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Isolation and extraction of Cholesterol Oxidase producers from the soil

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Abstract: *Cholesterol oxidase (CHO) is an enzyme, which catalyzes the oxidation of cholesterol and converts 5- cholesten-3_-ol into 4- cholesten-3-one. The objective of this study is to isolate extracellular cholesterol oxidase (CHO) producing micro-organisms from the soil sample and to obtain an abundant source of cholesterol oxidase (CHO) for industrial and medicinal needs. Cholesterol oxidase producing bacteria were isolated from soil samples from oil spillage areas. The isolate was tested for cholesterol oxidase activity by screening method. As the result of the screening, CHO producer strain was isolated and identified as Staphylococcus and Micrococcus species .The CHO enzyme was also further purified and its activity was measured by calorimetric assay method which was found to be 0.15 units/ml.*
Key-Words: *Cholesterol oxidase, 4-cholesten-3-one, Horseradish Peroxidase*

I. Introduction;

Cholesterol oxidase (CHO) is an enzyme which catalyzes the oxidation of Cholesterol and converts 5-Cholesten-3_- ol into 4-Cholesten 3-one1. Many bacteria can produce this enzyme including members of the genera Arthrobacter, Brevibacterium, Pseudomonas, Nocardia, Rhodococcus,Streptomyces,Corynebacterium and Shizophylum . Cholesterol oxidase enzyme has many applications in medicine agriculture, and pharmaceutical and so on. For instance, it can be used for production of diagnostic kits to detect blood Cholesterol , biological insecticide and precursors for steroid hormones . This enzyme can be secreted from a bacterium in 3 forms 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 . Many microorganisms have been determined which produce extracellular Cholesterol oxidase including Rhodococcus erythropolis, Streptomyces sp, Arthrobacter simplex, Brevibacterium sterolicum, Strerptomyces lividanse, Schizophylum commune, Micrococcus sp etc .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 chromomeric reaction catalyzed by horseradish peroxidase. In the present study, Staphylococcus sp., and Micrococcus sp was isolated from soil sample obtained from oil spillage area . The type of CHO enzyme produced by isolates was determined using an enzyme activity assay on supernatant of culture medium.

II. Materials and Methods.

2.1 Collection of soil samples:
Materials : zip lock bag, spatula.

The soil samples for the required cholesterol oxidase producing organisms was collected from the oil tanker station at Wadala.

2.2 Screening and enrichment of cholesterol oxidase producing organisms:
Materials: Soil sample 0.5 gm and Cholesterol broth
Principle of the cholesterol oxidase medium: CHO enzyme is able to convert cholesterol into cholest-4-en-3-one and hydrogen peroxide. 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
Method : Screening of cholesterol oxidase producing organisms was carried out by adding 0.5 grams of the soil sample in 100 ml Erlenmeyer flask containing 50 ml of cholesterol broth kept under shaker conditions at R.T for 24 hr. .

2.3 Isolation of Cholesterol oxidase producers from the enrichment medium
Materials: Cholesterol agar plates.
Method: The soil sample was enriched in cholesterol broth. Isolation was carried out by culturing and cholesterol agar plates The plates were incubated at R.T for 24-48 hours

2.4 Identification of the isolates

Identification of the isolates was carried out using Bergey's Manual of Determinative Bacteriology.

Materials: 1% Xylose , 1% Sucrose , 1% Inulin , 1% Glucose , 1% Mannitol , 1% Maltose , 1% Urease, Lysine decarboxylase, ornithine, Triple sugar iron agar slant (TSI), Tryptone water, Oxidase test disc, Methylene blue, Voges -Proskauer, Simmons citrate slant ,Catalase, Hydrogen peroxide, Kovac's Reagent.

Method: A loopful of the culture was Inoculated into the bio chemicals and incubated at 37^{o}C for 24 hours.. In case of oxidase disc place a loopful of the culture on the disc and observe the results in 2-3 minutes.

2.5 Enzyme assay

Extraction of the enzyme:

Materials: 24 hours old culture of cholesterol oxidase producer, 50% Acetone.

Method: For the extracellular enzyme extraction, 10ml of the 24 hour old culture was taken into a sterile centrifuge tube and allowed to spin at 5000 rpm for 20 minutes at 4^{o}C. The pellet was discarded and the supernatant was used to perform the assay. (Bholay et al) (2013) (Lashkarian H 2009) (Hameed. M 2010)

Cholesterol oxidase assay

Materials: Reagent A (Buffer): 50mM Potassium Phosphate Buffer, pH 7.5 at 25°C (Prepared 100 ml in distilled water using KH_2PO4. Adjusted to pH 7.5 with 1 M KOH); **Reagent B (ODA):** 1% (w/v) o-Dianisidine Solution (Prepared freshly in 5 ml distilled water using o- Dianisidine Dihydrochloride, Himedia); **Reagent C (Cholesterol):** 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); **Reagent D (POD):** Peroxidase Enzyme Solution (A solution was prepared immediately before use, using Horseradish Peroxidase, containing 100 units/ml in distilled water i.e. 0.1mg in 3.3ml); **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) (Bholay et al 2013) (Lashkarian H 2009)

Method:

A reaction cocktail was prepared by pipetting (in milliliters) the following reagents into a conical flask: Reagent A (Buffer) 40.0ml, Reagent B (ODA) 0.5 ml. 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. (Bholay et al 2013)

2.6 Purification of the Cholesterol oxidase enzyme

Materials: Cholesterol oxidase producing organism isolated from the soil sample, Cholesterol broth 50 ml, phosphate buffer pH 7.6, Dialysis bag (length 6 cm), thread, EDTA.

Method: The CHO producing organism isolated from the soil sample was grown in the cholesterol broth at stationary conditions at room temperature for 24 hours.10 ml of the 24 hours old culture was taken into a sterile centrifuge tube and was spined at 5000 rpm at 4^{o} C for 5-7 minutes ,the pellet was discarded and the supernatant was added into a treated dialysis bag and immersed into phosphate buffer pH 7.6 for 48 hours until sufficient turbidity was observed into the buffer solution indicating the purification to be occurred .

III. Results and Discussions

3.1 Isolation of Cholesterol oxidase (CHO) producing organism from the soil sample

The soil sample Oil spilled obtained from Wadala area gave isolates on incubation at RT for 48 hours, converting the cholesterol in the medium to cholest-4-en-3-one showing intense brown colour.

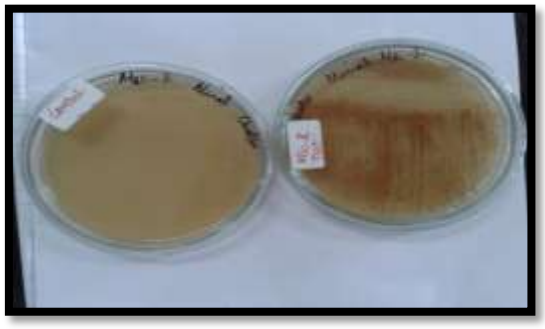


FIG 3.1: Isolated colonies of cholesterol oxidase producing organism

3.2 Colony Characteristics:

The Colony characteristics of the isolates was noted and it was found that the isolate obtained from oil spillage area was found to be gram positive cocci in tetrads.

3.3 Identification using Bergey's Manual of Bacteriology:

The routine Biochemical tests were performed for the isolates which were suspected to belong to the genus Staphylococcus and Micrococcus , the oil tanker area isolate was oxidase positive with bright blue colour change after 5 minutes and did not ferment any of the sugars for the TSI slant .Thus the two isolates identified were found to belong to the genus Staphylococcus and Micrococcus.

3.5 Cholesterol Assay

Reagents	Test		Blank	Control
	SET 1	SET 2		
Reaction cocktail	2.70	2.70	2.70	2.70
Reagent (C)	0.10	0.10	0.10	0.10
Reagent (POD)	0.10	0.10	0.10	0.10
Mixed by Inversion & equilibrated to 25 ° C monitored the A_{500} until constant reading				
INITIAL				
24 hrs	0.07	0.07	0.00	0.07
72 hrs	0.03	0.03	0.00	0.01
96 hrs	0.07	0.07	0.00	0.11
Reagent (A)	-	-	0.10	-
Reagent (E)	0.10	0.10	-	-
Supernant of un inoculated control	-	-	-	0.10
FINAL				
24 hrs	0.11	0.12	0.00	0.07
72 hrs	0.08	0.07	0.00	0.01
96 hrs	0.15	0.15	0.00	0.11

TABLE 3.1: Cholesterol oxidase assay

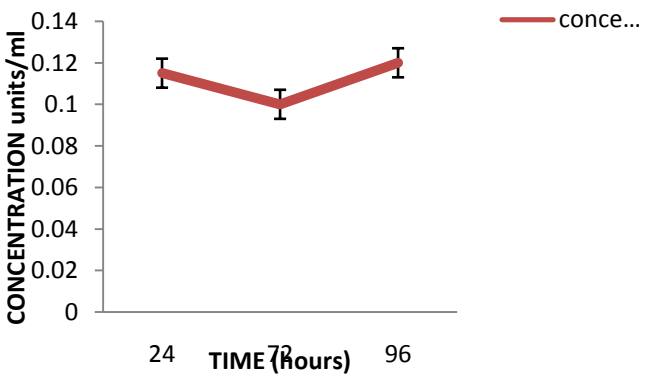
Formulae for units/ml CHO and units/mg protein

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

Calculation Table

	24 hours	72 hours	96 hours
SET 1	0.11	0.08	0.15
SET 2	0.12	0.07	0.15
AVERAGE	0.115	0.075	0.15
UNITS /ml ENZYME	0.153	0.1	0.12

TABLE 3.2 Calculation for CHO units/ml



GRAPH 3.1 Concentration of CHO Vs Time by Calculation

The enzyme activity for CHO was studied for various time intervals as observed in the graph. It was seen that the enzyme showed maximum activity at 96 hours.

IV. Conclusion:

Cholesterol oxidase enzyme has a great commercial value .Also along with Cholesterol oxidase another enzyme can also be extracted from the isolated strains that can be used for various applications. Cholesterol oxidase is also implicated in the manifestation of some of the diseases of bacterial (tuberculosis), viral (HIV) and non-viral prion origin (Alzheimer's). These applications and disease mechanisms have promoted the need of screening, isolation and characterization for future prospects.

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