ISOLATION AND IDENTIFICATION OF CHOLESTEROL OXIDASE PRODUCING *Pseudomonas Aeruginosa*

Hameed M. Jasim, Saddam Y. Diwan
Department of Biotechnology, College of Science, Al Nahrain University. Baghdad-Iraq.

Abstract

Cholesterol oxidase (EC:1.1.3.6) is industrially and commercially important for the application in bioconversions for clinical determination of total or free serum cholesterol, and in food industry, for this reason this study was aimed to isolate cholesterol oxidase producing *Pseudomonas aeruginosa* from different local sources, by taking 55 swap samples from different serological samples includes ears, urine, burns, wounds and sputum, in addition to another 35 environmental samples collected from soil and water from different locations in Baghdad governorate. From these samples 115 bacterial isolate were obtained, and among them only 60 isolate were identified as *P. aeruginosa* according to the results of biochemical tests and Api 20E system. Ability of these isolates in cholesterol oxidase production was screened in Lauria-Broth medium. Results showed that all of these 60 isolates were able to produce cholesterol oxidase with variable capacities, among them *P.aeruginosa* H48 was the most efficient isolate in enzyme production. The activity of crude enzyme in culture filtrate of this isolate was 1.71 U/ml. Cholesterol oxidase produced by *P.aeruginosa* H48 was purified by ammonium sulphate precipitation (70% saturation), dialysis and ion exchange chromatography using CM–cellulose.The enzyme activity of the partially purified enzyme was increased to 3.87 U/ml. Optimum pH and temperature for activity and stability of the partially purified enzyme were estimated. Results showed that the Optimum pH for enzyme activity and stability was pH7.0 and pH 6.5 respectively, while the optimum temperature for enzyme activity and stability was 35 and 37°C respectively.

Pseudomonas aeruginosa

حميد مجيد جاسم، صدام يحيى ديوان
قسم التقنيات الاحيائية، كلية العلوم، جامعة النهرين. بغداد-العراق.

الخلاصة

Pseudomonas aeruginosa

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P. aeruginosa H48

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P. aeruginosa H48

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Introduction

Pseudomonas spp are G-ve rods, motile by polar flagella, aerobic while some can grow unaerobically in the presence of nitrates and using it as an electron acceptor. Members of this genus are widely distributed in soil, water, crude milk, fishes and poultry [1]. This bacteria was clinically important because it was resistant to most antibiotics and they are capable of surviving in conditions that few other organisms can tolerate, they also produce slim layer to defend against phagocytosis in the blood stream of the host [2].

The genus *Pseudomonas* included two pigmentation groups as genetic characteristics, which are so called fluorescent and non fluorescent subgroups. Some species of *Pseudomonas* are plant pathogens like *P. putida*, others are opportunistic pathogenic for human and animals like *P. aeruginosa*, they are also play an important role in spoilage of foods, diary products, meat, poultry and eggs [3]. *Pseudomonas* spp characterized by its ability to produce many extracellular enzymes like esterase, alginase, collaginase and protease, which support these bacteria to infect body tissues [4].

One of the most important extracellular enzymes produced by *Pseudomonas* spp was cholesterol oxidase [5], which catalyses the oxidation of cholesterol (Cholest-5-en-3β-ol) at the C-3 position with the simultaneous isomerization of the Δ5- double bond to produce cholest-4-en-3-one as a final product [6].

Cholesterol Oxidase had received much attention owing to its medical application in the determination of cholesterol in blood serum and food and in the production of a starting material for the chemical synthesis of pharmaceutical steroid [7]. In addition, cholesterol oxidase can be employed to improve human health by degrading dietary cholesterol, which has been implicated in cardiovascular disease [8]. Also it is industrially and commercially important for application in the microanalysis of steroids in food samples and for distinguishing the steric configuration of 3-ketosteroids from the corresponding 3-β-hydroxysteroids [9]. According to these mentioned above, this study was aimed to isolate cholesterol oxidase producing *P.aeruginosa* from different clinical and environmental samples and study some of the enzyme biochemical characteristics.

Material and Methods

Isolation of *P.aeruginosa*

P.aeruginosa was isolated from different clinical samples collected from sputum, urine and ear, and from patients suffering from burns and injuries from different hospitals, in addition to environmental samples includes water and soil in Baghdad governorate.

Identification of the isolates

Different isolates obtained from clinical and environmental samples were cultured on cetrimide agar medium, then the grown colonies were identified according to Palleroni [10], by achieving biochemical tests, then full-identification using Api 20E system.

Production of Cholesterol oxidase

Production of cholesterol oxidase by the locally isolated *P.aeruginosa* was carried out in the production medium [11], by inoculating 100 ml of this medium with 1 ml of fresh culture of each isolate separately and incubated in shaker incubator at 30°C for 24 hrs.

Enzyme Assay

Assay of cholesterol oxidase was achieved according to Richmond [12], by measuring the conversion of cholesterol to 4-cholesten-3-one. 50ml of standard cholesterol solution was added to 2.5 ml of phosphate buffer and mixed by inversion. Spectrophotometer was calibrated against blank of the same buffer, then 10 ml of the enzyme solution was added and incubated for 1min. at 30 °C, then drop of triton X-100

Table 1: Bacterial isolates from different clinical and environmental samples.

Source of Sample	No. of Samples	No. of Isolates	Growth on Cetrimide agar
Urine	12	18	10
Burns	15	17	10
Wounds	15	23	13
Ears	5	5	2
Sputum	8	14	5
Soil	18	19	15
Water	10	11	3
Fruits	8	8	2
Total	91	115	60

Table 2: Biochemical characteristics of the locally isolated *Pseudomonas* spp

Test	Result
Gram stain	-ve
Catalase test	+ve
Oxidase test	+ve
Growth on King A.	+ve
Growth on King B.	+ve
Growth on Cetrimide.	+ve
Gelatinase test	+ve
Citrate utilization.	+ve
Growth at 4 Ċ	-ve
Growth at 42 Ċ	+ve

Ability of all these isolates in cholesterol oxidase production was examined in LB medium by determining enzyme activity (U/ml) in culture filtrate to select the efficient in enzyme production. Results indicated in table(3) showed that *Pseudomonas* spp H48 was the efficient in enzyme production, activity of the crude enzyme produced in its culture filtrate was 1.71 U/ml in comparison with the productivity of the other isolates. This isolates was full-identified using Api20E system, and the results showed that it was able to utilize arginine, citrate, gelatin and glucose, it was negative for β-galactosidase, lysine decarboxylase, ornithine decarboxylase, H_2S, urease, tryptophane deaminase, indole, and VP tests, while it was able to produce acid from rhamnose, sucrose, melibiose, amygdalin, arabinose mannitol,

was added to the mixture to stop the reaction, then enzyme activity was determined at 240nm according to the next formula. Activity unit was defined as the amount of enzyme oxidizing 1μmol of cholesterol to 4-cholesten-3-one at 30°C under the reaction conditions [13].

$$\text{Enzyme Activity (Unit / ml)} = \frac{\Delta\text{O.D.} \times \text{Total Volume} \times 0.082}{\text{Volume of Enzyme Taken}}$$

Determination of Protein concentration

Protein concentration was determined according to the method described by [14], using bovine serum albumin as a standard protein solution.

Purification of Cholesterol Oxidase

Cholesterol oxidase was purified first by ammonium sulphate precipitation at a saturation ratios ranged between 25% to 85%, dialysis and then ion exchange chromatography through CM-Cellulose column (22×1.5cm) equilibrated with phosphate buffer (0.25M, pH 7.0). After ammonium sulphate precipitation and dialysis,4 ml of crude enzyme was added to the column, washed and fractions were eluted with the same buffer containing gradual concentrations of sodium chloride ranging between (0.05 – 0.5 M) and the flow rate was(100 ml/ hour), 5 ml/ fraction.

Characterization of purified cholesterol oxidase

Purified cholesterol oxidase was charact-erized by determining the optimum pH and temperature for both activity and stability according to Whitaker [15].

Results and Discussion

Results indicated in table (1) showed that there is one hundred and fifteen bacterial isolate were obtained from different clinical and environmental samples. Among the total isolates, only 60 were able to grow on cetrimide agar plates, which indicates that these isolates may be belong to *Pseudomonas* spp.

These 60 isolates were identified according to its biochemical characteristics. Results indicated in table (2) showed that these isolates gave a positive result for production of oxidase ,catalase, and gelatinase, growing on king A and king B medium, and at 42°C , while they are negative for the other biochemical tests indicated in table (2).

of ammonium sulphate gave maximum activity of 3.7U/ml, specific activity was 5.44 U/mg of protein with a yield of 4.625% as it was mentioned in table(4).

Enzyme obtained from ammonium sulphate precipitation and dialysis was further purified by ion exchange chromatography using carboxymethyl cellulose. Results indicated in (figure 1) showed that cholesterol oxidase was eluted by 0.3M NaCl with specific activity of 10.46U/mg protein. This step demonstrated approximately 1.99 fold of purification with 30.21 % overall yield as it was mentioned in table (4).

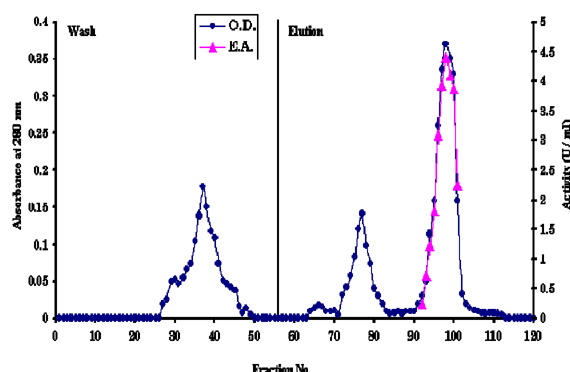

Figure 1: Ion Exchange Chromatography for Purification of Cholesterol oxidase from *P. aeruginosa* H48 by using CM-Cellulose.

Table 4:Steps of cholesterol oxidase purification from *P. aeruginosa* H48

Purification Step	Volume (ml)	Enzyme Activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/mg)	Total Activity (Unit)	Fold Purification	Yield (%)
Crude Enzyme	100	3.2	0.61	5.24	320	1	100
Ammonium Sulphate Precipitation	4	3.7	0.68	5.44	14.8	1.038	4.62
Ion Exchange	25	3.87	0.37	10.46	96.75	1.99	30.23

The purified enzyme was characterized by determining the optimum pH and temperature for both activity and stability respectively. Results indicated in (figure 2) showed that pH7.0 was the optimum for cholesterol oxidase activity, when the purified enzyme was added to substrate (cholesterol solution) incubated previously at a range of pH between 5.0 and 9.0 for 10 minutes at 32°C. At this pH, enzyme activity was 3.8 U/ml, and the activity was decrease at the acidic and basic pH values because of the conformational changes in enzyme configure-

inositol, and sorbitol. These results indicated that this isolate *P. aeruginosa,* according to [16].

Table 3: Activity of cholesterol oxidase produced from *Pseudomonas* spp isolates

Isolate No.	Source	Activity (U/ml)	Isolate No.	Source	Activity (U/ml)
H1	Urine	1.18	H31	Wounds	1.42
H2	Urine	1.02	H32	Wounds	1.47
H3	Urine	1.29	H33	Wounds	1.36
H4	Urine	1.26	H34	Ear	1.22
H5	Urine	0.72	H35	Ear	1.21
H6	Urine	1.37	H36	Sputum	1.21
H7	Urine	1.0	H37	Sputum	1.17
H8	Urine	1.41	H38	Sputum	0.4
H9	Urine	1.26	H39	Sputum	0.63
H10	Urine	1.21	H40	Sputum	0.65
H11	Burns	1.16	H41	Soil	1.17
H12	Burns	1.41	H42	Soil	1.33
H13	Burns	1.29	H43	Soil	1.33
H14	Burns	1.67	H44	Soil	1.45
H15	Burns	1.61	H45	Soil	1.28
H16	Burns	1.48	H46	Soil	1.41
H17	Burns	1.41	H47	Soil	1.19
H18	Burns	1.46	H48	Soil	1.71
H19	Burns	1.4	H49	Soil	1.46
H20	Burns	1.51	H50	Soil	1.11
H21	Wounds	1.56	H51	Soil	1.57
H22	Wounds	0.64	H52	Soil	1.13
H23	Wounds	0.6	H53	Soil	1.18
H24	Wounds	1.19	H54	Soil	1.14
H25	Wounds	1.32	H55	Soil	1.48
H26	Wounds	0.65	H56	Water	1.18
H27	Wounds	0.9	H57	Water	1.23
H28	Wounds	1.22	H58	Water	1.42
H29	Wounds	1.21	H59	Fruits	1.08
H30	Wounds	1.42	H60	Fruits	1.13

Cholesterol oxidase produced by *P.aeruginosa* H48 was purified first by ammonium sulphate precipitation using gradual saturation ratios ranging between 25 and 85%. It was found that precipitation of the enzyme with 70% saturation

38°C, in which the enzyme activity was 3.29 U/ml, and the remaining activity was 100%, then the activity was decreased gradually with the increase of the incubation temperature. Most enzymes often highly sensitive to high temperatures, while others which contain disulfide bonds are more stable in high temperatures than complex enzymes with high molecular weights as it was described by[18].

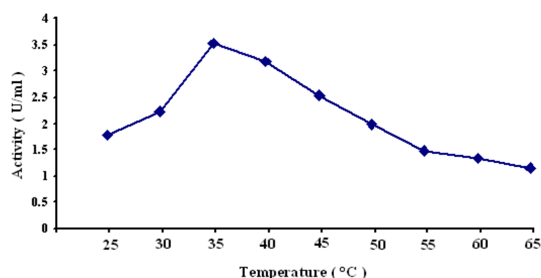

Figure 4: Effect of different temperatures on the activity of purified cholesterol oxidase from *P.aeruginosa* H48.

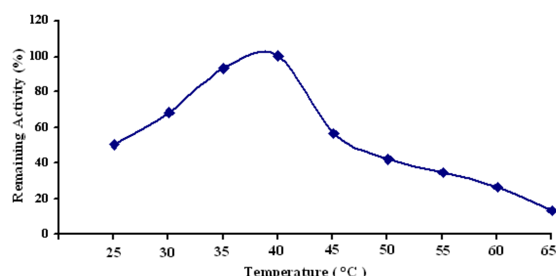

Figure 5: Effect of temperatures on the stability of the activity of purified cholesterol oxidase from *P. aeruginosa* H48.

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tion due to the changes in the ionizable groups located in the active sites of the enzyme as it was mentioned by Whitaker [15].

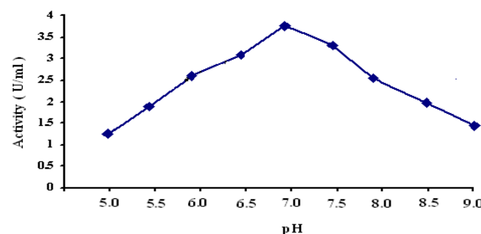

Figure 2: Effect of pH on the activity of the purified cholesterol oxidase from *P.aeruginosa* H48.

On the other hand, it was found that the optimum pH for the stability of cholesterol oxidase was pH 6.5 when the purified enzyme was incubated in test tubes containing 1ml of buffer solutions at a pH range between 5.0 and 9.0. At this pH, the enzyme activity was 3.62 U/ml, and the remaining activity was 100% as it was shown in (figure 3). Optimum temperature for cholesterol oxidase activity and stability was also determined. Results indicated in (figure 4) showed that the optimum temperature for enzyme activity was 35 °C when the enzyme was added to substrate (cholesterol solution)and incubated at different temperatures ranged between 25°C and 65°C for 10 min. At this temperature, the activity was 3.48 U/ml, and represents the optimum for enzyme activity because of the high effect on the reaction energy for both enzyme and substrate which leads to formation of enzyme–substrate complex and this will result in increasing the reaction speed as it was mentioned by Urban *et al.*[17] , hence enzyme activity was decreased above and below this temperature.

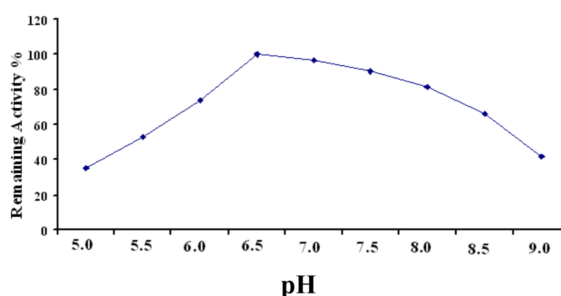

Figure 3: Effect of pH values on the stability of the activity of partially purified cholesterol oxidase from *P. aeruginosa* H48.

Effect of temperature on the stability of the purified cholesterol oxidase was also studied. Results indicated in figure(5) showed that the enzyme kept its activity for 10 minutes at 35 -

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