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PURIFICATION, CHARACTERIZATION AND ACTIVE GROUPS OF BACTERIAL CHOLESTEROL OXIDASE.

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ABSTRACT

Cholesterol oxidase (EC 1.1.3.6) was isolated and purified from *Staphylococcus epidermidis*. The enzyme was purified with 60% ammonium sulphate followed by DEAE-Cellulose and Sephadex-200. The specific activity was 62 U mg^{-1} protein and the purification fold was 43.4. The optimum temperature was 40°C and the optimum pH was 8.0. The K_m for cholesterol was 0.33 mM and for $CaCl_2$ was 0.23mM. The enzyme was inhibited by phenyl mercuric acetate (PMA), N-acetylimidazole (NAI), diethylpyrocarbonate (DEPC) and phenyl glyoxal (PG) indicating the presence of sulphhydryl, tyrosyl, histidyl and arginyl groups. The enzyme was activated by Ca^{2+} at 1.0 and 5.0 mM. Also Mn^{2+}, Mg^{2+} and K^{+} were activators. Ba^{2+} and Zn^{2+} were inhibitors at the higher concentrations, whereas Al^{3+} and Cu^{2+} were inhibitors at lower and higher concentrations.

Keywords: Cholesterol oxidase, Purification, K_m, Characterization

INTRODUCTION

Cholesterol decomposition ability is widespread among microorganisms that have been explored as free and immobilized cells (Lee *et al.,* 1992) or as enzyme source (Constantinidis, 1980) in steroid biotransformations. Cholesterol may be completely oxidized by microbial cells to carbon dioxide and water by the action of a complex enzyme system in which cholesterol oxidase is the first enzyme involved.

Cholesterol oxidase (EC.1.1.3.6) is a FAD-dependent enzyme that catalyses the oxidation of cholesterol (5-cholesten-3*β*-ol) to 4-cholesten-3-one with the reduction of oxygen to hydrogen peroxide.

$$\text{Cholesterol} + O_2 \xrightarrow{\text{Cholesterol oxidase}} \text{Cholest-4-en-3-one} + H_2O_2$$

Cholesterol oxidase was investigated in bacteria such as *Arthrobacter* (Liu *et al.,* 1988), and *Rhodococcus* (Johnson *et al.,* 1991)

Microbial cholesterol oxidases generally have neutral pH optima and possess stability over a wide pH range. The pH of cholesterol oxidase was found to be 6.0 for *γ-Proteobacterium* (Isobe *et al*., 2003) and *Bacillus* sp. (Rhee *et al*., 2002). Other bacterial cholesterol oxidase showed pH optimum 7.0 such as *Entrobacter* sp. (Ye *et al.,* 2008); *Chromobacterium* sp. (Doukyu *et al*., 2008) and *Streptomyces fradiae* (Yazdi *et al*., 2000). For *Brevibacterium* sp. (Salva *et al*., 1999) and *Arthrobacter simplex* (Liu *et al.,* 1988) the pH was 7.5. The cholesterol oxidases enzymes have temperature optima in the range of 40-60°C.

Cholesterol oxidase has a broad range of substrate specificity and can be used for the bioconversion of a number of 3*β*-hydroxysteroids, which in

turn can be used for the analysis of steroid hormones and other pharmaceutical steroids in the presence of organic solvents (Doukyu *et al*, 1996; Guo *et al,* 2003). Cholesterol oxidase can be used for the optical resolution of non-steroidal compounds, allylic alcohols in the presence of organic solvents (Dieth *et al,* 1995; Biellmann, 2001)

Cholesterol oxidase was purified from various bacteria such as *Rhodococcus* sp. (Machang'u and Prescott, 1991); *Streptomyces fradiae* (Yazdi *et al.,* 2001); *Rhodococcus* (Yazdi *et al.,* 2008) and *Entrobacter* sp. (Ye *et al.,* 2008).

Cholesterol oxidase has been used as a probe to investigate the interaction of cholesterol with phospholipids (Lange *et al,* 2005) and the eukaryotic membrane structure (Rouquette-Jazdanian *et al*, 2006). Cell-free enzymes and microbial cells have been investigated for reduction of cholesterol level in foods (Xiansheng *et al.,* 1990; Christodoulou *et al.*, 1994).

Many research groups demonstrated that chemical modification of enzymes could enhance its activity or stability by different dicarboxylic anhydrides, such as phthalic anhydrides (CA), maleic anhydride (MA), citraconic anhydride (CA) and succinic anhydride (Liu *et al*., 2006). The above mentioned anhydrides react specifically with the ε-amino group of lysine residues and change its charges from positive to negative.

MATERIALS AND METHODS

Culture conditions:

The control medium contained (g/L) NH_4NO_3 : 17g, K_2HPO_4: 0.25g, $MgSO_4.H_2O$: 0.25g, $FeSO_4$: 0.001g, NaCl: 0.005g, Cholesterol: 2g, Tween 20: 0.5ml, Agar: 20g and pH: 7.0.

Preparation of cell free extract:

Staphylococcus epidermidis was grown in cholesterol broth medium, buffered at the optimal pH 7.0. The cholesterol medium was incubated at the optimal temperature 30°C and 150 rpm for three days. The culture medium was centrifuged at 3500 rpm for 10 min and the clear supernatant was collected which represented the cell free extract.

Enzyme assay:

Cholesterol oxidase activity was measured at 37°C by a modification of the method of Allain *et al*. (1974). The assay buffer contained 400 mM KH_2PO_4, 360 mM KOH, 24 mM 4-hydroxybenzoic acid, 2.7 mM EDTA disodium salt, 4000 i.u./ml peroxidase, 2 g/l Triton X-100, 50 ml/l methanol and 0.7 mM 4-aminoantipyrine. The cholesterol solution was prepared in Triton X-100 according to the method of Lartillot and Kedziora (1990). The assay mixture contained 2.5 ml of the assay buffer and 0.125 ml of cholesterol solution. The reaction was started by addition of 50 µl of cholesterol oxidase solution. The appearance of red color was monitored continuously at 500 nm for 5 min. One unite of cholesterol oxidase is defined as the amount of enzyme that converts 1 nmol of cholesterol/min at 37°C.

Enzyme purification:

Cholesterol oxidase was prepared as described in Materials and Methods. The crude supernatant was subjected to purification procedure including treatment with 60% ammonium sulfate, DEAE-cellulose column and Sephadex G-200.

Estimation of total protein

Total protein in the extract was determined by the method of lowry *et al.* (1951).

Effect of pH on enzyme activity:

The effect of pH values (2−10) on enzyme activity was determined with appropriate buffers were used to determine the optimum pH of enzyme. The optimum pH values obtained from this assay were used in all subsequent experiments.

Effect of Temperature on enzyme activity:

In order to determine the optimum temperature of the enzyme reaction, the enzyme was incubated at the various temperature 20-80^{o}C. An aliquot of the enzyme solution was withdrawn from each tube for measuring enzyme activity using the assay method of that enzyme.

Effect of metal ions on cholesterol oxidase activity:

The effect of various metal ions namely Mg^{2+}, Mn^{2+}, Cu^{2+}, K^{+}, Al^{3+}, Ca^{2+}, Zn^{2+} and Ba^{2+} on cholesterol oxidase activity was studied at 1mM and 5mM using chloride salt of the various metals.

RESULTS AND DISCUSSION

Cholesterol oxidase was purified using schedule includes ammonium sulfate, DEAE-cellulose and Sephadex G200 (Table 1). The final specific activity was 62 Umg^{-1} protein with purification fold of 43.4 and yield 43.5%. Also, cholesterol oxidase was purified from *Rhodococcus* sp. (Wang *et al.*, 2008) with 53.1 fold and specific activity of 55.8 U mg^{-1} protein. It was observed that the specific activity increased continuously whereas the total protein and total activity declined gradually.

Table 1 : Purification of extracellular cholesterol oxidase.

Steps	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1}protein)	Purification (Fold)	Yield (%)
Culture supernatant	1500	2140	1.43	1	100
60% saturation $(NH_4)_2SO_4$	620	1800	2.90	2.03	84.1
DEAE-Cellulose	140	1200	8.57	6.0	56.1
Sephadex - 200	15	930	62	43.4	43.5

For studying the effect of pH on cholesterol oxidase activity, the enzyme activity was determined at pH range of 2-10. In fact, for such sort of study other factors, which may affect the enzyme activity, substrate concentration and the time of incubation were fixed. The results in Fig 1 indicate that cholesterol oxidase activity was increased continuously up to the optimal pH 8.0 after which a gradual decrease of the activity was observed. Li

et al. (2010) reported that the optimal pH value of cholesterol oxidase from *Bordetella* species was 8.0. Also, the pH of the enzyme from *Rhodococcus* sp. (Wang *et al.*, 2008) was 7.0.

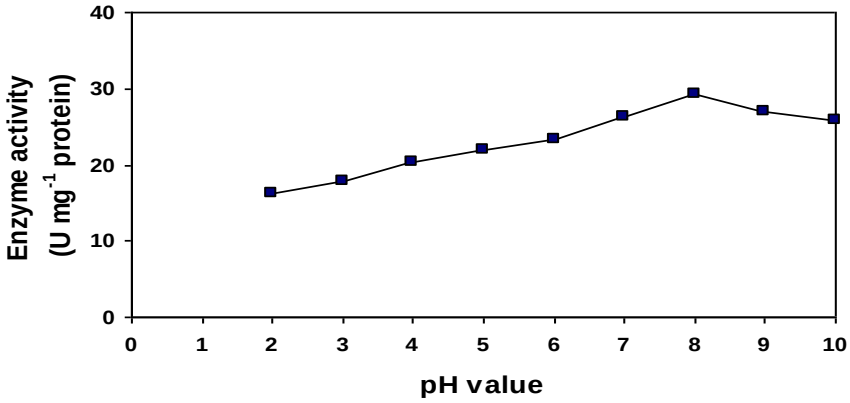

Fig. 1 : Effect of pH value on cholesterol oxidase.

For studying the effect of temperature of incubation mixture of cholesterol oxidase, the enzyme activity was determined at temperature range 20-80°C. The results in Fig 2 indicate that there was gradual increase in cholesterol oxidase activity with increasing the temperature up to 40°C after which there was a gradual decline in the activity. Cholesterol oxidase from *Bordetella* species (Liu *et al.*, 2010) expressed optimum temperature at 37°C.

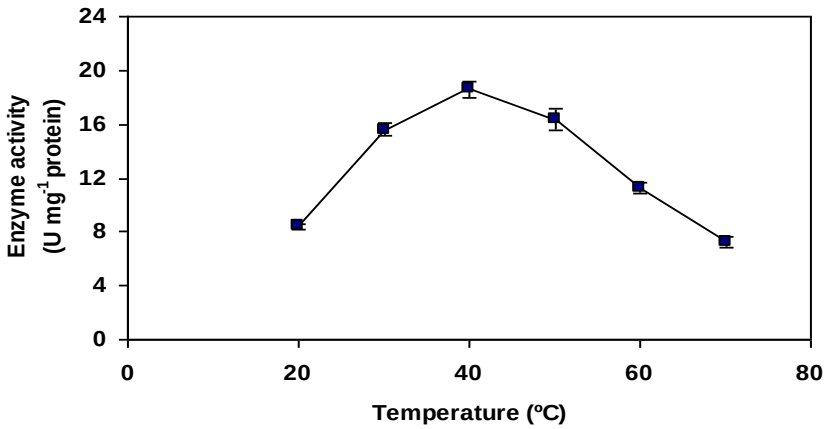

Fig. 2 : Effect of temperature on cholesterol oxidase.

The optimal temperatures of cholesterol oxidase from various sources were 25°C, 30°C, 37°C, 40°C, 42°C and 50°C for the enzyme from *Entrobacter* sp. (Ye *et al.*, 2008), *Pseudomonas* sp (Doukyu and Aono, 1998), *Pseudomonas* sp. (Lee *et al.*, 1989), *Bacillus* sp. (Rhee *et.al.*, 2002),

Corynebacterium cholesterolicum (Shirokane *et.al.,* 1977), *Arthrobacter* sp. (Wilmanska and Sedlaczek, 1988) and *Rhodococcus* sp. (wang *et al.,* 2008), respectively.

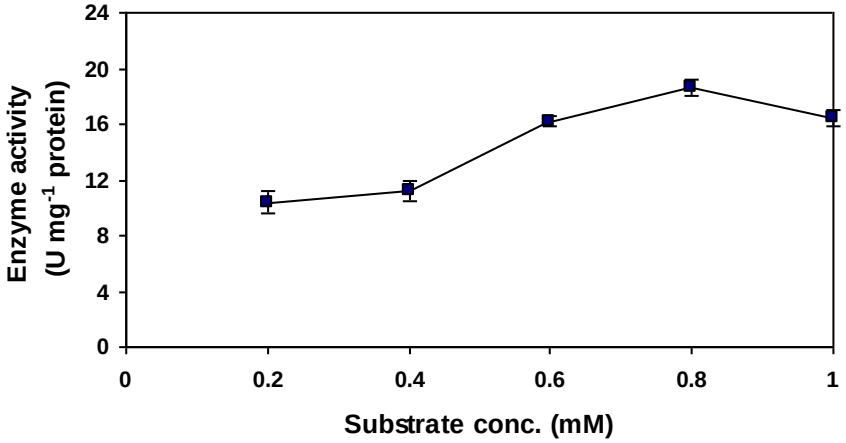

Fig. 3: Effect of substrate concentration on cholesterol oxidase.

The effect of cholesterol concentration on cholesterol oxidase was examined at constant temperature and pH. The cholesterol was examined at various concentrations 0.2-1 mM. The results in Fig 4 show that there was a continous increase in cholesterol oxidase activity with increasing cholesterol oxidase concentration up to 0.8 mM which is considered as optimal concentration.

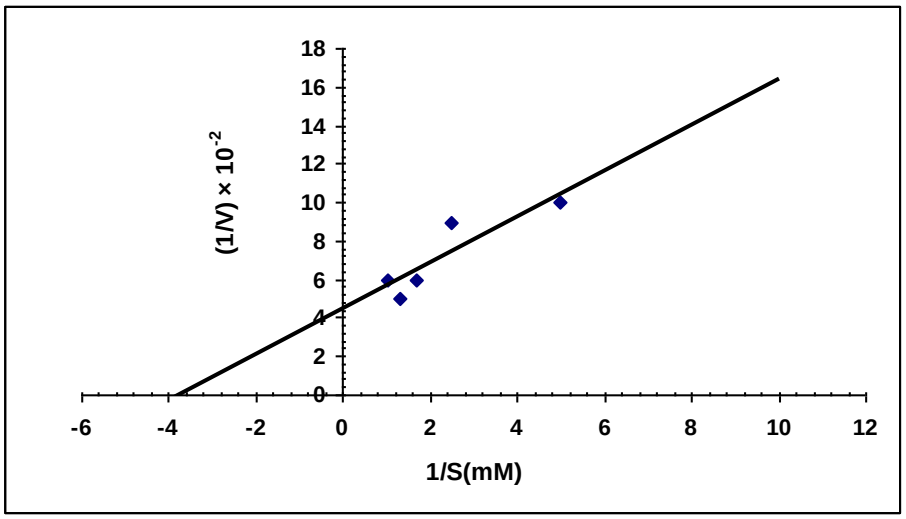

Fig. 4: Reciprocals of cholesterol oxidase velocity and cholesterol concentration.

It is possible to obtain the values of V_{max} and K_m directly from plotting the reciprocal of substrate concentration (S^{-1}) against velocity of cholesterol oxidase (V^{-1}). Lineweaver and Burk (1934) found that the reciprocal of both reaction velocity and substrate concentration from a straight line. In the present investigation the reciprocals of V and S of purified cholesterol oxidase were calculated and straight line is illustrated in the Fig 4. The intercept with the Y (V^{-1}) axis represents the reciprocal of the maximum velocity (1/V) of cholesterol oxidase. Additionally, the intercept of the sloping line with X (S^{-1}) axis represents the reciprocals of Mechaelis constant ($1/K_m$). Consequently, V_{max} and K_m values were calculated from Fig 4. The V_{max} was 23.8Umg^{-1} protein and K_m was 0.26 mM. Higher K_m value (0.566 mM) for cholesterol oxidase was reported for the enzyme from *Boredetella* species (Liu *et.al.*, 2010). Lower K_m value 55µM was reported by Wang *et al.* (2008) for the enzyme from *Rhodococcus* sp.

It is observed in Fig 5 that cholesterol oxidase was activated by Mn^{2+}, Mg^{2+}, Ca^{2+} and K^{+} at both concentrations 1 and 5 mM. However, Al^{3+} and Cu^{2+} were inhibitors. Zn^{2+} and Ba^{2+} were stimulators at 1mM and inhibitors at 5 mM. In support, Zn^{2+} and Cu^{2+} inhibited cholesterol oxidase from *Rhodococcus* sp. (Wang *et al.,* 2008). It is noted that Ca^{2+} was the best divalent cation to stimulate cholesterol oxidase. From Fig 6, the Km was 0.25

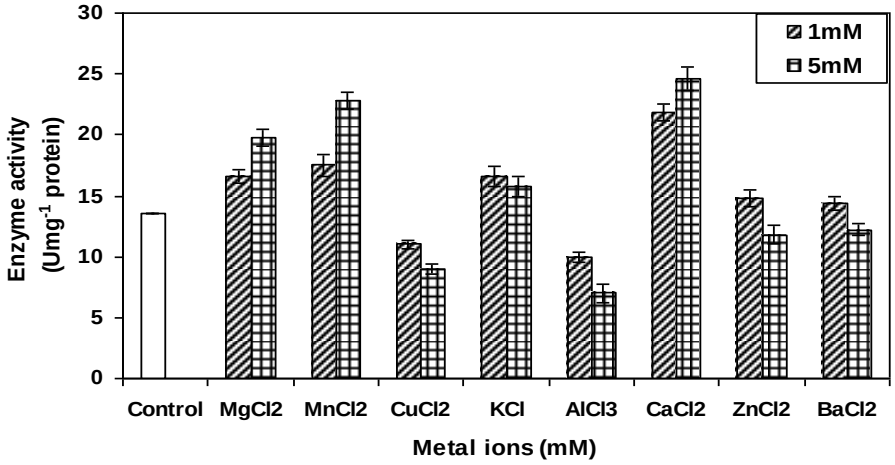

Fig. 5: Effect of metal ions on cholesterol oxidase.

The effect of phenyl glycoxal (PG), phenyl mercuric acetate (PMA), N-acetyl imidazole (NAI), diethyl pyrocarbonate (DEPC) on cholesterol oxidase activity were studied at various concentrations (0.2-1 mM) for various periods of time (20-100 min). The results in Figs 7,8,9,10 indicate that the enzyme activity was inhibited at various concentrations indicating the presence of arginyl, sulfhydryl, tyrosyl and histidyl as essential residues in the enzyme molecules. Also, Wang *et al.* (2008) suggested that sulfhydryl group may be involved in the catalytic activity of cholesterol oxidase from *Rhodococcus* sp.

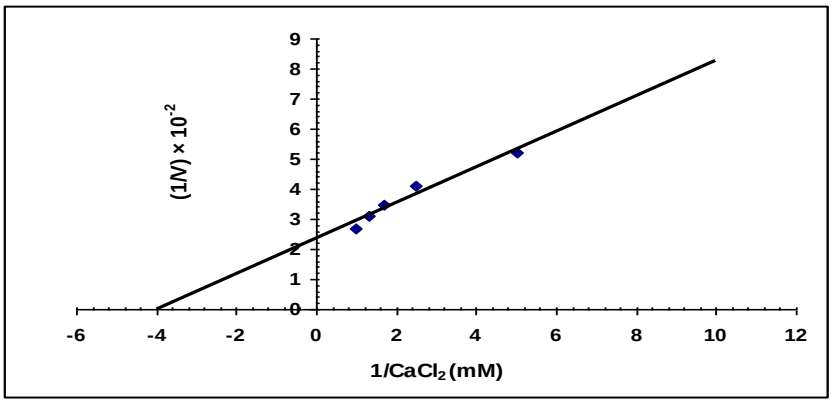

Fig. 6: Reciprocals of reaction velocity of cholesterol oxidase and calcium chloride concentration.

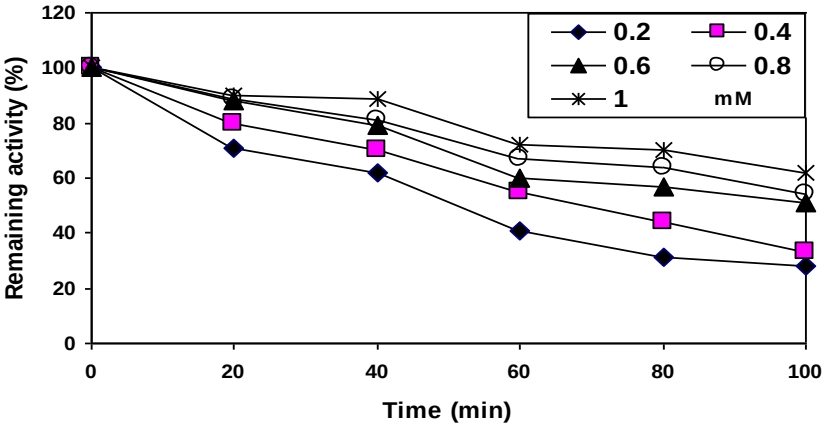

Fig. 7: Effect of phenylglyoxal on cholesterol oxidase activity.

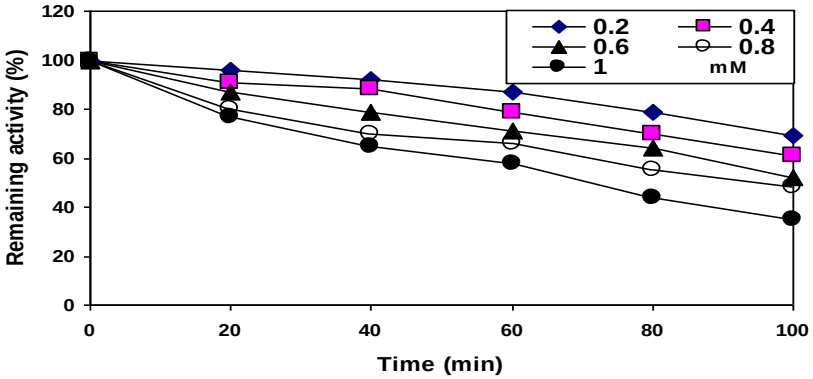

Fig. 8: Effect of PMA on cholesterol oxidase activity.

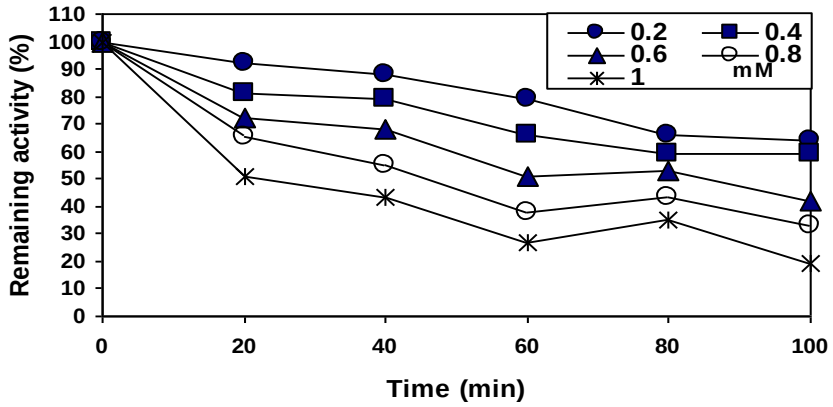

Fig. 9: Effect of N-acetylimidazole on cholesterol oxidase activity.

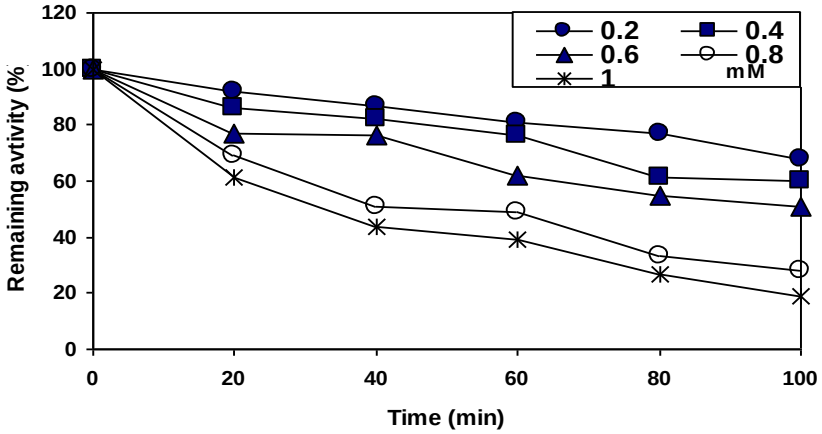

Fig. 10: Effect of Diethylpyrocarbonate on cholesterol oxidase activity.

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تنقية وخصائص والمجموعات النشطة لأكسيديز الكولستيرول البكتيرى.

حامد محمد الشورى ، دعاء بهاء درويش و مى محسن أحمد حسن طه.
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هدف هذا البحث عزل وتنقية أكسيديز الكولستيرول من *Staphylococcus epidermidis* . تم تنقية الانزيم بواسطة كبريتات الامونيوم (60 %) وتبع ذلك استخدام داى ايثيل اسيتات السليولوز وسيفادكس 200 . وقد لوحظ ان النشاط النوعى للانزيم كان 62 وحده انزيمية لكل جرام بروتين، ومعامل التنقية 43.4 . كانت درجة الحرارة $40^{\circ}C$ ، والرقم الهيدروجينى الامثل كان 8.0 . اظهر الكولستيرول ان مادة التفاعل الاساسية مع قيمة ثابت ميخائيل تساوى0.26.وبدراسة تأثير ايونات العناصر المختلفة اظهرت النتائج ان الكالسيوم كان افضل العناصر فى تحفيز النشاط الانزيمى وكذلك الماغنسيوم والمنجنيز والبوتاسيوم. بينما اظهر كل من الزنك و الالومنيوم والباريوم تأثير مثبط على النشاط الانزيمى. ثبط الانزيم بمعاملته بكل من PMA، N- استيل اميادازول ، داى ايثيل بيروكربونات ، فينيل جليكوزال وثبت من خلال ذلك وجود مجموعات سلفهيدريل ارجينيل هستيديل كمجموعات فعالة ولازمة للنشاط الانزيمى .

قام بتحكيم البحث

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