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Purification and Characterization of Cholesterol Oxidase from Novel Sources –*Aspergillus awamori* and *Aspergillus fumigatus*

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ABSTRACT

The research interest in exploring various natural habitats for discovering novel microbes as stable cholesterol oxidase producers is on the rise due to its broad range of clinical and industrial applications. A search was conducted to isolate cholesterol oxidase producing fungi. Cholesterol oxidase (CHOx) is a FAD-dependent enzyme of the oxido-reductase family, catalyses the oxidation of cholesterol to cholestenone. Screening was performed to isolate cholesterol oxidase producing organisms. The fungal isolate species producing the highest level of cholesterol oxidase was selected and identified to be *Aspergillus awamori* and *Aspergillus fumigatus*. Enzyme purification was performed by ammonium sulfate precipitation, ultrafiltration and ion exchange chromatography. The final specific activity was found out to be 13.42 units/mg for cell free *A. awamori* and 7.26 units/mg for membrane bound *A. awamori*, whereas the specific activity was found out to be 7.48 units/mg for cell free *A. fumigates* and 13.78 units/mg for membrane bound *A.fumigatus*, at a cholesterol concentration of 0.1%, pH 7 and temperature at 37°C. CHOx has a wide application in determining cholesterol level in various clinical and food samples.

KEY WORDS: Cholesterol oxidase, enzyme purification, *A. awamori*, *A. fumigates*

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INTRODUCTION

Cholesterol, a steroid belongs to the category of terpenoids lipid, has a chemical formula $C_{27}H_{46}O$. It is normally found in nature with an excellent relevance in biology, drugs and chemistry. It also plays an essential role as a structural component of animal cell membrane.

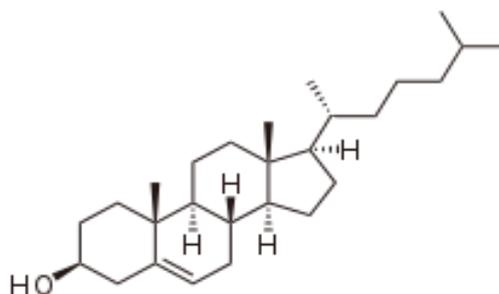


Figure1. Structure of cholesterol.

Cholesterol oxidase: Cholesterol oxidase (3- β hydroxysterol oxidase, EC 1.1.3.6) a member of flavin adenine dinucleotide (FAD) dependent enzymes super family, catalyses the oxidation of cholesterol (cholest-5-en-3 β -ol) to its 3-keto-4-ene derivative, cholestenone (cholest-4-en-3-one), with the reduction of oxygen to hydrogen peroxide. However, some bacterial cholesterol oxidases have also been reported to catalyse oxidation of cholesterol to 6 β -hydroperoxycholest4-en-3-one (HCEO) in place of cholest-4-en-3-one (CEO). Cholesterol oxidase exists in two different forms based on bonding between enzyme and FAD cofactor. At first, the enzyme is non-covalently linked to its FAD cofactor (class I) and secondly, the cofactor is covalently bound to the enzyme (class II)¹

Action of COX: COX catalyses not only the oxidation of D5-ene-3-hydroxysteroids with a Trans A-B ring junction to the corresponding D5-3-ketosteroid, but also to the D4-3 β -ketosteroid. The enzymes have a broad range of steroid specificities dependent on bacterial source and/or chemical modification, the presence of a 3 β -hydroxyl group was an essential requirement for substrate activity in all cases¹. The first isolation of the oxidation product was reported using an NAD⁺ and NADP⁺ independent soil *Mycobacterium*. Enzymes produced by *Nocardia* and other species are generally referred as oxidases because they require oxygen for their mode of action and others are known as dehydrogenases as they are true NAD dependent, e.g. the coupled enzyme 3 β -hydroxysteroid: NAD(P) oxidoreductase-3-ketosteroid D4, D5- isomerase (3 β -hydroxysteroid dehydrogenase: D5- isomerase)².

Organisms producing cholesterol oxidase: Microorganisms of both pathogenic and non-pathogenic nature are producers of cholesterol oxidase. These organisms include *Mycobacterium*, *Brevibacterium*, *Streptomyces*, *Corynebacterium*, *Arthrobacter*, *Pseudomonas*, *Rhodococcus*, *Chromobacterium* and *Bacillus* species. CHOx in most organisms is employed in the initial step of cholesterol metabolism. In pathogenic bacteria, CHOx acts as membrane-damaging factor and thus contributes as a virulence factor in the pathogenicity of these bacteria. In addition, *Streptomyces natalensis* produces a sterol molecule- cholesterol oxidase (PimE) that acts as a signaling macromolecule for the synthesis of an antifungal antibiotic, polyene macrolide pimaricin^{1,2,3}.

MATERIALS AND METHOD

Screening for cholesterol oxidase producer:

Source: Several higher fungi from our campus and lower fungi from soil samples were collected and were grown on potato dextrose agar plates. They were checked for cholesterol utilization by growing them in cholesterol media containing 0.1% cholesterol as the sole source of carbon. The organism showing maximum utilization was used for enzyme purification and characterization. The other organism that had the capability of cholesterol degradation was a contaminant growing in pure cholesterol stock.

Media used for growing the organism: K₂HPO₄ 2.5g/l, NH₄NO₃ 17g/l, MgSO₄.H₂O 2.5g/l, NaCl 0.05g/l, Cholesterol 1.0g/l tween-80 1.0 (ml/L). The pH of medium was adjusted to 7.0 prior to sterilization⁴.

Determination of cholesterol oxidase activity: The activity of cholesterol oxidase produced by the organism was assayed by the method described by Sasaki *et al*⁵. In this method residual cholesterol in the media (after utilization by the organism) in presence of oxygen is oxidized to 4-Cholesten-3-One and hydrogen peroxide is formed as a by-product. It then reacts with 4-aminoantipyrine and phenol catalytically in presence of peroxidase enzyme to form a dye Quinoneimine which is red in colour³. Intensity of colour formed is directly proportional to amount of cholesterol present. One unit (U) of COX is defined as the amount of enzyme required to produce 1mol of 4-cholestene-3-one (or H₂O₂) per minute under the assay conditions.

Protein estimation: Protein (enzyme) concentration was determined by Folin Lowry method using BSA as a standard for plotting a standard curve, measuring the absorbance at 640nm⁶.

1: Residual cholesterol (mg/dl) = Absorption after incubation / Absorption of standard × Concentration of standard

2: Amount of decomposed cholesterol (mg/dl) = Amount of cholesterol (steroid alcohol) in control – Amount of residual cholesterol of sample

3: Percentage of cholesterol decomposition = Amount of decomposed cholesterol/Amount of cholesterol in control × 100

Growth on indicator plates: Culture plates contained the same media as mentioned above along with 0.1% o-Dianisidine. The growth of organism on the indicator plate shows utilization of cholesterol as a result of cholesterol oxidase enzyme leading to production of cholestenone and hydrogen peroxide. Hydrogen peroxide will react with the o-Dianisidinedye and produces brown colour. Thus change in colour around the growth is an indication of the organism producing extracellular cholesterol oxidase.

Identification of the organism producing cholesterol oxidase: CHO producing isolates were identified by their morphological, cultural and biochemical characteristics by standard methods using “Illustrated genera of imperfect fungi Barnett & Hunter 4th edition” Molecular identification of the two fungal organisms was carried out by amplifying the 18S rRNA gene.

Purification of cholesterol oxidase produced by fungi: Fungal cells were grown in an optimized media. The medium was inoculated with 1 g of 24 h old inoculum and incubated for period of 72 hrs for isolate 1 and 96 hrs. for isolate 2. Cells were harvested and centrifuged at 10000 rpm for 15 min at 4°C. The supernatant obtained was used for the purification of extracellular enzyme. The pellet was treated with 50mM phosphate buffer containing TritonX to leach out the enzyme from the cell membrane and supernatant was collected.

Ammonium sulphate precipitation: The above cell free supernatant and membrane bound supernatant was subjected to ammonium sulfate (75% w/v) precipitation. The precipitated proteins were dialyzed against sodium orthophosphate buffer (0.05M; pH 7.0) overnight. The proteins were dissolved in Tris-HCl buffer (0.05M; pH 7.0) and stored at 4°C for further analysis

Ultrafiltration: The supernatant was subjected to ultrafiltration using ultrafiltration tubes of MWCO of 50 kDa. Specific activity was checked for the filter and filtrate respectively.

Ion exchange chromatography: Samples were loaded on DEAE-cellulose ion exchange column which was equilibrated with sodium phosphate buffer (0.05M; pH 7.0). The bound proteins were eluted with a linear gradient of NaCl (0.05-0.5M) in the same buffer. Protein content and cholesterol oxidase activity were determined at each step of purification.

Enzyme characterization: Various physiological parameters were assessed for enzyme characterization like pH, temperature, time of incubation/ reaction, concentration of substrate.

Determination of molecular weight and state of the enzyme: SDS-PAGE was performed according to the protocol described by Lammeli (1970). The resolving gel and the stacking gels were prepared for to 12.5 and 5% respectively. The purified protein sample was run on SDS-PAGE with a concurrent run of standard protein markers. The gel was carefully removed from the glass plates and was stained using the coomassie blue. The molecular mass of the purified protein was determined by comparing the relative mobility value of the unknown protein with known protein molecular weight markers.

RESULTS

Identification of fungi: Morphological and cultural characteristics of the fungi isolates were studied and by referring “Illustrated genera of imperfect fungi Barnett & Hunter 4th edition” they were classified to be of *Aspergillus* species.



Figure 2. *Aspergillus awamori*
Colour change on cholesterol plate



Figure 3. *Aspergillus fumigatus*
No colour change on cholesterol plate

Molecular identification by 18S gene sequencing: The gene sequence obtained with 18S rRNA gene of isolate 1 and isolate 2 was compared with other 18S rRNA gene sequences available in the Gen Bank database of NCBI with the aid of computation program, BLASTN homology analysis. The 18S rRNA gene sequence of the isolate 1 showed 99.08% similarity with sequence of *Aspergillus awamori*. Therefore, the isolate was identified as *Aspergillus awamori*. The 18S rRNA gene sequence of the isolate 2 showed 99.24% similarity with sequence of *Aspergillus fumigatus*. Therefore, the isolate was identified as *Aspergillus fumigatus*.

Cholesterol utilization: Flasks were incubated with cholesterol as sole source of carbon and checked for percentage of decomposition and specific activity. The flask with more specific activity has maximum decomposition.



Figure 5.a. Cholesterol utilization by *A. awamori*

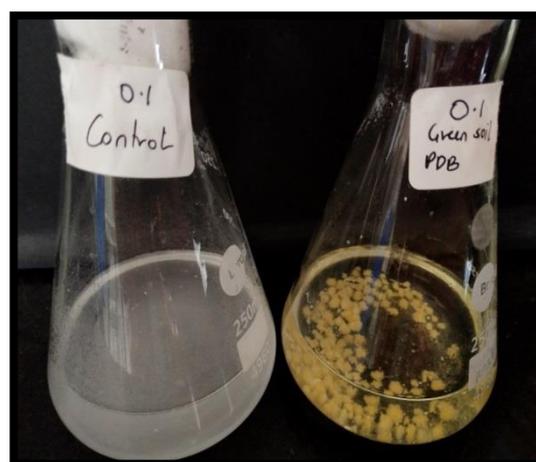


Figure 5.b Cholesterol utilization by *A. fumigatus*

Table: 1 Purification chart for extracellular choxby *A. Awamori*

Purification steps	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Fold purification	% yield
Homogenate	14.9	3.8	3.92	1	100
75% Ammonium sulphate precipitation	12.35	1.4	8.82	2.39	82.8
Ultrafiltration	11.51	1.07	10.76	2.74	77.24
DEAE cellulose	10.73	0.8	13.42	3.64	72.01

Table: 2 Purification chart for membrane bound choxby *A. Fumigatus*

Purification steps	Total activity (units)	Total protein (mg)	Specific activity (units/mg)	Fold purification	% yield
Homogenate	9.18	2.1	4.37	1	100
75% Ammonium sulphate precipitation	7.49	1.7	8.8	2.01	81.5
Ultrafiltration	6.63	1.34	8.97	2.05	72.22
DEAE cellulose	6.89	0.54	13.78	3.15	75.05

Enzyme characterization: Several parameters were checked and optimized to attain highest specific activity of the enzyme under investigation.

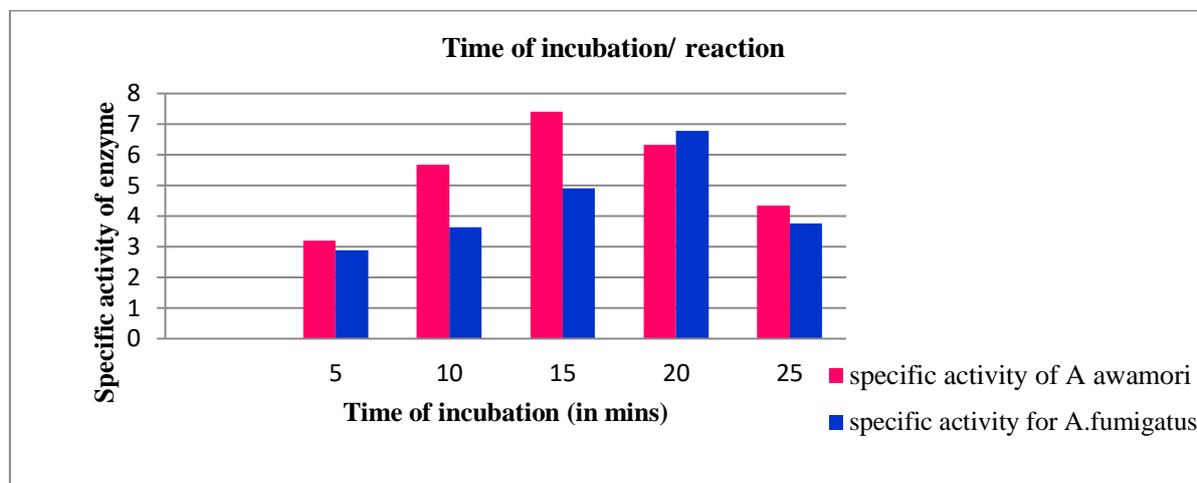


Fig6. Effect of incubation time

Effect of time of incubation was studied and it was observed that the maximum enzyme activity was after 15 minutes of incubation with cholesterol for *A. awamori* and 20 mins for *A. fumigatus*

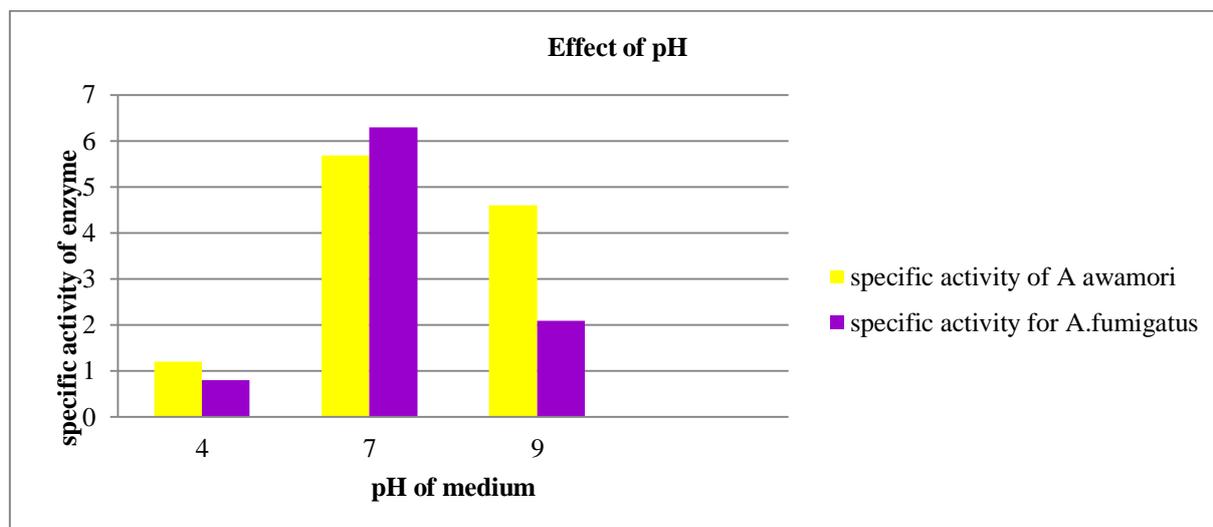


Fig7. Effect of pH

Effect of pH on medium was studied and it was observed that the maximum enzyme activity was at pH 7 for both the *Aspergillus* spp.

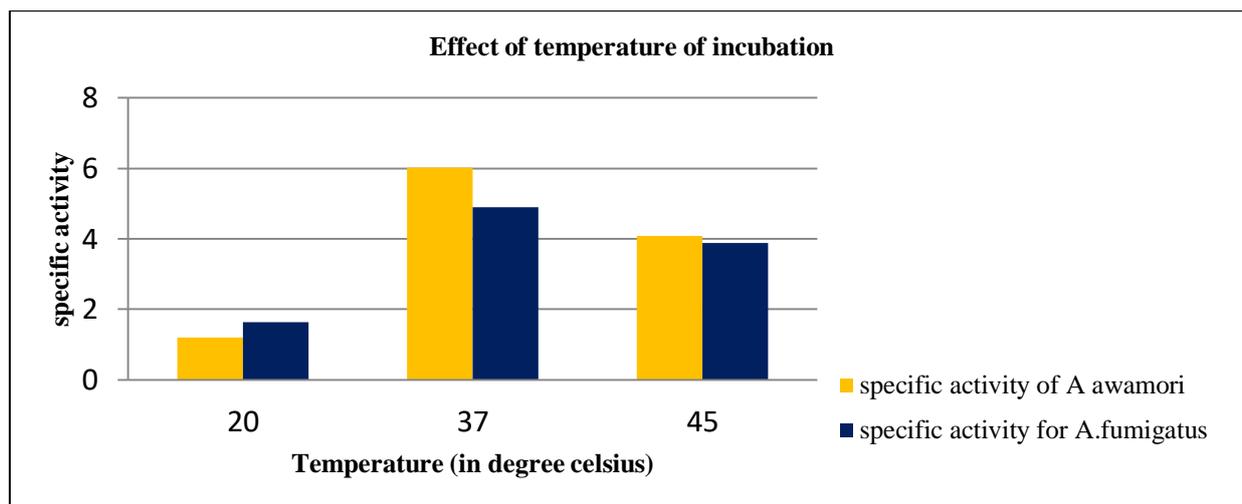


Fig8. Effect of temperature

Effect of temperature of incubation was studied and it was observed that the maximum enzyme activity was at temperature 37 for both of the *Aspergillus* spp.

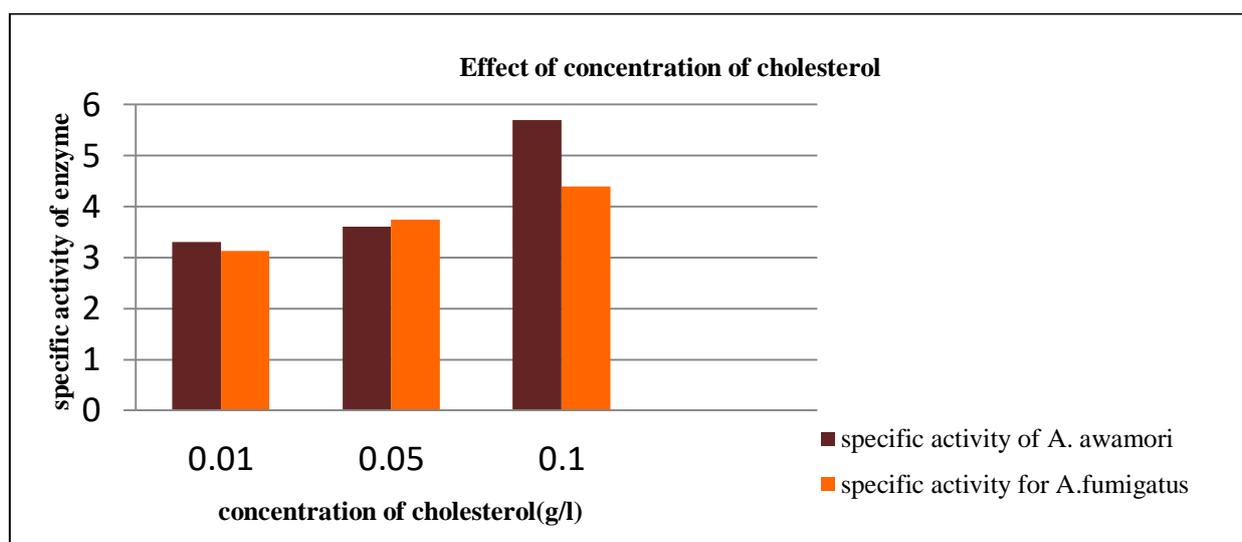


Fig9. Effect of substrate concentration

Effect of substrate concentration was studied and it was observed that the maximum enzyme activity was at a substrate concentration of 0.1g/dl for both the *Aspergillus* spp.

Molecular weight determination by SDS PAGE: On comparing with standard molecular marker the approx. weight was found out to be around 52 KDa.

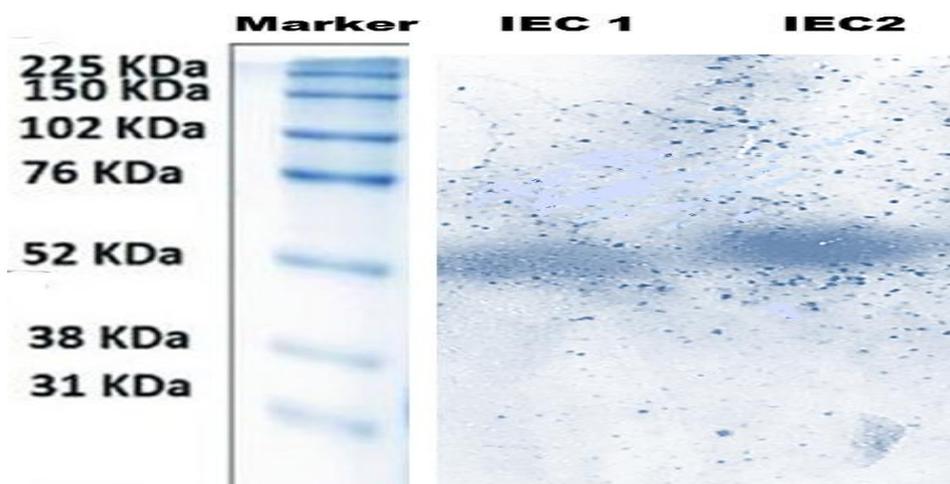


Fig10. Molecular weight determination by SDS PAGE

DISCUSSION

In the present investigation, isolation of fungi was carried out from different sample and were screened for the production of extracellular (cell free) CHOx and membrane bound CHOx both qualitatively and quantitatively. Quantitative detection involved assay of the percentage of substrate cholesterol decomposed (oxidized) by CHOx to cholest-4-en-3-one with simultaneous production of H₂O₂, yielding a chromogenic product (Quinineimine dye) with a maximum absorption at 505nm (Devi, S., & Kanwar, S. S. (2017))³ Purification of extracellular and membrane bound CHOx for both *A.awamori* and *A.fumigatus* was achieved by 80% ammonium sulphate precipitation followed by ion exchange chromatography. The specific activity, a measure of enzyme purity was estimated to be highest for cell free *A.awamori* (13.42 units/mg) and for membrane bound *A.fumigatus* (13.78 units/mg) at a substrate (cholesterol) concentration of 0.1%, pH 7 and temperature 37⁰C. Apart from fungi, various bacteria and actinomycetes have been reported as CHOx producers with differing specific activities (units/mg) which are as follows: *Chromobacterium sp.* 13.9, *Bacillus subtilis* 7.6, *Streptomyces parvus* 20 (Devi, S., & Kanwar, S. S. (2017)) *Pseudomonas sp.* 0.350, *Arthrobacter simplex* 3.6, *Streptomyces lavendulae* 1.140, *Rhodococcus* sp. 0.290 (Kumari, L., & Kanwar, S. S. (2012))² By comparing the specific activities of fungi with other organisms, fungi such as *A.awamori* and *A.fumigatus* were considered to be potent CHOx producers as they are novel sources of CHOx with a high specific activity of 13.42 units/mg (cell free *A.awamori*) and 13.78 units/mg (membrane bound *A.fumigatus*).

CONCLUSION

Aspergillus fumigatus isolated from soil and *Aspergillus awamori* isolated from contaminated stock solution had a great capacity to decompose cholesterol in a medium supplemented with

cholesterol. Under its optimal growth conditions at 37°C, pH 7 and 0.1g of cholesterol concentration, CHOx is an enzyme of great commercial value widely employed by laboratories routinely used for the determination of steroid alcohol (cholesterol) in food, blood serum and different clinical samples. Our preliminary work led to the conclusion that both *Aspergillus sps* might be considered as potentially interesting source of extracellular and membrane bound CHOx for clinical and commercial purposes. A perusal of literature has clearly shown that the existence of oxidase has not been reported from *A. awamori* and *A. fumigatus* which has also been identified as the hyper producer of cholesterol oxidase in the present study. Therefore, this study has provided a novel source for obtaining abundant amount of cholesterol oxidase to meet the needs of the industrial and medicinal fields. These results demonstrate the novelty of the source of cholesterol oxidase.

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