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ISOLATION AND CHARACTERIZATION OF CHOLESTEROL OXIDASE FROM *AMYCOLATOPSIS* STRAIN USING 16S rRNA PARTIAL SEQUENCE

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ABSTRACT: The aim of the present research work is to isolate and characterize microbial strain using partial sequencing of 16S rRNA gene region of *Amycolatopsis* strain K1. Five *Amycolatopsis* strains from soil samples were collected from the effluent sediments from Jajmau, Kanpur in U.P. India, and it was confirmed by polymerase chain reaction (PCR) that the isolates were different and not re-isolates of the same strain. Sequence analysis of the PCR-amplified 16S rRNA gene indicated that isolates showed maximum similarity to *Saccharopolyspora endophytica* strain YIM 61095. *Amycolatopsis* strain K1, which is a gram-positive bacterium, was cultured in a nutrient medium containing cholesterol at 30 °C for 24 h. A partial sequence of 16S rRNA gene was amplified by PCR and sequenced; the sequence data were submitted to NCBI. *Amycolatopsis* strain K1 partial sequence of 16S rRNA gene region resulted in 1366bp. The sequence data was submitted to NCBI, and the accession number obtained is SUB 6244430. The phylogenetic tree was constructed using the neighbor-joining through multiple sequence alignment program of Mega 4.

INTRODUCTION: Microorganisms play a very important role in our lives. They are found in almost every habitat present in nature like soil, water, air, spoiled bread, and various foodstuff. Microorganisms have been exploited in a number of ways by human beings for their benefit. They are used to produce a wide variety of pharmaceutical products and assist in maintaining and improving the environment [1]. The selection and use of microorganisms in industrial microbiology are challenging tasks that require an understanding of microbial growth as well as its interactions with other organisms.

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Screening of microbial sources for discovering novel molecules/activities is virtually an endless, rather expanding, area of exploited microbial diversity. Microbes and microbial metabolites have extensive applications in pharmaceutical, food, agricultural, waste treatment, leather, bioleaching, biofertilizers, and several another important commercial/industrial sectors of our society [2]. Microbial metabolites offer unique chemical and structural diversity of active compounds, which may be developed as lead therapeutic drug molecules [3].

Different metabolites are produced at various phases of the growth of the organism. Primary metabolites such as enzymes and peptides/proteins, which are essential for the life, are produced in the exponential phase of microbial growth, whereas secondary metabolites (antibiotics), which are not required for the life cycle, are produced under sub-optimal growth conditions near the end of the

exponential phase or in the stationary phase of growth [4]. The first reaction in the metabolism of cholesterol is its oxidation to 4-cholest-en-3-one [5]. After the breakdown of cholesterol by COD, the 4-cholestene-3-one is formed as the end product, with a molecular formula of $C_{27}H_{44}O$. It has clinical importance as an anti-obesity agent. It is also used for the prevention of various other diseases such as arteriosclerosis, cancers, and diabetes mellitus associated with obesity. Since it has a very good therapeutic importance, it is used by pharmaceutical industries as pills, capsules, tablets, powder, and suspensions. It is also used in food, where it is either added directly or as immersion [6]. Therefore, the study of cholesterol metabolism, the stability, and the substrate specificity of CODs *via* protein engineering techniques could be useful to understand its effect in pathological techniques and also the development of new organisms with potential use as biotechnological and pharmaceutical tools.

Cardiovascular diseases are major health problems with multifactorial etiology and need a quick, accurate diagnosis and therapeutic strategy[7]. Cholesterol in serum is a general biochemical marker for the prognosis of atherosclerosis and other lipid disorders. Higher levels of cholesterol in the blood are closely related to an increased risk of heart disease; hence for better therapy, an adult should be routinely screened for elevated cholesterol level [8]. The need for accurate cholesterol levels in serum has encouraged studies related to the development of methods for its routine assay that are fast, easy, and reproducible. An extensive range of the pharmacological assay is presently flooding the market for the detection of cholesterol levels in serum, but the operating costs of measurement are very high.

In order to overcome these effects, investigations have been extended for the search of novel microbes producing COD with improved efficacy and broader applications. Microbial COD is a very important enzyme at a commercial scale, generally employed by laboratories for the determination of cholesterol level in serum. For substantial commercial viability of the COD enzyme, technologies based on novel isolates and optimum fermentation conditions for producing COD needs to be investigated. The present study was aimed at studying the production, purification and characterization of COD from novel microbial isolates.

MATERIALS AND METHOD:

Chemicals: All the analytical grade chemicals used in this study were purchased from Sigma-Aldrich Chemicals Co. (USA). Components for bacteriological media were obtained from Hi-media (India). The organic and inorganic chemicals and solvents used were of analytical grade, obtained from Qualigens or SD-Fine chemicals (India). Protein molecular weight marker and DNA ladder were purchased from Genei (India). PCR master mix and primers were purchased from Xcleris Genomics (Ahmadabad, India). All the solutions were prepared with double distilled water just before use unless mentioned otherwise.

Screening and Isolation of Potential Microorganisms Producing Cholesterol Oxidase

***Screening of* Microorganisms Producing Cholesterol Oxidase:** Soil samples were collected from the effluent sediments from Jajmau, Kanpur in U.P. India. One gram of the soil sample was suspended in 10 ml of normal saline (NaCl 0.85%, w/v) and vortexed vigorously for 2 min. The soil particles were allowed to settle, and 1 ml of the supernatant was serially diluted, and 100µl of the sample was spread on cholesterol enrichment medium plates and incubated at 30 °C for 7 days. Isolated colonies were picked up and streaked on nutrient agar (HiMedia, India) for bacterial cultures and YMG agar **Fig. 1** for *Amycolatopsis* sp cultures. Single colonies that appeared on these plates were further screened.

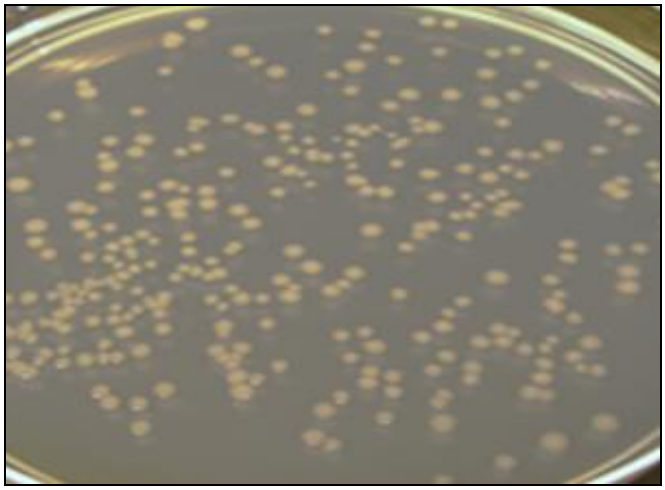

FIG. 1: COLONIES OF *AMYCOLATOPSIS* SPP

Cholesterol Indicator Plates: The microorganisms isolated from the primary screening were cultured on COD indicator plates **Fig. 2** (cholesterol 0.1%, triton X-100 0.1%, o-dianisidine 0.01%, horse-radish peroxides 1 U/ml, agar 1.5%) and further incubated for 7 days at 30 °C [9].

COD converts cholesterol into 4-cholesten-3-one and hydrogen peroxide. O-dianisidine of the medium reacts with hydrogen peroxide (H_2O_2) to form azo compound (Schiff's base) which turns the color of the medium into intense creamish color. Colonies showing intense creamish color in surrounding were picked up as COD producers and used for further study.

FIG. 2: ISOLATE (K1) ON AGAR PLATE (*AMYCOLATOPSIS* SPP)

Morphological Characterization of the Isolates: The isolates were classified morphologically as well as biochemically, and the genus assignments were done in accordance with the Bergey's manual of systematic bacteriology [10]. A systematic classification approach was applied for the identification and the physiological characterization of the COD producing isolates.

The plates were observed for macroscopic cultural and morphological characteristics such as colony diameter, colony color, and conidial color, nature of spore masses, mycelial color, colony reverse, colony texture, and color released in the substratum. Other tests for phenotypic characterization of microbial isolates included antibiotic susceptibility pattern, hydrolysis of citrate, casein, gelatin, utilization of arabinose, fructose, phenylalanine, and urea as (Appendix).

Identification of Cholesterol Oxidase Positive Bacterium: Molecular identification of the bacterial strain FH68 was carried out by amplifying the 16S rRNA gene. The forward primer: AGAGTTTGATCMTGGCTCAG and reverse primer: GYTACCTTGTTACGACTT were used. The product was sequenced using BDT v3.1 Cycle sequencing kit on ABI 3130 Genetic Analyzer. The consensus sequence of the 16S rRNA gene was generated from forward and reverse sequence data using aligner software. The 16S rRNA gene sequence was used to carry out BLAST analysis with the NCBI GenBank database. Based on the maximum identity score, the first ten sequences were selected and aligned using multiple alignment software program ClustalW. A distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4 software.

To identify the strain at molecular level, 16S rRNA homology studies were performed. Partial sequencing of 16S rRNA was done at Akaar biotech Lucknow, India. Closely related homologs were identified by comparing the above obtained partial 16S rRNA sequence of the isolated strains with the database of nucleotides at the National Center for Biotechnology Information (NCBI) webserver (http://www.ncbi.nlm.nih.gov), using the standard nucleotide BLAST program. Pairwise sequence alignment of strain with closely related (99%) homolog was studied through CLUSTALW v1.83 (http://www.ebi.ac.uk/clustalw).

Phylogenetic Analysis of Isolates: A phylogenetic analysis of strain was performed to predict the species level characterization of the isolates. The phylogenetic tree was constructed using the neighbor-joining method (Saitou and through multiple sequence alignment program of Mega 4 (http://www.kumarlab.net/publications). Detailed screening and isolation procedures for COD producing microorganisms led to the selection of one strain *Amycolatopsis* sp. (K1), for further studies.

RESULTS:

Soil Screening and Isolation of Potential Microorganisms Producing Cholesterol Oxidase: Soil samples were collected from the Jajmau, Kanpur, India, and used for primary screening of COD producing microorganism.

Screening of the soil samples led to the isolation of ten bacterial, which were able to grow on cholesterol medium containing cholesterol as the sole carbon and nitrogen source. A low number of microbial colonies were observed on cholesterol plates due to the ability to utilize cholesterol for their metabolic activities and growth. All the isolates were further subjected to secondary screening.

Secondary Screening for Cholesterol Oxidase Producing Microorganisms:

Cholesterol Oxidase Indicator Plate: During the secondary screening of COD producing microorganisms, *Amycolatopsis* sp. showed cream color pigmentation on cholesterol indicator plates due to the formation of Azo compound **Fig. 3.** These isolates were taken up for further studies. The results of primary screening and COD indicator plate assay are subjective and provide no information about the level of COD production by the microorganisms. The final selection of the microorganisms was based on quantitative measurements of the COD activity under submerged culture conditions.

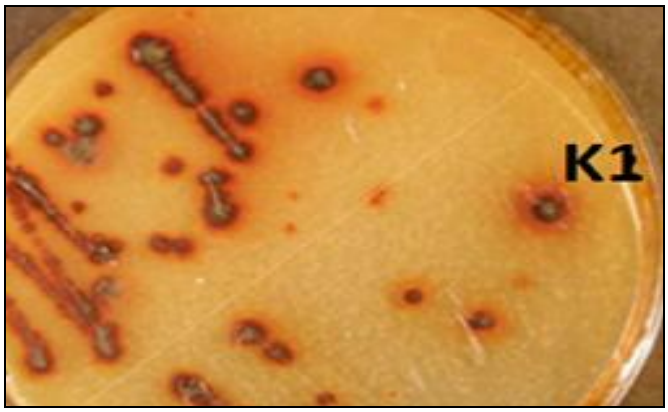

FIG. 3: SECONDARY SCREENING OF *AMYCOLATOPSIS* SPP

Culture Characterization of the Isolate (K1):

Morphological and Physiological Characteristics of the Isolate (K1): The selective screening experiments resulted in isolation of *Amycolatopsis* sp. form soil samples. The *Amycolatopsis* colonies were round, creamish colored and radiant **Fig. 1.** Colony morphology of *Amycolatopsis* exhibited similarity with *Streptomyces* and the genus description was matched [11]. Morphological and physiological characteristics confirmed that the isolated strain belongs to the genus *Amycolatopsis*.

Molecular Identification of the Isolates (K1): Genetic relatedness is provided with rapid and objective identification of the isolates compared to the traditional phenotypic characterization. Genes coding for rRNA are highly conserved, and their sequence analysis is used for inferring the evolution of taxa through billions of years. Further, species-level identification was performed through computational analysis. Results of Basic Local Alignment Search Tool (BLASTn) concluded that partial 16S rRNA gene sequence (1478 base pair) of the isolate was found to possess close similarity (99-98%) *Streptomyces rimosus* subsp. *rimosus* (NR024762.1) **Table 1**.

Finally, on the basis of evolutionary distances calculated through the neighbor joining method, the phylogenetic tree constructed with Mega 4 was categorized into two major clads, **Fig. 4**. Both clad were further divided into two sub-clads *viz*., sub clad 1.1, 1.2, and 2.1, 2.2, respectively. The sub-clad 1.2 and 2.1 leads *Amycolaptosis japonica* strain MA 417CF17 and *Amycolapotasis reqifeucin* strain GY080 alone, respectively, whereas sub-clad 1.1 and 2.2 further divided into two branches, 1.1.1 containing *Amycolatopsis decaplanina* strain DSM 44594 and *Amycolatopsis keratiniphila* strain D2. The above phylogenetic tree confirmed the maximum similarity of the isolate with *Amycolatopsis orientalis* strain IMSNU 20058. The *Amycolatopsis orientalis* strain IMSNU 20058 has been deposited at Akaar Biotech Lucknow, India, and its 16S rRNA sequence has been submitted to the NCBI GenBank database under accession number SUB 6244430 **Fig. 4.**

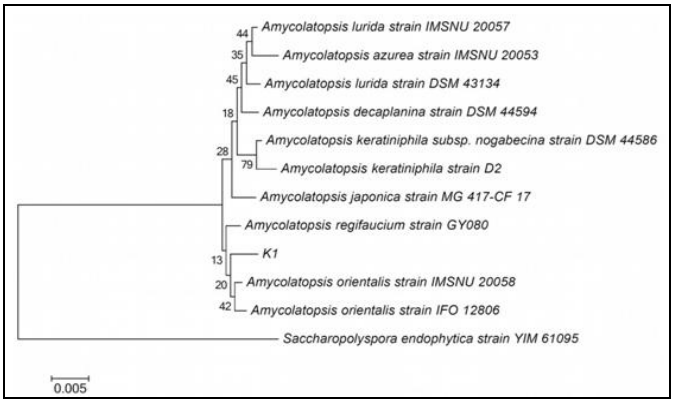

FIG. 4: PHYLOGENETIC TREE OF CHOLESTEROL OXIDASE PRODUCING *AMYCOLATOPSIS* SP. (K1) POSSESSING SIMILARITY OF 99% WITH *SACCHAROPOLYSPORA ENDOPHYTICA*

TABLE 1: THE ALIGNMENT RESULTS OF NUCLEOTIDE SEQUENCE OF SAMPLE AM WITH RESPECT TO 10 CLOSELY RELATED HOMOLOGOUS SEQUENCES DURING NCBI NBLAST SEARCH

Description	Max Score	Total Score	Query Cover	E Value	Ident.	Accession
Amycolatopsis orientalis strain IMSNU 20058	2182	2182	100%	0	99%	NR 042104.1
Amycolatopsis japonica strain MG 417-CF 17	2172	2172	100%	0	99%	NR 025561.1
Amycolatopsis keratiniphila subsp. nogabecina strain DSM 44586	2159	2159	100%	0	98%	NR 025563.1
Amycolatopsis keratiniphila strain D2	2152	2152	100%	0	98%	NR 036923.1
Amycolatopsis regifaucium strain GY080	2148	2148	98%	0	99%	NR 042747.1
Amycolatopsis lurida strain IMSNU 20057	2145	2145	100%	0	98%	NR 042040.1
Amycolatopsis decaplanina strain DSM 44594	2145	2145	100%	0	98%	NR 025562.1
Amycolatopsis lurida strain DSM 43134	2137	2137	100%	0	98%	NR 114905.1
Amycolatopsis orientalis strain IFO 12806	2134	2134	100%	0	98%	NR 115645.1
Amycolatopsis azurea strain IMSNU 20053	2128	2128	100%	0	98%	NR 042103.1
Saccharopolyspora endophytica strain YIM 61095	1801	1801	99%	0	93%	NR 132591.1

DISCUSSION: Cholesterol oxidase (COD) is a FAD-containing flavor-oxidase that catalyzes the oxidation of the C3-OH group of cholesterol to give cholest-5-en-3-one and its isomerization to cholest-4-en-3-one [12]. COD is used to determine cholesterol levels in food and blood serum by coupling of the enzyme with peroxidase [13]. Increasing demand for specific estimation of steroids in clinical samples has enhanced the importance and need for COD in the pharmaceutical industry. It is also used in the production of precursors of hormonal steroids like estrogen, progesterone, and testosterone from cholesterol. It is currently being used in organic synthesis and in the oxidation of non-steroidal compounds. In addition, COD has also been reported to show insecticidal properties, bacterial pathogenesis [14,] and biosynthesis of macrolide antifungal antibiotics [15.]

Some *Amycolatopsis* species are also reported for glycopeptide antibiotic vancomycin [16]. COD produced by *Amycolatopsis* sp. has been reported to have insecticidal activity against insects like boll weevil larvae [17]. These studies indicate that *Amycolatopsis* sp. is a commercially important source for COD production. In t

he present study, screening of soil samples from Jajmau, Kanpur lead to the isolation of ten bacterial cultures, which were able to grow on cholesterol medium containing cholesterol as the sole carbon and nitrogen source. Among K1 was found to be good producer of COD. Production of COD was further verified by indicator plates assay showing the intense cream color pigmentation due to the presence of o-dianisidine of the medium reacts with hydrogen peroxide (H_2O_2) to form Azo compound (Schiff‟s base), which turns the color of the medium into intense cream color [9]. *Amycolatopsis* isolates (K1) have been further identified by morphological and physiological studies [11.]

Finally, species-level identification was done by 16S rRNA gene homology studies. Woese (1987) [18] has reported rRNA as the ultimate molecular chronometers to establish the phylogenetic relationship of microorganisms.

Their large size and multiple domains allow the comparison of accumulation of sequence changes, which can be defined as the rate at which mutations become fixed over time. Genes of rRNA shows a high degree of functional constancy, as they occur in all organisms, but different domains in their sequence change at very different rates, allowing most phylogenetic relationships (including the most distant) to be measured [18].

Partial 16S rRNA gene sequence of the isolate K1 was found to possess close similarity (99-98%) with *Amycolatopsis lurida* IMSNU20057, *Amycolatopsis azurea* IMSNU20057. Isolation and physiological characterization of the *Amycolatopsis* (K1) have been reported by Padma *et al.* (2012) [16]. *Amycolatopsis orientalis* has also been reported for the production of a wide range of metabolites like vancomycin, an antibacterial agent.

CONCLUSION: In the present study, the potential of *Amycolatopsis* K1 for cholesterol oxidase production has been investigated. Sequence analysis of the PCR-amplified 16S rRNA gene indicated that isolates showed maximum similarity

to *Saccharopolyspora endophytica* strain YIM 61095 *Amycolatopsis* K1 could be used as a promising, efficient source for cholesterol oxidase production.

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CONFLICTS OF INTEREST: The authors declare that there are no conflicts of interest.

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