ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 12, December 2013

Isolation and characterization of Cholesterol Oxidase Producing Soil Bacterium and *In-vitro* Assay of Enzyme

Salman Dinakar M[1], Amruthashree BG[1]., Kusuma BC[1]. and Megha S[2].

Lecturer, Department of Biotechnology, SIET, Tumkur, Karnataka, India-572106[1]

U.G. Student, Department of Biotechnology, SIET, Tumkur, Karnataka, India-572106 [1 & 2]

Abstract: Cholesterol oxidase (COD) is an enzyme of great commercial value widely employed by laboratory for the determination of cholesterol in food, serum and other clinical samples.COD producing bacterium was isolated from the soil sample by cultivating in a Luria Bertoni agar medium containing cholesterol and confirmed by colony staining method. Different biochemical tests were performed on isolated bacterium to identify its properties. The extracellular COD activity was assessed in serum at different temperatures and pH conditions using Silver nanoparticles by comparing with COD produced by *Rhodococcus equi*. The activity of COD was optimum at 45^0C and pH 8 in bound form.

Key words: Cholesterol, Cholesterol oxidase, Luria Bertoni agar medium, Colony staining method, Silver nanoparticles and *Rhodococcus equi*

I. INTRODUCTION

Cholesterol oxidase (COD) is an enzyme that converts cholesterol into cholest-4-en-3-one [5]. Many microorganisms such as *Nocardia rhodocorus*, *Arthobacter simplex*, *Pseudomonas sp*, *Rhodococcus sp*, *Streptomyces hygroscopius* and *Brevibacterium* and few other fungal species have been reported to produce COD [7]. COD enzyme has many applications in medicine, agriculture, industry and pharmaceutical purposes. For instance, it can be used for production of diagnostic kits to detect blood cholesterol, biological insecticide and precursors for steroid hormones [5].The enzyme has been used in the determination of serum cholesterol and in the clinical diagnosis of arteriosclerosis and other lipid disorders [2]. It participate in bile acid biosynthesis.COD is used in the production of precursors of hormonal steroids from cholesterol.COD exhibits potent insecticidal activity that is very important and vital for pest control strategies employing transgenic crops [7]. This enzyme belongs to the family of oxido-reductases, specifically those acting on the CH-OH group of donor with oxygen as acceptor. The systematic name of this enzyme class is cholesterol: oxygen oxidoreductase. Other names in common use include cholesterol- O_2 oxidoreductase, 3beta-hydroxy steroid oxidoreductase, and 3beta-hydroxysteroid: oxygen oxidoreductase. This enzyme participates in bile acid biosynthesis. The substrate-binding domain found in some bacterial cholesterol oxidizes is composed of an eight-stranded mixed beta-pleated sheet and six alpha-helices. This domain is positioned over the isoalloxazine ring system of the Flavin adenine dinucleotide (FAD) cofactor bound by the FAD-binding domain and forms the roof of the active site cavity, allowing for catalysis of oxidation and isomerisation of cholesterol to cholest-4-en-3-one. Cholesterol oxidase is a bacterial-specific flavoenzyme that catalyzes the

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 12, December 2013

oxidation and isomerisation of steroids containing a 3beta hydroxyl group and a double bond at the Delta5-6 of the steroid ring system. The enzyme is a member of a large family of Flavin-specific oxidoreductases and is found in two different forms: one where the flavin adenine dinucleotide (FAD) cofactor is covalently linked to the protein and one where the cofactor is non-covalently bound to the protein. These two enzyme forms have been extensively studied in order to gain insight into the mechanism of Flavin-mediated oxidation and the relationship between protein structure and enzyme redox potential. More recently the enzyme has been found to play an important role in bacterial pathogenesis and hence further studies are focused on its potential use for future development of novel antibacterial therapeutic agents. In this review the biochemical, structural, kinetic and mechanistic features of the enzyme are discussed. In the present study effects pH and temperature on the activity of COD from soil microbes were investigated.

II. MATERIALS AND METHODS

A. SCREENING FOR COD PRODUCING BACTERIUM

I]. ***ISOLATION:***

Luria Bertoni agar medium (LB agar medium) was purchased from Himedia (Mumbai, India). Ninety-nine percent of pure cholesterol was purchased from Thomas Baker. Soil waste of oil refinery was collected from NR refinery oil industry, Tumkur, India. Sample was inoculated on to LB agar medium containing 0.5% cholesterol (0.5 g of cholesterol butanol) after appropriate dilution (10^{-2} to 10^{-12}). The plates were incubated at 37^0C for 5 days. The production of COD was identified by using colony staining method [5]

II]. COLONY STAINING METHOD:

Filter papers were dipped into a solution containing 0.5% cholesterol, 1.7% 4-aminoantipyrin; 6% phenol and 3000U/l horseradish peroxidase (HRP) in 100 mm potassium buffer phosphate. Soaked filter paper was placed on grown colonies and then observed for color change of filter paper to red after incubation at 37^0C for 24 hours.

B. CHARACTERIZATION OF BACTERIUM

The isolated bacterium was characterized by colony characteristics on LB agar medium and broth and performed several biochemical test [3]

C. *PREPARATION OF CRUDE ENZYME*

Isolate was cultivated in Luria Bertoni broth supplemented with 0.1% cholesterol (0.1 g in 100 ml of ethanol). The flasks were kept on an orbital shaker for 5 days at room temperature. The broth was filtered using muscline cloth and the culture filtrate thus obtained was treated as crude enzyme.

D. COD QUANTIFICATION BY LOWRY'S METHOD

The protein content of crude enzyme was estimated by Lowry's method using BSA as standard. Zero, 0.2, 0.4, 0.6, 0.8, and 1.0 ml standard BSA solution was pipetted into 6 test tubes. For the 7^{th} and 8^{th} test tube, 0.5 and 1.0ml of crude COD was add. The volumes of all the tubes were made to 1.0ml with distilled water. Then 5ml of alkaline copper reagent was add to each test tube and allowed to stand for 10 min at room temperature and then 0.5ml of 1:1FC reagent was added to each test tube and mix immediately. Then all the test tubes were allowed to stand for 20 min at room temperature in a dark place. Optical density was taken at 660 nm.

E. IN VITRO CHARACTERIZATION OF COD

I]. EFFECT OF pH:

The effect of pH on the activities of the free and bound COD in the Substrate was investigated in the pH range of 4, 5, 5.6,6,7 and 8 at 37°C [4]. DNS solution , 99 % Cholesterol (Thomas baker) and 5 ml of Serum as substrates, crude enzyme

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 12, December 2013

and buffer solutions (0.1M Sodium acetate buffer (pH- 4.0, 5.0, 5.6) and 0.1M Phosphate buffer (pH-6.0, 7.0 and 8.0), silver nanoparticles and three sets of test tubes were used to characterize COD under different pH. One ml of Water was added to the first tubes of each set which serve as blank and 0.5 ml of different pH 4.0, 5.0, 5.6, 6.0, 7.0 and 8.0 buffers to the remaining 6 tubes of each set. Then 0.8 ml of enzyme was added to the each tube except the blank. Then 0.2 ml of 99 % Cholesterol, serum and serum plus silver nanoparticles were added as substrates to the different sets of test tubes followed by incubation at room temperature for 5 minutes. Then 1ml of DNS reagent was added to each tube and incubated in boiling water bath for 5 minutes. Then 5ml of water was added to all the test tubes and read the OD at 540 nm.

II]. SILVERNANOPARTICLE PREPARATION:

Silver nitrate solution was prepared by dissolving 8.2 mg silver nitrate in 50 ml of deionized water. Twenty ml of silver nitrate was added to 20 ml of broth which had COD. The solution was incubated in dark at room temperature for 24 hrs.

III]. EFFECT OF TEMPERATURE:

The thermal stability of free and bound COD in the substrate was investigated in the temperature range 25,35,45,55 and 65°C [4]. DNS solution , 99 % Cholesterol (Thomas baker) and 5 ml of Serum as substrates, crude enzyme and buffer solutions (0.1M Sodium acetate buffer) silver nanoparticles and three sets of test tubes were used to characterize COD under different temperatures (25^0C,35^0C,45^0C,55^0C and 65^0C). To the first tube of each set, 0.2ml of 99 % Cholesterol, serum and serum plus silver nanoparticles were added respectively. Then 1.0 ml of buffer solution was added to them. Then 0.5 ml of buffer was added to the remaining 5 test tubes of each set. All the tubes were incubated at different temperature for 5 minutes (25^0C, 35^0C, 4^{50}C, 55^0C and 65^0C). After incubation 0.8 ml of enzyme to each tube except first one which was used as blank. Further incubated all the tubes for 15 minutes and add 1.0 ml of DNS reagent to each test tube. Again all the test tubes were incubated in boiling water bath for minutes and allowed to cool. Five ml of distilled water was added to all the test tubes before taking the reading at 540 nm.

III. RESULTS

COD producing bacterium was identified by red color formation after colony staining due to the formation of quinoneimine dye (fig-1). Isolated bacterium formed white colonies on LB agar medium. It was a gram negative, non motile bacillus. It showed positive results for various biochemical tests (table-1). The concentration of COD produced from soil bacterium and R.equi in broth was 168 and 192 µmol (fig-2). The enzyme activity of soil bacterium and R.equi was 1.976 and 2.25 µmol/ml/min respectively. The optimum pH for COD was 4.5 in cholesterol and serum. The optimum pH of COD in bound form without centrifugation was 6 and 8 when checked after centrifugation (fig 3). The optimum temperature of enzyme without with silver nanoparticles was 35^0C and 45^0C respectively. The optimum temperature was similar before centrifugation and after centrifugation with silver particles (fig4).

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 12, December 2013

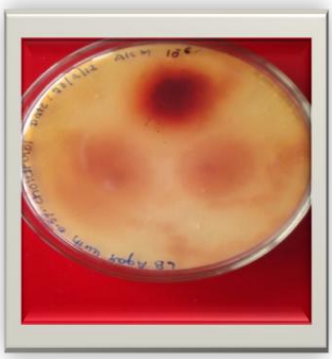

Figure-1: formation of red color on the filter paper after colony staining

Table-1: Biochemical and colony characteristics of isolated bacterium

Sl.no	Biochemical tests	Result
1	Indole	+
2	Methyl red	+
3	Voges	-
4	citrate	+
5	Amylase Production Test	-
6	Urease	+
7	Catalase	+
8	Cellulase	-
9	Gram staining	-

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 12, December 2013

10	Lactose	-
11	Hydrogen production sulfide test	+
	Other characteristics	
12	Negative Staining	bacilli
13	Shape	Rod
14	Motility	Non motile
15	Nutrition	Heterotrophic
16	Colonies	White

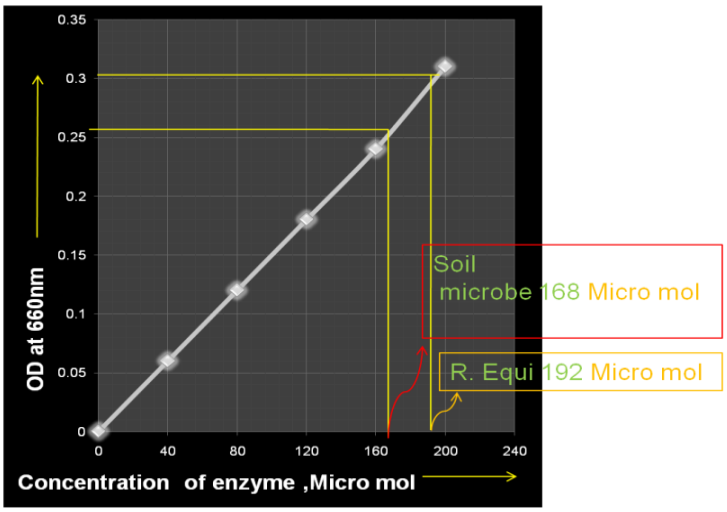

Figure- 2: Concentration of COD of soil bacterium and *R.equi* with standard BSA curve

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 12, December 2013

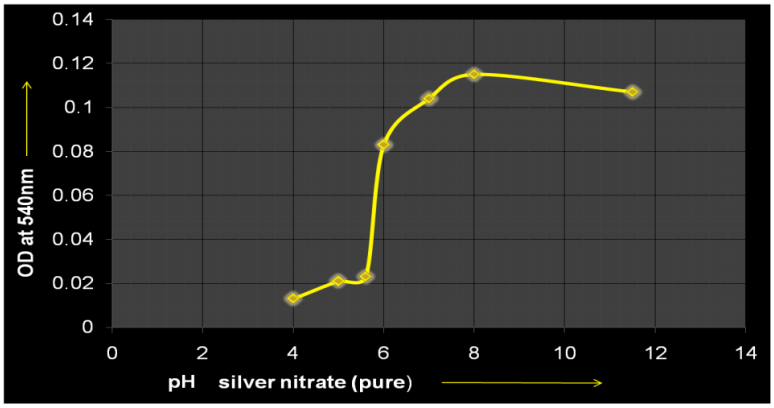

Figure-3: optimum pH of COD in bound form after centrifugation

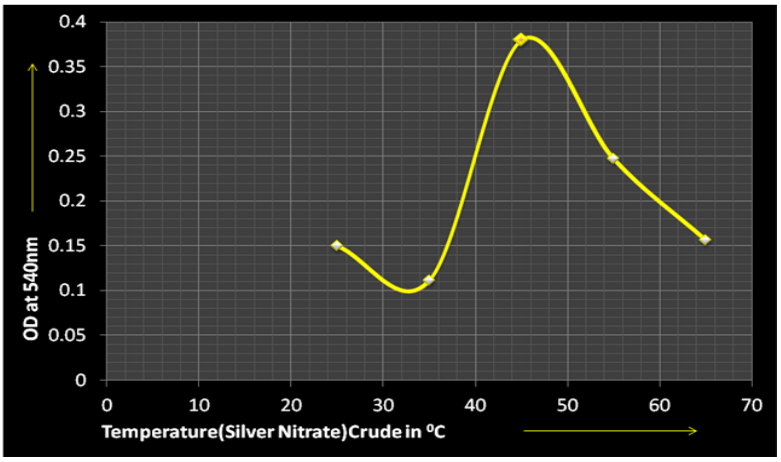

Figure-4: Optimum temperature of COD along with silver nanoparticles without centrifugation.

IV.CONCLUSION

COD producing bacterium isolated from soil collected from oil waste was gram negative bacterium. The enzyme activity was changed under different pH and temperature in free and bound form when different substrates were used. COD activity was increased in bound state when compared to free form. The optimum activity of enzyme was shifted from acidic to alkaline. The optimum pH of COD was 8 in the bound form. Thermal stability of COD increased from 35 to 45^0C in the

ISSN: 2319-8753

International Journal of Innovative Research in Science, Engineering and Technology

(An ISO 3297: 2007 Certified Organization)

Vol. 2, Issue 12, December 2013

bound form when compared to free form. There is no variation in optimum activity of COD with centrifugation and without centrifugation. The enzyme concentration obtained from isolated gram negative bacterium was less compared to *Rhodococcu equi.*

REFERENCES

[1]. Yoshiaki, N., Naok, H., Shin-ichi, T., Mitsuo, Y., Isao, F., Noriaki, H., and Yoshikatsu., "Improvement of thermal stability of Streptomyces cholesterol oxidase by random mutagenesis and a structural interpretation", Protein Engineering, vol.10, pp.231-235, 1997.

[2]. Alice, V., Kimberley, Q., Sandro, G., and Rene, C., "Oxygen Acess to the active site of cholesterol oxidase through a narrow channel is gated by an Arg-Glu pair". J Biol chem., vol. 276(32), pp. 30435-41, 2001.

[3]. Aneja, K.R., "Isolation and characterization of bacteria. Experiments in microbiology plant physiology and biotechnology", New age international private limited, Fourth edition, 2003.

[4]. Gillies, K.K., Gregory, M.C., and Joseph, I., "Examination of cholesterol oxidase attachment to magnetic nanoparticles", Journal of Nanobiotechnology, vol.3, pp1, 2005.

[5]. Hamed, L., Jamshid, R., Kiana, S., Hossein, S., and Mehdi,S., "Extracellular Cholesterol oxidase from *Rhodococcus sp*. Isoation and molecular characterization", Iranian Biomedical Journal, vol.14, pp.49-57, 2010.

[6]. Thirumalai, A., Prabhu, D., and Soniya, M., "Stable silver nanoparticles synthesizing methods andits applications".J.Biosci, Res. Vol. 1(14), pp.259-270,2010.

[7]. Aifa Rodrigues, Delcy Correia, Kanchana, Prachi gawde, Sangeetha Sarkar. "Production and partial characterization of Cholesterol oxidase from *Mucococcus species* isolated from Goa, India", Journal of Applied Biology and Pharmaceutical Technology, vol. 2, pp.393-398, 2011.