Int.J.Curr.Microbiol.App.Sci (2023) *12*(04): 48-62

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 12 Number 4 (2023)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2023.1204.006

Purification and Kinetics of Cholesterol Oxidase from *Penicillium chrysogenum*

Nessma M. El-Zawawy[1*], Mohammed S. Yousef[1], Hamed M. El-Shora[2], Metwally A. Metwally[1] and Sally M Metwally[1]

[1]Department of Botany, Faculty of Science, Tanta University, Egypt
[2]Department of Botany, Faculty of Science, Mansoura University, Egypt
**Corresponding author*

Keywords

Cholesterol oxidase, Purification, Metal ions, Chelating agents and Active groups

Article Info

Received:
05 March 2023
Accepted:
04 April 2023
Available Online:
10 April 2023

A B S T R A C T

Cholesterol oxidase (CO) was purified from *P. chyrsogenum* by 85% ammonium sulphate precipitation, DEAE-Cellulose and Sephadex G-200. The final specific activity was 300 units mg^{-1} protein and 230-fold. CO was purified to homogeneity as indicated by SDS-PAGE which expressed a single band at 43 KDa. The optimal cholesterol concentration was 1mM. K_m and V_{max} values were 0.53 %w/v and 51.5 Umg^{-1} protein, respectively. The optimal incubation time for enzyme catalysis was 15 min. The optimal temperature was 40^oC and the optimal pH was 7. At 1 mM and 5mM Ca^{+2} activated CO whereas Cu^{+2}, mg^{+2}, Hg^{+2}, B^{+2}, and Fe^{+2} inhibited CO activity. Hg^{+2} was the most potent inhibitor which abolished 69.1% and 86.8% a t 1 mM and 5 mM, respectively. Ethylenediaminetetra acetate (EDTA), ethyleneglycoltetra acetate (EGTA), *0*-phenanthroline and α-α-dipyridyl inhibited CO activity with IC_{50} of 9.7, 7.6, 20.8 and14.2 mM, respectively indicating that CO is a metalloenzyme. N-ethylmaleimide (NEM), dansyl chloride (DnsCl), N-bromosuccinimide (NBS) and diethylpyrocarbonate (DEPC) and butandione (BD) inhibited CO of from *P. chrysogenum* in concentration-dependent manner with IC_{50} values 5.1, 5.8, 6.7, 8.1 and 8.2 mM, respectively indicating the essentiality of sulfhydryl, lysyl, histidyl, tryptophanyl and arginyl groups, respectively.

Introduction

Studying of the industrial enzymes and their uses is known as enzyme technology. The benefits of using enzymes are endorsed to numerous properties. They are specific to their substrates; they cause fewer environmental contamination, act at slight temperatures as well as neutral pH. They produce unwanted by-products (Adrio and Demain, 2014). The wide range of microbial enzymes made them interesting tools for application in different areas including chemical industry, agricultural purposes, food industry, textile industry, pharmaceuticals, wood industry, and cosmetics (Liu and Kokare, 2017).

Commercial applications of enzymes are classified into various groups including food feed and detergents (Sharma *et al.*, 2010). Feed and food signifies the largest section for industrial enzymes. However, detergents signify the other group of industrial enzymes (Adrio and Demain, 2014).

Cholesterol is essential for different metabolic pathways in mammal's bodies. Cholesterol is involved in transport of fat in blood stream and is a part of the structure of cell membranes (Doukyu and Ishikawa, 2020).

Cholesterol is the important in synthesis of vitamin D. One of the main roles of cholesterol is its involvement in synthesis of bile acids. They have role in absorption of fats from the intestine (Sagermann, *et al.,* 2010; Kumari and Shamsher, 2015; Kalaivani and Suja, 2020).

Cholesterol oxidase (CO, EC.1.1.3.6) is bi-functional enzyme belongs to the first group of enzymes which are oxidoreductases (El-Naggar *et al.,* 2018; Devi *et al.,* 2019; Perdani *et al.,* 2020). The enzyme was found in fungi (Perdani *et al.,* 2019) and actinomycetes (Kumari and Shamsher, 2012).

CO oxidizes cholesterol and converts 5-cholesten-3β-ol into 4-cholesten-3-ones (Bholay *et al.,* 2013). Cholest-4-en-3-one is an essential synthetic intermediate in transformation of many steroids. Moreover, cholest-4-en-3-one is involved in synthesis of drugs (Wu *et al.,* 2015). CO is produced by different microorganisms as an extra-cellular or intra-cellular enzyme (Lashgarian *et al.,* 2016; Perdani *et al.,* 2019). The present work aimed to purify CO from *P.chyrsogenum* and studying its properties.

Materials and Methods

Experimental Organism

Penicillium chyrsogenum was provided by Prof. Metwally A. Metwally, Prof. of Microbiology, Botany Department, Faculty of Science, Tanta University, Egypt.

Preparation and determination of CO activity

The enzyme extract was prepared and assayed by the method of Ruiz-Herrera and Starkery (1969).

Purification of CO

CO precipitation by ammonium sulphate

Ammonium sulphate was used to precipitate the extracellular CO from the fungal culture, in ice bath, till desired saturation (85% w/v) according to Bollag *et al.,* (1996).

Ion-Exchange chromatography

DEAE-cellulose was made by soaking 3g of DEAE-cellulose in phosphate buffer (pH 7.0) for 24h, then packing the column (2×40cm). The column was equilibrated by phosphate buffer (pH 7.0). The flow rate was 1 ml/5 min and the concentrated enzyme (5ml) dialysate was uploaded to the column of DEAE-cellulose.

The enzyme in the gel was equilibrated by 30 ml phosphate buffer (pH 7.0). CO was then eluted by NaCl concentrations (100, 150, 200 mM). The activity of CO as well as the protein content was measured in each fraction. The active fractions were pooled and concentrated by dialysis against polyethyleneglycol (PEG).

Gel-filtration chromatography

The dialysis was fractionated by gel filtration chromatography using Sephadex G_{200} column. Sephadex G_{200} particles (2g) were soaked in 100 ml potassium phosphate (pH 7.0) overnight at 4ºC, then carefully packing to the column (2×40cm).

The column was pre-equilibrated with potassium phosphate (pH 7.0) and the rate of buffer flow was assessed. The column was maintained at 4ºC in refrigerator during the fractionation process. CO was eluted by potassium phosphate buffer (pH 7.0) at 1 ml/ 10min flow rate.

The activity of CO and protein content of the fractions were measured. The active fractions were pooled and consumed for investigating the biochemical properties.

Biochemical characterization of the purified CO

Effect of substrate concentration on CO activity

The effect of cholesterol concentration as a substrate for CO activity was studied using cholesterol at various concentrations (0.2, 0.4, 0.6, 0.8 and 1.0 mM). The Michaels constant (Km) and the maximum velocity (V_{max}) of CO was evaluated by plotting the relation between the reciprocal of substrate (S) against the reciprocal of velocity (V).

Effect of incubation time on CO

To study the effect of incubation time on CO activity, the enzyme mixture was incubated for 5,10, 15, 20, 25 and 30 min followed by determination of CO activity.

Effect of incubation temperature on CO activity

The enzyme activity was measured at temperature range 20-60oC. The other factors affecting the enzyme activity such as substrate concentration, enzyme concentration and pH were kept constant.

Effect of pH on CO activity

Effect of pH of reaction mixture on CO activity was determined at different pH values (4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8 and 8.5) using various buffers: 50 mM acetate buffer (pH 3.0-5.0), 50 mM phosphate buffer (pH 5.0-8.0) and 50mM sodium carbonate buffer (pH 9.0-10.0).

Effect of some cations on CO activity

The effect of various metal ions on CO activity from *P.chrysogenum* was tested. The CO activity was measured in the presence of Ca^{+2}, Cu^{+2}, Mg^{+2}, Hg^{+2}, Ba^{+2} and Fe^{+3} as chloride salt at 1mM and 5 mM in the assay mixture and CO activity was determined.

Effect of chelating agents on CO activity

Effect of EGTA and EDTA on CO activity

The effect of EGTA (ethylene glycol tetraacetate**)** and EDTA (ethylene daiamine tetraacetate**)** on CO activity was investigated. They were tested at 2, 4, 6, 8, and 10 mM in the reaction medium followed by determination of enzyme activity and calculating the relative activity (%of the control value).

Effect of *o*-phenanthroline and α-α-dipyridyl on CO activity

The chelating agents of *0*-phenanthroline and α-α-dipyridyl were tested at 5, 10, 15, 20, 25 and 30 mM in the reaction medium followed by enzyme assay as mentioned before.

Effect of various reagents of active groups in enzyme protein

Effect of N-ethylmaleimide (NEM) on CO activity

Purified CO was incubated for 1 h at 25 ºC in 100 mM Tris-HCl (pH 5.0). NEM was tested at 2, 4, 6, 8 and 10mM. After 60 min of the incubation aliquot of the mixture was withdrawn and assayed for CO activity.

Effect of dansyl chloride (DnsCl) on CO activity

Stock solution of DnsCl in acetonitrile was prepared. CO (0.5 ml) was incubated for 60 min at 4 ºC with different concentrations of dnsCl (2, 4, 6, 8, and 10 mM) in 100 mM Tris (pH 7).

The reaction was terminated by 20 mM βmercaptoethanol and 30 mM lysine (El-Shora *et al.*, 2015).

Effect of diethyl pyrocaronate (DEPC) on CO activity

Dimethyl pyrocaronate (DEPC) was prepared (Hirs and Timasheff, 1977).The purified CO (0.5ml) incubated with 0,2,4,6,8 and 10 mM DEPC at 25 ºC in assay buffer for 5 min prior to enzyme assay and subsequently the activity of CO determined and the results were depicted as graph.

Effect of N-bromosuccinamide (NBS) on CO activity

Tryptophanyl group of CO was modified using NBS according to Williams (1975). Aliquots of each concentration of NBS (2, 4, 6, 8, and 10 mM) were added to 5 ml of CO in 15 mM phosphate buffer (pH7.0). The modified CO was dialyzed against distilled water after 1 h of stirring at 28± 2 ºC for 24 h and the enzyme was assayed.

Effect of butanedione (BA) on CO activity

The modification was carried out at room temperature according to Willassen and little, (1989) and Bihzad and EL-Shora (1996). BA solution was tested at various concentrations (2, 4, 6, 8 and 10 mM) added to CO (5 ml) in 50 mM sodium borate buffer (pH 8.0) to start the modification reaction. CO incubated with the buffer alone represents the untreated control and the activity was measured.

Results and Discussion

Purification of CO

CO was isolated and purified by 85% ammonium sulphate, DEAE-Cellulose and Sephadex G-200. The results are summarized in Table 1. The results indicate that CO was purified with specific activity of 300 units mg^{-1} protein. The yield was 12.1% and the purification fold was 230. The purity of CO was confirmed by SDS-PAGE which showed a single band at 43 KDa (Fig.1)

Effect of cholesterol concentration on CO activity

The effect of cholesterol as substrate concentration on CO activity was examined using various concentrations of cholesterol (0.2, 0.4, 0.6, 0.8 and 1.0 mM). CO activity was determined in presence of each concentration. The results in Fig. 2A demonstrates that there was continuous increase in the enzyme activity with increment in substrate concentrations up to 1.0 mM where the activity was 16.5 units mg^{-1} protein after which there was steady increase in the enzyme activity at the higher concentration of cholesterol. Plotting the reciprocal of substrate concentration (1/S) against the reciprocal of velocity of enzyme (1/V) gave straight line Fig 2B. The K_m and V_{max} values are calculated from this figure and their values were 0.53 % (w/v) and 51.5 units mg^{-1} protein, respectively.

Effect of incubation time on CO activity

The effect of incubation time on CO activity was carried out at various time intervals (5, 10, 15, 20, 25 and 30 min). The results are shown in Fig. 3. The results indicate that increasing the time of incubation from 5 to 15 min led to increase in CO activity. The activity after 15 min was 15.8 units mg^{-1}protein increasing the time after 15 min caused reduction of CO activity continuously and reached 11.0 units mg^{-1}protein after 30 min.

Effect of temperature CO activity

The effect of incubation temperature on CO activity was investigated at various temperatures (20, 25, 30, 40, 45, 50, 55 and 60^{o}C). The other factors affect the enzyme activity was retained constant including pH, enzyme concentration and substrate concentration.

The obtained results show that there was continuous increment with the increase in the incubation temperature Fig. 4. The optimum temperature was 40^{o}C after which the activity declined gradually.

Effect of pH CO activity

For studding the effect of pH on CO activity, the other factors which affect the enzyme activity were kept constant such as temperature, enzyme concentration and substrate concentration. Various pH values were tested (4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8 and 8.5) for their effect on CO activity. The results demonstrate that there was continuous increase in CO activity with the increase in pH up to 7 which is the optimum pH for the enzyme catalysis, and after which continuous decrease in CO activity at the extreme pH values (Fig. 5).

Effect of some cations on CO activity

The effect of various cations including Ca^{+2}, Cu^{+2}, Mg^{+2}, Hg^{+2}, $Ba^{+2}$and Fe^{+3} on CO activity was investigated. These cations were tested at both 1mM and 5 mM in the form of chloride salts. The results are shown in Fig. 6A. It is apparent that Ca^{+2} was activator at both 1 mM and 5 mM, where it activated the enzyme with 22% and 34%, at 1mM and 5mM, respectively. Mg^{+2} did not show any effect on CO activity. On the other hand, the remained cations including Cu^{+2}, Hg^{+2}, Ba^{+2} and Fe^{+3} inhibited the enzyme at both tested concentrations with different rates.

Effect of Ca^{+2}concentrations on CO activity

Since Ca^{+2} was the most effective cation in enhancing CO activity and the results are shown in Fig.6B. Plotting the relation between the reciprocals of Ca^{+2} (1/S) concentrations and the enzyme velocity (1/V), it is apparent that this relation follows Lineweaver-Burk plot. The values of V_{max} and K_m were 16.9 units mg^{-1} protein and 0.29 %w/v, respectively.

Effect of chelating agents on CO activity

Ethylene daiamine tetraacetate (EDTA) and ethylene glycol tetraacetate (EGTA)

EDTA and EGTA as chelating agents were tested at various concentrations (2- 10 mM). The results were illustrated in Fig. 7A and Fig. 7B. The activity of CO decreased gradually with increasing EDTA the concentration. The IC_{50} of EDTA was 7.7 mM and the IC_{50} of EGTA was 5.8mM.

Effect of *o*-phenanthroline and on α- α dipyridyl CO activity

The effect of *o*-phenanthroline and α-α dipyridyl as chelating agents were examined at various concentrations (2, 4, 6, 8 and 10 mM) on CO activity was investigated. The relative activities were calculated. The results in Fig.7C indicate apparent reduction of CO with increasing *o*-phenanthroline concentration. IC_{50} of *o*-phenanthroline was 8.5 mM.

Also, there was continuous decrease in CO with increasing in α- α dipyridyl concentration (Fig. 7D) and the IC_{50} of α- α dipyridyl was 5.7 mM. The inhibition of CO by EDTA, EGTA, *o*-phenanthroline and α-α dipyridyl reveals that CO is a metalloenzyme.

Effect of various reagents of active groups in enzyme protein

Effect of N-ethylmaleimide (NEM) on CO activity

The effect of NEM as a reagent for sulfhydryl group on CO was investigated and the results are shown in Fig.8A.The enzyme activity decreased gradually in a concentration dependent manner. The IC_{50} of NEM was5.1mM.

Effect of dansyl chloride (DnsCl) on CO activity

DnsCl is a reagent used for detection of lysyl group in protein EL-Shora *et al.*, (2015). The effect of DnsCl on CO activity was investigated at various concentrations (2, 4, 6, 8 and 10 mM).

The relative activity was calculated. The results in Fig. 8B indicate continuous decrease in the enzyme activity with the increase in DnsCl concentrations. IC_{50} of DnsCl was 5.8 mM.

Effect of diethylpyrocarbonate (DEPC) on CO activity

DEPC is used as a reagent for histidyl group in the enzyme molecule. This compound was tested at various concentrations (2-10 mM) and CO activity was determined for each concentration. The results indicate continuous reduction of the CO activity with the increase of DEPC concentration and the IC_{50} value was 6.7 mM Fig. 8C.

Effect of N-bromosuccinimide (NBS) on CO activity

N-bromosuccinimide is an indicator for tryptophanyl group in protein EL-Shora *et al.*, (2015). Therefore, this experiment aimed to investigate whether tryptophanyl group is essential for CO catalysis. This compound is tested at various concentrations (2- 10 mM). The results are shown graphically in Fig. 8D. The results show that there was continuous reduction in CO activity in presence of NBS and the reduction was concentration-dependent and the IC_{50} of NBS was 6.1 mM.

Table.1

Step	Total protein (mg)	Total units	Specific activity ($unitsmg^{-1}protein$)	Yield (%)	Fold of purification
Crude extract	475	617.5	1.3	100	1
Ammonium sulphate (85%)	108	432	4.0	69.9	3.1
DEAE-Cellulose	6.4	160	25.0	25.9	19.2
Sephadex G-200	6.25	75	300.0	12.1	230

Fig.1 SDS-PAGE of CO from *P. chrysogenum.*

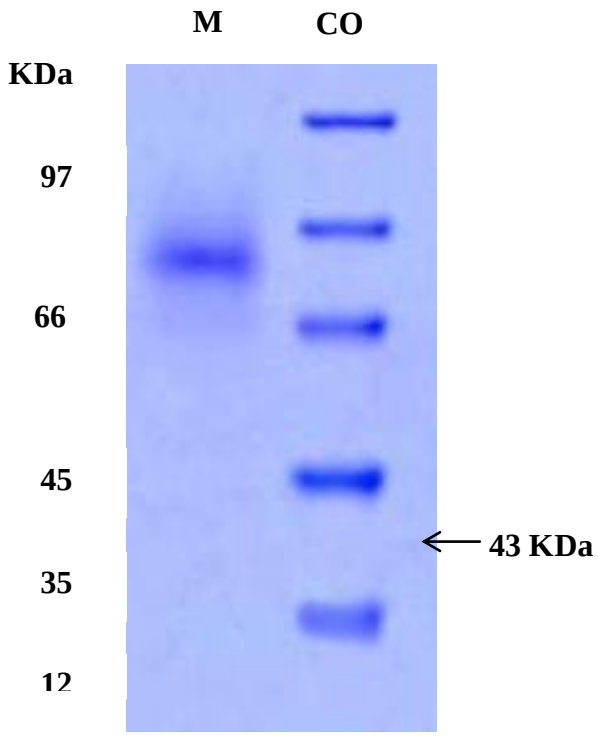


Fig.2 A) Effect of substrate concentration on CO activity and B) Lineweaver-Burk plot of CO.

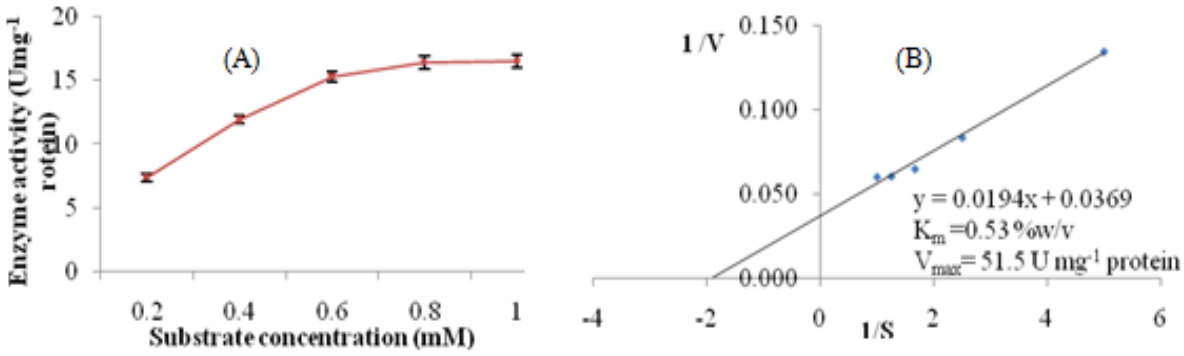


Fig.3 Effect of incubation time on CO activity.

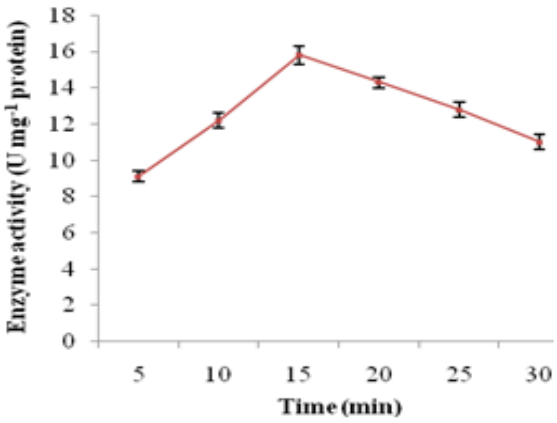


Fig.4 Effect of temperature on CO activity.

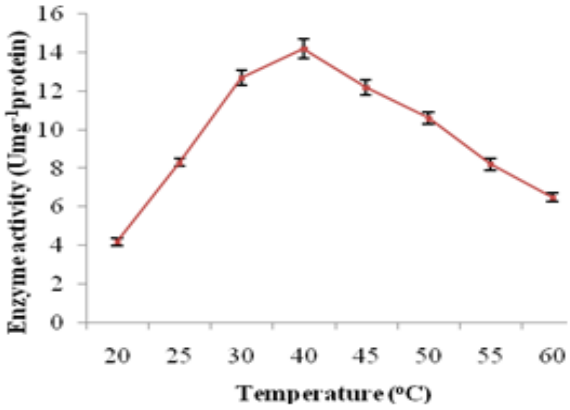


Fig.5 Effect of pH on CO activity.

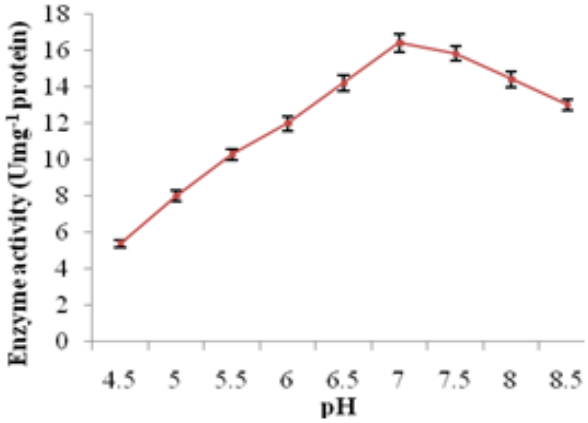


Fig.6A Relative activity of CO in presence of some cations.

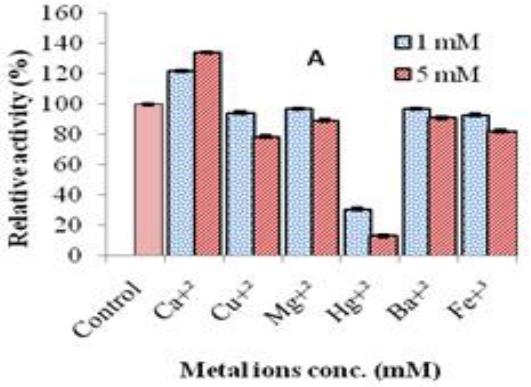


Fig.6B Lineweaver-Burk plot with Ca^{2+}.

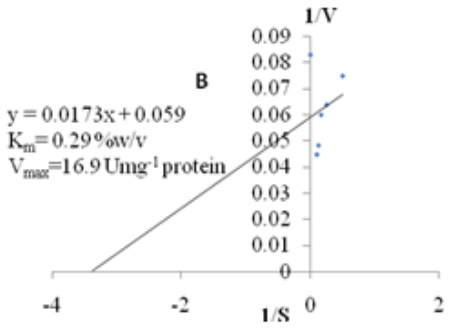


Fig.7A The relative activity of CO in presence of EDTA

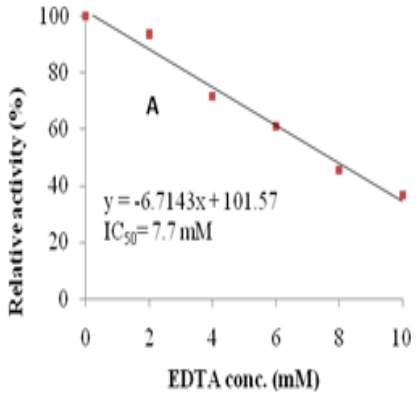
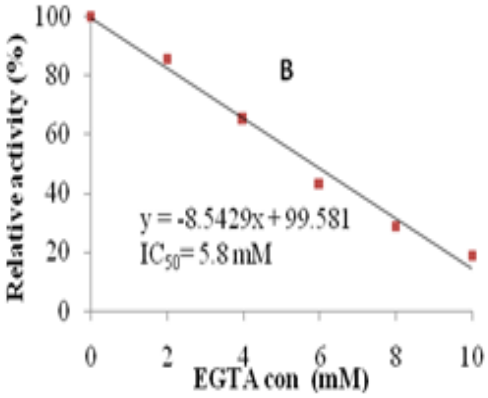


Fig.7B EGTA



Effect of butanedione (BD) on CO activity

In this experiment the influence of BD as a reagent of arginyl group EL-Shora *et al.*, (2019) on CO activity was studied. The results are illustrated in Fig. 8E and indicate that BD inhibited CO activity gradually depending on the concentration and the IC_{50} of BD was 8.2 mM.

CO was purified from *P. chrysogenum* by 85% ammonium sulphate precipitation, DEAE-Cellulose and Sephadex G-200. The final specific activity was

300 units mg^{-1} protein and 230 fold. CO was purified to homogeneity as indicated by SDS-PAGE which expressed a single band at 43 KDa. The specific activity of purified CO from *Streptomyces sp.* AKHSS was 1.99 units mg^{-1} with 16.58-fold purification and 23.68% yield (Kavitha and Savithri, 2019). El-Naggar *et al.*, (2018) reported the purification of CO from *Stryptomyces aegyptia* NEAE 102 through ammonium sulphate precipitation, dialysis and DEAE Sepharose CL-6B ion exchange chromatography with specific activity 16.08 units mg^{-1}protein and 3.14-fold. Yehia *et al.*, (2015) reported that the partial purification of the enzyme from *Enterococcus hirae* increased the enzyme's specific activity from 53.30 to 124.87 units mg^{-1}protein, which corresponds to 2.3-fold purification and 79 % yield on Sephadex G-100 purification step. The specific activity of the obtained pure CO from *Staphylococcus epidermidis* was 62 units mg^{-1} protein with43.4 fold. However, the specific activities from *Entrobacter sp.*, *Bordetella sp*. and *Rhodococcus sp*. were 25.2, 21.0 and 5.5 units mg^{-1}protein, respectively (Elalami *et al.*, 1999; Ye *et al.*, 2008; Lin *et al.*, 2010). These results revealed that there was an increase in CO activity with the increase of incubation till 15 min which was the optimal time. The results also reveal that there was continuous increase in the enzyme activity with increasing of substrate concentrations up to 1.0 mM where the activity was 16.5 units mg^{-1} protein after which there was steady increase in the enzyme activity at the higher concentration of cholesterol as substrate. After the initial period, the rate of reaction decreases and shows that the substrate is present in excess. The explanation of this phenomenon is the reduction of enzyme activity after a period of time. This may be due to the effect of heat of incubation on the tertiary structure of the enzyme or to formation of side-product of the reaction, which inhibits the enzyme activity (Lizotte *et al.*, 1990). The obtained relationship between the velocity (V) and concentration (S) of CO reaction is in accordance with the typical enzyme reaction (Michaels and Menten, 1913). Also, it was apparent that the typical addition of excessive amounts of the substrate resulted in lowering the velocity of enzyme reaction. This may be due to the fact that higher substrate concentration may cause an inhibition of the enzyme reaction (Palmer, 1995). K_m and V_{max} values of CO from *P. chrysogenum* were 0.53 mM and 51.5 units mg^{-1} protein, respectively. Also, The $K_m$of CO was 0.026 and 0.556 mM from *Chromobacterium sp*. (Doukyu *et al.*, 2008) and *Bordetella* sp. (Lin *et al.*, 2010), respectively. Generally, the advantages of knowing K_m value for an enzyme of interest. Firstly, we can predict the need of cells to more enzymes or more substrate to speed up the reaction. Some enzymes are usually not saturated by substrates (E-Shora *et al.*, 2016). Second, K_m value represents approximate inverse measure of the affinity of the enzymes that can catalyze reactions with two similar subrates, the substrate for which the enzyme has the lower K_m is one most frequently acted upon the cell (Devasena, 2010).

Fig.7C *o*-phenanthroline

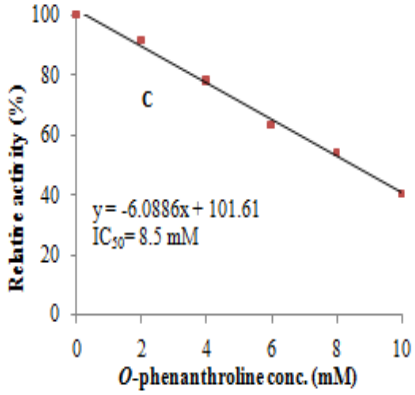


Fig.7D α-α dipyridyl

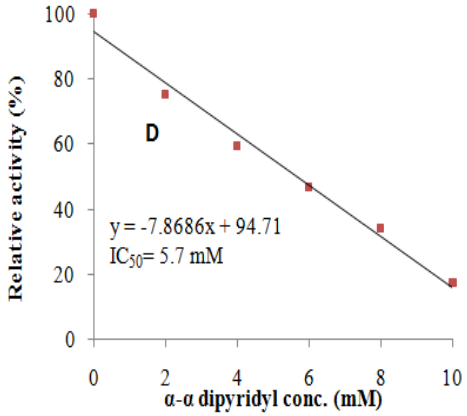
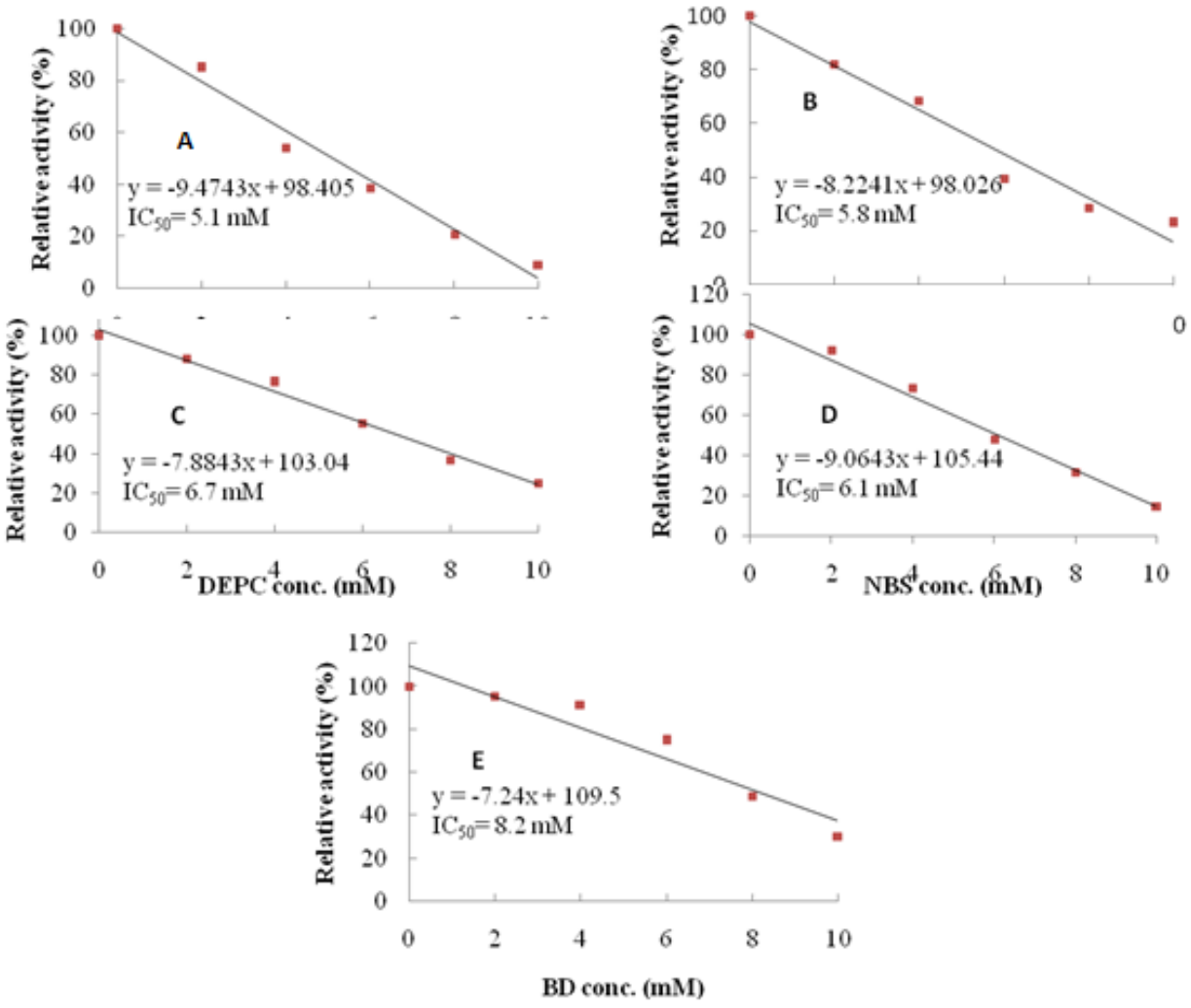


Fig.8 The relative activity of CO in presence of NEM (Fig. 8A), DnsCl(Fig. 8B), DEPC (Fig. 8C),NBS (Fig. 8D) and BD (Fig. 8E).



Thirdly, the K_m gives approximately measure of the cell in which reaction occurs. For example, enzymes can catalyze reactions with the relatively concentrated substrates usually have relatively high K_m values for their substrates and enzymes that react with substrates present in very low concentrations have much lower K_m values for their substrates (EL-Shora *et al.,* 2015).

The results elucidated that the optimum temperature CO from *P.chrysogenum* was 40°C after which the activity declined gradually at the higher temperatures. This result was in agreement with results obtained from *Staphylococcus epidermidis* when the optimum temperature of CO was 40°C (EL-Shora *et al.,* 2011). Also, it is similar to that reported for the enzyme from *Rhodococcus equi* (Richmond, 1973). Other optimum temperature was reported for the enzyme from *Proteobacterium* (Isobe *et al.,* 2003), *Brevibacterium sterolicum* (Fujishiro *et al.,* 2002) and *Chromobacterium* sp. (Doukyu *et al.,* 2008). The heat stability of CO is useful when supplying it in a process involving cholesterol degradation, e.g. food-cholesterol degradation (Wang *et al.,* 2008).The heat stability of an enzyme is affected by two factors. The first one is the primary structure of the enzyme. The high content of hydrophobic anion acids provides a compact structure, which cannot denaturants easily by change in the external environment.

The optimum pH for CO from *P.chrysogenum* was 7.0. This result was harmony with results obtained by (Yehia *et al.,* 2015). Generally, enzymes are sensitive to changes in pH value. The pH can influence the enzyme activity in a number of ways. 1: it can change the ionization of enzyme substrate complex. 2: it can change the ionization of various group of enzyme molecule which way affect the affinity of the enzyme for its substrate. 3: It changes the ionization of the substrate which way affect binding of substrate to enzyme.4: At the extreme PH it can brings changes in protein structure (Palmer, 1995).

On the other hand, the remained cations including Cu^{+2}, Mg^{+2}, Hg^{+2}, Ba^{+2} and Fe^{+3} inhibited the enzyme at both tested concentrations with different rates. This inhibition of CO by Cu^{+2} and Hg^{+2} might be due to oxidation of sulfhydryl groups of the enzyme. Shah and Dubey (1997) reported that heavy metal ions could replace the essential metal ions in the active center of enzymes, thus inhibiting the enzyme activity.CO activity was markedly inhibited by Hg^{+2} and Cu^{+2} (Doukyu, 2009). $FeCl_3$ remarkably inhibited the activity of CO from *Streptomyces violascens* (Tomioka *et al.,* 1976). Cu^{+2} inhibited CO from *Streptoverticillium cholesterolieum* (Inouye *et al.,* 1982). Ca^{+2} was activator at both 1 mM and 5 mM, where it activated CO with 22% and 34%, respectively. Mg^{+2} did not show any effect on CO activity. These results were in harmony with those obtained by EL-Shora *et al.,* (2011) for CO from *Staphylococcus epidermidis*. The activation of CO by Ca^{2+} could be attributed to the strengthening of interactions inside CO molecules and by the binding of Ca^{2+} to the autolysis site. Ca^{2+} activated other enzymes such as phytase (El-Shora and Abo-Kassem, 2000). The increase in the activity of the enzyme in the presence of Ca^{+2} may be attributed to stabilization of enzyme in its active conformation rather than involving in the catalytic reaction. It is possible to act as a salt or ion bridge through a cluster of carboxylic groups as has been suggested to maintain rigid conformation of the molecule (EL-Shora *et al.,* 2009)

The inhibition of CO by EDTA, EGTA, α- α dipyridyl and *o*-phenanthroline seems likely to be mediated through chelation of the metal present in CO molecule converting it to non-active enzyme (EL-Shora *et al.,* 2015). Other microbial enzyme have been reported to be inhibited by EDTA such as *β*-lactamase (Marchiaro *et al.,* 2008), protease (El-Shora *et al.,* 2009) and *β*-glucanase (EL-Shora *et al.,* 2019). The inhibition of CO activity by the four chelating compounds indicates that CO is a metalloenzyme.

NEM, DnsCl, NBS, DEPC and BD inhibited CO of from *P. chrysogenum* in concentration-dependent manner. The inhibition of CO by these compounds

reveals the necessity of sulfhydryl, lysyl, histidyl, tryptophanyl and arginyl groups for enzyme catalysis (EL-Shora *et al.*, 2015; EL-Shora *et al.*, 2016; EL-Shora *et al.*, 2019). Generally, it is remarkable that the chemical modification studies could selectively target the enzyme residues specifically at the active site. In fact, these results illustrate the usefulness of such study in preliminary identification of the groups in enzyme molecules responsible for catalysis.

References

Adrio, J. L. and Demain, A. L. (2014). Microbial enzymes: Tools for biotechnological processes. *Biomolecules*, 4: 117–139. https://doi.org/10.3390%2Fbiom4010117

Bholay A. D., Gadekar D. J., Sawant S. K., and Sonawane S. M. (2013). Bacterial extracellular cholesterol oxidase and its pharmaceutical perspectives. *International Journal of Current Microbiology and Applied Science*, 2: 19-28.

Bollag, D. M., Rozycki, M. D. and Edelstein, S. J. (1996). Protein methods, 2nd edn. Wiley-liss, Indiana, 156-172.

Devasena, T. (2010). Enzymology.Oxford University Press.

Devi S., Sharma B., Kumar R. and Kanwar S. S. (2019). Purification, characterization and biological cytotoxic activity of the extracellular cholesterol oxidase produced by *Castellaniella sp*. COX. *Journal of Basic Microbiology*, l:1-15.

Doukyu N, and Ishikawa M (2020). Cholesterol oxidase from *Rhodococcuserythropolis* with high specificity toward *β*-cholestanol and pytosterols. PLoS ONE 15(10): e0241126. https://doi.org/10.1371/journal.pone.0241126

Doukyu N, Shibata K, Ogino H and Sagermann M (2008). Purification and characterization of *Chromobacterium*sp.DS-1 cholesterol oxidase with thermal, organic solvent, and detergent tolerance. *Applied Microbiology and Biotechnology*, 80: 59 -70.

Doukyu, N. (2009). Characteristics and biotechnological applications of microbial cholesterol oxidases. *Applied Microbiology and Biotechnology*, 5: 825-837. https://doi.org/10.1007/s00253-009-2059-8

Elalami, A., Kreit, J., Filali-Maltouf, A., Boudrant, J., and Germain, P. (1999). Characterization of a secreted cholesterol oxidase from *Rhodococcus sp*. GKI (CIP 105335). *World Journal of Microbiology and Biotechnology*, 5: 579-585. https://doi.org/10.1023/A:1008958008333

El-Naggar N. E., Soliman H. M. and El-Shweihy N. M. (2018). Extracellular cholesteroloxidase production by *Streptomyces aegyptia*, *in vitro* anticancer activities against rhabdomyosarcoma, breast cancer cell-lines and *in vivo* apoptosis. *Scientific Reports*, 1: 2706 https://doi.org/10.1038/s41598.

El-Shora, H. M. ;Doaa B. Darwish and Mai M. Taha (2011). Purification, characterization and active groups of bacterial cholesterol oxidase. *Journal of Agricultural Chemistry and Biotechnology*. Mansoura University, 2: 205-215. https://dx.doi.org/10.21608/jacb.2011.57269

El-Shora, H. M., and Abo-Kassem, E. M. (2000). Regulation and characterization of phytase activity in *Latuca sativa* cotyledons. *Bull.* Faculty of Science. Assiut University, 29: 389-403.

El-Shora, H. M., and Metwally, M. (2009). Effect of phytohormones and group selective reagents on acid phosphatase from *Cladosporium cladosporioides*. *Asian Journal of Biotechnology*, 1:1-11. https://doi.org/10.3923/ajbkr.2009.1.11

El-Shora, H. M. EL-Shobaky, A. M and AL-Atrozy, M. M (2016). Activity of purified bacterial myrosinase and its essential residues. *International Journal Current Microbiology and Applied Science*, 2: 567-578. http://dx.doi.org/10.20546/ijcmas.2016.501.064

El-Shora, H. M. Ghada, S. F. and Amany, H. S. (2015a). Purification, thermosatability and chemical modification of pullulanase from

sunflower. *International Journal Current Microbiology and Applied Science,* 12: 284-296.

El-Shora, H. M., Metwally, M and Abd El-Rheem, A. M. M (2019). Biochemical characteristics of *β*-glucanase from *Penicillium chrysogenum*. *Research Journal of Pharmaceutical, Biological and Chemical Sciences,* 10: 3-37. https://doi.org/10.33887/rjpbcs/2019.10.3.37

El-Shora, H. M., Metwally, M. A., and Khalaf, S. A. (2009). Essential groups and stability of α-glucosidase of *Penicllium notatum*. *Annls of Microbiology,* 2: 285-291. https://doi.org/10.1007/BF03178330

Fujishiro K, Uchida H, Shimokawa K, Nakano M, Sano F Ohta T, Kayahara N, Aisaka K, Uwajima T (2002) Purifiation and properties of a new *Brevibacterium sterolicum* cholesterol oxidase produced by *E. coli* MM294/pnH10. FEMS Microbiol Lett. 215: 243–248. https://doi.org/10.1111/j.1574-6968.2002.tb11397.x

Hirs, C. H. W. and Timasheff, S. N. (1977). Modification of histidyl residues in protein by diethylpyrocarbonate. *Methods Enzymology,* 47: 431-441. https://doi.org/10.1016/0076-6879(77)47043-5

Inouye, S., Soberon, X., Franceschini, T., Nakamura, K., Itakura, K., and Inouye, M. (1982). Role of positive charge on the amino-terminal region of the signal peptide in protein secretion across the membrane. *Proceedings of the National Academy of Sciences,* 11: 3438-3441. https://doi.org/10.1073%2Fpnas.79.11.3438

Isobe K, Shoji K, Nakanishi Y, Yokoe M, Wakao N (2003) Purifiation and some properties of cholesterol oxidase stable in detergents from gamma-proteobacterium Y-134. J BiosciBioeng. 95: 257–263. https://doi.org/10.1016/s1389-1723(03)80026-9

Kalaivani, G. J. and Suja, S. K. (2020). Cholesterol oxidase immobilized inulin based nanocomposite as the sensing material for cholesterol in biological and food samples. *Enzyme and Microbial Technology,* 140: 109631. https://doi.org/10.1016/j.enzmictec.2020.109631

Kavitha, A. and Savithri, H. S. (2019). Characterization of cholesterol oxidase from a marine *Streptomyces sp.* and its cytotoxicity. *Process Biochemistry,* https://doi.org/10.1016/j.procbio.2019.10.024

Kumari L. and Kanwar S. S. (2012). Cholesterol oxidase and its applications. *Scientific Research,* 2: 49-65 http://dx.doi.org/10.4236/aim.2012.22007

Kumari, L. and Shamsher, K. S. (2015). Cholesterol oxidase: Role in biotransformation of cholesterol. *Applied Microbiology and Biotechnology,* 3: 53-65. http://dx.doi.org/10.7324/JABB.2015.3609

Lashgarian H. E., Jahanbakhsh S. and Shahzamani K. (2016). Molecular identification of cholesterol oxidase enzyme producing streptomyces bacteria in soil of Lorestan Province, Iran. *International Journal of Medical Research and Health Sciences*, 5:54-62.

Lin Y L, Fu J A and Song X (2010). Purification and characterization of an extracellular cholesterol oxidase from a *Bordetella species*. *Process Biochemstery*. 45: 1563–1569. http://dx.doi.org/10.1016/j.procbio.2010.06.005

Liu, X., and Kokare, C. (2017). Microbial enzymes of use in industry. In: *Biotechnology of Microbial Enzymes*, 267-298. Academic Press. http://dx.doi.org/10.1016/B978-0-12-803725-6.00011-X

Lizotte, P. A., Henson, C. A. and Duke, S. H. (1990). Purification and characterization of pea epicotyl β-amylase. *Plant Physiology*, 3: 615-621. https://doi.org/10.1104%2Fpp.92.3.615

Marchiaro, P., Tomatis, P. E., Mussi, M. A.,

Pasteran, F., Viale, A. M., Limansky, A. S., and Vila, A. J. (2008). Biochemical characterization of metallo-β-lactamase VIM-11 from a *Pseudomonas aeruginosa* clinical strain. *Antimicrobial Agents and Chemotherapy*, 6: 2250-2252. https://doi.org/10.1128%2FAAC.01025-07

Michaelis, L. and Menten, M. L. (1913). Die kinetik der invertinwirkung. *Biochem*, 49*:* (333-369) https://doi.org/10.1021/bi201284u

Palmer, T. (1995). Understanding Enzymes. John Wiley and Sons, New York. pp 55–71.

Perdani M. S., Sahlan M., Farida S., Putri D. N., Soekanto S. A. and Hermansyah H. (2019). Kinetic study of cholesterol oxidation by cholesterol oxidase enzyme as application for cholesterol biosensor. AIP Conference Proceedings 2092, Issue 1. https://doi.org/10.1063/1.5096731.

Perdani, M. S., Sahlan, M., Yohda. M. and Hermansyah. H. (2020). Immobilization of cholesterol oxidase from *Streptomyces Sp*. on magnetite silicon dioxide by crosslinking method for cholesterol oxidation. *Applied Biochemistry and Biotechnology*, 191: 968–980. https://doi.org/10.1007/s12010-020-03241-w

Richmond, W. (1973). Preparation and properties of a cholesterol oxidase from Nocardia sp. and its application to the enzymatic assay of total cholesterol in serum. *Clinical chemistry*, *19*(12), 1350-1356.

Sagermann, M., Ohtaki, A., Newton, K., and Doukyu, N. (2010). Structural characterization of the organic solvent-stable cholesterol oxidase from *Chromobacterium sp*. DS-1. *Journal of Structural Biology*,1: 32-40. https://doi.org/10.1016/j.jsb.2010.01.012

Shah, K., and Dubey, R. S. (1997). Effect of cadmium on proline accumulation and ribonuclease activity in rice seedlings: role of proline as a possible enzyme protectant. *Biologia Plantarum*,1: 121-130. https://doi.org/10.1023/A:1000956803911

Sharma, V. K., Kumar, N., Prakash, T. and Taylor, T. D. (2010). MetaBioME: a database to explore commercially useful enzymes in metagenomic datasets. *Nucleic Acids Research*, 38: D468–D472. https://doi.org/10.1093/nar/gkp1001

Tomioka, H., Kagawa, M., and Nakamura, S. (1976). Some enzymatic properties of 3β-hydroxysteroid oxidase produced by *Streptomyces violascens*. *The Journal of Biochemistry*, 5: 903-915. https://doi.org/10.1093/oxfordjournals.jbchem.a131158

Wang, C., Li, L., Chi, S., Zhu, Z., Ren, Z., Li, Y.,... & Cao, G. (2008). Thorium-doping–induced superconductivity up to 56 K in Gd1− xThxFeAsO. *EPL (Europhysics Letters)*, *83*(6), 67006. https://doi.org/10.1209/0295-5075/83/67006

Willassen, N. P. and Little, C. (1989). Effect of 2,3 butanedione on human myeloperoxidase. *International Journal of Biolochemistery*, 7: 755-759. https://doi.org/10.1016/0020-711x(89)90207-3

Williams, M. N. (1975). Effect of N-bromosuccinimide modification on dihydrofolate reductase from a methtrexate-resistant strain of *Escherchia coli*. Activity, spectrophotometric, fluorescence and circular dichroism studies. *Journal of Biological Chemistry*,1: 322-330. https://doi.org/10.1016/S0021-9258(19)42018-8

Wu K., Wei Li, Jianrui Song 2,3 and Tao Li. (2015). Production, purification and identification of cholest-4-en-3-one produced by cholesterol oxidase from *Rhodococcussp*. in Aqueous/Organic Biphasic System. *Biochemistry Insights*, 8: 1–8. https://doi.org/10.4137/BCI.S21580

Ye, D., Lei, J., Li, W., Ge, F., Wu, K., Xu, W. and Yong, B. (2008). Purification and characterization of extracellular cholesterol oxidase from *Entrobacter* sp. *World Journal of Microbiological Biotechnological*, 24: 2227–2233. https://doi.org/10.1007/S11274-008-9734-2

Yehia, B. R., Rebeiro, P., Althoff, K. N., Agwu, A. L., Horberg, M. A., Samji, H. and North American AIDS Cohort Collaboration on Research and Design (NA-ACCORD) (2015). The impact of age on retention in care and viral suppression. *Journal of Acquired Immune Deficiency Syndromes,* 4: 413. https://doi.org/10.1097/QAI.0000000000000489

How to cite this article:

Nessma M. El-Zawawy, Mohammed S. Yousef, Hamed M. El-Shora, Metwally A. Metwally and Sally M Metwally. 2023. Purification and Kinetics of Cholesterol Oxidase from *Penicillium chrysogenum*. *Int.J.Curr.Microbiol.App.Sci.* 12(04): 48-62. **doi:** https://doi.org/10.20546/ijcmas.2023.1204.006