

## ISOLATION AND IDENTIFICATION OF CHOLESTEROL OXIDASE PRODUCING ACTINOMYCETES FROM EGYPTIAN SOIL FOR A LARGE SCALE FERMENTATION IN SUBMERGED CULTURES

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### ABSTRACT

Isolation and identification of new microbial isolates capable of producing cholesterol oxidase enzyme (CHO) is of great potential in the field of biotechnology of industrial enzymes. Four producing strains of actinomycetes were isolated from Egyptian soils demonstrated high activity in cholesterol degradation compared to the other isolates. Based on different standard morphological, biochemical as well as molecular techniques using 16S rRNA sequencing, these actinomycetes were identified as *Streptomyces* sp. strain NHIA\_CH3 MK680299, *Streptomyces* sp. strain NHIA\_CH5 MK680300, *Streptomyces griseus* strain NHIA\_CH6 MK680301 and *Streptomyces drozdowiczii* strain NHIA\_CH10 MK680302. In order to optimize the CHO enzyme activity, different fermentation media were tested in submerged cultures at shake flask level. Maximum activity (23.7 U/mL) was observed in culture of *Streptomyces* sp. strain NHIA\_CH5 which was scaled up to the 5L volume of the stirred tank bioreactor. Results of the batch fermentation showed an earlier production of CHO enzyme with 4% increase in activity compared to that obtained from shake flask cultures indicated that *Streptomyces* sp. strain NHIA\_CH5 could be a potential source of CHO enzyme for commercial purpose.

**Keywords:** isolation, *Streptomyces* sp., cholesterol oxidase, optimization, scale up

### INTRODUCTION

Cholesterol oxidase (CHO) is a flavoenzyme, oxidizes cholesterol and converts 5-cholesten-3 $\beta$ -ol into 4-cholesten-3-one. This reaction is a first step in the microbial degradation of cholesterol and its derivatives (Saranya *et al.*, 2014). CHO is an important precursor for the synthesis of hormones and steroidal drug intermediate (Fazaeli *et al.*, 2018) and has a broad range of applications, i.e. cholesterol determination in food and medicine, cholesterol biotransformation into valuable products, preparation of food stuffs and inhibition of cotton weevil growth. Furthermore, cholesterol oxidases were involved in the appearance of Alzheimer's disease, tuberculosis and HIV (Srivastava *et al.*, 2015). Hence, researches on microbial CHO have received much attention in recent years. Microbial conversions of cholesterol are more regio- and stereo-selective when compared to chemical reactions and have been used for a long time in the production of pharmaceutical products (Saranya *et al.*, 2014). Studies have reported extracellular CHO production in shake flasks from microorganisms, e.g., *Pseudomonas* sp., *Arthrobacter* sp., *Rhodococcus equi*, *Brevibacterium sterolicum*, *Mycobacterium* sp., as well as some *Streptomyces* strains, i.e. *S. violascens* (Srivastava *et al.*, 2018a). *Streptomyces* are Gram-positive soil microorganisms capable of producing a variety of industrially important enzymes. Furthermore, compared to other producers, *Streptomyces*-produced CHO are generally more stable, have longer storage times, and are cost-effective (Elsayed & Abdelwahed, 2020). Production of CHO in submerged fermentation is greatly influenced by medium composition and strain capacity. This could minimize the cost for overall production process. Different parameters such as pH, medium composition, temperature, aeration, agitation and controlling of the dissolved oxygen (DO) was achieved previously during a scale-up study for CHO production (Thiry & Cingolani, 2002). The present study was aimed to isolate the most potent cholesterol degrading actinomycete strains to evaluate maximum ability for CHO production in the selected suitable medium containing cholesterol as inducer in submerged cultures at shake flask level followed by a large-scale bioprocess in 5L bioreactor to achieve more production yield and help in providing information to apply in future industrial process scale-up.

### MATERIALS AND METHODS

#### Isolation of actinomycetes

Actinomycetes screened in this study were isolated from various soil samples collected from different localities in Egypt. One hundred grams of soil samples were taken in sterile Petri dishes individually and placed in hot air oven at 70 °C for 10 min. The treated samples were serially diluted and plated on starch-nitrate agar medium of the following composition (g/L): Starch 20; KNO<sub>3</sub> 2; K<sub>2</sub>HPO<sub>4</sub> 1; MgSO<sub>4</sub>·7H<sub>2</sub>O 0.5; agar 20 and distilled water up to 1 L [supplement with Nalidixic acid (10 µg/mL) and Amphotericin B (20 µg/mL)] using the spread plate technique then the plates were incubated at 30 °C for 8–10 days. Morphologically distinct actinomycetes isolates were picked up, streaked, cultivated a number of times on starch-nitrate agar medium plates, and then incubated at 30°C for 8–10 days to check its purity. The pure actinomycete isolates were maintained on starch-nitrate agar medium slants. For long-term storage, the cultures were preserved in 20% (v/v) glycerol at -80 °C (Shepherd *et al.*, 2010).

#### Screening for CHO producing organisms

For screening of CHO enzyme producing actinomycetes, colonies were selected on suitable indicator agar plate containing (g/L): 2 cholesterol, 1.0 Triton X-100, 0.1 O-dianisidine, 1000 U/L peroxidase and 20 agar. Plates were incubated for 4 days at 30 °C. Cholesterol penetrates into actinomycete cells where it can be converted into hydrogen peroxide by CHO. Reagents that exist in the medium react with hydrogen peroxide to form azo compound which turns the color of the medium to intense brown color (Srivastava *et al.*, 2018). These isolates were maintained on slopes containing starch-nitrate agar medium. The isolates were stored as spore suspensions in 20% (v/v) glycerol at -80 °C for subsequent investigation.

#### Cultural, morphological and biochemical analysis

Only actinomycete isolates that gave a high result for CHO enzyme production

were analyzed for cultural characteristics which described the growth, color of the aerial mass, soluble pigment and the substrate mycelium of the strain on different ISP media observed by naked eye. These characteristics of actinomycete isolates were observed in mature cultures grown on various media on the 7<sup>th</sup>, the 14<sup>th</sup>, and the 21<sup>st</sup> day followed the guidelines adopted by the International *Streptomyces* Project (ISP) (Shirling & Gottlieb, 1966). both spore chains and spore surface of the isolated strain were determined to discover the morphological properties of strains by a light microscope (Olympus CX41 optical microscope model LC20, GMBH, Münster, Germany) with digital camera output connected to a computer monitor. This was done using the cover slip technique as described by Shirling & Gottlieb (1966). The spore surface of these isolated strains from the culture on 21 days of incubation was observed under transmission electron microscope (TEM) Zeiss EM 10 (Zeiss, Oberkochen, Germany). These characters and photos of the *Streptomyces* isolates were compared to the similar *Streptomyces* species in Bergey's Manual of Determinative Bacteriology Ninth edition (Cross, 1989). Carbon utilization was determined on plates containing ISP basal medium. Separately-sterilized carbon sources were added to a final concentration of 1.0%. The plates were incubated at 30°C and the growth using glucose as positive control was noticed after 7, 14 and 21 days (Ismail et al., 2017; Awad & Germoush, 2017).

**Molecular identification and phylogenetic analysis of the isolated strains**

Strains were grown on a slant of the actinomycete isolation agar medium for 7-10 days at 30°C. Two ml of spore suspension were inoculated in the ISP 2 broth medium and incubated on incubator shaker Innova 4080 (New Brunswick, NJ, USA) at 200 rpm and 30°C for 24h to form pellets of vegetative cells. Total genomic DNA extraction and purification were carried out according to the method of Lee et al. (1989). The polymerase chain reaction (PCR) amplification and sequencing were carried out as described by Rintala et al., (2001). The nucleotide sequences of the 16S rRNA gene of the isolated strains have been deposited in NCBI database of the GenBank under different accession numbers. Phylogenetic data were obtained by aligning the nucleotides of different 16S rRNA retrieved from the Basic Local Alignment Search Tool (BLAST) algorithm available through the National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov>), using the CLUSTAL W program version 1.8 with standard parameters.

**Effect of different media for the production of CHO**

Different production liquid media described before from literatures to support CHO production representing a broad range of nutrient sources were investigated for the primary evaluation of medium optimization process. Fifty mL starch-nitrate broth medium as mentioned above was inoculated with a loop full from slant culture to prepare the seed culture, then incubated at 30 °C for 48 h with shaking at 200 rpm. A two percent (v/v) from the seed culture was transferred to each 250 ml Erlenmeyer flask containing 50 mL of the production media. The screening production media that were inoculated and incubated in an orbital shaker at 200 rpm and 30 °C for 5 days composed of (g/L) were as follow:

Medium No.	Composition (g/L)	Reference
1	Glucose, 4; malt extract, 10 ; yeast extract, 4; CaCO <sub>3</sub> , 2	(Niwas et al., 2013)
2	Malt extract, 2; yeast extract, 4; glucose, 15 ; peptone, 5; MgSO <sub>4</sub> .7H <sub>2</sub> O, 0.5 ; K <sub>2</sub> HPO <sub>4</sub> , 1; NaCl, 0.5	(Niwas et al., 2013)
3	Soluble starch,10; peptone, 6; glucose, 10; yeast extract, 5; MgSO <sub>4</sub> .7H <sub>2</sub> O, 0.5; K <sub>2</sub> HPO <sub>4</sub> , 1; NaCl, 0.5	(Tabatabaei et al., 2001)
4	Soluble starch, 15; malt extract, 2; yeast extract, 4 peptone, 0.5; MgSO <sub>4</sub> .7H <sub>2</sub> O, 0.5 ; K <sub>2</sub> HPO <sub>4</sub> , 1; NaCl, 0.5	(Varma et al., 2003)
5	Soluble starch, 10; peptone, 30; glucose, 10; beef extract, 30; MgSO <sub>4</sub> .7H <sub>2</sub> O, 1; NaCl, 7.5	(Kajiya et al., 1991)
6	(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> , 2; glycerol, 5; K <sub>2</sub> HPO <sub>4</sub> , 2; MgSO <sub>4</sub> .7H <sub>2</sub> O, 0.2 and peptone, 2	(Richmond, 1973)

Cholesterol was added to each of the production media as 2 g in 1% Triton X100 per liter, pH 7

The fermentation profile was studied by harvesting triplicate flasks at intervals of 24 h. Supernatants of the fermented cultures were obtained by centrifugation of the media at 5,000 rpm for 10 min at 4°C for protein determination and enzyme assay.

**Up scaling of CHO production in 5L stirred tank bioreactor**

Cultivations in bioreactor level were carried out using 5L-stirred tank bioreactor (Bioflo 310, New Brunswick Scientific, New Brunswick, NJ, USA), with a working volume of 3L. Filtered sterile air was introduced to the bioreactor at an aeration rate of 0.5 v/v/min. The concentrations of dissolved oxygen were measured using polarographic electrode (Ingold, Switzerland). The pH of the bioreactor cultivations was initially adjusted at 7.0 using standard NaOH/HCl solutions, and was kept uncontrolled throughout the bioreactor run. Agitation was performed using a two 6-bladed Rushton turbine impellers (di (impeller diameter)=75 mm; dt (tank diameter)=215 mm; di/dt = 0.35) at 300 rpm. If applicable, foam was suppressed using silicon antifoam reagent (Fluka, Switzerland). Temperature was adjusted at 30°C. All the samples were analyzed for three times and the average mean of the data are represented.

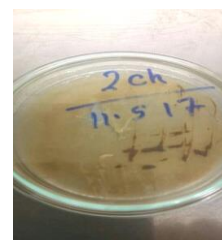
**Determination of CHO activity**

The enzymatic activity assay of CHO is based on the conversion of cholesterol to 4-cholestene-3-one. The assay was performed by following the method of Allain et al. (1974). One unit of CHO has been defined as the quantity of the enzyme required to produce 1.0 μmol of 4-cholesten-3-one per min at pH 7.0 and 37 °C. The concentration of the protein/enzyme was measured by the method of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard.

**RESULTS AND DISCUSSION**

**Actinomycetes isolation and screening for CHO activity**

It is of great importance to obtain the required isolate in a pure culture to ease the following optimization and production processes. Generally, CHO indicator agar method is used to confirm the ability of the isolated strain to produce CHO. During the screening, out of ten actinomycete isolates from soil samples were collected from various areas in Egypt, four showed promising activities to degrade cholesterol as noticed by the intense brown color on indicator agar plates having cholesterol as the only C-source. Accordingly, cholesterol was converted into 4-cholesten-3-one and H<sub>2</sub>O<sub>2</sub> to form azo-compound (Schiff 's base) as represented in figure 1. The 4 potent isolates were selected and subjected to identification (Nagasawa et al., 1969; Srivastava et al., 2018).



**Figure 1** Formation of AZO component because of CHO production by one of the isolated actinomycete

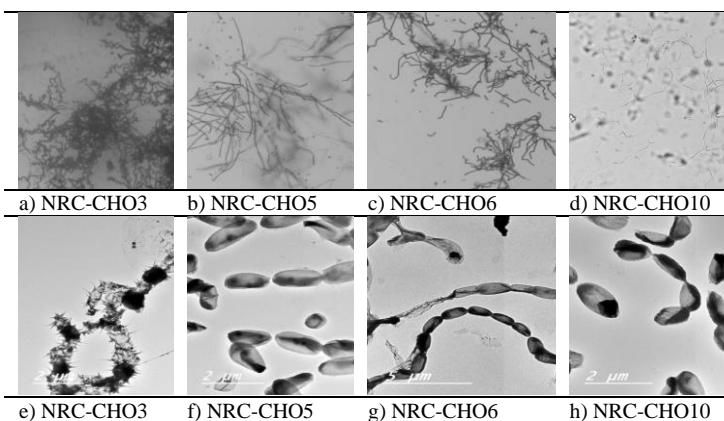
**Cultural and microscopic characteristics of CHO producers isolates**

In this study, the identification of the four CHO enzyme producing isolates coded as NRC-CHO 3, NRC-CHO 5, NRC-CHO 6 and NRC-CHO 10 were obtained through colony morphology observation on starch-nitrate agar medium . Colonies are dry, compact, chalky-like, heaped, folded, slow growing, aerobic with aerial and substrate mycelia of different colors with an earthy odor. The isolated strains were able to grow and spread on ISP medium including both defined and undefined components. Furthermore, all selected strains grew moderately to heavily on ISP1, ISP2, ISP3, ISP4, ISP5, ISP6 and ISP7. It was observed that the abundance and the color of aerial mycelium depended on the medium composition and the age of the culture (Table 1).

**Table 1** Cultural characteristics of isolate coded NRC-CHO 3, NRC-CHO 5, NRC-CHO 6 and NRC-CHO 10 in different media

Medium	Growth	Color of			
		Aerial mycelium	Substrate mycelium	Diffusibile pigments	
<b>Isolate NRC-CHO 3</b>					
1	Tryptone yeast extract broth (ISP 1)	Weak	Gray	Beige	None
2	Yeast -malt extract agar (ISP 2)	Abundant	Gray	Light Brown	None
3	Oat meal agar (ISP 3)	Abundant	Gray	Dark Beige	Beige
4	Inorganic-trace salt- starch agar (ISP4)	Abundant	Gray	Brown	None
5	Glycerol asparagine agar (ISP 5)	Abundant	Gray	Beige	None
6	Peptone yeast extract iron agar (ISP 6)	Abundant	Gray	Gray	None
7	Tyrosine agar (ISP 7)	Abundant	Gray	Beige	None
8	Nutrient agar	Abundant	Gray	Brown	None
9	Czapek's agar	Moderate	Gray	Beige	None
<b>Isolate NRC-CHO 5</b>					
1	Tryptone yeast extract broth (ISP 1)	Abundant	Light Beige	Beige	None
2	Yeast -malt extract agar (ISP 2)	Abundant	Gray	Brown	None
3	Oat meal agar(ISP 3)	Abundant	Gray	Beige	None
4	Inorganic-trace salt- starch agar (ISP4)	Abundant	Gray	Brown Beige	None
5	Glycerol asparagine agar (ISP 5)	Abundant	Gray	Beige	None
6	Peptone yeast extract iron agar (ISP 6)	Abundant	Gray	Gray	None
7	Tyrosine agar (ISP 7)	Abundant	Light Gray	Beige	None
8	Nutrient agar	Abundant	Gray	Light Brown	None
9	Czapek's agar	Abundant	Gray	Light Gray	None
<b>Isolate NRC-CHO 6</b>					
1	Tryptone yeast extract broth (ISP 1)	Abundant	Beige gray	Beige	None
2	Yeast -malt extract agar (ISP 2)	Abundant	Gray	Light Brown	None
3	Oat meal agar (ISP 3)	Abundant	Gray	Beige	None
4	Inorganic-trace salt- starch agar (ISP4)	Abundant	Gray	Light Brown	None
5	Glycerol asparagine agar (ISP 5)	Abundant	Light gray	Beige	None
6	Peptone yeast extract iron agar (ISP 6)	Abundant	Gray	Beige	None
7	Tyrosine agar (ISP 7)	Moderate	Light Gray	Light Beige	None
8	Nutrient agar	Abundant	Gray	Light Brown	Light Yellow
9	Czapek's agar	Abundant	Gray	Beige	None
<b>Isolate NRC-CHO 10</b>					
1	Tryptone yeast extract broth (ISP 1)	Abundant	Light Gray	Beige	None
2	Yeast -malt extract agar (ISP 2)	Abundant	Gray	Brown	None
3	Oat meal agar(ISP 3)	Abundant	Gray	Beige	None
4	Inorganic-trace salt- starch agar (ISP4)	Abundant	Gray	Dark Brown	None
5	Glycerol asparagine agar (ISP 5)	Abundant	Gray	Beige	None
6	Peptone yeast extract iron agar (ISP 6)	Moderate	Gray	Beige	None
7	Tyrosine agar (ISP 7)	Abundant	Gray	Beige	None
8	Nutrient agar	Abundant	Gray	Brown	None
9	Czapek's agar	Abundant	Gray	Brown	None

The actinomycete isolates vegetative hyphae were found to be branched and not fragmented. The characterization of these strains depended on the color of different mycelia, pigments, as well as spore form and surface. Under microscope, spore chain architecture changed according to strain type. These isolates were examined with light microscopy (100× magnification), on the ISP-2 to ISP-5, showed the spiral nature of the spore hyphae chains (S) as in isolate NRC-CHO3 and NRC-CHO6 (Fig. 2a,c). While, the rectus-flexibilis (RF) was observed in isolates NRC-CHO5 and NRC-CHO10 (Fig. 2b,d). The spore surface of the selected isolates NRC-CHO3 was spiny (Fig.2e) and smooth spore surface was observed in NRC-CHO5, NRC-CHO6 and NRC-CHO10 (Fig. 2f, g, h). Furthermore, **Salim et al. (2017)** observed similar hyphae and colony structures for their isolated strains. Colonies formed long spore chains with more than ten spores/ chain. We suggested that the isolated strains belong to the genus *Streptomyces* by comparing their properties and features with those reported for known species of actinomycetes as in **Bergey's manual of systematic bacteriology (Whitman et al., 1998)**. Further confirmation was obtained by studying their acid-fastness, Gram-staining pattern, where all 4 isolates showed negative acid-fastness and positive Gram-staining (**Taddei et al., 2006**).



**Figure 2** Micro-morphology of aerial hyphae and spore chain structure: a, c, d) spiral type, b) rectus flexible (RF) using light microscope 100x magnification. Spore surface: e) spiny as in isolate NRC-CHO3, f, g, h) smooth as in isolates NRC-CHO5, NRC-CHO6 and NRC-CHO10 (×40.000) after 14 days of the incubation on Bennett's agar medium at 30°C.

For the biochemical studies 9 tests were considered as a confirmatory identification to genus was achieved according to the work of **Shirling & Gottlieb (1966)**. Isolates were able to utilize most of the carbon sources with different degree based on the medium composition. The results were presented in table 2.

**Table 2** Sugars utilization of isolates coded NRC-CHO3, NRC-CHO5, NRC-CHO6 and NRC-CHO10

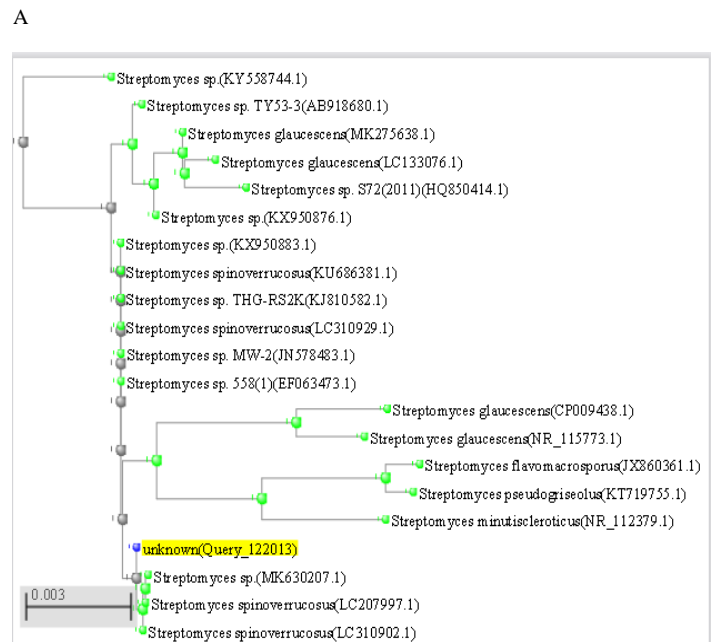
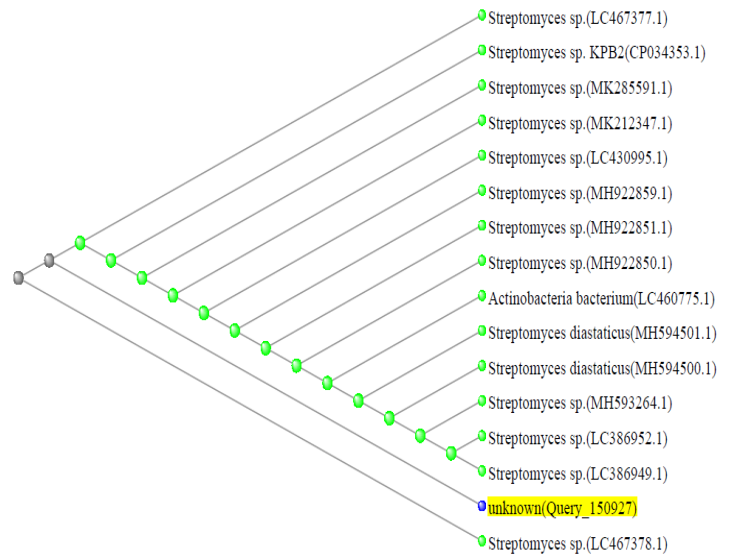
Sugars utilization	Isolate NRC-CHO3	Isolate NRC-CHO 5	Isolate NRC-CHO 6	Isolate NRC-CHO10
No carbon	-	-	-	-
D-glucose	+	+	+	+
D-xylose	+++	+++	+++	+++
L-arabinose	++	++	++	+
D-fructose	++	+++	+	+
Raffinose	+++	+++	+++	++
D-Manittol	+++	+++	+++	+++
Meso-	+++	+++	+++	+++
Salicin	+++	++	+++	++
Sucrose	+	++	++	+

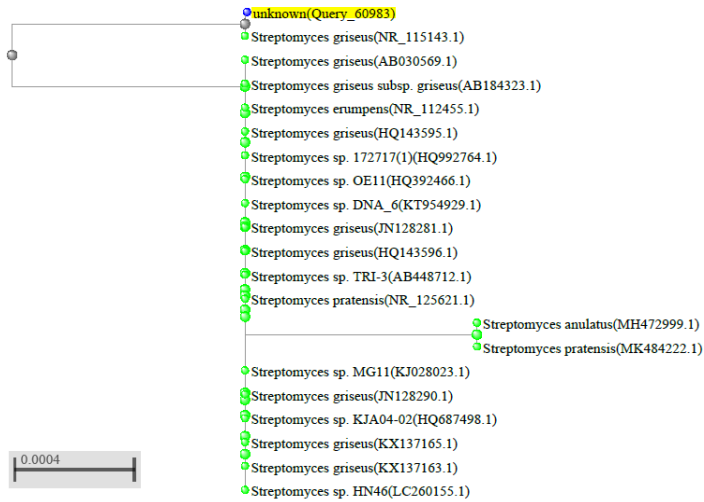
**Molecular Identification and Phylogenetic**

Molecular identification techniques are usually applied for their efficiency and rapidity (Boudemagh et al., 2005). Accordingly, 16S rRNA sequences were analyzed to confirm the identity of the isolated strains (Fig. 3). Results showed that the four isolated strains NRC-CHO3, NRC-CHO5, NRC-CHO6 and NRC-CHO10 with nucleotide sequence 841 bp ; 1133 bp; 1281 bp and 735 bp respectively were subjected to match with the 16S rRNA reported gene sequences in the gene bank database. The BLAST analysis clearly assigned the isolates to the *Streptomyces* genus, with 98% similarity and the isolate NRC-CHO 3 was assigned the nomenclature of *Streptomyces* sp. strain NHIA\_CH3 and its nucleotides were deposited at the GenBank under the accession number of (MK680299). Similarly NRC-CHO5, NRC-CHO6 and NRC-CHO10 were assigned the nomenclature of *Streptomyces* sp. strain NHIA\_CH5 (MK680300), *Streptomyces griseus* strain NHIA\_CH6 (MK680301) and *Streptomyces drozdowiczii* strain NHIA\_CH10 (MK680302). Maleki et al. (2013) reported that 16S rRNA identification is simpler and more efficient in identification of new *Streptomyces* strains. It is well reported that *Streptomyces* species usually constitute higher percentages of total actinomycete strains responsible for different bioactivities as well as industrial enzymes (Singh et al., 2016).

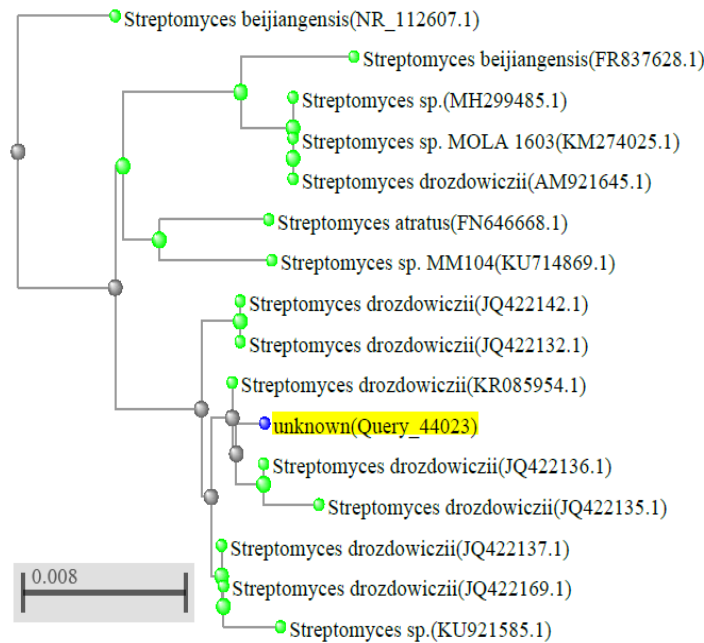
**Media selection for production optimization**

The selection of a suitable production medium is an important prerequisite for fermentative production of CHO. Cultivation medium provides generally growing cells with required energy sources and nutrients, which are then integrated within different cellular metabolic pathways (Singh et al., 2017). In the present study, six different fermentation media described previously for CHO production were tested. It has been previously shown that CHO production by *Streptomyces* is strain-dependent (Yamada et al., 2019). Here, screening of different production media with the four potent isolated strains was carried out. Figure 4 represents the effect of using different cultivation media on CHO levels over different incubation periods. It can be noticed that enzyme activity was increased linearly from 24 to 96 h and thereafter remained more or less constant in all culture media under test. Generally, the highest enzyme production was obtained after 96 h for the selected potent isolates. Medium 6 was found to be the most suitable for the production of CHO by *Streptomyces* sp. strain NHIA\_CH3 and *Streptomyces drozdowiczii* strain NHIA\_CH10 recorded the highest activity of 14.9 and 10.876 U/mL respectively whereas *Streptomyces griseus* strain NHIA\_CH6 showed the highest activity of enzyme attained 12.54 U/mL in medium 1. In case of *Streptomyces* sp. strain NHIA\_CH5, the present investigation revealed that, the highest CHO enzyme activity of 23.7 U/mL was achieved in culture media 5. Complex nutrients, i.e. yeast extract, cholesterol, potato starch, etc., were found to enhance CHO production (Yazdi et al., 2001). Moreover, Chauhan et al. (2009) reported that all organic nitrogen sources gave promising results whereas inorganic nitrogen sources supported very low biomass and CHO production. This is mainly due to the presence of different essential amino acids and growth factors in yeast extract, which enhances cellular performance, metabolism and CHO production consequently. In contrast, Yehia et al. (2015) found that inorganic N-sources, e.g. NaNO<sub>3</sub> and NH<sub>4</sub>NO<sub>3</sub>, C-sources e.g. glycerol and xylose are the most suitable nutrients for CHO production by *Pseudocardia compacta* S-39. Furthermore, Collins et al. (2013) used glycerol for higher biomass and CHO production by *E. coli* BL21 (DE3). However, cholesterol can also be consumed by the CHO producing microbe for energy and carbon sources (Arima et al., 1969). In this study, the optimum media obtained from shake-flask experiments using *Streptomyces* sp. strain NHIA\_CH5 gave the highest activity compared to the other potent isolate under investigation, subsequently validation in 3L working volume bench top bioreactor was investigated.





C



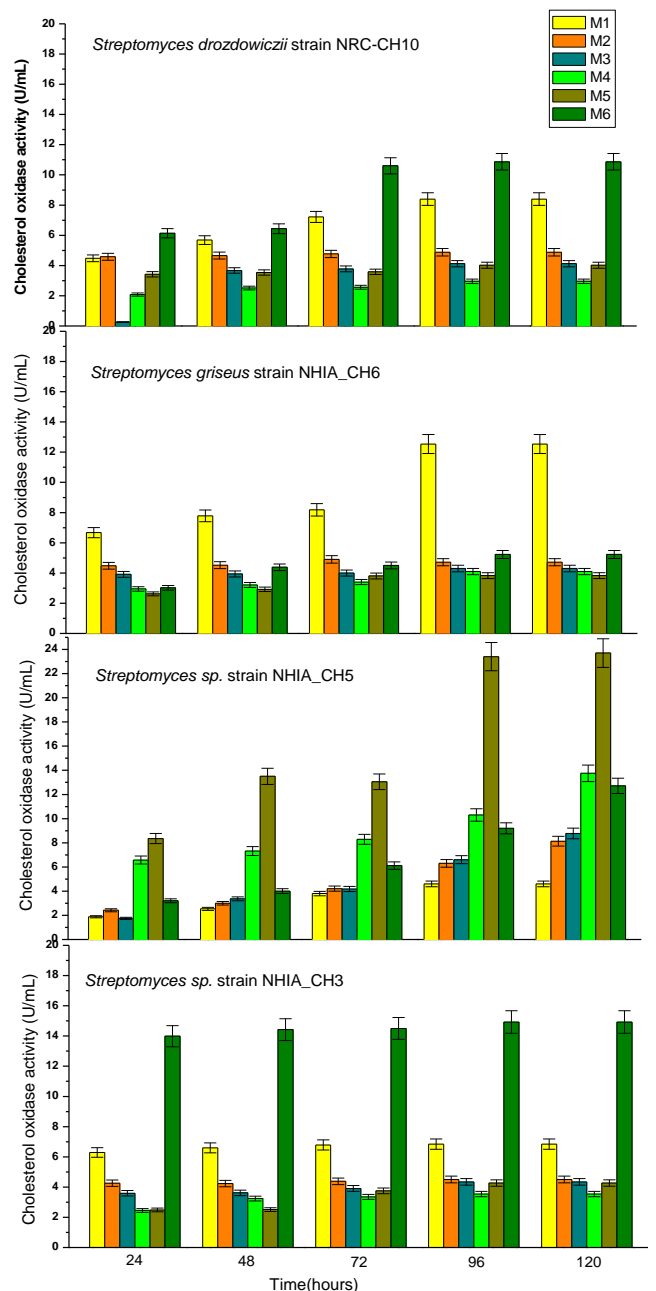
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**Figure 3** Phylogenetic tree of the 16S rRNA gene sequence of a) *Streptomyces* sp. strain NRC-CHO3 (Query\_150927), b) *Streptomyces* sp. strain NRC-CHO5 (Query\_122013), c) *Streptomyces* sp. strain NRC-CHO6 (Query\_60983) and d) *Streptomyces* sp. strain NRC-CHO10 (Query\_44023) with other closely related gene sequences. GenBank accession numbers are mentioned within the parentheses.

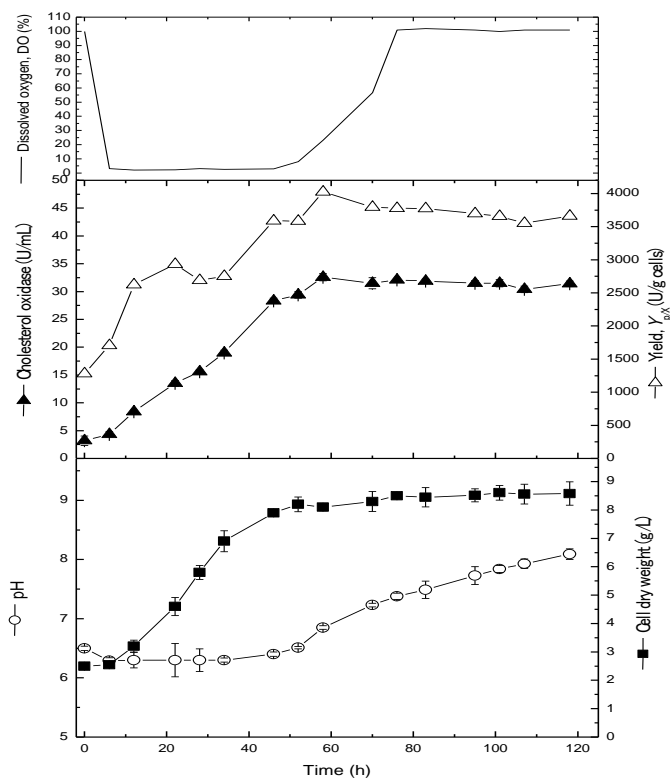
**Production of cholesterol oxidase in 5L-stirred tank bioreactor**

Microbial production processes for valuable industrial enzymes are generally performed using stirred tank vessels. The homogeneity of growth environment in bioreactors allows better and adequate growth because of homogeneous distribution of oxygen and nutrients. Furthermore, bioreactors avoid the presence of measurable differences in hydrostatic pressures inside cultivation vessels. Additionally, fluid and viscosity characteristics of culture broths are enhanced, which provides growing cells with more favorable cultivation conditions. These conditions are reflected on the improved oxygen and nutrient assimilation rates, when compared to shake-flask cultivations. Accordingly, CHO production was performed in 5L-BioFlo 310 bench top bioreactor (working volume of 3 L), where the final optimized cultivation medium and the most potent producer isolate were used. It can be seen that cells grown in bioreactor had enhanced growth and production kinetics (Fig. 5). Up to 40 h, cellular growth followed normal exponential growth pattern, where cellular growth rate averaged about 0.18 g/L/h. Accordingly, cells reached their maximal (7.9 g/L) at 46 h. Afterwards, cellular growth was more or less stable, with a decreased in growth rate (0.01 g/L/h). Additionally, it can be seen that cells consumed the provided oxygen at their highest rates during the exponential growth phase. Up to 40 h, oxygen consumption rate reached 2.11%/h. When cellular growth was stabilized, oxygen consumption continued for another 40 h. Accordingly, oxygen consumption

decreased step-wisely and ceased by 80 h, where the cultivation was saturated by oxygen. For CHO production, cells started to produce CHO during the exponential growth phase, meaning that enzyme production is growth associated. This was moreover confirmed when enzyme production started to become constant by entering the stationary growth phase. The CHO production rate in bioreactor averaged about 2.14 U/mL/h during first 46 h, and the maximum CHO production reached 29.4 U/mL at 52 h. This concentration was higher by 4% and earlier by 44 hours than that obtained during shake-flask cultivation (23.7 U/mL) at 96 h. Afterwards, enzyme production was almost stable until cultivation was ended. Concomitantly, CHO specific yield coefficient ( $Y_{P/X}$ ) recorded its maximum (4024.69 U/g cells) at 58 h, and then ranged from 3551.4 to 3795.2 U/g cells by the end of cultivation. In our findings, results revealed that a scale-up process greatly enhances CHO production, when compared to shake-flask level. Similarly, **Srivastava et al. (2018a)** has revealed 4.75-fold increase in CHO production from *Streptomyces rimosus* using 3L bioreactor as compared to shake-flask production. The larger scale cell cultivations were carried out previously by **Prasad et al. (2014)** used stirred tank bioreactors to produce industrial enzymes. Also, Felse and Panda (2000) produced chitinase by *Trichoderma harzianum* in stirred tank bioreactor. On the other hand, **Ueda et al. (2011a)** produced FR901379-acylase by *Streptomyces* sp. 6907 and **Rahulan et al. (2011)** produced leucine amino peptidase by *Str. gedanensis* in a large-scale bioreactor whereas the production of peroxidase by *Streptomyces* sp. strain BSII#1 was reported by **Musengi et al. (2014)**.



**Figure 4** CHO production profile for *Streptomyces* isolates in different media containing cholesterol as inducer at shake flask level in submerged cultures



**Figure 5** Kinetics of cells growth and CHO production by *Streptomyces* sp. strain NHIA\_CH5 in 5L stirred tank bioreactor

**CONCLUSION**

In the present study, prominent isolates of *Streptomyces* for the biosynthesis of CHO enzyme were isolated from soil samples of Egypt. They were identified based on colony characters, pigmentation profile, physiological, biochemical and microscopic features and were characterized as *Streptomyces* based on conventional taxonomy and confirmed by molecular identification. The production of CHO enzyme by *Streptomyces* isolates on different media was varied depending on the medium composition and the isolate potential. The most powerful isolate with the suitable medium for maximum production were chosen for the scale-up study in a 5L laboratory-scale bioreactor. Results showed that the CHO enzyme production was enhanced by 4% activity reaching up to 29.4 U/mL at 52 h in large scale production as compared to the shake flask level with the maximum activity of 23.7 U/mL at 96h incubation time. In the present investigation, *Streptomyces* sp. strain NHIA\_CH5 is favored with the capability to degrade cholesterol and represented a good source for CHO that can be exploited for potential biomedical, environmental, and industrial applications.

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