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PRODUCTION AND PARTIAL CHARACTERIZATION OF CHOLESTEROL OXIDASE FROM *MICROCOCCUS SP* .ISOLATED FROM GOA, INDIA

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ABSTRACT : Seven bacterial strains that expressed inducible extracellular cholesterol oxidase (COD) were isolated from soil samples of Goa, India. One of the bacterial strains, *Micrococcus* sp. was found to produce the highest level of cholesterol oxidase (3.68 U /ml). The optimum pH for COD was found to be 7.0 and it was stable for at least 30 min at pH 7.0 by exhibiting more than 75% of the initial activity. The optimum temperature was found to be 50°C and the enzyme retained more than 70% of the initial activity after 60 min of heat treatment at 50°C. This appears to be the first report on the production of COD by *Micrococcus* sp. isolated from Goa, India.
Key words: Enzyme; COD; *Micrococcus* sp., optimum pH

INTRODUCTION

Cholesterol oxidase (COD, EC 1.1.3.6) catalyses the oxidation of cholesterol (5-cholesten-3β-ol) to cholestenone (4-cholesten-3-one). An increasing need for specific estimation of steroid in clinical samples has enhanced the importance and demand of COD in the pharmaceutical industry. The enzyme can also be used in the production of precursors of hormonal steroids from cholesterol. COD also exhibits potent insecticidal activity that is very important and vital for pest control strategies employing transgenic crops. For these reasons, a variety of COD producing micro-organisms have been isolated (Doukyu, 2009). Many micro-organisms such as *Nocardia rhodocorus*, *Arthrobacter simplex*, *Pseudomonas* sp, *Rhodococcus* sp, *Coryneform bacterium*, *Actinomyces lavendulae*, *Streptomyces hygroscopius* and *Brevibacterium* and a few other fungal species have been reported to produce COD (Varma and Nene, 2003).

Cholesterol decomposition ability is widespread among micro-organisms that have been explored as free and immobilized cells or as enzyme source in steroid bio transformations. Cholesterol may be completely oxidized by microbial cells to carbon dioxide and water by the action of a complex enzyme system in which cholesterol oxidase is the first enzyme involved. Cell-free enzymes and microbial cells have been investigated for reduction of cholesterol level in foods, for precursor production in manufacturing pharmaceutical steroids from cheap sterols and for clinical assay of serum cholesterol (MacLachlan *et al*., 2000). Microbial cholesterol oxidases have received much attention in recent years, mainly due to its large use in medical practice for determination of free and bound cholesterol (Terezinha *et al*., 1999). In view of the above, the present study is aimed at screening and isolation of micro-organisms from soil samples for the production of enzyme Cholesterol Oxidase and its partial characterization.

MATERIALS AND METHODS

All chemicals used were of analytical grade and obtained from Hi-Media/ Merck/ Qualigens (India) or Sigma Chemical Co. (USA). In all experiments, the measurements were carried out with duplicated parallel cultures.

Primary screening for COD producing bacteria

Soil samples were collected aseptically from mangroves and coastal habitats of Goa, India. They were spread plated on medium containing agar (1.5%) and cholesterol (0.5%) as sole source of carbon and incubated at 37°C for a period up to 4 days after appropriate dilution. The production of COD by bacteria was confirmed by the appearance of clearance zones on the plates.

Preparation of crude enzyme

Isolates wherein COD production was detected qualitatively were raised in production medium (Luria Bertoni broth supplemented with 0.1% cholesterol). The flasks were kept on an orbital shaker for 48-72 h at room temperature. The broth was then centrifuged and the culture filtrate thus obtained was treated as crude enzyme and stored at 4^{o} C until further use.

Secondary screening of novel bacterial isolates producing COD

The above said crude enzyme preparations were used to determine enzyme activity in order to select a novel isolate exhibiting maximum enzyme activity.

Enzyme assay

The activity of the extracellular enzyme was determined according to the method described by Inouye et a*l* (1982). One unit of cholesterol oxidase activity (U) was defined as that which brings about the formation of 1 µg of 4-cholesten-3-one per minute at 37°C. Protein content of the crude enzyme was estimated by Lowry's method (1951) using BSA as standard.

Identification of bacterial strains

The selected bacterial strain was identified based on cellular morphology, staining and biochemical tests.

Characterization of COD

The optimum temperature of the crude enzyme was determined by carrying out the enzyme assay in the temperature range from 25°C to 55°C. Heat tolerance of the enzyme was determined by incubating aliquots of crude enzyme at its optimum temperature for a time period of 1 h and measuring residual activity. Enzyme activity without heat treatment is taken as 100%.

To evaluate the effect of pH on the enzyme activity at its optimum temperature, enzyme assay was carried out using buffer of pH range from 6.0 to 9.0 (for pH 6.0 ,6.5 & 7.0, 0.2M phosphate buffer and for pH 7.5, 8.0,8.5 & 9.0, 0.125M Tris-HCl). The pH stability of the cholesterol oxidase was determined by the pre incubation of the enzyme with buffer of optimum pH for 1 h at 30^{0}C and measuring residual activity. Enzyme activity without incubation is taken as 100%.

RESULTS AND DISCUSSIONS

Screening for COD producing bacterial strains

Around 7 bacterial colonies were isolated on primary screening (Table 1.). These cultures were further screened quantitatively for the final selection of a novel bacterial isolate exhibiting maximum COD activity. Among the bacterial cultures tested, the isolate M2 showed highest zone of clearance with COD activity of 3.68 U /ml (Fig 1, Table 1.).

Identification of the selected bacterial strain

Using morphological and biochemical characteristics, the genus of the selected bacterial (M2) strain was identified as *Micrococcus*. Similar report of cholesterol oxidase production from *Micrococus sp* was reported by Verma *et a*l., (1992). Details of the identification are given in Table 2 a & 2 b.

Table 1. Screening of COD-producing bacterial strains

Isolate	Ratio =clearance zone diameter/colony diameter	Activity (U/ml)
M1	5	3.18
M2	8	3.68
M3	4	3.39
M4	4	2.71
M5	3	1.19
M6	4	1.42
M7	6	1.76

Table 2 a. Colony Morphology of *Micrococcus*

Colony Morphology	Result
Shape	Punctiform
Elevation	Convex
Shape of Edge of Margin	Entire
Surface Texture	Smooth, Shiny

Table 2 b. Biochemical Characteristics of *Micrococcus*

Biochemical characteristics	Results
Gram's Staining	+
Catalase	+
Spores	-
Motility	-
Urea	-
Glucose	-
Lactose	-
Sucrose	-
Starch Hydrolysis	-
Gelatin Hydrolysis	+
Citrate Utilization	+
Nitrate Reduction	-
Oxidase	+
Methyl Red	-
Voges Prokauer Test	-
Indole	-
Hydrogen Sulphide Production	-

Note: + = Positive; - Negative

Characterization of COD

Effect of temperature on enzyme activity and stability

Thermal stability of an enzyme is an important property for industrial applications. The effect of temperature on activity and stability of extracellular COD enzyme from *Micrococcus* strain was assessed under a standard condition. It is seen that optimum temperature was found to be 50°C (Fig 2.A.) and it retained more than 70% activity at 50°C after 60 min of heat treatment (Fig2.B).

Fig.1 Cholesterol agar plate showing clearance zones by COD producing bacteria

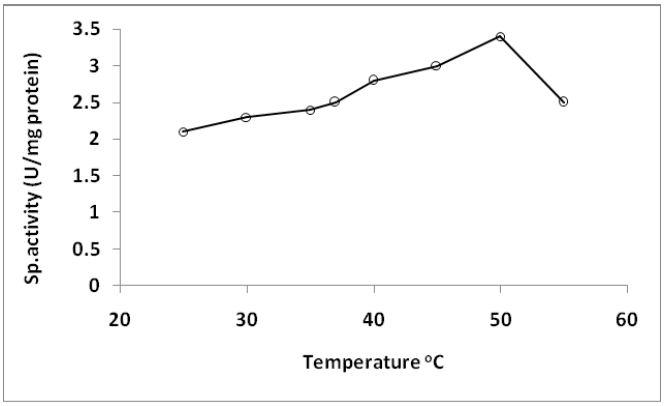

Fig .2.A. Effect of temperature on enzyme activity

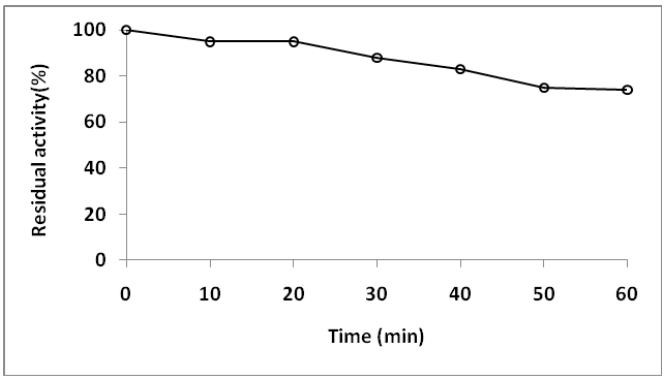

Fig. 2.B. Effect of temperature on enzyme stability

These values are higher than the optimum temperature for the enzyme from *Rhodoccocus equi* and from *Corynebacterium cholesterolicum*, with maximum activity at 47ºC and 40ºC, respectively (Terezinha *et al*., 1999) and are in accordance with the earlier reports for *Streptomyces fradiae* and *Brevibacterium* sp. which produced COD with optimum activity and stability for 30 min at 50°C and 53°C respectively (Tabatabaei *et al*., 2001; Fujishiro *et al.*, 2002).

Effect of pH on enzyme activity and stability

The study of effect of pH on enzyme activity and stability is shown in Fig 3.A. and 3.B. respectively. The enzyme is pH dependent with maximum activity at around 7.0. Enzyme activity was found to be stable for at least 60 min at pH 7.0 by exhibiting more than 75% activity. The optimum pH for cholesterol oxidase from *Micrococcus* sp. was similar to that found in literature for the same enzyme from other micro-organisms (Doukyu, 2009). Usually the optimum pH for the enzyme activity is between 7.0 and 8.0, as can be seen for the enzymes from *Actinomyces lavendulae* mycelium, *Corynebacterium cholesterolicum*, *Streptoverticillium cholesterolicum*, *Rhodococcus equi* and *Streptomyces violascens* (Lashkarian *et al*., 2010).

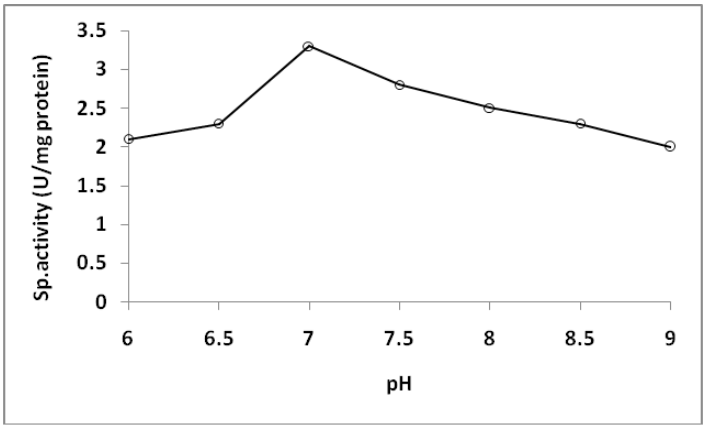

Fig.3.A. Effect of pH on enzyme activity

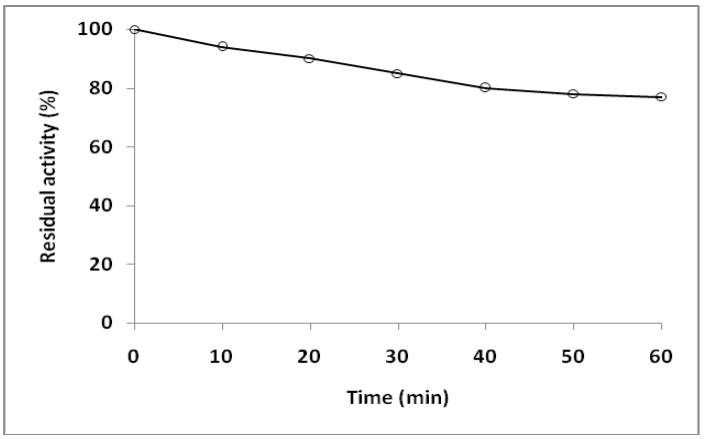

Fig. 3.B. Effect of pH on enzyme stability

CONCLUSION

Cholesterol oxidase is an enzyme of great commercial value widely employed by laboratories routinely devoted to the determination of cholesterol in food, serum and other clinical samples (Sojo et al., 1997). For this reason, a diversity of micro-organisms, which are capable of producing high levels of this enzyme have been isolated. Taking into account the extracellular production, its efficient recovery, pH tolerance and a good thermal stability, COD produced by *Micrococcus* sp. should prove to be an industrially important enzyme. Our preliminary work led to the conclusion that *Micrococcus* sp might be considered as potentially interesting sources of extracellular cholesterol oxidase for clinical and commercial purposes. This appears to be the first report on production of COD by *Micrococcus* sp isolated from Goa, India.

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