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Original Article

PRODUCTION AND OPTIMIZATION OF CHOLESTEROL OXIDASE FROM *RHODOCOCCUS* SPECIES

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ABSTRACT

Objective: Optimization of conditions for cholesterol oxidase production by the microorganism isolated from urban compost and dairy soil samples.

Methods: Isolates were obtained on the basis of their capability of growing on isolation medium A and B and their cholesterol oxidase (CHO) production was estimated. CHO production was optimized by the optimization of temperature, pH, carbon sources, and organic and inorganic nitrogen sources.

Results: 3 isolates out of 22 were found to secrete extracellular CHO as detected by cholesterol oxidase indicator plate A and were designated as cholesterol oxidase producing isolate 1, 2 and 3 (COP 1, COP 2 and COP 3). Results showed that the strain COP 2 belonging to the genus *Rhodococcus* sp. based on morphological, cultural and biochemical characteristics recorded highest cholesterol oxidase activity. Optimum temperature and pH for CHO activity were found to be 35 °C and 7.5 respectively. Steroidal substrate cholesterol produced a significant increase in CHO level (0.502 IU/ml). Organic and inorganic nitrogen sources were supplemented in combinations leads to increase in CHO production as compared to individual components. $(NH_4)2HPO_4$ and yeast extract supported the highest enzyme production (0.574 IU/ml).

Conclusion: The isolate COP 2 produced significant levels of cholesterol oxidase extracellularly in optimized medium as compared to cell bound CHO, and can be easily produced on an industrial scale.

Keywords: Cholesterol oxidase, *Rhodococcus* sp, Biochemical, Optimization.

INTRODUCTION

Cholesterol oxidase (COX, EC 1.1.3.6) a monomeric bi-functional FAD-containing (flavo enzyme) enzyme belongs to the family of oxidoreductases, specifically those acting on the CH-OH group of donors with oxygen as acceptor. Cholesterol oxidase (CHO) enzyme catalyzes the oxidation of cholesterol and converts 5-cholesten-3-ol into 4-cholesten-3-one [1]. The systematic name of this enzyme class is cholesterol: oxygen oxidoreductases and other common names in use are 3β-hydroxy steroid oxidoreductases, and 3β-hydroxy steroid: oxygen oxidoreductases. CHO was first isolated and characterized from *Rhodococcus erythropolis* and has many applications in agriculture, medicinal industry and pharmaceutical sectors. For instance, it can be used for the production of diagnostic kits to detect blood cholesterol, biological insecticide and precursors for steroid hormones [2, 3]. Cholesterol oxidase enzyme is simple, specific, and highly sensitive; its use has become widespread in the determination of serum cholesterol that has direct implications in atherosclerosis, coronary heart disease and other lipid disorders, and for determining the risk of heart attack and thrombosis. This enzyme shows potent insecticidal activity has been used to track cell cholesterol and has also been found to be a potent parricide [4, 5]. It has also been developed as a pest control in the agricultural industry especially in transgenic crops [4]. Biochemical and structural, studies of enzyme revealed the involvement of an enzyme in interaction with lipid bilayer [6, 7]. With only the exception of glucose oxidase, CHO is the most widely used enzyme in clinical laboratories. Many bacteria can produce this enzyme including members of the genera *Arthrobacter, Brevibacterium, Pseudomonas, Nocardia, Rhodococcus, Streptomyces, Corynebacterium* and *Shizophylum* [8]. 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 major importance [9]. Because of the commercial value of cholesterol oxidase there is wider interest in producing this enzyme from microbial cells. Thus, there is need to isolate newer bacterial cultures to produce cholesterol oxidase optimally under physiological conditions and to study the important biochemical properties of this enzyme.

Therefore, keeping in mind the importance of cholesterol oxidase enzyme the present study was planned to isolate CHO enzyme producing bacteria from urban compost and dairy soil sample, to determine the type (extracellular or cell bound) of CHO enzyme produced by the isolates and to optimize cholesterol oxidase production by optimizing the cultural conditions.

MATERIALS AND METHODS

Collection of soil samples

26 different urban compost and dairy soil samples were collected from Himachal Pradesh, Jammu & Kashmir and Punjab in a sterile polythene bags and brought at the laboratory for further studies.

Isolation of bacteria

For the isolation of microorganisms, 1 g of soil samples from different places were suspended in 100 ml of distilled water and was shook vigorously for 30 mins by placing it in an incubator shaker. A volume of 100 µl of supernatant was inoculated onto the medium "A" (contained (g/l): agar, 3.0%; K_2HPO_4, 0.25; NH_4NO_3, 17; $MgSO_4$. H_2O 0.25 % ; $FeSO_4$, 0.001; NaCl, 0.005; cholesterol, 0.1 % and Tween 80, 0.5 ml) at pH 7. The inoculated plates were placed in the incubator for 7-10 days at 30 ˚C. After incubation, plates were observed for colony characteristics. Larger fast growing colonies were sub cultured in secondary medium containing cholesterol as the only source of carbon as well as yeast extract [9-10].

Screening of CHO producing bacteria

CHO producing colonies were selected on cholesterol oxidase 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 of peroxidase to one l of agar medium. Bacterial colonies were cultured on these plates and incubated at 30 ˚C. Cholesterol penetrates into bacterial cells where it can be converted into hydrogen peroxide by cholesterol oxidase. Reagents that exist in the medium react with hydrogen peroxide (H_2O_2) to form azo compound which turns the color of medium into intense brown color [11-13].

Identification of isolates

CHO producing isolates were identified by their morphological, cultural and biochemical characteristics by standard methods using Bergey's manual of systematic bacteriology [14].

Assessment of enzyme activity

CHO activity of isolated bacteria was detected by hydrogen peroxide generated during cholesterol oxidation process and was measured [15]. In this reaction, hydrogen peroxide was coupled with 4-aminoantipyrine and phenol by peroxides to produce quinoneimine dye with maximum absorption in 500 nm. Dissolved cholesterol in non-ionic detergent triton X-100 was used as the substrate of the reaction. The reaction mixture was consisted of 3 µmol of cholesterol in 1.0 ml of 1% triton X-100, 300 µmol of phosphate buffer (disodium phosphate-monopotassium phosphate), pH 7.0, 0.1 ml of enzyme solution, 1.2 µmol of 4-aminoantipyrine, 21 µmol of phenol and 20 U of horseradish peroxidase (HRP) in a final volume of 3 ml. Reaction was performed at 37 ˚C for 10 min with shaking during incubation period. This reaction was terminated by heating at 100 ˚C for 3 min. One unit of enzyme was defined as the amount of enzyme that forms 1 µmol of H_2O_2 per minute at 37 ˚C.

$$\text{Cholesterol} + O_2 \longrightarrow \text{4-cholesten-3-one} + H_2O_2$$

$$2H_2O_2 + \text{4 - Aminoantipyrene} + \text{Phenol} \longrightarrow \text{Quinoneimine dye} + 4H_2O$$

To determine the cell bound and extracellular CHO activity, bacterial cultures were centrifuged in 10,000 x g for 5 min and supernatant was used as an extracellular enzyme source. In order to find the cell bound CHO, cellular pellet was washed twice with 0.1 M phosphate buffer (pH 7.2). After resuspending cellular pellet in the same buffer containing 0.1% of triton X-100, the supernatant was incubated at 30 ˚C for 30 mins, centrifuged in 10,000 x g and used for detection of cell bound CHO [9].

Effect of pH on CHO activity

To evaluate the optimum pH for the enzyme activity, the enzyme solution was incubated from pH 4.5 to 9 for 24 h and enzyme activity were measured under standard conditions. Acetate buffer was used for pH 4.5 to 5.5, phosphate buffer for pH 6 to 7 and Tris-HCl for pH 8 to 9 [8, 15].

Effect of temperature on CHO activity

In order to determine the effect of temperature on enzyme activity Sasaki's method was performed on an enzyme solution under standard conditions except for reaction temperature. The enzyme solution was incubated at 20, 25, 30, 35, 40, 45 and 50 ˚C for 24 h [8, 15].

Effect of carbon sources

Carbon sources (0.2% w/v) glucose, steroidal compound (cholesterol) and non-steroidal hydrophobic substrate (n-hexanoate) were supplemented as individual components to the production media to check the effect of these sources on enzyme production [16].

Effect of nitrogen sources

Combination of inorganic (0.2% w/v) and organic (0.5% w/v) nitrogen sources as well as an individual organic component (yeast extract) and inorganic component $(NH_4)_2HPO_4$ (Diammonium hydrogen phosphate) were checked for CHO production [16].

RESULTS AND DISCUSSION

22 bacterial isolates were obtained from 26 urban compost and dairy site soil samples collected from various sites based on their capability to grow on an isolation medium A containing cholesterol as the sole carbon source. Among them, only 3 isolates were found to cholestrol oxidase producing (COP) and designated as COP 1, COP 2 and COP 3. Then, these strains were examined for CHO production and results showed that the isolated strain COP 2 had the highest ability for CHO production (1.162 U/ml) in comparison with the other isolates in extracellular form as compared to cell bound (Table-1). Therefore, strain COP 2 was chosen for further studies. The cells of COP 2 were gram positive, irregular rods but eventually presented as coccoid forms as growth continued changed and results of biochemical characteristics are shown in table 2. From these observations, it is clear that isolate COP 2 belongs to *Rhodococcus* species. In previous studies also a high amount of CHO enzyme produced by *Rhodococcus* is extracellular form and only a low amount of CHO is membrane bound or intracellular type [17].

Table 1: Extracellular and cell bound CHO activity of isolates

S. No.	Isolate	Extracellular activity (U/ml)	Cell bound activity (U/ml)
1.	COP 1	0.786±0.08	0.452±0.05
2.	COP 2	1.162±0.09	0.964±0.05
3.	COP 3	0.926±0.04	0.654±0.03

Values are means±SD of three replicates (n=3)

Table 2: Biochemical properties of isolate COP 2

Test	Result	Test	Result
Gram reaction	Positive	H_2S production test	Variable
Catalase	Positive	Lactose fermentation	Negative
Haemolysis	Negative	Sucrose fermentation	Negative
Oxidase	Positive	Maltose fermentation	Negative
Blood agar culture	Mucoid colonies	Xylose fermentation	Negative
Indole production	Negative	Glucose fermentation	Negative
Methyl Red test	Negative	Mannitol fermentation	Negative
Vogus Proskaur test	Negative	Motility	Negative
Nitrate reduction test	Negative	Endospore	Absent
Urease production test	Negative	Gelatin hydrolysis	Negative

Effect of temperature

Enzyme activity was measured under standard conditions except for reaction temperature. Optimum CHO activity was seen at 35 ˚C from *Rhodococcus* sp. (fig. 1). Results of the present study were compatible with an earlier study conducted by Kumari and Kanwar [18]. Temperature 35 ˚C was found to be optimum for *Bacillus licheniformis* [19] and the optimum activity and stability of CHO from *Streptomyces fradiae* and *Brevibacterium* sp. were reported at 50 ˚C and 53 °C for 30 min. respectively [9, 20]. Optimum temperature for maximum enzyme activity from *Rhodoccocus equi* and from *Corynebacterium cholesterolicum*, was 47 ˚C and 40 ˚C respectively [21] and the optimum enzyme production in the basal medium was 30 °C [16].

Effect of pH

The optimum pH for production of CHO enzyme was recorded to be pH 7.5 as maximum CHO production (0.498 U/ml) was recorded at this pH (fig. 2). Results of the present study were compatible with

previous studies in which pH 7 was noted optimum for enzyme production [19, 20, 16]. The optimum pH for CHO production in bound form without centrifugation was 6 and after centrifugation were 6 and 8 respectively [22].

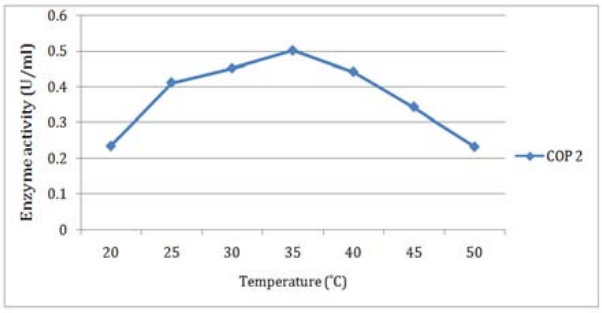

Fig. 1: Effect of incubation temperature on enzyme production

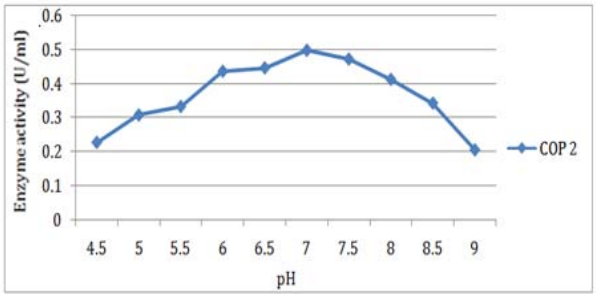

Fig. 2: Effect of pH on enzyme production

Effect of carbon sources

CHO was produced with all the carbon sources tested; however, steroidal substrate cholesterol (0.502U/ml) only showed a significant increase in CHO levels (fig. 3). Results of the present investigation were similar to the results of Ahmad and Goswami [16] who also observed steroidal substrate to increase levels of CHO production. *Rhodococcus erythropolis,* under appropriate conditions, can show a significant CHO activity when grown in a mineral medium containing cholesterol as a sole carbon source [23].

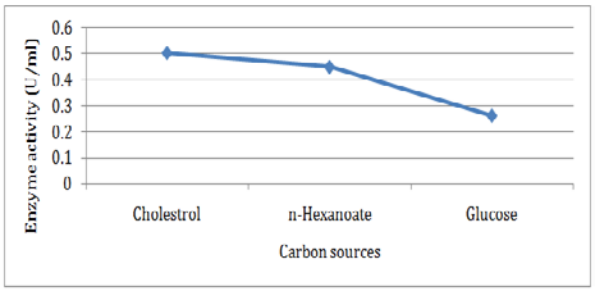

Fig. 3: Effect of carbon sources on enzyme production

Effect of nitrogen sources

Different combinations of inorganic and organic nitrogen sources were supplemented to CHO production and it was observed that yeast extract with the combination of $(NH_4)2HPO_4$ give maximum CHO production (0.574I U/ml). Organic (yeast extract) and inorganic (DAPH) nitrogen sources when supplemented as individual components to the production media leads to decrease in enzyme production (0.286 and 0.25 U/ml respectively) (fig. 4). Earlier study also reported that the combination of $(NH_4)2HPO_4$ and yeast extract leads to the highest enzyme production (0.353 U/ml) as compared to individual organic and inorganic components [16].

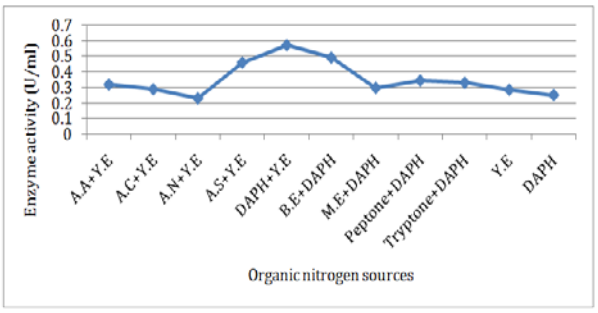

Fig. 4: Effect of inorganic and organic nitrogen sources on enzyme production

A. A (ammonium acetate), A. C(ammonium chloride), A. N (ammonium nitrate), A. S (ammonium sulphate), DAHP (di ammonium hydrogen phosphate), Y. E(yeast extract), B. E (beef extract), M. E (malt extract)

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. *Rhodococcus* species which are capable of producing cholesterol oxidase have been isolated from urban compost and dairy soil samples. Bacterial isolate COP 2 showed maximum enzyme production and production was increased by optimization of cultural conditions *viz.,* temperature, pH, carbon and nitrogen sources. Our work led to the conclusion that isolated *Rhodococcus* sp. might be considered as potentially interesting source of cholesterol oxidase for commercial purpose.

CONFLICT OF INTERESTS

Declared None

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