In enzyme assay, the purity of enzyme was checked by measuring specific enzyme activity and was found to be 226.32 units/ml as compaired to crude which was 213.74 units/ml and the fold purification was 1.29 (Noryuki *et al.*, 1998).

Optimization of physical parameters

After optimization of physical parameter for maximum CHO yield, the pH 7.0 and temperature 35°C was found to be optimum for *Bacillus licheniformis* (Fig.1 & Fig.2). The enzyme activities were measured under standard conditions (Kanchana *et al*., 2011).These values are lower than the optimum temperature for the enzyme from *Rhodoccocus equi* and from *Corynebacterium cholesterolicum*, with maximum activity at 47°C and 40°C respectively (Terezinha *et al*., 2000) and are in accordance with the earlier reports for *Streptomyces fradiae* and *Brevibacterium* sp. which produced CHO with optimum activity and stability for 30 min at 50°C and 53°C respectively.

Figure.1 Effect of pH on CHO yield

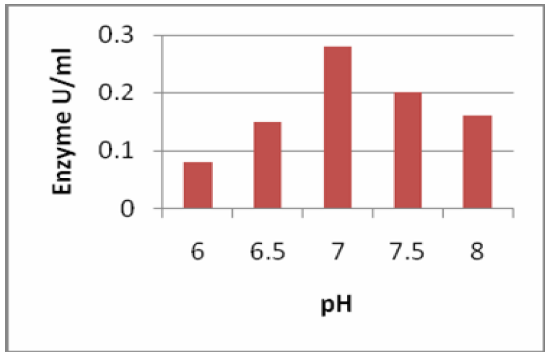
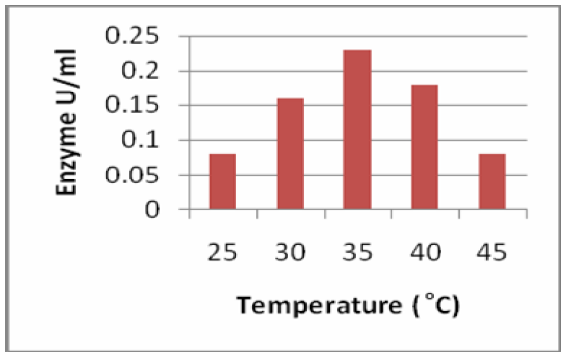


Figure. 2 Effect of temperature on CHO yield



Detection of COP by TLC

It was observed that the specific activity of precipitate was greater than that of crude extract and supernatant, which indicated the purification of the enzyme in precipitate fraction. The percent yield of enzyme after purification was 68%. For the reaction products of enzyme sample the hR_f ($R_f \times 100$) value by TLC was 59.49. Therefore by comparing with standard hR_f chart the product is 20α-Hydroxycholesterol in the enzymatic reaction (Lebovics, 2002). Cholesterol oxidation by the membrane-bound enzyme from *Brevibacterium* sp. resulted in 4-cholesten-3-one as main product (Terezinha *et al*., 2000).

Applications of purified enzyme

The antimicrobial activity assay the purified enzyme showed 21 mm and 19 mm zone of inhibition against *Micrococcus* and *Bacillus subtilis*, while for other organisms the zones were less than 10 mm. The percent mortality rate of *Cryptolaemus montrouzieri* after 15min was 88% showing bioinsecticidal activity. The previous study showed CHO from *Streptomyces* culture showed lethal concentration (LC50) at 20.9µgm/ml (Purcell *et al*., 1993). The effect of enzyme on the serum cholesterol, *in vitro* was also studied and it was found that reduction in serum cholesterol level was 71.56% and 60.52% for concentrated and diluted enzyme samples respectively. LDL incubation with 1 unit/ml of CHO from *Proactinomyces erythropolis, Nocardia* resulted in the formation of CO-LDL. CO-LDL demonstrated a significant reduction (85%) in its UC content which was the result of its conversion to cholestenone in mouse.

Cholesterol oxidase enzyme has a great commercial value in widely employed laboratories routinely for determination of cholesterol in food, serum and other clinical samples (Sojo *et al*., 1997).

Therefore as per need of industry a various type of micro-organisms, which are capable of producing high levels of this enzyme have been isolated. Taking into study the extracellular production, its efficient recovery, pH tolerance and a good purified enzyme activity, CHO produced by *B. lichiniformis* should prove to be an industrially important enzyme. The COP was also determined to be 20α-Hydroxycholesterol. Our preliminary work led to the conclusion that *B. lichiniformis* might be considered as potentially sources of extracellular cholesterol oxidase for clinical and commercial purposes.

Acknowledgments

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