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Isolation and identification of extracellular cholesterol oxidase producing microorganisms from various sources

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Abstract

Cholesterol oxidase (EC1.1.3.6; CHO) is an enzyme, which catalyzes the oxidation of cholesterol and converts 5-cholesten-3β-ol into 4- cholesten-3-one. The objective of this study is to isolate extracellular cholesterol oxidase (CHO) producing microorganisms to obtain an abundant source of cholesterol oxidase (CHO) for industrial and medicinal needs. Cholesterol oxidase producing bacteria were isolated from waste of regional oil mill, soil and compost. Twenty-five isolates are tested for cholesterol oxidase activity by screening method. As the result of the screening, CHO producer strain was isolated and identified as Streptomyces sp., Arthrobacter sp. and Aspergillus sp. CHO activity of isolates were measured by spectroscopic assay method. Purification and characterization of CHO enzyme is under way.

Key-Words: Cholesterol oxidase, 4-cholesten-3-one, Horseradish peroxidase

Introduction

Cholesterol oxidase (CHO) is an enzyme which catalyzes the oxidation of Cholesterol and converts 5-Cholesten-3β- ol into 4-Cholesten 3-one[1]. Many bacteria can produce this enzyme including members of the genera *Arthrobacter*, *Brevibacterium, Pseudomonas, Nocardia, Rhodococcus, Streptomyces, Corynebacterium and Shizophylum* [2, 3.] Cholesterol oxidase enzyme has many applications in medicine [4], agriculture, and pharmaceutical [5] and so on. For instance, it can be used for production of diagnostic kits to detect blood Cholesterol [6], biological insecticide [7] and precursors for steroid hormones [8]. This enzyme can be secreted from a bacterium in 3 types including intracellular, extracellular and membrane-bound. Due to wide spectrum applications of Cholesterol oxidase, screening and isolation of bacterial strains producing extracellular form of Cholesterol oxidase is of great importance [9]. Many microorganisms have been determined which produced extracellular Cholesterol oxidase including *Rhodococcus equi, Rhodococcus erythropolis*[10], *Streptomyces sp, Arthrobacter simplex, Brevibacterium sterolicum, Strerptomyces lividanse, Schizophylum commune, Micrococcus sp* etc [11, 12,13].

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Enzymatic properties of cholesterol oxidase from *Rhodococcus* strains (some of which named formerly as *Nocardia*) are particularly suitable for use in the analytical determination of cholesterol, in which the hydrogen peroxide formed is used in a chromogenic reaction catalyzed by horseradish peroxidase. In the present study, *Streptomyces sp*., *Arthrobacter sp*. and *Aspergillus sp* was isolated from waste of regional oil mill, soil and compost. The type of CHO enzyme produced by isolates was determined using an enzyme activity assay on supernatant of culture medium.

Material and Methods

Isolation of microorganisms

Cholesterol oxidase producing microorganisms were isolated by following procedure. 1 g of various samples was suspended in 100 ml of distilled water. The suspension was vigorously shaken for 30 min. A volume of 100 µl of supernatant was inoculated in medium (medium A) containing cholesterol as the sole carbon source. A medium 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. The pH of medium was adjusted to 7.0. The inoculated plates were incubated at 30°C for 7-12 days. After incubation period was completed, abscission colonies were appeared on the plate surface. For fast growing and generating, larger colonies were sub cultured in secondary medium (medium B) containing cholesterol as the only source of carbon as

well as yeast extract [9, 14] . This medium contained yeast extract, 0.3 g; $(NH_4)_2HPO_4$, 0.1 g; cholesterol, 0.15; Tween 80, 0.05 ml; pH – 7; agar, 3.0 % and distilled water, 100 ml. Each colony on medium A was cultured in medium B and incubated at 30°C for 24 h. Then, larger colonies generated on medium B were used for further identification. Identification of isolated microorganisms was performed by microbiological examination and biochemical tests [9,11] .

Screening of CHO producing organism

CHO is able to convert Cholesterol into Cholest-4-en-3-one and hydrogen peroxide. 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, 0.1g o-dianisidine and 1000 Units of peroxidase to 1 liter 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 [15, 17, 18].

Identification of isolates

Identification of isolates was carried out by studying their morphological, cultural, biochemical and molecular characteristics by standard method. Bacterial and fungal isolates were identified by using Bergey's manual of systematic bacteriology, 2nd edition and llustrated genera of imperfect fungi, 4th edition by Barnet &Hunter respectively.

Determination of CHO activity

CHO activity was measured by centrifuge the medium at 10,000 rpm for 20 min. at 4°C by modified method based on the study of Allain et.al [4, 12]. In this reaction, hydrogen peroxide generated during Cholesterol oxidation process was coupled with 4-aminoantipyrine and phenol by peroxidase to produce quinoneimine dye with maximum absorption in 500nm. The reaction mixture was consisted of 1mM 4-aminoantipyrine , 5 mM phenol, 5 U/ml of horseradish peroxidase and sodium phosphate buffer (20 mM, pH 7.0). 50 µL of 6 g/L Cholesterol dissolved in dimethyl formamide containing 5% (v/v) Triton X-100 was added to 1ml of reaction mixture, Which was then pre incubated for 3 min. at 30°C. The reaction was initiated by adding 20 µL of enzyme sample and was continued for 5 min at 30°C. The assay mixture was boiled in a water bath for 2 min. to stop the reaction, and then place in an ice bath for 2 min. Absorbance of the reaction solution was monitored at 500 nm. (Systronic 2203, Japan). The assay mixture containing inactivated enzyme was used as the blank. One unite of CHO activity was defined as the amount of enzyme that converts 1µmol of cholesterol in to 4-cholesten - 3 - one per minute at 30°C.

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

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

Results and Discussion

A 25 samples each from three different sources (waste of regional oil mill, compost and soil) were collected. 20 isolates were obtained from these samples on their capability on growing on isolation medium A containing cholesterol as the sole carbon source. Among them 2 isolates from each sample were found to secrete extracellular CHO were detected by cholesterol oxidase indicator plate. The result of microscopic observation and growth characteristics of these isolates is shown in table-1**.** Two isolates of waste from regional oil mill RO-3 & RO-10 were identified as *Arthrobacter sp.* and *Streptomyces sp.* respectively from their gram staining, morphological, biochemical and colony characteristics. The results of microbiological and biochemical properties of RO-3 is shown in table-2. The cells of RO-3 were irregular rods but eventually presented as coccoid forms as growth continued. The result of growth on medium B is shown in figure-1. Cholesterol oxidase from *Streptomyces hygroscopicus* and the recombinant enzyme from *Brevibacterium sterolicum* expressed in *Escherichia coli* have been characterized for their chemical, physical, and biochemical properties by Giovanni Gadda et al. [16].Isolates C-7 and C-4 from compost was identified as *Streptomyces sp.* from their gram staining, morphological and colony characteristics. The results of screening for CHO producing organism on cholesterol indicator plate was shown in figure-2**.** Two fungal isolates from soil S-2 and S-6 were identified as *Aspergillus sp.* CHO activity of isolates was performed by modified method based on the study of Allain et.al. [4, 12] . Among the six isolates RO-10 shows the highest activity of 1.6 U/ml. Result of activity is shown in table-3.

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. A diversity of micro-organisms, which are capable of producing high levels of this enzyme have been isolated. Our preliminary work led to the conclusion that Streptomyces sp. might be considered as potentially interesting source of extra cellular cholesterol oxidase for clinical and commercial purposes.

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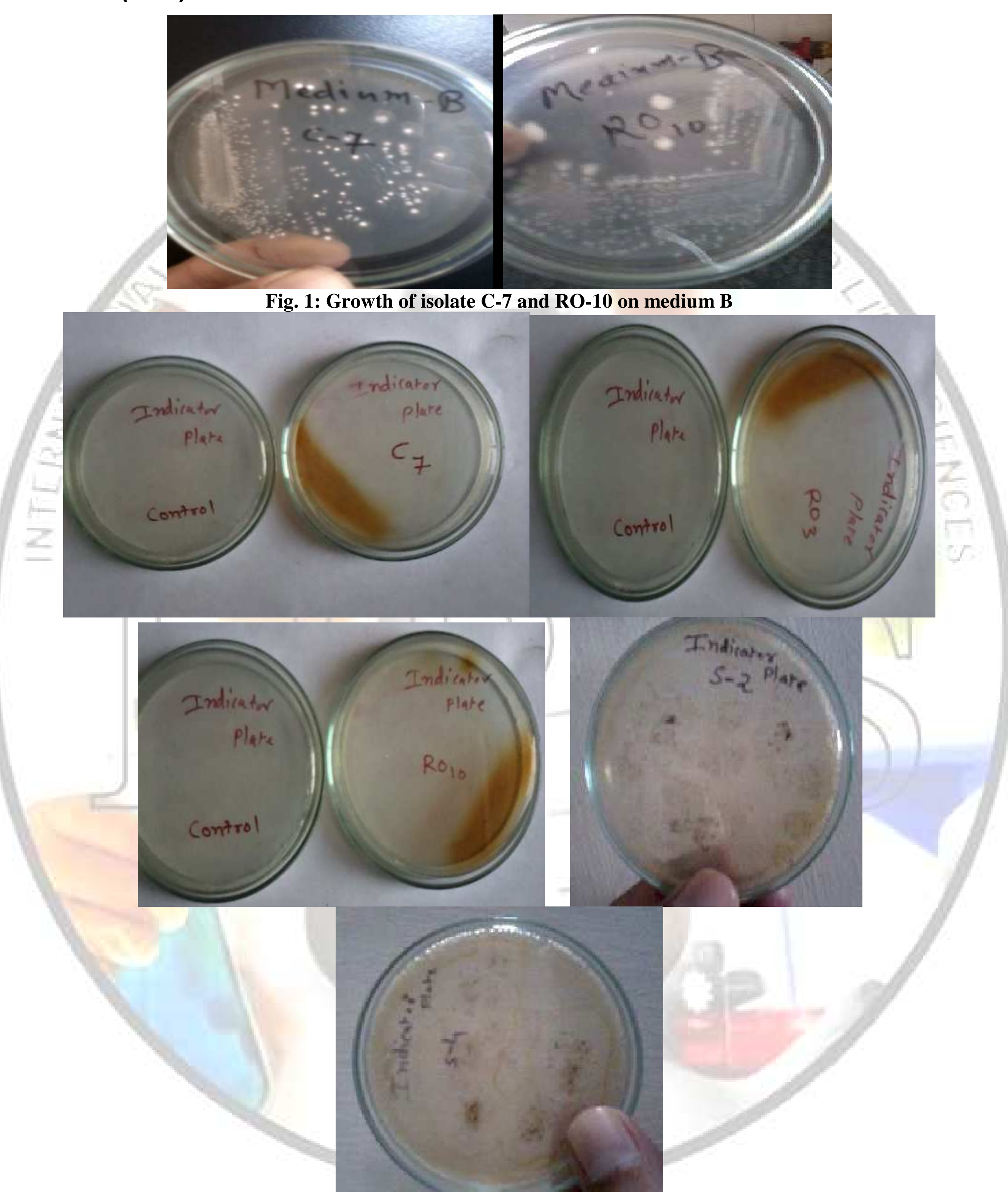

Fig. 1: Growth of isolate C-7 and RO-10 on medium B

Fig. 2: Growth on Cholesterol oxidase indicator plates

Table 1: Morphological and colonial characteristics of isolates from waste of regional oil mill,compost and soil

Sample	Isolate No.	Medium	Colony / growth characteristics	Morphology	Figure
waste of regional oil mill	RO-3	Medium B	Small, creamy white, elevated colony	Gram positive, short coco bacilli rods.	
	RO-10		White, cottony, raised and chalky colony	Gram positive, filamentous organism	
Compost	C-4		Off white, cottony, raised and dry colony	Gram positive, filamentous organism	
	C-7		White, cottony, raised and chalky colony	Gram positive, filamentous organism	
Soil	S-2		Green mycelia colony	Conidiophore with septate mycelium	
	S-3		Brown mycelia colony	Conidiophore with septate mycelium	

Table 2: Biochemical and microbiological properties of isolate RO-3.

Test	Result	Test	Result
Catalase	Positive	Motility	Non motile
Gelatinase	Positive	Endospore	absent
Indole production	Negative	Lactose	Acid & Gas production
MR	Negative	Sucrose	Acid & Gas production
VP	Negative	Maltose	Acid & Gas production
Citrate utilization	Positive	Mannitol	Acid & Gas production
Nitrate reduction	Positive	Xylose	Acid & Gas production
Urease	Negative	Glucose	Acid & Gas production
H_2S production	Negative		

Table 3: Extracellular CHO activity of isolates

Isolate	Activity (Units/ ml)
RO-3	0.91
RO-10	1.6
C-4	1.1
C-7	1.26
S-2	1.03
S-3	0.97