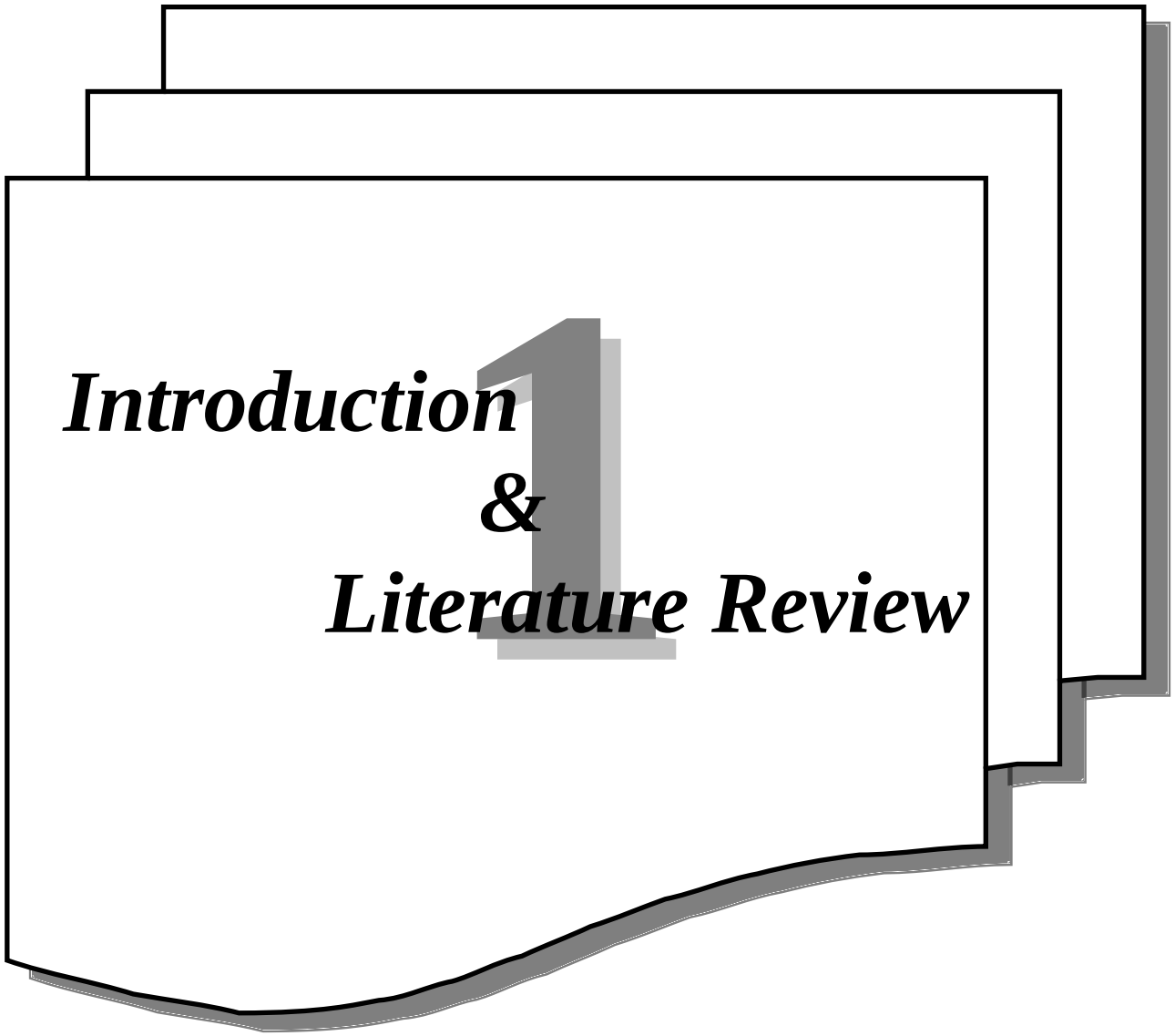
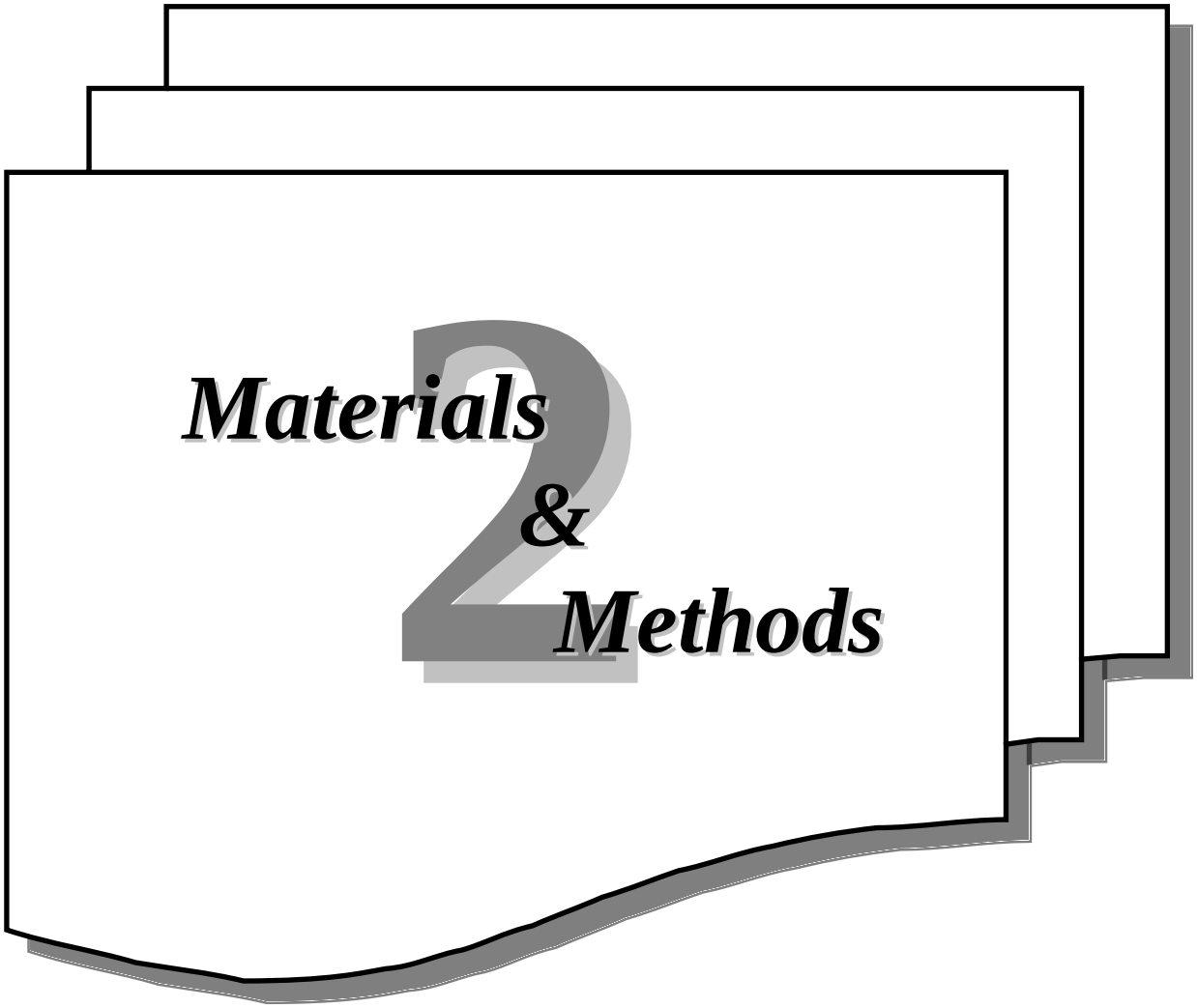
Chapter One

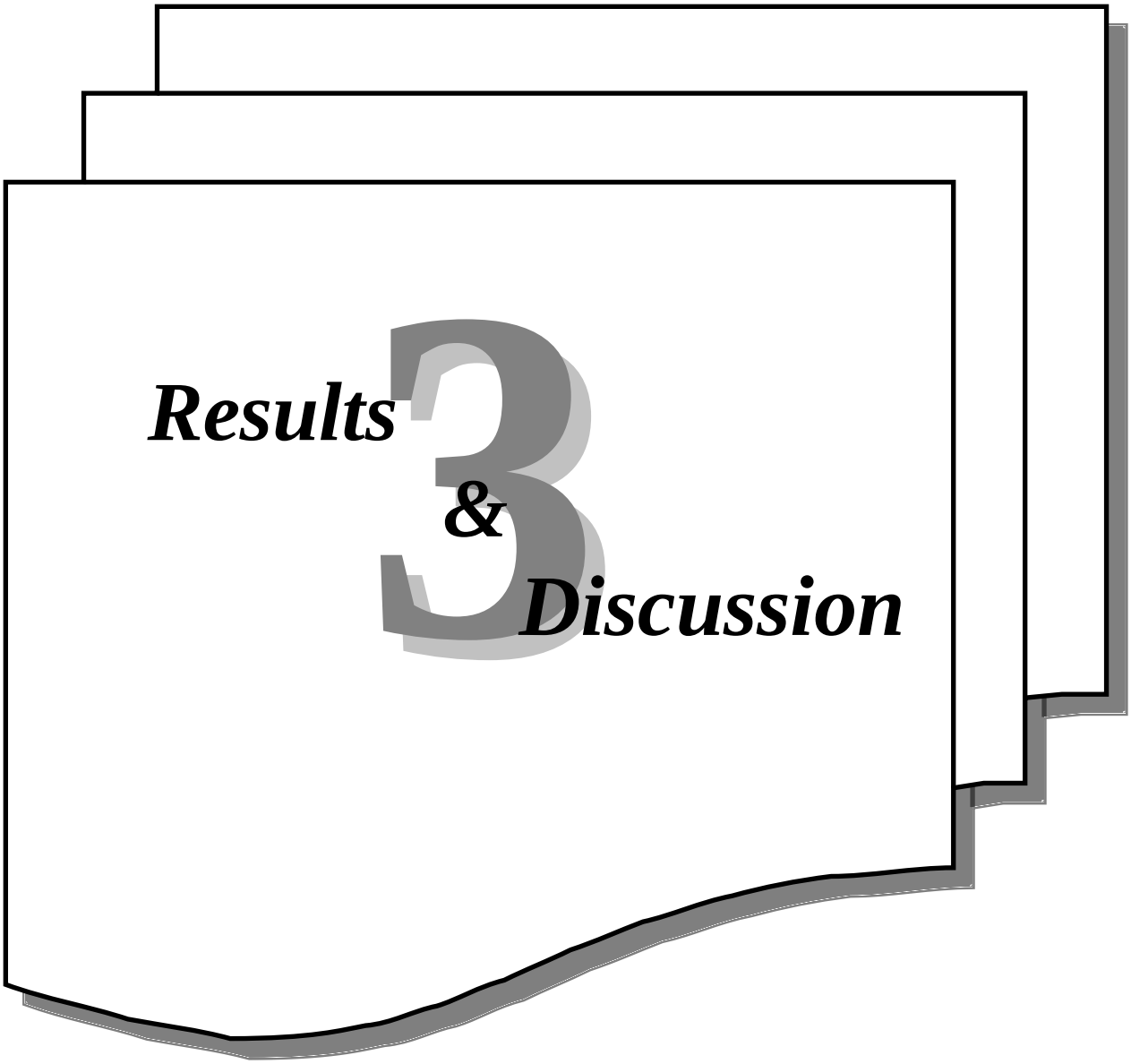


Introduction & Literature Review

Chapter Two



Chapter Three



Conclusions & Recommendations

References

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Yours Truly,

Saddam Yahya.

1: INTRODUCTION AND LITERATURE REVIEW

1.1: Introduction

Microbial cholesterol oxidase (EC 1.1.3.6) catalyzes the oxidation and isomerization of cholesterol to 4-cholesten-3-one. An oxidized form of the cholesterol oxidase dehydrogenates cholesterol, probably to the 5-en-3-one derivative. A reduced forms of the enzyme, yielded from the cholesterol dehydrogenation reaction, deoxygenated cholest-5-en-3-one to 6ß-hydroperoxycholest-4-en-3-one (Doukyu and Aono, 1999).

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 (Watanabe *et al*., 1986). In addition, cholesterol oxidase can be employed to improve human health by degrading dietary cholesterol, which has been implicated in cardiovascular disease (Kaunitz, 1978). It has been shown that cholesterol oxidase from *Pseudomonas fluorescens* is responsible for the production of 6ß-hydro-peroxycholest-4-en-3-one but not cholest-4-en-3-one (Teng and Smith, 1996).

Cholesterol oxidase was isolated and characterized from numerous bacterial sources. Cholesterol oxidases can be intrinsic membrane bound enzymes located on the outside of the cell, as produced by *Nocardia rhodochrous*, *Nocardia erythropolis*, and *Mycobacterium* sp. (Cheetham *et al.*, 1982),or can be isolated from broth filtrate as in cultivation of *Streptomyces violascens, Brevibacterium sterolicum, Streptoverticilium cholesterolicum,*

Rhodococcus equi no 23, *Mycobacterium* ATCC 19652 and *R. erythropolis* (Smith *et al.*,1993).

According to (Lee *et al*., 1989), a cyclohexane-tolerant and cholesterol-converting bacterium, *Pseudomonas* sp. had been isolated. Cholesterol oxidase produced by these bacteria has been purified and characterized. Because of the importance of this enzyme in laboratory experiment, this study was aimed to:

- Isolation of cholesterol oxidase producing *pseudomonas* sp. from different environmental sources and clinical cases.
- Screening the ability of the bacterial isolates in cholesterol oxidase production and select the efficient producer.
- Determining the optimal conditions for cholesterol oxidase production.
- Extraction, partial purification and characterization of cholesterol oxidase.

1.2: Literature Review

1.2.1: The Genus *Pseudomonas*

Pseudomonas sp. are gram negative rods, motile by one polar flagella, aerobic while some can grow unaerobically in the presence of nitrates and using it as an electron acceptor. These bacteria do not ferment sugars but utilize it oxidatively as other organic compounds. Member of this genus are variable in their metabolic activity and widely distributed in soil, water, waste water, crude milk, fishes and poultry (Harry *et al*., 1992).

Members of this genus are classified into five groups based on ribosomal RNA homology. These bacteria are clinically important because they are resistant to most antibiotics and they are capable of surviving in conditions that few other organisms can tolerate. They also produce a slime layer that is resistant to phagocytosis. The genus *Pseudomonas* is often encountered in hospital and clinical work because it is a major cause of hospital acquired (nosocomial) infections. Its main targets are immunocompromised individuals, burn victims, and individuals on respirators or with indwelling catheters. Additionally, these pathogens colonize the lungs of cystic fibrosis patients, increasing the mortality rate of individuals with the disease. Infection can occurs at many sites and can lead to urinary tract infections, sepsis, pneumonia, pharyngitis, and a lot of other problems (Stolp and Gudkari, 1984). Species of this genus like *P. fragi*, *P. putida*, and *P. fluorescence* produce unsoluable pigments like Pyoveridine, which is a yellow pigment.

Other species like *P. aeruginosa*, which produce phenazine pyocyanin, which is a red pigment. Also *P. aeruginosa* and *P. putida* can produce pyomelanin, which is a dark brown pigment (Holt *et al.*, 1994; Lennet, *et al.*,

1985). The genus *Pseudomonas* included two pigmentation groups as genetic characters, which are so called fluorescent and non-fuorescent subgroups. Some species of *Pseudomonas* are plant pathogens like *P. Putida*, others are opporunistics pathogenic for human and animals like *P. aeruginosa*, they are also play an important role in spoilage of food, dairy product, meat, poultry and eggs. If spoilage develops after storage under refrigerated conditions, *Pseudomonas* usually prevails (Brooks *et al.*, 1995).

Pseudomonas species characterize by its ability to produce many enzymes like (esterase, alginase, colaginase, elastase and protease) which support this bacteria to infect body tissues (Ding *et al*., 1985; Linker and Evans, 1984; Holder and Neely, 1989; Marne and Vandel, 1988).

One of the most important extracellular enzymes produced by *Pseudomonas* sp. is cholesterol oxidase (Lee *et* al., 1989).

1.3: Cholesterol

Cholesterol (cholest-5-en-3*b*-ol) is principle sterol of higher animals. Cholesterol is practically insoluble in water, but it is soluble in aqueous solutions of bile salts. It is found in all body tissues, especially the brain and spinal cord, and in animal fats or oils. Cholesterol is considered a key intermediate in the biosynthesis of fecal sterols, bile acids, and steroid hormones in animals. Turfitt, (1942) first demonstrated the formation of 4-cholesten-3-one from cholesterol by *Proactiomyces erythropolis* (*Nocardia erythropolis*) and showed that this oxidation of the 3*b*-hydroxy group to the keto group of cholesterol was the initial step in the aerobic catabolism of the sterol. On the other hand, in the intestinal tract of humans, cholesterol can be anaerobically metabolized by bacterial

microflora to coprostanol 65% and coprostanone 10% and to a lesser extent to cholestanone and epicoprostanol Figure (1-1).

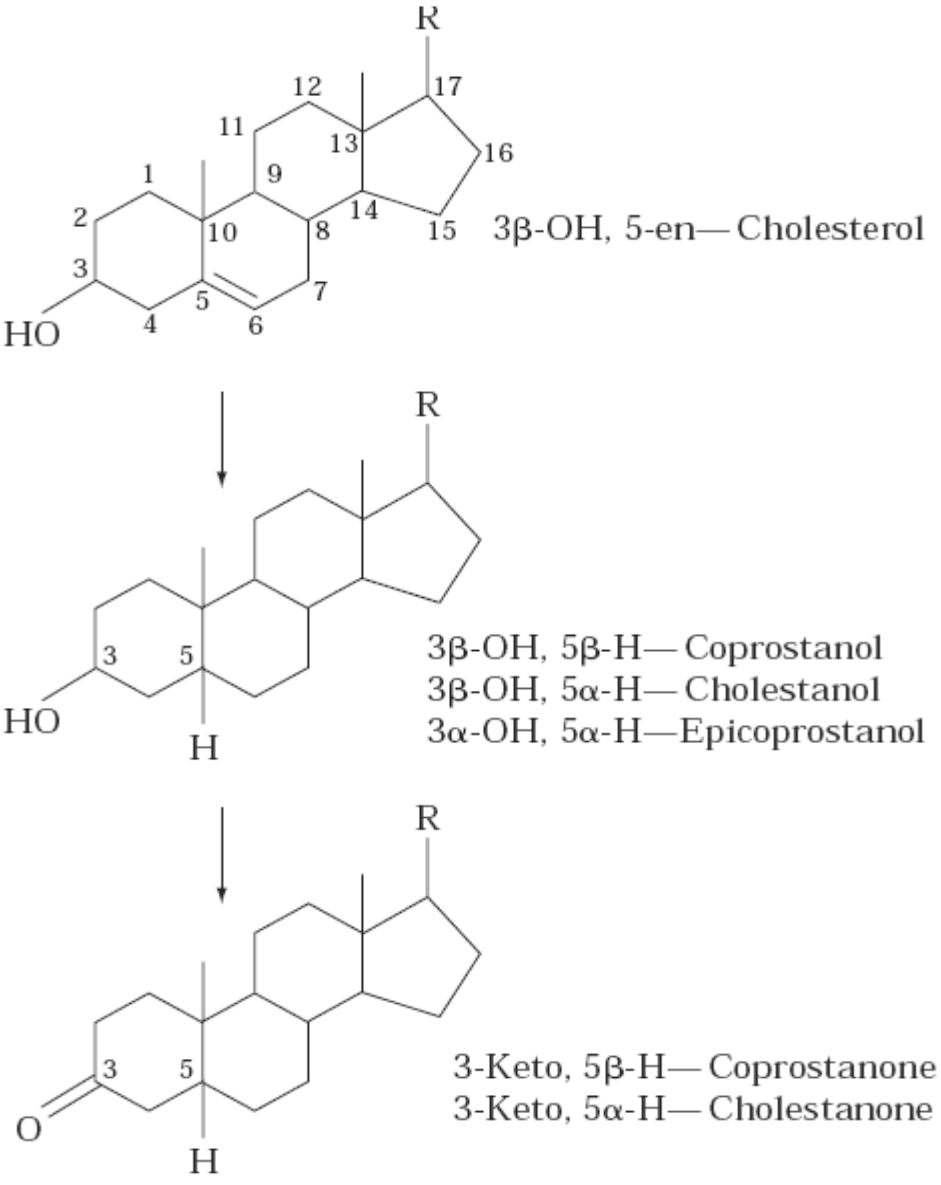


Figure (1-1) Anaerobic metabolism of cholesterol. (MacDonald *et al.*, 1983).

1.4: Cholesterol oxidase

This enzyme is one of the oxidoreductases which catalyses various types of oxidation - reduction reactions (Mckee and Mckee, 1996).

Cholesterol oxidases (cholesterol: oxygen oxidoreductase), catalyses the oxidation of cholesterol (cholest-5-en-3β-ol) to cholest-4-en-3-one (Smith and Brooks, 1974; Uwajima *et al*., 1974). The cholesterol oxidation step with cholesterol oxidase is the oxidation of the hydroxy group at the C-3 position with the simultaneous isomerization of the Δ^5-double bond to produce cholest-4-en-3-one as a final product as shown in figure (1-2). Cholesterol oxidases were produced and characterized from numerous bacterial sources; most of them were shown to contain 1 mole of tightly bound FAD per mol of protein as a prosthetic group (Kamei *et al*., 1987).

A cyclohexane-tolerant and cholesterol-converting bacterium, *Pseudomonas* sp. had been isolated. This microorganism effectively oxidizes cholesterol; extracellular cholesterol oxidase produced by this bacterium has been purified and characterized previously (Doukyo and Aono, 1998). This enzyme was stable in the presence of organic solvents and showing high activity. Long-term oxidation of cholesterol with the oxidase generates several products besides cholest-4-en-3-one. It has been shown that the cholesterol oxidase of *Pseudomonas fluorescens* is responsible for the production of 6β-hydroperoxycholest- 4-en-3-one but not cholest-4-en-3-one.

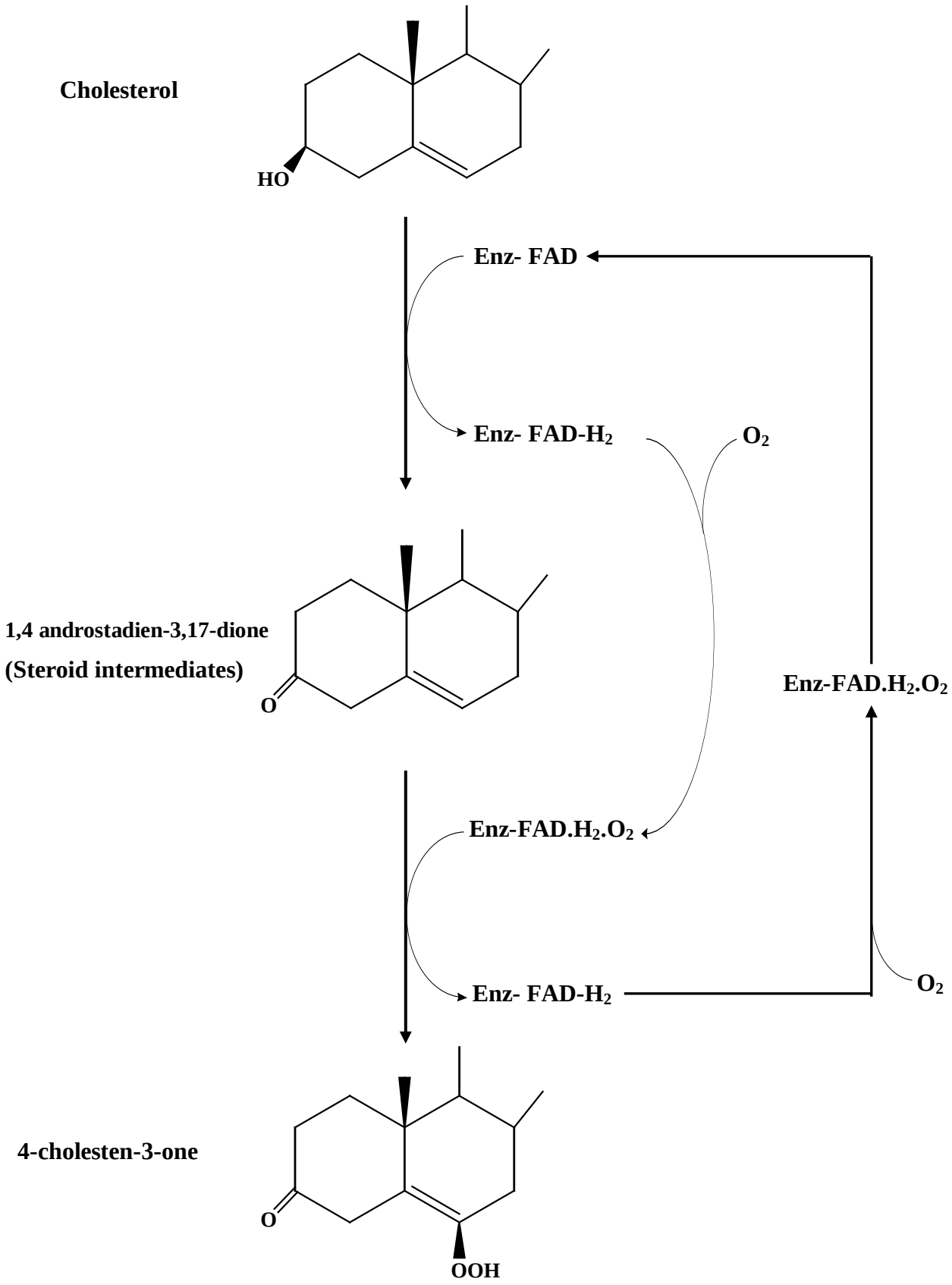


Figure (1-2): Oxidation of cholesterol with cholesterol oxidase from *Pseudomonas sp*. (Teng and Smith, 1996)

Cholesterol oxidase is a flavo-enzyme (with a FAD prosthetic group) that produces hydrogen peroxide according to the following reaction:

$$\text{Cholesterol} \xrightarrow{O_2} \text{4 - Cholesten - 3 - one} + H_2O_2$$

Cholesterol Oxidase is not likely to be inducible by cholesterol because the amount of the cholesterol oxidase activity produced by the microorganism *Pseudomonas* sp. was the same as that when grown in medium containing cholesterol or with out it (Doukyu and Aono,1998).

The structure of cholesterol oxidase reveals deeply buried active sites occupied by water molecules in the absence of its steroid substrates (Yue *et al.*, 1999).

(a) The application and uses of cholesterol oxidase

Cholesterol oxidase is industrially and commercially important for application in bioconversions for clinical determination of total or free serum cholesterol (Allain *et al.*, 1974). The enzyme is also used in the microanalysis of steroids in food samples and for distinguishing the steric configuration of 3-ketosteroids from the corresponding 3β-hydroxysteroids (Pollegioni *et al.*, 1999).

- **Diagnosis**

Cholesterol oxidase is used in the determination of serum cholesterol concentration for the assessment of arteriosclerosis and other lipid disorders and of the risk of thrombosis.

Richmond, (1972) and Flegg, (1973) first showed the possibility of using cholesterol oxidase from microorganisms as a basis for a specific assay for serum cholesterol. In the procedure, 4-cholesten-3-one produced was assayed by its UV absorption after solvent extraction of the reaction mixture. However, their procedures were tedious and time consuming.

Allain *et al*., (1974) developed the excellent method in which the hydrogen peroxide produced during the cholesterol oxidase reaction was oxidatively coupled with 4-aminoantipyrine and phenol by peroxidase to form a quinoneimine dye. Figure(1-3) shows the scheme for the enzymatic determination of total cholesterol. First, cholesterol esters are able to be converted to free cholesterol by the action of cholesterol esterase. Then, the liberated cholesterol can be determined by cholesterol oxidase and peroxidase. This enzymatic method is simple and specific under extremely mild conditions. The results with serum samples agreed very well with those obtained by chemical procedures based on the Liebermann-Burchard reaction. This assay system is marketed as a serum cholesterol determination kit; worldwide annual sales amount to 100$ to 200$ million.

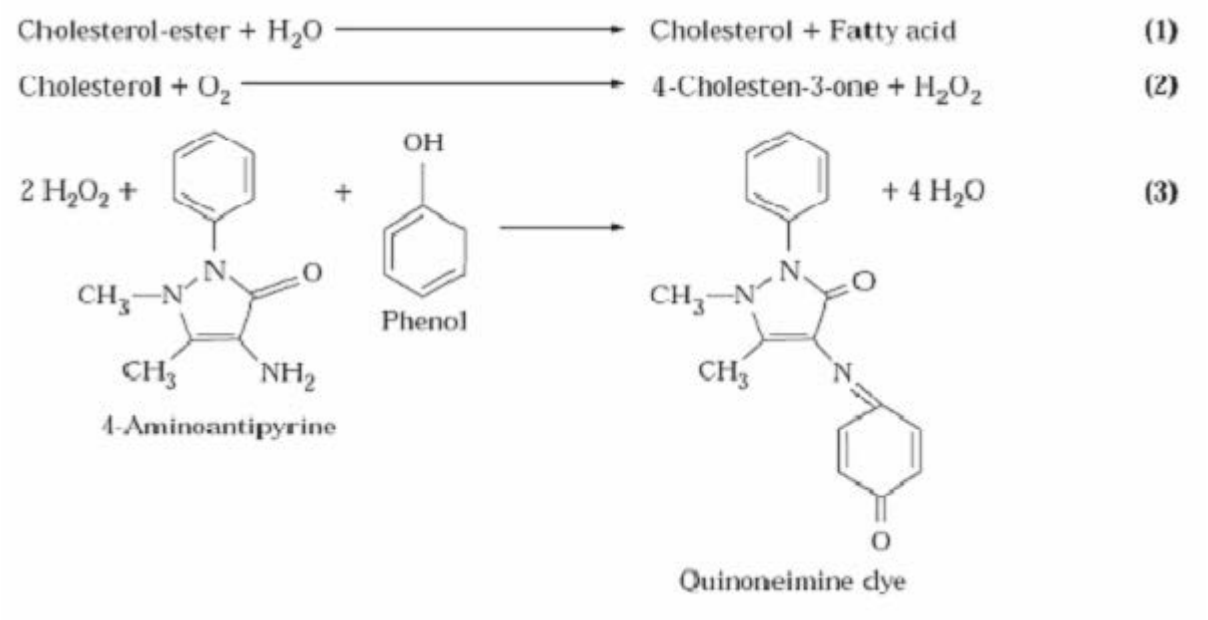


Figure (1-3) Scheme for enzymatic determination of total cholesterol. Reactions 1, 2 and 3 are catalyzed by cholesterol esterase, cholesterol oxidase and peroxidase respectively. (Watanabe *et al*., 1986)

- **Food industry**

Dietary cholesterol has been implicated as one of the causes of coronary artery disease. Because of this, reducing dietary cholesterol is said to cause a gradual reduction in serum cholesterol and regression of atherosclerotic plaque. The National Cholesterol Education Program coordinating board recommends a daily cholesterol intake in the American diet of 300 mg or less. As a result, reduction of cholesterol in food items relatively rich in cholesterol (dairy foods, eggs) has become a challenging problem.

Some organisms have been shown to degrade cholesterol, as described earlier, but only a few do so without accumulating any steroid intermediates. Watanabe *et al*., (1987) described the degradation of cholesterol in egg yolk and

lard by crude cholesterol oxidase in *Rhodococcus. equi* no. 23 and the bacterial cell suspension. *R. equi* no.23 degraded cholesterol with little or no accumulation of steroid intermediates such as 4-cholesten-3-one and 1,4-androstadiene- 3,17-dione, which are recognized as key intermediates in a comprehensive pathway of cholesterol degradation. A biotechnological approach to reducing the cholesterol content of milk involves the genetic engineering of starter culture bacteria (*lactococci*, *lactobacilli*, *streptococci*), leading to the expression of phenotypic traits that are related to cholesterol metabolism. These cultures are then used in the production of fermented dairy foods (yogurt and cheeses).

Somkuti *et al*., (1991) have investigated the possibility of introducing the cholesterol oxidase gene from *Streptomyces* sp. SA-COO into a common dairy starter culture bacterium such as *Streptococcus thermophilus* and *Lactobacillus casai*. The biosynthesis of a functional cholesterol oxidase in *Streptococcus thermophilus* was confirmed by TLC analysis of reaction products after incubation of sonically disrupted cells with cholesterol. However, unlike *Streptomyces lividans, Streptococcus thermophilus* did not secrete cholesterol oxidase into the culture medium. At present, the use of cholesterol-degrading enzymes in the food industry remains unrealistic because of the lack of toxicological data on enzyme extracts and the intermediate products of cholesterol degradation (Corbin *et al*., 1994).

- **Agriculture**

The discovery of novel insecticidal proteins is vital for pest control strategies using transgenic crops. Filtrates from several thousand microbial fermentations were assayed for insecticidal activity against crop-damaging insects. Two *Streptomyces*-culture filtrates killed boll weevil larvae in feeding

studies, and the bioactive component was identified as cholesterol oxidase (Purcell *et al*., 1993). The enzyme was active in a dose-dependent manner, and the LC50 against neonate boll weevil larvae was 20.9 μg/ml after 6 days in diet incorporation bioassay. *Bacillus thuringensis* subsp. Kurstaki protein has an LC50 of 1 μg/mL against tobacco budworm and 37 μg/ml against European corn borer in similar diet-incorporation bioassay. Thus, cholesterol oxidase is active against the boll weevil in the same concentration range that *B. thuringiensis* protein is active against lepidopterans.

Morphological changes induced by ingestion of cholesterol oxidase suggest that the enzyme has a direct effect on the midgut tissue of boll weevil larvae. Thus, cholesterol oxidase disrupted the midgut epithelium at low doses and lysed the midgut cells at higher doses.

The cholesterol oxidase gene from *Streptomyces* sp. was expressed in tobacco and tomato, and successful control of boll weevil was achieved by the Monsanto Company (Corbin *et al*., 1994).

Cholesterol biosensor based on entrapment of cholesterol oxidase in a silica sol-gel matrix at a Prussian blue modified electrode has been developed (Li *et al*., 2003). However, this method of enzyme immobilization raises concerns on reduced surface area for enzyme binding and pore-diffusion resistance (Berry *et* al., 2003).

One of the intriguing aspects of cholesterol oxidase function is its association with the lipid bilayer and its importance in bacterial metabolism and pathogenesis. In recent years, non-aqueous enzymology has emerged as a major area of biotechnology research and development. It has been reported that enzymes behave quite differently in organic media and have striking new properties with longer stability. The enzyme stability in organic solvents is not

only totally different from that in water, but the reaction can be controlled, or even reversed, by the solvent. The main feature of the non-aqueous enzyme-immobilization technique is that the non-soluble enzyme can be recovered after the reaction. Thus solvent engineering provides an alternative approach to protein synthesis (Koskinen and Kilbanov, 1996). The use of enzymes for diagnostic purposes is very restricted, owing to high costs, limited availability and the stability of enzymes at room temperature. During the past decade, immobilization has generally been accepted in the biosensors field as a complementary method to the existing analytical tools. A number of enzyme-immobilization methods have been proposed for the purpose of making enzyme sensors or enzyme reactors, especially for clinical analysis. Immobilized enzymes, therefore, offer a tremendous scope in this regard because they can be separated easily from the solvent system and re-used over a longer period of time (Broun *et al*., 1973; Sundaram *et al*., 1979).

DeCuyper and Jonian (1990) designed phospholipids coated in organic colloids to fix cytochrome ***c*** oxidase to the carrier surface. Strasser and co-workers immobilized mitochondrial inner membrane on concanavalin A–Sepharose. It had been developed a technique for the immobilization of cholesterol oxidase on formvar. Formvar, a polyvinyl resin, and enzymes were immobilized by the entrapment method, with the help of chloroform and ethylene dichloride. The immobilized enzyme was used without any cross-linking agent and the leaching was negligible.

The suitability of the enzyme as an analytical reagent gives several approaches for the assay of cholesterol in serum is therefore possible:

- Measurement of oxygen consumption with an oxygen electrode.

- Measurement of hydrogen peroxide production, enzymetically or otherwise.
- Measurement of Δ4-cholestenone, either directly or as a derivative. (Tammes and Nordschow, 1968).

(b) The structure of cholesterol oxidase

Cholesterol oxidase is a monomeric flavoenzyme, which catalyzes the oxidation and isomerization of cholesterol to cholest-4-en-3-one. The enzyme interacts with lipid bilayers in order to bind its steroid substrate.

The X-ray structure of the enzyme from *Pseudomonas* sp. revealed two loops, comprising amino acid residues 78-87 and residues 433-436, which act as a lid over the active site and facilitate binding of the substrate (Vrielink *et al*., 1991). It was postulated that these loops must open, forming a hydrophobic channel between the membrane and the active site of the protein and thus sequestering the cholesterol substrate from the aqueous environment. Here it had been describe the three-dimensional structure of the homologous enzyme from *Streptomyces* refined to 1.5 Å resolutions**.** Structural comparisons to the enzyme from *Pseudomonas* sp. reveal significant conformational differences in these loop regions; in particular, a region of the loop comprising amino acid residues 78-87 adopts a small amphipathic helical turn with hydrophobic residues directed toward the active site cavity and hydrophilic amino acid residues directed toward the external surface of the molecule. It seems reasonable that this increased rigidity reduces the entropy loss that occurs upon binding substrate. Consequently, the *Streptomyces* enzyme is a more efficient catalyst. In addition,

it had been determined the structures of three active site mutants which have significantly reduced activity for either the oxidation (His447Asn and His447Gln) or the isomerization (Glu361Gln). This structural and kinetic data indicate that His447 and Glu361 act as general base catalysts in association with water and Asn485. The His447, Glu361, H_2O541, and Asn485 hydrogen bond network is conserved among other oxidoreductases. This catalytic tetrad appears to be a structural motif that occurs in flavoenzymes that catalyze the oxidation of unactivated alcohols (Coulombe, 2001).

however the structure of the enzyme is composed of 12 α-helices and 17β -strands and can be subdivided into two functionally distinct domains:(1) an FAD-binding domain and (2) a substrate-binding domain. Although cholesterol oxidase (2) carries out an identical catalytic reaction to that of cholesterol oxidase (1), the structural folds for the two enzymes are vastly different. In particular, the nucleotide binding fold commonly seen in the FAD-binding domain is not present in cholesterol oxidase(2); rather it adopts a fold seen in the structures of vanillyl alcohol oxidase (VAO),the molybdenum-containing enzyme carbon monoxide dehydrogenase , and *p*-cresol methylhydroxylase . This fold has recently been described as a new family of structurally related oxidoreductases (Coulombe, 2001).

(c) The active site of cholesterol oxidase

The active site consists of a cavity sealed off from the exterior of the protein. A model for the steroid substrate, cholesterol, can be positioned in the pocket revealing the structural factors that result in different substrate binding affinities between the two known forms of the enzyme. The structure suggests that Glu^{475}, located at the active site cavity, may act as the base for both the oxidation and the isomerization steps of the catalytic reaction. A water-filled channel extending toward the flavin moiety, inside the substrate-binding cavity, may act as the entry point for molecular oxygen for the oxidative half-reaction. An arginine and a glutamate residue at the active site, found in two conformations are proposed to control oxygen access to the cavity from the channel. These concerted side chain movements provide an explanation for the biphasic mode of reaction with dioxygen and the ping-pong kinetic mechanism exhibited by the enzyme (Coulombe *et al.*, 2001).

(d) The mechanism of cholesterol oxidase activity

To understand the mechanism of the enzyme, it is necessary to first understand how substrate is bound and what physical forms of substrate are preferred. Being very hydrophobic, cholesterol partitions preferentially into the lipid bilayer, although it does have a measurable rate of spontaneous transfer via the aqueous milieu between membranes. The spontaneous rate of cholesterol leaving the lipid bilayer is insufficient to account for the rate of cholesterol oxidation catalyzed by the enzyme. Thus, the enzyme must form a complex with the lipid bilayer that allows cholesterol to move directly from the membrane into the active site, without aqueous solvation of cholesterol. Examination of all the

X-ray crystal structures revealed active sites that are deep cavities and sequestered from bulk solvent by protein loops that cover the entrance to the cavity. It was postulated that in the lipid bilayer protein complex, the loops open to allow active site access to lipidbilayer (Vasodevan and Zhou, 1996).

Generally mode of action of the enzyme was demonstrated by (Allain *et al*., 1974) to include the following steps:

1. Association of substrate with the enzyme.
2. Chemical reactions.
3. Releasing of the product.

Activity of Cholesterol Oxidase can be observed by the followings:

1. Appearance of the conjugated ketones.
2. Formation of hydrogen peroxide in a coupled test with peroxidase.
3. Measuring the oxygen consumption polarographically.

Methods for determining the activity of the enzyme varied between the producing microorganisms and different experiments using the enzyme.

The method used in this study was according to Richmond (1972), which include adding the crude enzyme to a phosphate buffer (0.1ml containing 0.5ml of triton×-100 and at pH 7.0) and the substrate (Cholesterol) and the reading the absorbancy at 240 nm and readings of the spectrophotometer are used in equation for determining the enzyme activity.

1.4.1: Microbial cholesterol oxidases

Cholesterol oxidase is produced by a wide range of microorganisms, and because of its importance for oxidation of cholesterol which has been linked with the development of colon cancer, have led researchers to hypothesize a likely

role for them in cardio vascular diseases in human (Kaunitz,1978), so it had been studied and identified from numerous microorganisms.

The degradation and assimilation of cholesterol by various microorganisms are well known in natural environments. However, knowledge about the degradation pathway of cholesterol is still very limited (Gilliland *et al*., 1985).

Kim *et al*., (2002) demonstrated the microorganisms, which produce cholesterol oxidase, are:

- *Nocardia erythropolis*.
- *Arthrobacter*.
- *Mycobacterium*.
- *Bacillus sphaericus*.
- *Bacillus subtitles.*
- *Brevibacterium.*
- *Corynebacterium.*
- *Rhodococcus erythropolis*.
- *Schizophylum*.
- *Streptomyces* sp.
- *Pseudomonas* sp.

1.4.2: The factors affecting the productivity of the enzyme

Enzymes are proteins, living organisms limit its production, and it's different from other proteins by its specific activity, which make it as a co-factor in biological reactions by lowering the activation energy. Because all enzymes are a protein materials, then any one of high temperature, strong acids or bases,

organic solvents may cause an enzyme denaturation which may led to loss the enzyme activity (Smith and brooks,1974).

There are many conditions, which affect the productivity of cholesterol oxidase, which include carbon source, nitrogen source, phosphate source temperature, pH, and time of incubation.

(a) Effect of pH in cholesterol oxidase productivity

Enzyme productivity and continuing of metabolic pathway is highly affected by pH of the medium. Microorganisms are variable in their pH, eg. Molds are preffers growing in neutral and basic conditions of pH, while fungi can grow in wide range of pH (Park, 1968). There are types of microorganisms grows only in basic range of pH (8 - 11) and their growth and production for enzymes is weak in neutral and acidic ranges of pH (Emtseva and Konovalov, 1979).

(b) Effect of temperature on cholesterol oxidase productivity

Temperature is considered as one of the most important factors affecting enzyme productivity and the growth of the microorganisms. The optimum temperature for the productivity of cholesterol oxidase differs according to the producing microorganism. According to (Salva *et al*., 1999), who found that the optimum temperature for the enzyme productivity from *Brevibacterium* sp. was 53°C and this value is higher than the optimum temperature for the *Rhodoccocus equi*. and from *Corynebacterium cholesterolicum*, which produce the enzyme at 47°C and 40°C respectively. Also, the optimum temperature for cholesterol oxidase produced from *Sreptomyces violascens* is 50°C with a plateau between

40°C and 60°C. The optimum temperature for the production of the cholesterol oxidase from *Pseudomonas* sp. is 30°C (Kim *et al*., 2002).

(C) Effect of time of incubation on cholesterol oxidase productivity

Time of incubation is one of the important parameters affecting the productivity of cholesterol oxidase, and this parameter is different between producing microorganisms, for example incubation period for production of the enzyme from *Nocardia cholesterolicum* was 18–40 hrs, while optimal incubation period for enzyme production from *Brevibacterium* sp. was 7 days (Salva *et al*., 1999).

(d) Effect of nutritional factors on cholesterol oxidase production

i. Effect of carbon source

Microorganisms differ in their needs to carbon sources according to their nutrient nature; the use of pure carbon sources like (glucose, sucrose, and fructose) is expensive from the economical case, so the industrial fermentation try to use cheap carbon sources especially industrial and agricultural by-products such as date extract, beet molasses and cane molasses (Anderson *et al*., 1987; Horan *et al*., 1983).

The optimum carbon source is different between microorganisms for the higher production of cholesterol oxidase, it has been established that soluble starch was the optimal carbon source for the production of the enzyme from *Bacillus circulans* and *Streptomyces* sp., while the maltose is the best carbon source for *Bacillus licheniformis* (Sztajer and Maliszewiska, 1988).

ii. Effect of Nitrogen Source

Nitrogen Source is one of the most important part of the components of the culture medium for the producing microorganism such as inorganic nitrogen salts or organic sources like amino acids and proteins, and these nitrogen sources can be used either as oxidized form ($NO^{-}_{٣}$, NO^{-}_{2}) or as reduced form (NH_2, NH^{+}_{4}). Amino acids and ammonium salts considered as nitrogen sources, which can be used easily by the microorganisms.

Generally the effect of nitrogen sources varies in productivity of the enzyme between different microorganisms (Martin and Demain, 1980).

It was observed that peptone 5% is the best nitrogen source for the production of cholesterol oxidase by *Streptomyces* sp., while soya bean is the best nitrogen source for the enzyme production by *Bacillus* sp. (Sztajer and Maliszewiska, 1988).

iii. Effect of phosphate source

Mineral salts have an effect on the production of cholesterol oxidase by different microorganisms, and there is several studies find that the use of low concentration of inorganic phosphate (8 – 32 mg/l) lead to decrease cholesterol oxidase production comparing with the use of high concentration (1 g/l). It was found that the presence of phosphate in the media may have negative or positive effect on the reactions involve in the biosynthesis of cholesterol oxidase, it was found that the increases in the phosphate concentration lead to inhibit some of these reactions (Horan *et al*., 1983).

1.5: Enzyme Purification

In order to make any study about the enzyme to understand its characteristics and its role in any reaction, it is very important for the enzyme to be partially purified to get exact results during the study. Enzyme purification process means separation of the enzyme from another enzymes and materials presents in the crude filtrate, which include a gradual steps in which the ratio of the enzyme activity / protein concentration is increasing and led to the purification of the enzyme until the homogenization (Kornberg, 1990).

Different methods for purification of cholesterol oxidase from microorganisms were achieved for partial and complete purification. One of the methods used for purification of cholesterol oxidase was achieved in the presence of organic solvents because this enzyme shows stability and also the enzyme activity will enhanced 3 to 3.5 fold in the presence of organic solvents (Doukyu and Aono, 1998).

Other method for extraction and purification of cholesterol oxidase from *Rhodococcus erythropolis* by using of Triton x-114 for extraction and purification of the enzyme, the method depends on the fact that the detergents are able to disrupt lipid – protein association and release of cell linked cholesterol oxidase. The use of Triton x-100 has been largely accepted but other polyoxyethylene type non-ionic detergents whose cloud point is in the biocompatible range can be used for cholesterol oxidase solubilization and purification (Sojo *et al.,* 2002).

3: RESULTS AND DISCUSSION

3.1: Isolation of *Pseudomonas* species

One hundred and fifteen bacterial isolates were obtained from different clinical samples; urine, burns, wounds, ear swaps and sputum and from natural environments; water, soil and fruits as indicated in table (3-1).

Table (3-1): 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

Among the total isolates, 60 isolates were able to grow on cetrimide agar plates, which gives an indication that these isolates are belong to *Pseudomonas* sp., these 60 isolates were further identified according to the cultural, morphological and biochemical tests.

3.2: Identification of bacteria

Local isolates that were able to grow on cetrimide agar plates, which may be suspected to be *Pseudomonas* sp., were further identified according to morphological characteristics and biochemical tests. For the former, colonies of each isolate that were plated on nutrient agar showed different morphological characteristics of *Pseudomonas* sp. such as mucoidal growth, smooth in shape with flat edges and elevated center, whitish or creamy in color and has fruity odor.

3.2.1: Morphological characteristics

Colonies which were suspected to be belong to the genus *Pseudomonas* were grown on nutrient agar, and characterized by mucoidal properties and most of them were produce Pyocyanin and have a grapelike odor and the shape of the colony appears like a fried egg shape, these results are reasonable with the results demonstrated by (Collee *et al*., 1996; Holt *et al*., 1994**).**

3.2.2: Microscopical Examination

Microscopical examination of each isolate showed that they were all motile, non-spore forming, gram negative and rod shape. These results were agreed with (Holt *et al*., 1994) which certify the identification.

3.2.3: Biochemical Tests

The isolates that were suspected to be belongs to *Pseudomonas* sp. were subjected to a number of biochemical tests.

Results indicated in table (3-2) showed that these isolates gave a positive result for oxidase and catalase which indicate that these isolates belongs to *Pseudomonas* sp. in addition to its morphological characteristics which were identical to this genus. After making the steps of the screening of these isolates

to choose the best isolate in cholesterol oxidase production, five isolates showed higher enzyme activity than the others table (3-3).

Table (3-2): Morphological and biochemical characteristics of the locally isolated *Pseudomonas* sp.:

Test	Result
Colony color	Green
Cell shape	Rod
Gram stain	-ve
Catalase Production	+ve
Oxidase Production	+ve
Growth on King A.	+ve
Growth on King B.	+ve
Growth on Cetrimide.	+ve
Gelatin Hydrolysis.	+ve
Citrate utilization.	+ve
Growth at 4 Ċ	-ve
Growth at 42 Ċ	+ve

3.3: Screening of *Pseudomonas* Isolates for Cholesterol oxidase Production:

In order to select the efficient isolate in cholesterol oxidase production, the ability of these isolates in enzyme production was assayed using (LB Medium) by determining enzyme activity (U/ml) of the crude enzyme in culture filtrate using enzymatic assay procedure mentioned in (2.2.6.). Results indicated in table (3-3) showed that five isolates were more efficient in enzyme production

more than the other isolates, activity of the crude enzyme of these five isolates were ranged between (1.56 to 1.71) U/ml.

Table (3-3): Activity of cholesterol oxidase produced from *Pseudomonas* isolates.

Isolate No.	Source	Enzyme Activity U/ml	Isolate No	Source	Enzyme Activity U/ml
H1	URINE	1.18	H 31	WOUNDS	1.42
H 2	URINE	1.02	H 32	WOUNDS	1.47
H 3	URINE	1.29	H 33	WOUNDS	1.36
H 4	URINE	1.26	H 34	EARS	1.22
H 5	URINE	0.72	H 35	EARS	1.21
H 6	URINE	1.37	H 36	SPUTUM	1.21
H 7	URINE	1.0	H 37	SPUTUM	1.17
H 8	URINE	1.41	H 38	SPUTUM	0.4
H 9	URINE	1.26	H 39	SPUTUM	0.63
H 10	URINE	1.21	H 40	SPUTUM	0.65
H 11	BURNS	1.16	H 41	SOIL	1.17
H 12	BURNS	1.41	H 42	SOIL	1.33
H 13	BURNS	1.29	H 43	SOIL	1.33
H 14	BURNS	1.67	H 44	SOIL	1.45
H 15	BURNS	1.61	H 45	SOIL	1.28
H 16	BURNS	1.48	H 46	SOIL	1.41
H 17	BURNS	1.41	H 47	SOIL	1.19
H 18	BURNS	1.46	H 48	SOIL	1.71
H 19	BURNS	1.4	H 49	SOIL	1.46
H 20	BURNS	1.51	H 50	SOIL	1.11
H 21	WOUNDS	1.56	H 51	SOIL	1.57
H 22	WOUNDS	0.64	H 52	SOIL	1.13
H 23	WOUNDS	0.6	H 53	SOIL	1.18
H 24	WOUNDS	1.19	H 54	SOIL	1.14
H 25	WOUNDS	1.32	H 55	SOIL	1.48
H 26	WOUNDS	0.65	H 56	WATER	1.18
H 27	WOUNDS	0.9	H 57	WATER	1.23
H 28	WOUNDS	1.22	H 58	WATER	1.42
H 29	WOUNDS	1.21	H 59	FRUITS	1.08
H 30	WOUNDS	1.42	H 60	FRUITS	1.13

From these results it was obvious that the isolate H48 was the efficient in enzyme production, activity of the crude enzyme produced by this isolate was

1.71 U/ml. These five isolates were further identified by Api20E system as shown in figure (3-1); they were able to utilize arginine, citrate, gelatin and glucose. They gave negative results for β-galactosidase, lysine decarboxylase, ornithine decarboxylase, H_2S, urease, tryptophane deaminase, indole, VP, mannitol, inositol, sorbitol, rhamnose, sucrose, melibiose, amygdalin and arabinose. These results indicated that these five isolates were belongs to the species *Pseudomonas aeruginosa,* these results are in agreement with those mentioned by (Mandell *et al*., 1995; Collee *et al*., 1996).

Figure (3-1): API 20 E systems for identification of *P. aeruginosa.*

3.4: Optimum conditions for production of cholesterol oxidase

3.4.1: Effect of Carbon Source:

Six carbon sources (Fructose, Galactose, Xylose, Glucose, Maltose and Sucrose) were used as a sole source of carbon and energy to determine the favorable one for cholesterol oxidase production from *Pseudomonas aeruginosa* H48.

Results shown in figure (3-2) showed that the maximum production of the cholesterol oxidase was gained when glucose was used as a sole carbon and energy source. Enzyme activity of cholesterol oxidase in the crude filtrate was 1.8 U/ml, while the specific activity was 2.19 U/mg proteins. This was reasonable with Kim *et al*,(2002) who found that the optimal medium composition for cholesterol oxidase production from *Bacillus subtilis* containing

glucose (2%) in addition to cholesterol (0.2%) as inducer for enzyme production, while other carbon sources used (fructose, sucrose, galactose, maltose and xylose) gave the following enzyme activities (1.37, 1.3, 1.18, 0.93 and 0.39 U/ml) respectively and specific activities (1.8, 1.74, 1.73, 1.09 and 0.84 U/mg) respectively.

As mentioned above, the optimal carbon source for enzyme production was glucose, and this may be because it is an important carbohydrate source and the best carbon and energy source for the enzyme production and can be used easily by the microorganism.

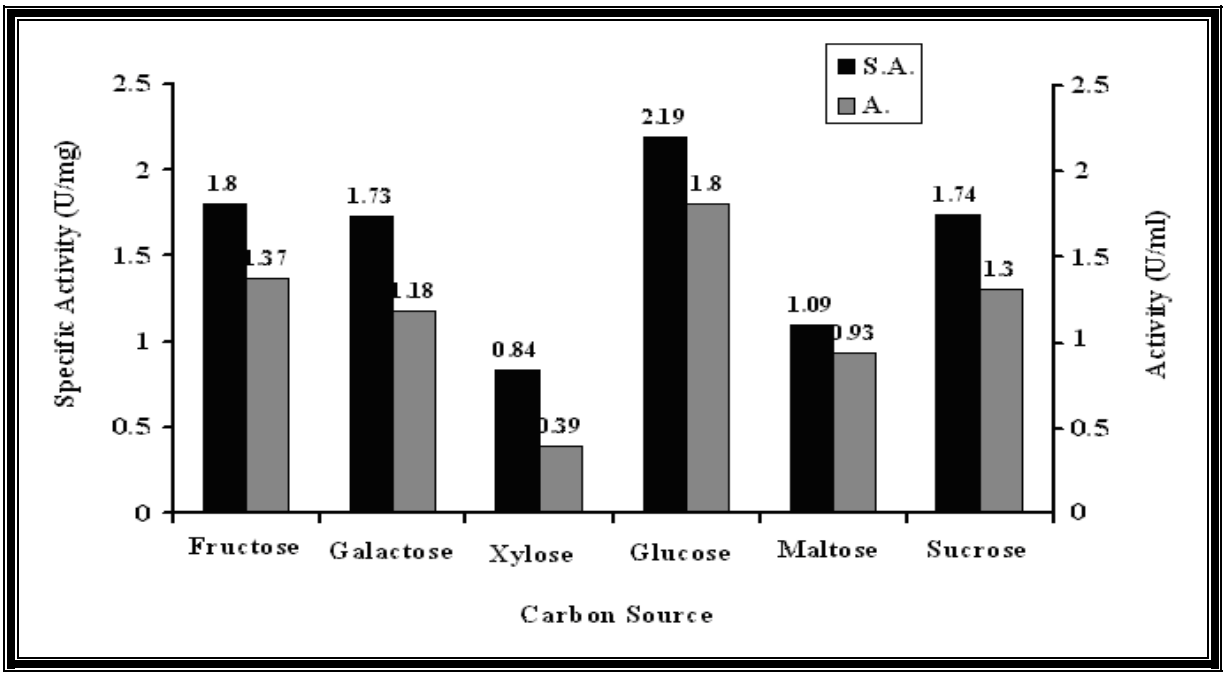


Figure (3-2): Effect of different carbon sources on cholesterol oxidase production by *P. aeruginosa* H48 after incubation at 37°C for 24 hr in shaker incubator with 150 rpm.

Sztajer and Maliszewiska, (1988) demonstrated that soluble starch was the optimal carbon source for the production of the cholesterol oxidase from *Bacillus circulans* and *Streptomyces* sp., while the maltose is the best carbon source for *Bacillus licheniformis*.

Cholesterol was used in this study to detect its effect in enzyme production. The results obtained in the presence of cholesterol as a carbon source was 0.6 U/ml enzyme activity and 1.03 U/mg specific activity.

According to this result the optimum carbon source (Glucose) was used in the next experiments for cholesterol oxidase production.

- **Effect of carbon source concentrations**

Different concentrations of Glucose were used to determine the optimum concentration for the production of the cholesterol oxidase by the *P. aeruginosa* H48. Results indicated in Fig.(3-3) showed that the maximum production of the enzyme was obtained when glucose was added in a concentration of 2.5% the specific activity of the crude enzyme was 2.43 U/mg in the crude filtrate, while other concentrations of glucose showed less activity and specific activity especially the concentrations (1% and 1.5%) which gave specific activities (0.6 and 0.65 U/mg) respectively, then specific activity increased until reaches its maximum at 2.5% which may indicate that this concentration of carbon source is the best for providing the microorganism with the needed energy for growth and maximum production of the enzyme then the specific activity begins to decrease at a concentration 3% of glucose which was 1.58U/mg.

Other studies indicated that the optimum carbon and energy source for the enzyme production varies between different microorganisms, for example Lee *et al.*, (1997) referred that the optimum carbon source for cholesterol oxidase production by *Rhodococcus equi* no. 23 was cholesterol at concentration (0.1 %), while Yazdi *et al.*, (2004) found that the optimum carbon source for *Rhodococcus* sp. was also the cholesterol but in a concentration of (0.15%).

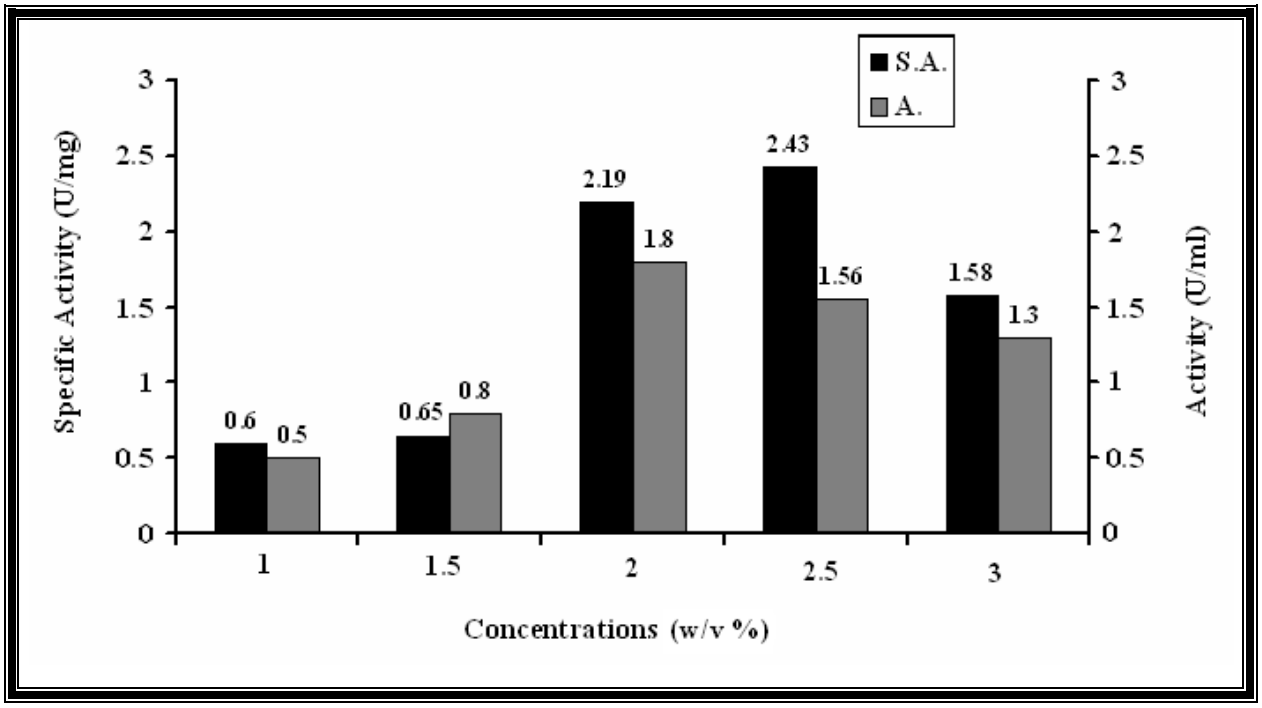


Figure (3-3): Effect of different concentrations of glucose in cholesterol oxidase production by *P. aeruginosa* H48 after incubation at 37°C for 24 hr in shaker incubator with 150 rpm.

٣,٤,٢: Effect of nitrogen source

Different nitrogen sources were added to production media to determine the optimum source for production of cholesterol oxidase from *P. aeruginosa* H48.

Five nitrogen sources (Ammonium nitrates, ammonium chloride, ammonium sulphate, peptone and meat extract), were added to the production medium at a concentration of 1.5% , these nitrogen sources were added to the production medium instead of (Tryptone 1% and yeast extract 0.5%).

Results indicated in figure (3-4) showed that the optimum nitrogen source for cholesterol oxidase production by *P. aeruginosa* H48 was meat extract at which the specific activity of the crude enzyme was 2.53 U/mg. According to this result it is considered the best among the other nitrogen sources; this may because meat extract provides nitrogen, vitamins, amino acids and carbon for microbiological culture media according to Acumedia order (2007). This may

supplements the microorganism with requirements for growth and enzyme production in the culture medium. Other nitrogen sources which are (NH_4NO_3, NH_4Cl, $(NH_4)_2SO_4$ and Peptone) gave different specific activities 2.13, 1.95, 1.8 and 2.11 U/mg respectively, and this is may be because these nitrogen sources do not provide the optimal conditions in the medium for growth of the microorganism and production of the enzyme under study. On the other hand, results indicated in figure (3-4) showed that, organic nitrogen sources are better than inorganic nitrogen sources in cholesterol oxidase production. Generally the effect of nitrogen sources varies in productivity of the enzyme between different microorganisms (Martin and Demain, 1980).It was observed that peptone 5% is the best nitrogen source for the production of cholesterol oxidase by *Streptomyces* sp., while soya bean is the best nitrogen source for the enzyme production by *Bacillus* sp. (Sztajer and Maliszewiska, 1988).

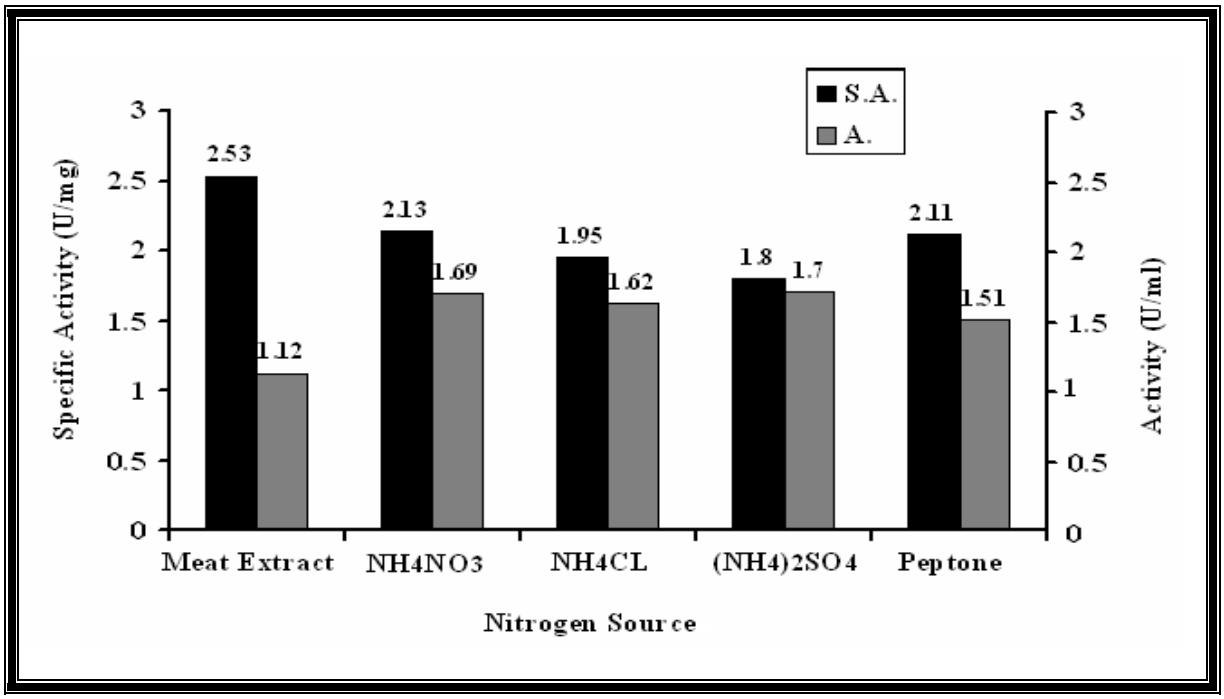


Figure (3-4): Effect of different nitrogen sources in cholesterol oxidase production by *P. aeruginosa* H48 after incubation at 37°C for 24 hr in shaker incubator with 150 rpm.

According to these results, meat extract was used in the next experiment of optimization for cholesterol oxidase production.

- **Effect of nitrogen source concentrations**

Five concentrations of the optimum nitrogen source (Meat Extract), 0.5%, 1%, 1.5%, 2% and 2.5% was used to determine the optimum concentration for cholesterol oxidase production by *P. aeruginosa* H48. Results indicated in figure (3-5) showed that the optimal concentration of meat extract was 0.5% w/v, the specific activity of crude enzyme in crude filtrate was 3.18 U/mg, this result indicating that the composition of meat extract which include nitrogenous source, vitamins and carbon source will be compatible with the presence of glucose 2.5% a carbon source which providing the microorganism with essential nutrients, while the specific activity was decreased with the increasing of meat extract concentration in the production medium (3.03, 2.53 and 2.81 U/mg) then at concentration 2.5% the specific activity was 2.89 U/mg.

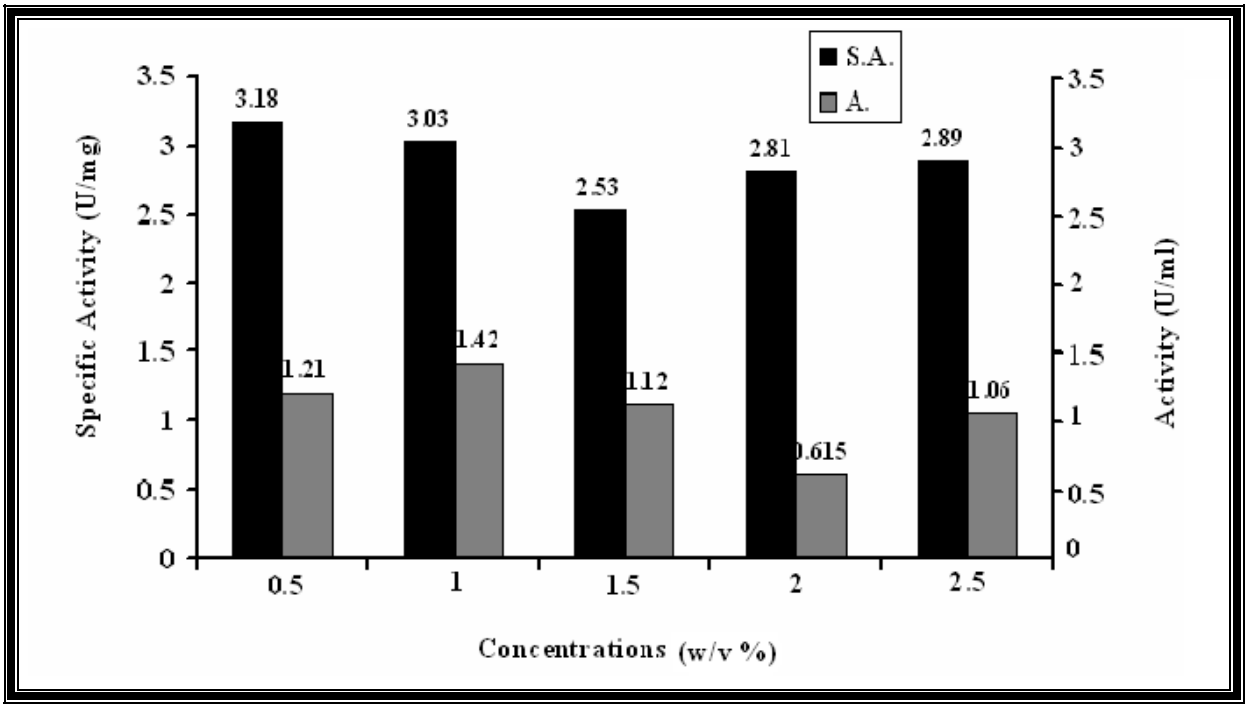


Figure (3-5): Effect of different nitrogen concentrations on cholesterol oxidase production by *P. aeruginosa* H48 after incubation at 37°C for 24 hr in shaker incubator with 150 rpm.

Other studies concerning the production of the cholesterol oxidase from other microorganisms indicated that the best nitrogen source in the production medium from *Bacillus subtilis* was yeast extract in a concentration of 0.5% (Kim *et al*., 2002). While, Lee *et al*., (1997) demonstrated that the optimum nitrogen source for enzyme production from *Rhodococcus equi*. Was yeast extract in a concentration ranged between 0.4 and 0.5%, while addition of NH_4Cl enhancing cholesterol oxidase production, to maximal at 0.1% (w/v). Yazdi *et al*., (2004) mentioned that the optimum nitrogen source for the production medium of cholesterol oxidase from *Rhodococcus* sp. was yeast extract at a concentration of 3% w/v.

According to these results, meat extract was used in a concentration of 0.5% in the next experiments of optimization for cholesterol oxidase production.

3.4.3: Effect of phosphate source

Different phosphate sources were also studied to determine the optimum for production of cholesterol oxidase by the locally isolated *P. aeruginosa* H48. Two types of phosphate sources (KH_2PO_4 and K_2HPO_4) were added to the production medium at a concentration of 0.025% and a mixture of them (K_2HPO_4 0.006% & KH_2PO_4 0.018%, K_2HPO_4 0.018% & KH_2PO_4 0.006% , K_2HPO_4 .0.0125% & K_2HPO_4 0.0125%) was also used.

Results indicated in figure (3-6) showed that the optimum phosphate source for production of cholesterol oxidase by the locally isolated *P. aeruginosa* H48 at which the enzyme gave its maximum specific activity was a mixture of K_2HPO_4 (0.018%) and KH_2PO_4 (0.006%) at which the enzyme activity was 1.47 U/ml and specific activity of the crude enzyme was 3.31 U/mg this is may be due to the effect of this concentration of the mixture of phosphate sources on buffering capacity which has a direct effect on the pH of the production medium, buffering capacity maintain a specific pH value which means that the more molecules of the buffer components that are present, the

larger number of H^+ and OH^- ions that can be absorbed without changing the pH value (McKee 1996), while the other concentrations of the phosphate sources used showed specific activities appears near to the result mentioned previously but it was lower values and as shown in figure (3-6).

Kim *et al*., (2002) mentioned that the optimum phosphate source in production medium of cholesterol oxidase from *Bacillus subtilis* was K_2HPO_4 (0.025%). Salva *et al*., (1999) showed that production medium of cholesterol oxidase from *Brevibacterium* was consist of K_2HPO_4 (0.025%) as phosphate source.

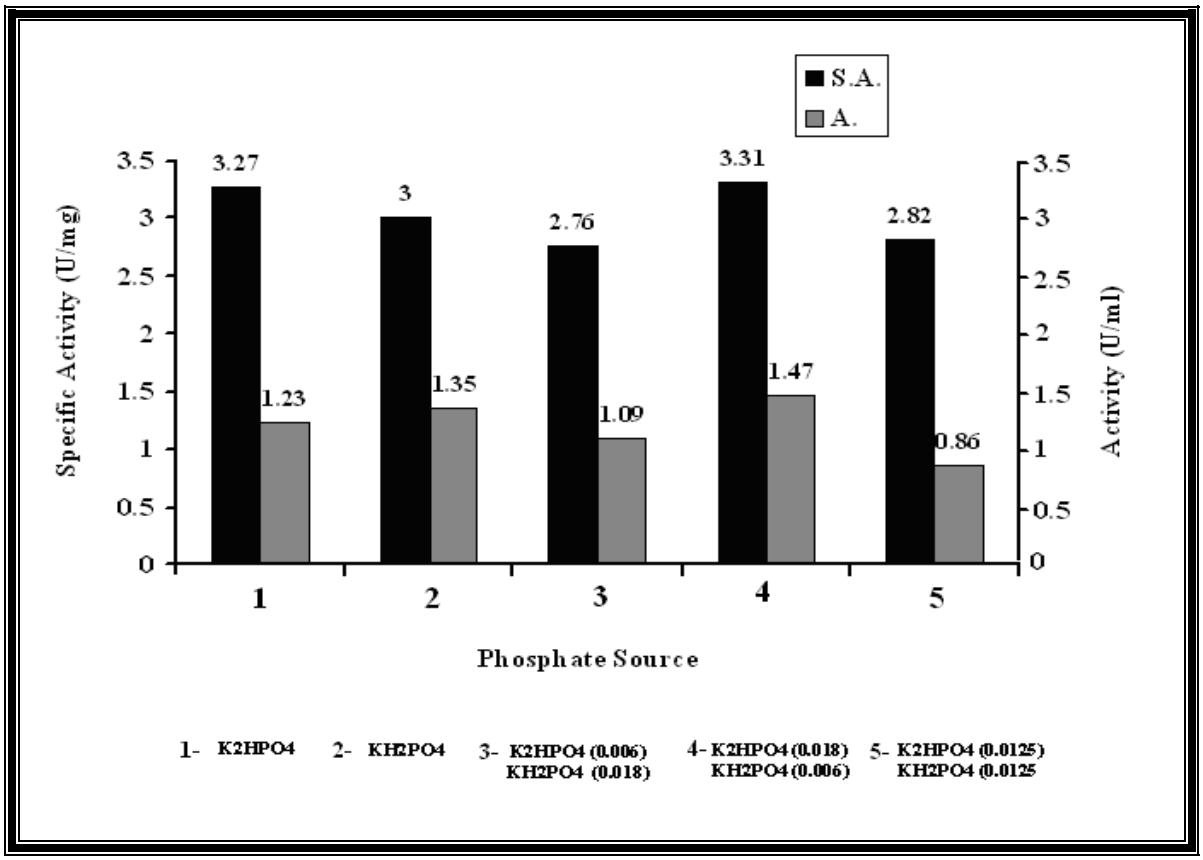


Figure (3-6): Effect of different phosphate sources on cholesterol oxidase production by *P. aeruginosa* H48 after incubation at 37°C for 24 hr in shaker incubator with 150 rpm.

3.4.4: Effect of pH

Different pH values were used to determine the optimum for production of cholesterol oxidase by *P. aeruginosa* H48.

As shown in figure (3-7) it was found that the maximum production of the enzyme was obtained when the pH value of the growth medium was adjusted to 7.0, at which the enzyme activity was 1.47 U/ml and specific activity of the crude enzyme was 3.31 U/mg this result was agreed with Doukyu and Aono (1999). This may because of this pH value may achieve the optimum conditions for bacterial growth and enzyme production.

On the other hand, it was found that the optimum pH value for production of extracellular cholesterol oxidase depends on the bacterial species, but it was almost near from neutral pH for the most bacteria (Kim *et al*., 2002). Lartillot and Kedztora (1990) also demonstrated that the optimum pH for the enzyme production from *streptomyces* sp. was 7.0. Aono *et al.*, (2003) demonstrated that the optimum pH for production of the enzyme was ranging between 5.0 – 8.5. Doukyu and Aono (1998) demonstrated that cholesterol oxidase extracted from *Pseudomonas* sp. showing optimal activity at pH 7.

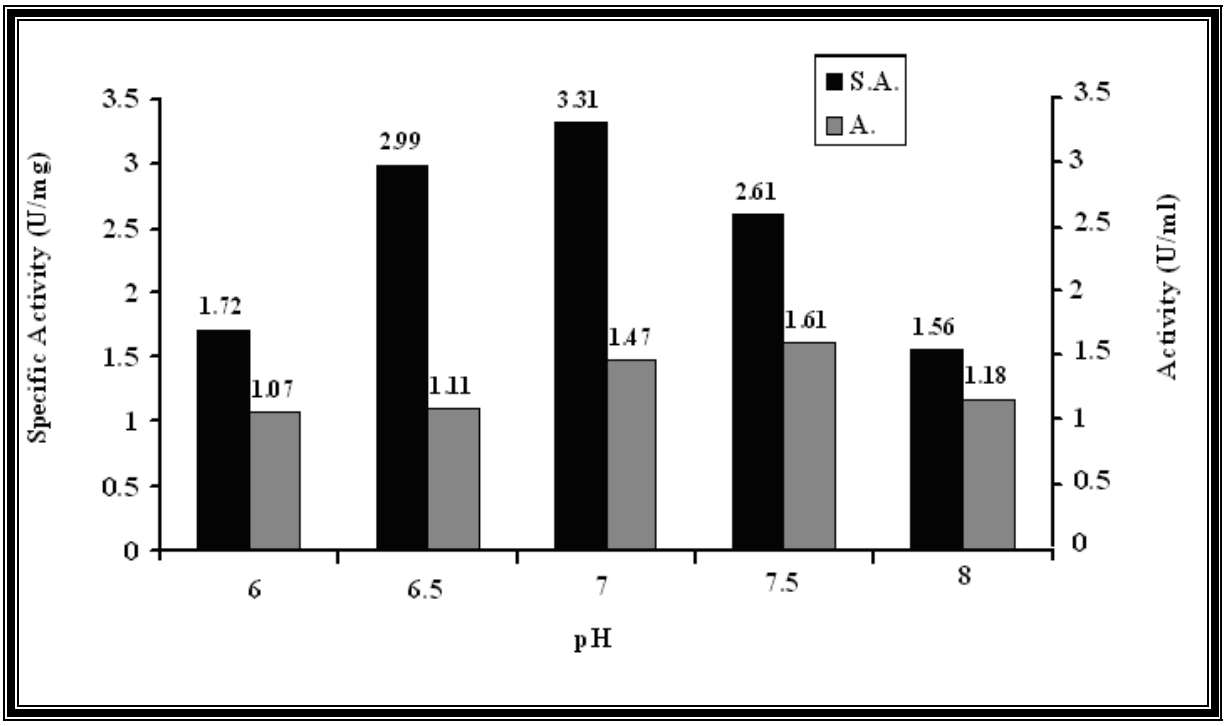


Figure (3-7): Effect of different pH values on cholesterol oxidase production by *P. aeruginosa* H48 after incubation at 37°C for 24 hr in shaker incubator with 150 rpm.

The effect of pH value on enzymes productivity and activity is due to two reasons, which are:

- Its effect on the properities of the culture medium including the solubility of the nutrients molecules transport and ionization.
- pH value affects the stability of the enzyme. (Bull and Bushnel, 1976).

3.4.5: Effect of temperature

In order to determine the optimum incubation temperature for cholesterol oxidase production by *P. aeruginosa* H48, different incubation temperatures (28, 32, 37, 40 and 42°C) were used for this purpose.

It was found that the maximum production of cholesterol oxidase appeared at (32°**C**), in this temperature the enzyme activity was 1.45U/ml and specific activity of the enzyme from filtrate was 3.4 U/mg, this may because that this

temperature was the optimum for growth of the producing microorganism and this result was close to the result observed by Doukyu and Aono (1998) who incubated the bacteria at 30°C.

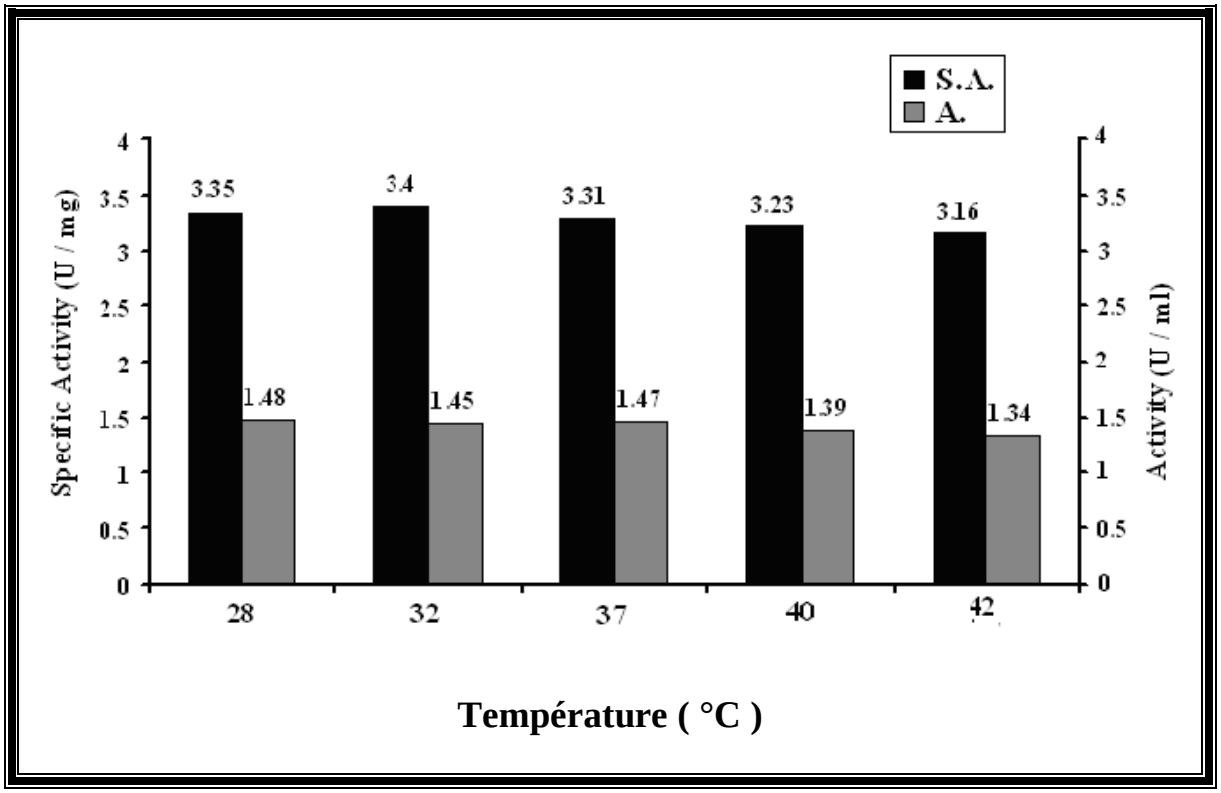


Figure (3-8): Effect of different incubation temperatures on cholesterol oxidase production by *P. aeruginosa* H48 after incubation at 37°C for 24 hr in shaker incubator with 150 rpm.

Low temperatures may lead to decreasing in enzyme production because these low temperatures will cause lowering in the growth of the microorganism, while continuing production of the enzyme at 45Ċ may be due to the ability of this genus to grow at this temperature (Urban *et al*., 2001; Gilbert *et al*., 1991). Optimum temperatures for production of the enzyme depending on the producing microorganism, Salva *et al*., (1999) reported that the temperature profile of cholesterol oxidase from *Brevibacterium* sp. pointed out optimum temperature for enzyme activity at around 53°C and this value was higher than the optimum temperature for the enzyme purified from *Rhodococcus equi.* and

from *Corynebacterium cholesterolicum*, with maximum activity at approximately 47°C and 40°C, respectively. It was closed to that for the enzyme purified from *Streptomyces violascens*, with optimum temperature at around 50°C, with a plateau between 40°C and 60°C. On the other hand Chang *et. al*.,(2004) mentioned that the maximum cholesterol oxidase production by *Rhodococcus equi.* No.23 was at 30 and 25°C, respectively.

3.4.6: Effect of time of incubation

Different periods of incubation (24, 48, 72, 96 and 120 hrs) were applied to estimate the optimum one for production of the enzyme from *P. aeruginosa* H48.

In this study, the optimum incubation period was 48 hrs at which the enzyme activity was 1.43 U/ml and specific activity of the enzyme was 3.48 U/mg as indicated in figure (3-9) this result may be because this incubation period is enough for the microorganism to grow and to consume the nutritional compounds in the production medium perfectly for maximum production of the enzyme. Others showed specific activities similar to that found after 48hr and also it is clear that the enzyme activities were more than that of incubation for 48 hr, for example the enzyme activity after the incubation for 24 and 72 hr were (1.45 and 1.48 U/ml) respectively, which was more than the incubation for 48 but the specific activity was decreased (3.4 and 3.43 U/mg), then the specific activity decreased continuously after (96 and 120 hr) of incubation with specific activity (3.36 and 3.31 U/mg), respectively. It was obvious that the specific activity was decreased gradually and this may be resulted from the consumption of the medium compounds completely by the microorganism.

Incubation periods for the production of cholesterol oxidase varies from one microorganism to another, for example, incubation period for production of the enzyme from *Nocardia cholesterolicum* was 18 – 40 hrs, while optimal

incubation period for enzyme production from *Brevibacterium* sp. was 7 days (Salva *et al*., 2000).

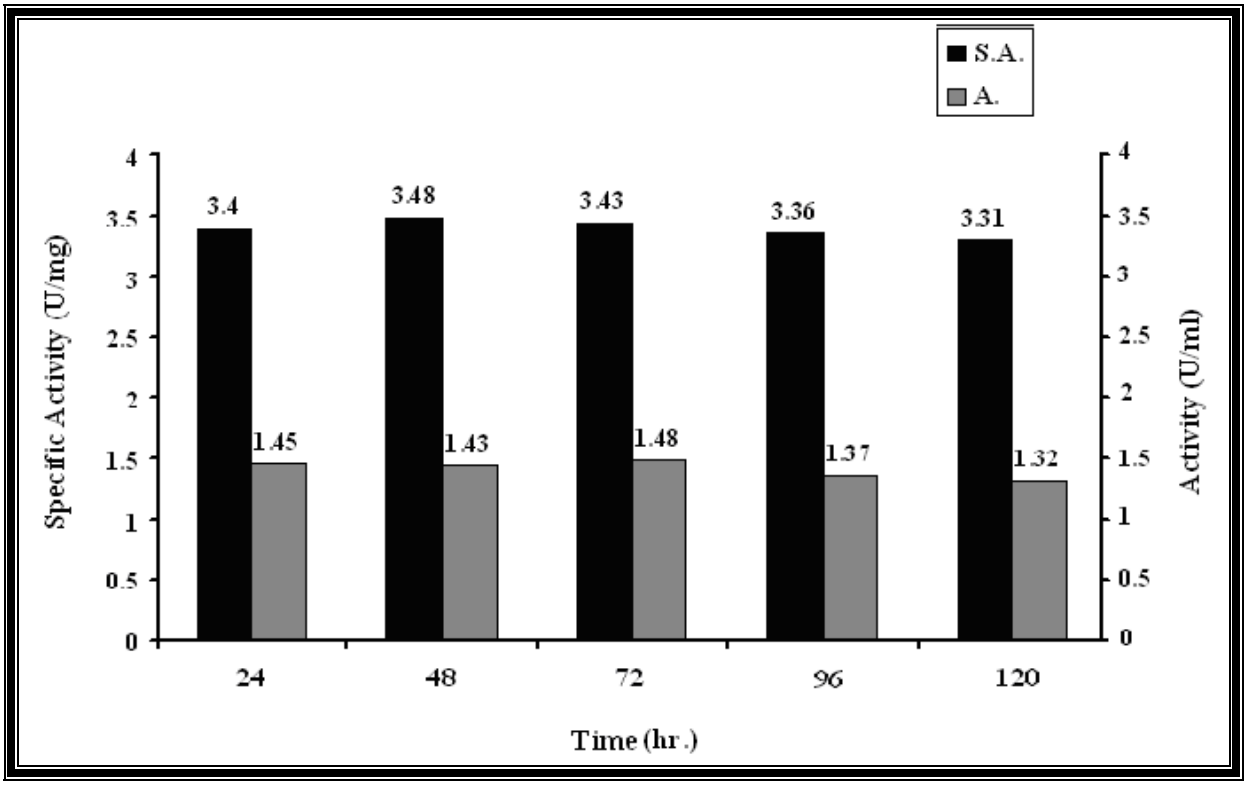


Figure (3-9): Effect of different incubation periods on cholesterol oxidase production by *P. aeruginosa* H48 after incubation at 37°C in shaker incubator with 150 rpm.

3.5: Enzyme Purification

3.5.1: Concentration of the crude enzyme by ammonium sulphate precipitation:

In this study, ammonium sulphates were used in a gradual saturation ratios ranging between 25 - 85%.

It has been found that the precipitated enzyme reaches its maximal activity 3.7 U/ml and specific activity 5.44 U/mg of protein with 4.625% yield at the saturation ratio 60-70% of the ammonium sulphate and this was reasonable with Doukyu and Aono (1998), who reported that this protein can be extracted with 70% saturation of ammonium sulphate.

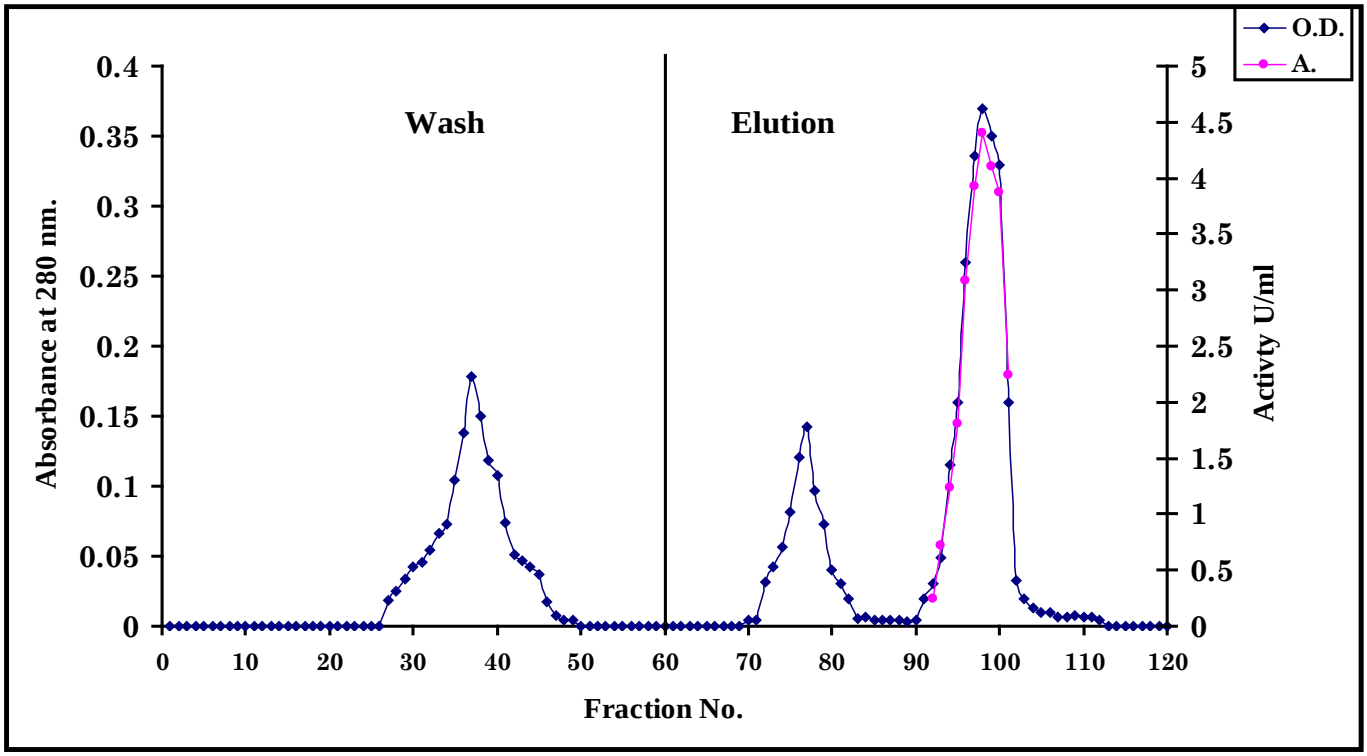
Ammonium sulphates were used at different saturation ratios to concentrate cholesterol oxidase produced by other microorganisms.

Cholesterol oxidase produced from *Nocardia rhodocrous* was precipitated with 50% of ammonium sulphate and the specific activity of the enzyme was 5.4 U/mg with 14.6 % yield (Buckland *et al.*,1975) , and at 50% and 55% of ammonium sulphate from *Brevibacterium* and *Streptomyces* respectively, (Pollegioni, *et al.*,1999).

3.5.2: Purification of cholesterol oxidase by ion exchange chromatography:

Cholesterol oxidase obtained from ammonium sulphate precipitation step was further purified by ion exchange chromatography using carboxymethyl cellulose column with dimensions (22×1.5cm) and equilibrated with phosphate buffer (pH 7.0, 0.25 M), 4 ml of precipitated enzyme was added, and the column was 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. Cholesterol oxidase was eluted with NaCl (0.3 M) which is used as the elution buffer as shown in figure (3-10), and the procedure demonstrated approximately fold of purification was 1.99 with 30.21 % overall yield as shown in table (3-4).

The partially purified cholesterol oxidase showed a final specific activity of approximately 10.6 U/mg of protein and the results shown in figure (3-10) shows CM-cellulose elution fractions containing most of cholesterol oxidase activity. The partially purified enzyme was further characterized in order to determine the optimum pH for enzyme activity, optimum pH for stability, optimum temperature for enzyme activity and optimum temperature for stability.



Figure(3-10): Ion Exchange Chromatography for Purification of cholesterol oxidase from the locally isolated *P. aeruginosa* H48 by using CM- Cellulose.

Table (3-4): Steps of cholesterol oxidase purification from locally isolated *P. aeruginosa* H48:

No.	Purification Steps.	Volume (ml).	Enzyme Activity (U/ml).	Protein Conc. (Mg/ml).	Specific Activity (U/mg).	Total Activity (Unit).	Fold Purification.	Yield %
1	Crude Enzyme.	100	3.2	0.61	5.24	320	1	100
2	Precipitation with Ammonium Sulphate (70%).	4	3.7	0.68	5.44	14.8	38	4.62
3	IonExchange chromatography With CMC (elution)	25	3.87	0.37	10.46	96.75	1.99	30.23

3.6: Factors affecting partially purified cholesterol oxidase:

3.6.1: Optimum pH for activity of partially purified cholesterol oxidase from locally isolated *P. aeruginosa* H48:

Activity of partially purified cholesterol oxidase from *P. aeruginosa* H48 was studied in different pH values to determine the optimum. This step was accomplished by adding 1.8 ml of cholesterol solution to 1 ml of phosphate buffer with range of pH value (5.0 – 9.0), then incubated for 10 min at 32Ċ, then 0.2 ml of purified enzyme was added then enzyme activity was determined.

As shown in figure(3-11), the optimum pH was 7.0 for cholesterol oxidase activity at which the enzyme activity was 3.8 U/ml , and the activity at this pH value was higher than those of the acidic and basic values because the effect of the pH of the reaction medium on the ionizable groups located in the active sites of the enzyme or as a result of changing the ionic form of the enzyme – substrate (ES) complex and enzyme – product (EP), this effect depends especially on the (ES) when the concentration of the substrate is greater than Michalis constant (Km < S), while in the case of the concentration of the substrate is lower than Michalis constant (Km > S) the effect will depends on the enzyme, (Whitaker, 1972).

Salva *et al.*, (2000) demonstrated that the optimum pH value for cholesterol oxidase from *Brevibacterium* sp. was 7.5.

Richmond, (1972), certified that the optimum pH for the activity of the enzyme is 7.0; the optimum pH may differ according to the type of the producing microorganism.

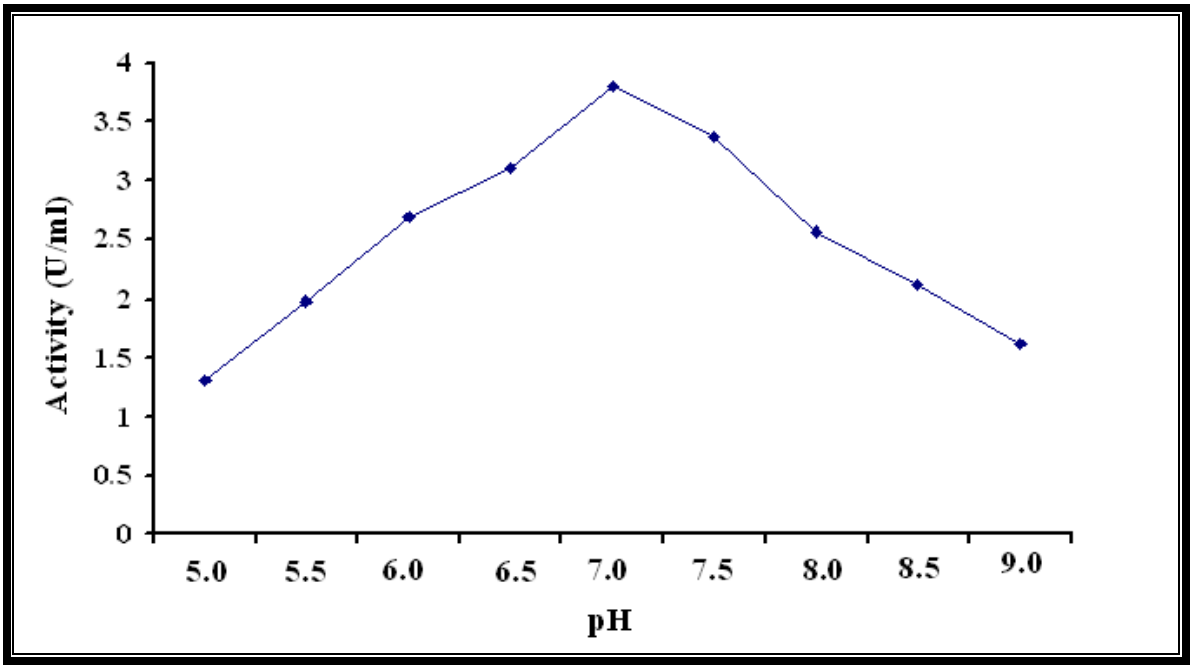


Figure (3-11): Effect of pH on the activity of partially purified cholesterol oxidase by *P. aeruginosa* H48.

3.6.2: Stability of partially purified cholesterol oxidase at different pH values:

Stability of cholesterol oxidase was studied at different pH values by addition of 0.2 ml of the purified enzyme to test tubes containing 1 ml of the buffer solutions with pH ranging between (5.0 - 9.0), then incubated for 30 min at 32Ċ, then 0.2ml was taken from each tube and added to test tubes containing 1.8 ml of the cholesterol solution, then enzyme activity was determined.

It has been found that enzyme activity was 3.62 U/ml at the pH 6.5, and it is also stable at pH range of 6.0 – 8.5.

Stability of cholesterol oxidase in different pH values varies between producing microorganisms.

It has been found that the enzyme obtained from *Rhodococcus* sp. is stable at a wide range of pH values but its optimum pH for stability was 7.0 and also keeps its stability at pH values between 6.0 – 8.0 (Lee *et al*., 1997).

Cholesterol oxidase produced by *Pseudomonas* sp. shows stability over a rather wide pH range of 4.0 – 11.0 (Lee *et al*., 1989).

Salva *et al*., (1999) showed that cholesterol oxidase obtained from *brevibacterium* sp. has a wide pH range of stability which ranging between (5.0 – 9.0).

Results in figure (3-12) include the optimum pH for the stability of the enzyme activity and shows the pH range of stability.

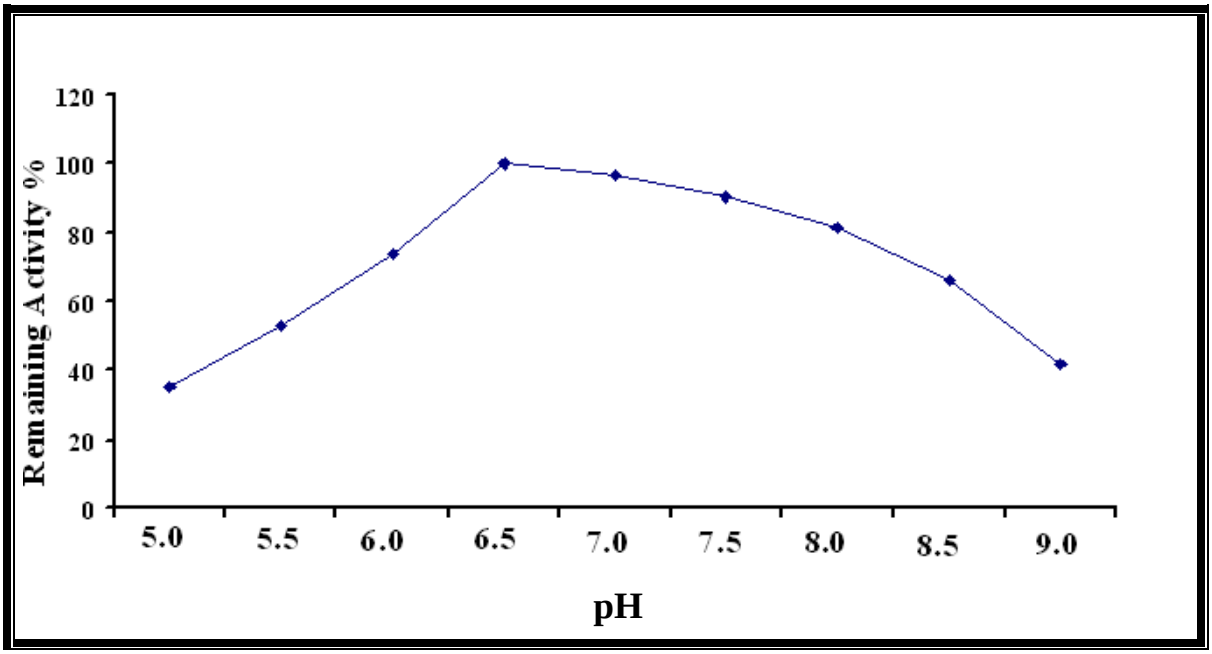


Figure (3-12): Effect of pH values on the stability of the activity of partially purified cholesterol oxidase from locally isolated *P. aeruginosa* H48.

3.6.3: Optimum temperature for cholesterol oxidase activity from locally isolated *P.* aeruginosa H48

Stability of the purified enzyme was studied by incubation of 1.8 ml cholesterol solutions at different temperatures ranging from 25°C to 65°C for 10 min. then 0.2 ml of the purified enzyme was added and left for 10 min then enzyme activity was determined. The results in the figure(3-13) indicated that the activity of the enzyme reaches the maximum (3.48 U/ml) at 35°C, and decreased to (1.1 U/ml) at 65°C, which may be attributed to the denaturation of the enzyme because of the effect of high temperatures at the structure of the protein and changing the structure of the active site which results in unsuitability of the enzyme for binding with the substrate and reaction, this result is reasonable with that obtained by Wang, (1999). The increase in enzyme activity with temperature is due to the elevating in reaction energy for the enzyme and the substrate which leads to the formation of enzyme – substrate complex and this will result in increasing the chemical reaction speed (Urban *et al*., 2001). Chang *et al*., (2004) observed that the optimum temperature for activity of

cholesterol oxidase from *Rhodococcus* sp. was 30°C. Salva *et al.,* (1999) demonstrated that the optimum temperature for cholesterol oxidse activity from *Brevibacterium* was 45°C.

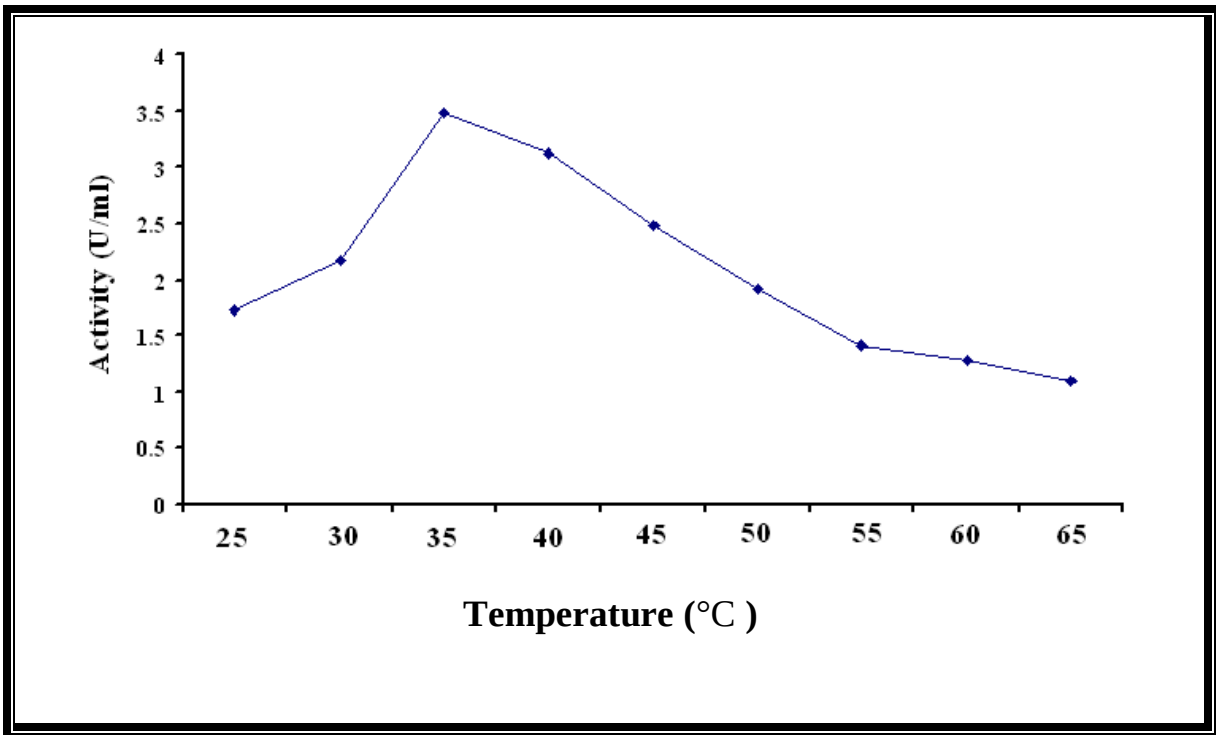


Figure (3-13): Effect of different temperatures on the activity of partially purified cholesterol oxidase from locally isolated *P. aeruginosa* H48.

3.6.4: Stability of partially purified cholesterol oxidase from locally isolated *P. aeruginosa* at different temperatures:

As indicated in figure (3-14), cholesterol oxidase keeps its activity when incubated at different temperatures for 10 min especially at 35 - 40°C, at which the enzyme activity was 3.17 and 3.42 U/ml respectively, then the activity decreased gradually. This decreasing of enzyme activity with the increase of temperature is obvious in most enzymes. Enzymes sensitivity differs toward temperatures, many enzymes are highly sensitive to high temperatures and lose its activity at 35Ċ like Catalase extracted from cows liver (Godfrey,1983), while other enzymes still active for few minutes at 100Ċ like Ribonuclease. Some enzyme features (Molecular weight and Complexity) have a relationship with enzyme sensitivity towards temperature. Mostly, enzymes with low molecular

weights which composed of single polypeptide chain and contain disulfide bonds (s – s bonds) are more stable in high temperatures than complex enzymes with high molecular weights. The environmental conditions also contribute in increasing and decreasing the enzymes sensitivity to temperatures, like pH, ionic strength and the presence of other materials with the enzyme (Segel, 1976; Whitaker, 1972). In this study, the enzyme shows activity at different temperatures but was stable obviously at 40Ċ, and this result was reasonable with Vasudevan and Zhou (1996) who demonstrated that the enzyme is stable at 40-45.Other studies showed that the stability of the enzyme is different between the producing microorganisms, Salva *et al.*, (1999) demonstrated that the enzyme from *Brevibacterium* is stable at 53°C.

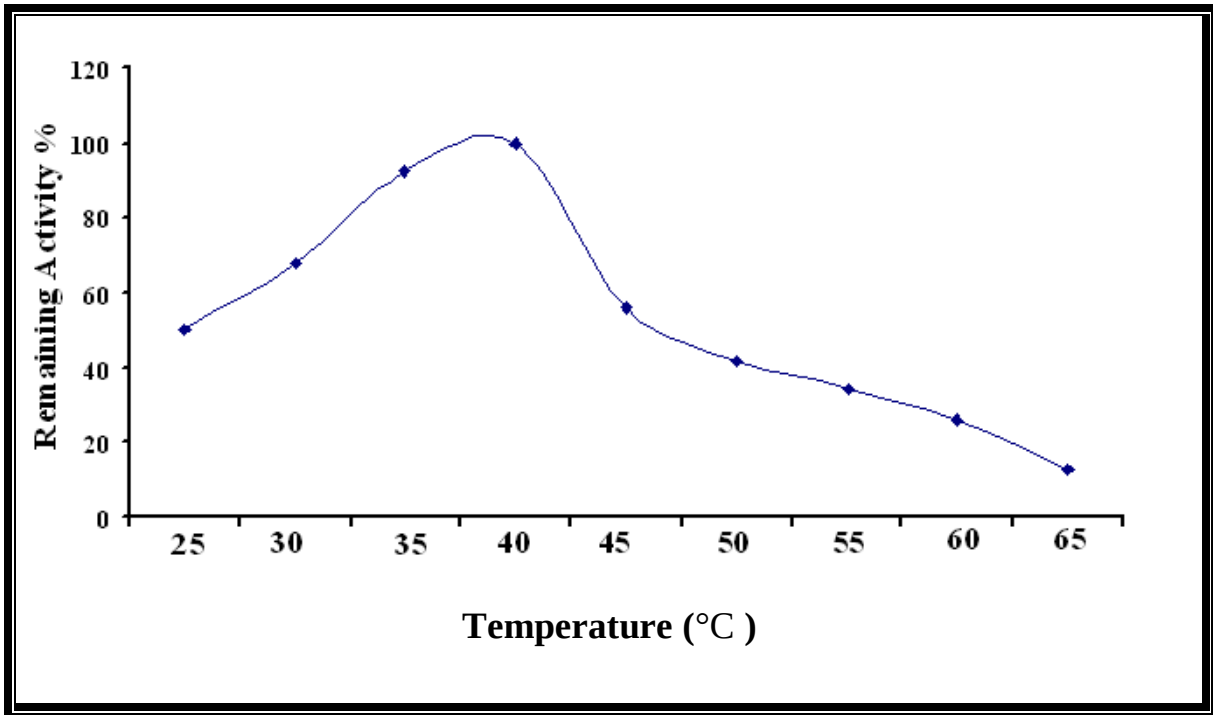


Figure (3-14): Effect of temperatures on the stability of the activity of partially purified cholesterol oxidase from locally isolated *P.* aeruginosa H48.

2: MATERIALS AND METHODS

2.1: Materials

2.1.1: Equipment and Apparatus

The following equipments and apparatus were used in this study:

Equipment	Company
Autoclave	Karl Kolb/Germany
Compound Light microscope	Olympus (Japan)
Refrigerator centrifuge	Harrier (U.K.)
Distillator	Gallenkamp (England)
Hot plate with magnetic stirrer	Gallenkamp (England)
Incubator	Gallenkamp (England)
Electrical Oven	Gallenkamp (England)
pH-meter	Gallenkamp sanyo (U.K.)
Sensitive balance	Mettler/Swizerland
Shaker incubator	Sartorius (Germany)
UV- Spectrophotometer	GFL (Germany)
Spectrophotometer	Aurora instruments Ltd.(England)
Vortex	Stuart scientific (U.K.)
Water bath	Memmert (Germany)
Portable Centrifuge	Hermle labortechnik(Germany)
Micropipette	Witey (Germany)

2.1.2: Chemicals

The following chemicals were used in this study:

Chemicals	Company
K_2HPO_4, KH_2PO_4, $MgSO_4.7H_2O$, $CaCl_2.2H_2O$, NaH_2PO_4, Na_2HPO_4, $Na_2MO_4.2H_2O$, $MgCl_2$, K_2SO_4, Glucose, Sucrose, Fructose, Na_2CO_3, $CaCl_2$, NaOH, Maltose, Ammonium sulphate.	BDH (England)
Gelatin	Biolife (Italy)
Agar, Hydrogen peroxide, N,N,N,N-tetramethyl-p-phynylene-diamine dihydrochloride	Difco(U.S.A)
NaCl, crystal violet, Cholesterol.	Fluka(Switzerland)
Peptone, Meat Extract	Oxoid (U.K)
Cetrimide, Glycerol, Phenol, Ammonium chloride	Riedel-Dehaeny-(Germany)
Bovine serum albumin,	Sigma (U.S.A)
Tris(Hydroxymethylaminoethane)	Sigma(Germany)
(CMC_ Cellulose)	LKB (Sweeden)

2.1.3: API 20E Kit (API Bio Merieux, Lyon, France)

API 20E Kit consists of:

(a) Galleries: the gallery is a plastic strip with 20 microtubes containing dehydrated reactive ingredients.

(b) Api 20E reagents:

- Oxidase reagent (1% tetra-methyl-p-phenyle-diamine)
- Kovac's reagent (p-dimethyl aminobenzaldehyde at 4% in HCl isoamyl alcohol).
- Voges-Proskauer reagent:
  - Vp1 (40% potassium hydroxide).
  - Vp2 (6% alpha-nephthol).
- Ferric chloride 10%.

2.1.4: Media

(a) Ready to Use Media

These media were prepared as recommended by manufacturing Companies. The pH was adjusted to 7.0 and sterilized by autoclaving.

- MacConkey agar (Oxoid-England).
- Nutrient agar (Oxoid-England).
- Nutrient broth (Oxoid-England).
- Simmon citrate agar (Difco-U.S.A).
- Trypticase soy agar (Biolife).

(b) Synthetic Media

- **Cetrimide agar Medium** (Stolp and Gadkari, 1981)

  This medium was consisting of the following components:

Component	Concentration(g/L)
Peptone	20
$MgCl_2$	1.5
K_2SO_4	10
Cetrimide	0.3
Agar	15

These components were dissolved in 950 ml of distilled water, pH was adjusted to 7.2- 7.4 then volume was completed to 1000 ml and sterilized by autoclaving (121 ºC 15 Ib/in^2).

- **King A medium (**Starr *et al*.,1981**)**

  This medium was consisting of the following components:

Component	Concentration(g/L)
Peptone	20
K_2SO_4	10
$MgCl_2$.	1.4
Glycerol	10
Agar	15

These components were dissolved in 950 ml of distilled water, pH was adjusted to 7.2 then volume was completed to 1000 ml and sterilized by autoclaving (121 ºC 15 Ib/in^2).

- **King B medium (**Starr *et al.*,1981**)**

  This medium was consisting of the following components:

Component	Concentration(g/L)
Peptone	20
$MgSO_4.7H_2O$	3.5
K_2SO_4	1.5
Glycerol	10
Agar	15

These components were dissolved in 950 ml of distilled water, pH was adjusted to 7.2 then volume was completed to 1000 ml and sterilized by autoclaving (121 ºC 15 Ib/in^2).

- **Starch Agar (**Atlas *et al.*,1995)

  This medium was consisting of the following components:

Component	Concentration(g/L)
Agar	12
Soluble starch	10

These components were dissolved in 950 ml of distilled water, and pH was adjusted to 7.5 then volume was completed to 1000 ml and sterilized by autoclaving (121 ºC 15 Ib/in^2).

- **Gelatin medium (**Stolp and Gadkari,1984**):**

  This medium was consist of the following components

Component	Concentration(g/L)
Gelatin	4
K_2HPO_4	0.5
KH_2PO_4	0.5
Glucose	0.05

These components were dissolved in 950 ml of distilled water, pH was adjusted to 7.0 then volume was completed to 1000 ml and sterilized by autoclaving (121 ºC 15 Ib/in^2).

- **Laurie Broth medium (L.B.).(**Doukyu and Aono, 1998**).**

  This medium was consisting of the following components:

Component	Concentration(g/L)
Tryptone	10
Yeast Extract	5
NaCl	10

These components were dissolved in 950 ml of distilled water, and pH was adjusted to 7.0 then volume was completed to 1000 ml and sterilized by autoclaving (121 ºC 15 Ib/$in^{2)}$.

2.1.5: Reagents

- **Catalase reagent (**Atlas *et al.*,1995**):**

  This reagent composed of (3%) hydrogen peroxide.

- **Oxidase reagent (**Atlas *et al.*,1995**):**

This reagent is composed of 1gm of N, N, N; N-tetramethyl-p-phynylene-diamine dihydrochloride dissolved in 100 ml distilled water and kept in dark bottle at 4°C.

- **Crystal violet stain (**Atlas *et al.*,1995**):**

This stain was prepared by dissolving 2 gm of crystal violet in 20 ml of 95% ethanol then the volume was completed to 100 ml with distilled water and filtered using Whatman filter paper No. 2 and stored at 4Ċ

- **Sufranin counter stain (**Atlas *et al.*,1995**):**

This stain was prepared by dissolving 0.25 gram of sufranin in 10 ml of 95% ethanol, and then the volume was completed to 110 ml with distilled water, and allowed to stand for several days and filtered through filter paper wattman No. 1 and kept at 4°C.

2.1.6: Solutions and Buffers

- **Phosphate buffer saline:** (Cruikshank *et al.*,1975)

This solution was prepared by dissolving 8g of NaCl, 0.2g KCl, 0.2g of KH_2PO_4, and 1.15g of Na_2HPO_4 in 1000 ml of distilled water; pH was adjusted to 7.3 and sterilized by autoclaving.

- **Coomassie Brilliant Blue G-250**

This solution was prepared by dissolving 100mg of coomassie blue G-250 in a mixture containing 100 ml of phosphoric acid (٪85) and 50 ml of ethyl alcohol, then the volume was completed to 1 litter by disttilled water, and the solution was then filtered through watman filter paper No. 1 and stored at 4°C.

- **Sodium Hydroxide solution (1 M)**

This solution was prepared by dissolving 4g of sodium hydroxide in 100 ml distilled water.

- **Bovine serum albumin solution (1mg / m**l):

This solution was prepared by dissolving 0.1g of bovine serum albumin in quantity of distilled water then the volume was completed to 100ml with distilled water.

2.2: Methods

2.2.1: Sterilization methods (Collins and Lyne, 1987)

- **Moist heat sterilization**

Solutions and Culture media, and solutions were sterilized by autoclaving at 121°C, 15 1b/in^2 for 15 minutes.

- **Dry heat sterilization**

Glass wares were sterilized in an oven at 180 – 200 °C for 2 hours.

2.2.2: Isolation of *Pseudomonas* sp.

(a) Samples collection

Two types of samples were collected in order to isolate *Pseudomonas* sp. which they were:

i. Clinical Samples

Different clinical samples were collected from sputum, urine, and ear, in addition to samples collected from patients suffering from burns and injuries from different hospitals in Baghdad governorate.

ii. Environmental Samples

Water and soil samples were collected from different locations in Baghdad governorate.

(b) Samples preparation

Clinical samples were grown directly in nutrient broth containing test tubes and immediately transferred to the department of biotechnology labs, then they were platted on MacConkey agar plates and incubated overnight at 37°C, while environmental samples from water and soil were diluted using sterilized distilled water and as follows:

i. Water Samples

One-milliliter of each sample was diluted by 9ml of sterilized distilled water in test tubes, mixed thoroughly, serial dilutions for each tube were done separately, then 100µl aliquots from the appropriate dilution (10^{-3}) were taken out and spreaded on MacConkey agar plates and incubated at 37°C for 18 hours.

ii. Soil Samples

One-gram of each soil sample was added to 9ml of sterilized distilled water in test tubes, mixed thoroughly and serial dilution for each tube were done separately, then 100µl aliquots from the appropriate dilution (10^{-3}) were taken and spreaded on MacConkey agar plates and incubated at 37°C for 16 hours.

After incubation, the resultant colonies were selected and streaked on cetrimide agar plates (as a selective medium for *Pseudomonas* sp.) and incubated at 37°C for 24 hour.

2.2.3: Identification of *Pseudomonas* isolates

Different isolates from clinical and environmental samples were taken and grown on cetrimide agar medium to make sure that this type of bacteria may belong to the genus of *Pseudomonas*, and then they were identified according to their staining ability, shape, color, size, production of pigments, transparency and mucoid properties of the colonies growing on nutrient agar and MacConkey agar plates, then some biochemical tests were achieved as follows:

(a) Cultural and Morphological Study

i. Morphology of colonies

Shape, color and viscosity of the colonies were studied on nutrient agar plates medium after incubation at 37°C for 24 hr.

- **Gram's stain (**Harely and Prescott,1996**)**

Single colony of each isolate was transferred and smeared on a clean slide and fixed by heat. The smear was stained with crystal violet (for 1 minute) and excess stain was washed then treated with iodine(for 1 min.) and

the excess was washed with distilled water, then decolorized with absolute alcohol and the excess was washed, and then counterstained with safranine(for 30 second) and the excess was washed, then examined under light microscope.

- **Growth at 4°C and at 42°C (**Collins and Lyne,1987**)**

One of the tests used for differentiation between species of the genus *Pseudomonas* is achieved by growing the bacterial isolates at 4°C and at 42°C .At this test bacterial isolates (or the suspected *Pseudomonas* isolates) were grown on nutrient agar plates and incubated at 4°C and at 42°C for 24 hr.

(b) Biochemical testes

i. Catalase teste (Maza *et al*.,1997**)**

This test was performed by adding few drops of hydrogen peroxide H_2O_2 (3%) on a single colony of each isolate grown on nutrient agar. The production of gaseous bubbles indicates a positive result.

ii. Oxidase test (Harely and Prescott, 1996**)**

Filter paper was saturated with oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride), then single colony of each bacterial isolate was rubbed on the filter paper with a sterile wooden applicator stick. An immediate color change to a deep blue indicates a positive result.

iii. Citrate utilization test (Atlas *et al*., 1995**)**

This test was used to examine the ability of bacterial isolates to utilize citrate as a sole source of carbon and energy. In this test, a colony of each

isolate was inoculated simmon citrate slant and was incubated overnight at 35°C. Appearance of blue color indicates a positive result.

iv. Gelatin hydrolysis test (Atlas *et al.*, 1995**)**

This test was performed to demonstrate the ability of bacterial isolate to hydrolyze gelatin (collagen).

Nutrient broth- gelatin tube were inoculated with bacterial isolates and incubated at 37°C for 24- 48 hour. After incubation, tubes were placed in a refrigerator for 30- 60 minutes. After incubation, liquefaction of nutrient broth- gelatin media indicates a positive result.

v. Growth on cetrimide agar (Stolp and Gadkari, 1981**)**

This medium was used as selective medium for *Pseudomonas* sp.; bacterial isolates were streaked on citrimide agar plates and incubated at 37°C for 24 hr.

vi. Growth on King A medium (Cruikshank *et al.*, 1975**)**

This test was performed to study the production of pyocyanin pigment. Single colony of each isolate was streaked on kings A medium and incubated at 37°C for 24 hr.

vii. Growth on King B medium (Cruickshank *et al.*, 1975**)**

This test was performed to study the ability of production of fluorescin pigment. Single colony of each isolate was streaked on King B medium and incubated at 37°C or 24 hr. Then the plates were exposed to U.V. light to detect the presence of fluorescin.

2.2.4: Identification of *Pseudomonas* Isolates using API 20 E system

Local isolates that have the features and characteristics of *Pseudomonas* sp. on nutrient agar plates subsequently identified using biochemical tests were further characterized using Api20 E system as a standardized characterization system for Enterobacteriaceae and other non-fastidious Gram-negative rods. The system consists of 20 microtubes containing dehydrated substrates. These tests are inoculated with a bacterial suspension, which reconstitutes the media. During incubation, metabolism produces color changes that are either spontaneous or revealed by the addition of reagents.

Biochemical tests included in this system are:

1. ONPG: Beta-galactosidase test.
2. ADH: Arginine dehydrolase test.
3. LDE: Lysine decarboxylase test.
4. ODC: Ornithine decarboxylase test.
5. CIT: Citrate utilization test.
6. H_2S: Hydrogen sulphide test.
7. URE: Urease test.
8. TDA: Tryptophane deaminase test.
9. IND: Indol test.
10. VP: Voges Proskauer test.
11. GEL: Gelatin liquifaction test.
12. GLU: Glucose fermentation test.
13. MAN: Mannitol fermentation test.
14. INO: Inositol fermentation test.
15. SOR: Sorbitol fermentation test.
16. RHA: Rhamnose fermentation test.
17. SAC: Sucrose fermentation test.

18. MEL: Melibiose fermentation test.
19. AMY: Amygdalin fermentation test.
20. ARA: Arabinose fermentation test.

(a) Preparation of the strip

Five-milliliter of distilled water dispensed into wells of the strip in order to provide a humid atmosphere during incubation.

(b) Preparation of the inoculum

Single, pure isolated colony was picked up from plating medium of nutrient agar. This colony was suspended in a test tube containing 5 ml of distilled water and mixed thoroughly.

(c) Inoculation of the strip

According to the manufacture instructions, both the tube and cupule section of CIT, VP and GEL tests were filled with the bacterial suspension. Other tests, only the tubes were filled. The tests ADH, LCD, ODC, URE and H_2S were overlaid with mineral oil to create anaerobic conditions. After inoculations, the plastic lid was placed on the tray and inoculated at 37°C for 24 hr.

(d) Reading the strip:

After incubation, the following reagents were added to the corresponding microtubes:

1. One drop of VP reagent to VP microtube and wait for 10 min then the result was recorded immediately.
2. One drop of 10% ferric chloride to TDA microtube then the result was recorded immediately.

3. One drop of Kovac's reagent to the IND microtube.

The results were recorded and compared to that of identification table, identification of isolates was performed using analytical profile index.

2.2.5: Maintenance of Bacterial Isolates

Maintenance of bacterial isolates was performed according to Maniatis *et al*.,(1982) ; Atlas *et al*., (1995), as following:

(a) Short-term storage

Bacterial isolates were maintained for period of few weeks on nutrient agar plates. The plates were tightly wrapped in parafilm and stored at 4°C.

(b) Medium term storage

Isolates of bacteria were maintained by streaking on slants of nutrients agar medium for period of few months. Such medium was prepared in screw-capped vials containing 15-20 ml of the medium. The isolates were streaked on these slant media and were incubated at 37°C for 24 hour. After that, the slants were taken and wrapped with parafilm and stored at 4° C.

(c) Long term storage

Bacteria can be stored for many years in medium containing 1.5% glycerol at low temperature without significant loss of viability. This was done by adding 1.5 ml of sterilized glycerol to an exponential growth of bacterial isolates in a screw-capped bottle with final volume 10 ml and stored at -20°C.

2.2.6: Detection of the ability of *Pseudomonas* sp. isolates in production of cholesterol oxidase

To examine the ability of local isolates of *Pseudomonas* sp. in enzyme production, the production medium described by **(**Doukyu and Aono, 1998**)** was used for this purpose, by inoculating 100 ml of the production medium with 1 ml of fresh culture of each isolate separately and incubated in shaker incubator (150 rpm) at 30°C for 24 hrs.

2.2.7 : Assay of cholesterol oxidase

The assay of cholesterol oxidase activity is based on the conversion of cholesterol to cholest- 4- en- one, which has an absorption maximum at 240 nm owing to the conjugated carbonyl group in ring A as it was described by Richmond (1972).

50µl of 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 10µl of the enzyme solution was added and incubated for 1min at 30°C then triton x-100 was added to the mixture to stop the reaction then the absorbency was determined at 240nm, then the rate of the change in absorbance was measured and the enzyme activity was determined as the following equation mentioned by Richmond, (1972).

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

The enzyme unit of cholesterol oxidase was defined as the amount of enzyme oxidizing 1µmol of cholesterol to 4-cholesten-3-one min^{-1} at 30°C. (Kim *et al*., 2002)**.**

2.2.8: Quantitative Estimation of Protein

Protein concentration was estimated according to the method described by (Bradford, 1976) by using Coomassie blue G-250 and Bovine serum albumin (BSA) to draw standard curve and estimate protein in concentrated filtrate.

- **Standard Curve of Bovine Serum Albumin**

1. Bovine serum albumin (BSA) solution was prepared by dissolving 0.1 g of BSA in a quantity of D.W. and the volume was completed to 100 ml D.W.
2. Different concentrations of BSA (2, 4, 6, 8, 10, 12, 14 and 16 µg/ml) were prepared.
3. A portion of 20 µl from each concentration was transferred to sterile test tube, and then 50 µl of 1N NaOH was added.
4. A quantity of Coomassie blue reagent 1 ml was added to these tubes. After well mixing, it was left at room temperature for 5 min followed by reading the absorption for each concentration at wavelength of 595 nm. Absorption was drawn against protein concentration Figure (2-1).

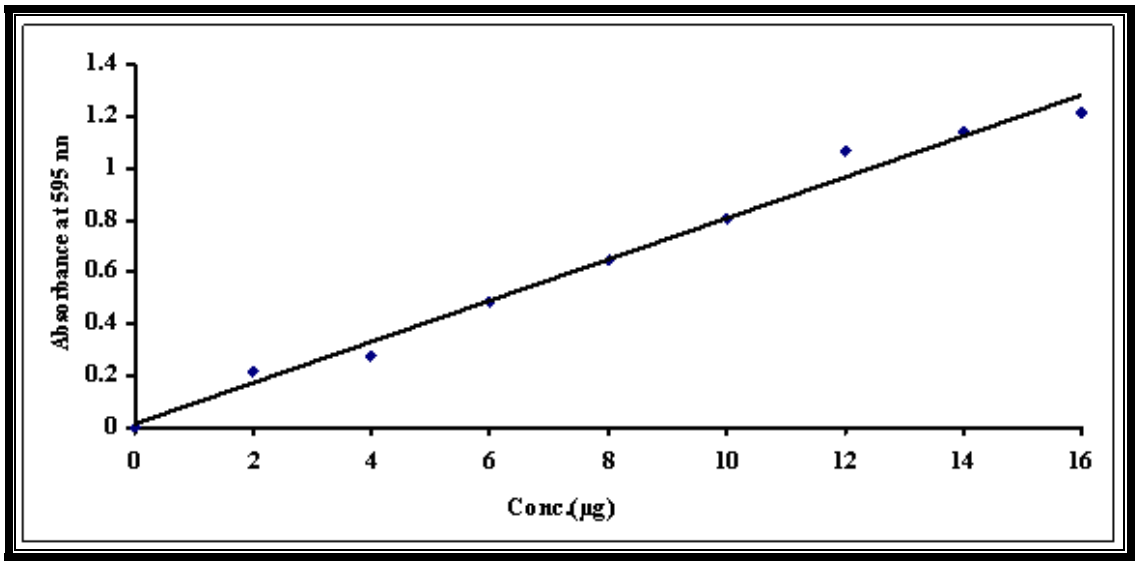


Figure (2-1): Standard Curve for Quantitative Estimation of Protein.

2.2.9: Determination of optimum conditions for cholesterol oxidase production

In order to determine the optimum conditions for cholesterol oxidase production, the selected isolate that was the best among the different isolates in enzyme production, was used for production. Optimum conditions include type and concentration of carbon source, type and concentration of nitrogen source, type of phosphate source, temperature, pH, and incubation time.

(a) Effect of Carbon source

Six different carbon sources (fructose, galactose, xylose, glucose, maltose and sucrose) were used to determine the optimum for cholesterol oxidase production. These carbon sources were added in a concentration of 2% w/v.

- **Concentration of carbon source (Glucose)**

Glucose which was the best carbon source was used for cholesterol oxidase production by the selected isolate was used in different concentrations (1%, 1.5%, 2%, 2.5% and 3%) to determine the optimum concentration in the medium for production of the enzyme.

(b) Effect of Nitrogen source

Five nitrogen sources were used to determine the optimum in enzyme production, which are (Meat extract, Peptone, NH_4NO_3, NH_4Cl and $(NH_4)_2SO_4$. These sources were added to the medium at a concentration of 1.5% w/v.

- **Concentration of nitrogen source**

A different concentration of meat extract which is the best in the enzyme production was used to determine the optimum concentration for the production medium. Five concentrations of the appropriate nitrogen source (0.5%, 1.0%, 1.5%, 2.0%, 2.5%w/v) were used to determine the optimum.

(c) Effect of Phosphate Source

In order to determine the optimum phosphate source for cholesterol oxidase production, two types of phosphate sources (KH_2PO_4 0.025% and K_2HPO_4 0.025%) and mixture of them (KH_2PO_4 0.018% and K_2HPO_4 0.006%, KH_2PO_4 0.006% and K_2HPO_4 0.018%, KH_2PO_4 0.0125 % and K_2HPO_4 0.0125%) were used.

(d) Effect of Temperature

In order to determine the optimum incubation temperature for cholesterol oxidase production by the producing microorganism, five different temperatures (28, 32, 37, 40 and 42°C) were used for this purpose.

(e) Effect of pH

In order to determine the optimum pH for production of cholesterol oxidase, the following pH values were used for this purpose: (6.0, 6.5, 7.0, 7.5 and 8.0).

(f) Effect of Incubation time

In order to determine the optimum incubation period for the production of cholesterol oxidase from the locally isolated *Pseudomonas aeruginosa* H48, different periods were applied which are (24, 48, 72, 96, and 120 hrs). These incubation periods includes the conditions mentioned previously.

(g) Extraction of enzyme

10ml of cultured broth of *Pseudomonas aeruginosa* H48 was inoculated in 1L of Laurie broth medium after adding the optimum nutritional factors and incubated at 32°C for 48 hr. Then the cultured broth was centrifuged

using cooling centrifuge at 8000 rpm for 15 min. Then the enzyme activity and protein concentration was determined.

2.2.10: Purification of Cholesterol Oxidase

(a) Precipitation with ammonium sulphate

10 ml of crude enzyme was added to test tube then ammonium sulphate crystals were added to the crude enzyme gradually with continuous mixing at saturation ratios ranging from 25% - 85%, then the solution was centrifuged at 8000rpm for 15 min after each addition the enzyme activity and protein concentration was determined for each precipitate, and this procedure was applied for each precipitation ratio after dissolving with 2 ml of phosphate buffer (pH 7.0, 0.25M).

(b) Dialysis of Enzymes

After enzyme had been precipitated, the solution will contain a lot of residual ammonium sulfate which was bound to the enzyme. One way to remove this excess salt is to dialyze the enzyme against a buffer low in salt concentration. The precipitate at 70% Ammonium sulphate shows the maximum enzyme activity and used for further purification in dialysis tube. Dialysis tube prepared by soaking in distilled water for 24 hr and then the concentrated enzyme solution is placed in the dialysis membrane, which allows water and salt to pass out of the bag while enzyme is retained. Next, the dialysis tube is placed in a phosphate buffer for 16 to 24 hours. When this process of equilibration repeated several times (replacing the buffer solution with low salt solution each time), the enzyme in the bag will reach a low salt concentration.

2.2.11: Ion Exchange Chromatography

(a) Solutions

1. Sodium phosohate buffer (0.25M), pH 7.

2. NaCl 0.25M in NaOH (0.25M) solution.

3. HCl (0.25M).

4. Sodium Phosphate- Sodium Chloride Solution:

Gradual concentrations of NaCl were prepared ranging between 0.05-0.5M by dissolving it in sodium phosphate solution.

(b) Column Preparation

The column of the ion exchange (CM-Cellulose) was prepared according to the method described by Whitaker, 1972, and as follows:

30 - 40g of dry weight of the ion exchange material was resuspended in a quantity of distilled water in graduated cylinder and then left to stand for a while for stability of the crystals then filtered in Buckner funnel, and then the resin is suspended in 500 ml of 0.5M NaCl – 0.5M NaOH and filtered again. Then the filtrate was washed five times with 500 ml of deionized water then suspended in 500 ml 0.1M of the buffer pH (7.0) to be used. The exchanger was placed in a glass column (1.5 ×22cm and the column was left to the next day with leaving some of the solution on the exchanger surface to prevent its solidification.

(c) Method of ion exchange chromatography

Enzyme solution was added to the column which is equilibrated previously, the enzyme eluted by the NaCl solution with range of molar concentration between 0.05M to 0.5M and the fractions were collected (5 ml/fraction), the flow rate was 100 ml/hour. Then the absorbency for each

fraction was measured at 280 nm. Enzyme activity was determined for each fraction.

2.2.12: Determination of the optimal conditions for the enzyme activity and stability

(a) Determination of the optimal pH for the cholesterol oxidase Activity

1.8 ml of the substrate (Cholesterol) solution was added to 1 ml of the buffer prepared with different pH values (5.5 to 9.0) and the test tubes were placed in a water path (32°C) for 10 minutes then 0.2 ml of the partially purified enzyme solution was added to the test tubes and then incubated for 10 min. the reaction was stopped by adding 0.5 ml of Triton x-100 then enzyme activity was calculated.

(b) Determination of the optimal pH for the stability of cholesterol oxidase Activity

0.2 ml of the partially purified enzyme was added to test tubes containing 1 ml of the buffers solution with different pH values (5.5 - 9.0) and incubated for 30min. at 32°C. Then the test tubes were placed in ice path. Then 0.2 ml was taken from each tube and placed in test tubes containing 1.8 ml of the substrate solution (Cholesterol pH 7.0) and incubated for 10 min. at 35°C then the reaction stopped by adding 0.5 ml of triton X-100 and the enzyme activity for each sample was calculated.

(c) Determination of the optimal temperature for the cholesterol oxidase activity

1.8 ml from the substrate solution (Cholesterol solution, pH 7.0) was incubated in different temperatures ranging from 25°C to 65°C for 10 min.

then 0.2 ml of the partially purified enzyme was added and left for 10 min. then the reaction was stopped by adding 0.5 ml triton X-100 and then enzyme activity was determined.

(d) Determination of the optimal temperature for the stability of cholesterol oxidase activity

0.2 ml of the partially purified enzyme was incubated in different temperatures ranging from 25, 30, 35, 40, 45, 50, 55, 60 to 65°C for 15 min. After that the test tubes were placed in ice bath then transferred to water path at 32°C then 2.0 ml of the substrate solution (Cholesterol solution, pH 7.0) was added and incubated for 10 min. then the reaction was stopped by adding triton X-100, then the remained activity for the enzyme was calculated for each tube.

Committee Certification

We, the examining committee, certify that we have read this thesis and examined the student in its contents and that, according to our opinion, is accepted as a thesis for the degree of Master of Science in Biotechnology.

Signature:
Name:
Scientific Degree:
(Chairman)

Signature:
Name:
Scientific Degree:
(Member)

Signature:
Name:
Scientific Degree:
(Member)

Signature:
Name: Dr. Hameed Majeed Jasim
Scientific Degree: Assit. Prof.
(Member/ Supervisor)

Approved by the Dean of College of Science / AL – Nahrain University.

Signature:
Name: Dr. LAITH ABDUL-AZIZ AL-ANI
Scientific Degree: Assist. Prof.
Title: Dean of the College of Science

Date:

Conclusions:

- *Pseudomonas* sp. isolated from different clinical and environmental samples were able to produce cholesterol oxidase.
- Locally isolated *P. aeruginosa* H48 was the best in cholesterol oxidase production.
- Optimum conditions for cholesterol oxidase production by the locally isolated *P. aeruginosa* H48 involved the use of glucose (2.5%), Meat extract (0.5%), dipotassium hydrogen phosphate (0.018 %), and potassium dihydrogen phosphate(0.006 %) at pH 7.0, and incubation at 32Ċ for 48 hrs.
- Cholesterol oxidase produced by the locally isolated *P. aeruginosa* H48 can be partially purified by two steps of purification (precipitation by 60-70 % saturation of ammonium sulphate, and separation by ion exchange chromatography using CM-Cellulose).
- Partially purified cholesterol oxidase was active at pH 7.0 at 35 Ċ and it was stable at pH 7.5 at 40 Ċ.

Recommendations:

- Determination of the genetic loci responsible for cholesterol oxidase production by the locally isolated *P. aeruginosa* H48.
- Genetic modification of *P. aeruginosa* H48 to enhance its ability for cholesterol oxidase production.
- Determination the optimum conditions for cholesterol oxidase production by the locally isolated *P. aeruginosa* H48 using solid state fermentation.
- Studying the kinetics of cholesterol oxidase produced by *P. aeruginosa* H48.

CONTENTS

CHAPTER TWO: MATERIALS AND METHODS

List of Abbreviations

Abreviations	Words
Api	Analytical Profile Index
CMC	CarboxyMethyl cellulose
E.A.	Enzyme Activity
EP	Enzyme and Product
ES	Enzyme and Substrate complex
L.B.	Laurie Broth
LC	Lethal Concentration
S.A.	Specific Activity
P. aeruginosa	*Pseudomonas aeruginosa*
VAO	Vanillyl alcohol oxidase

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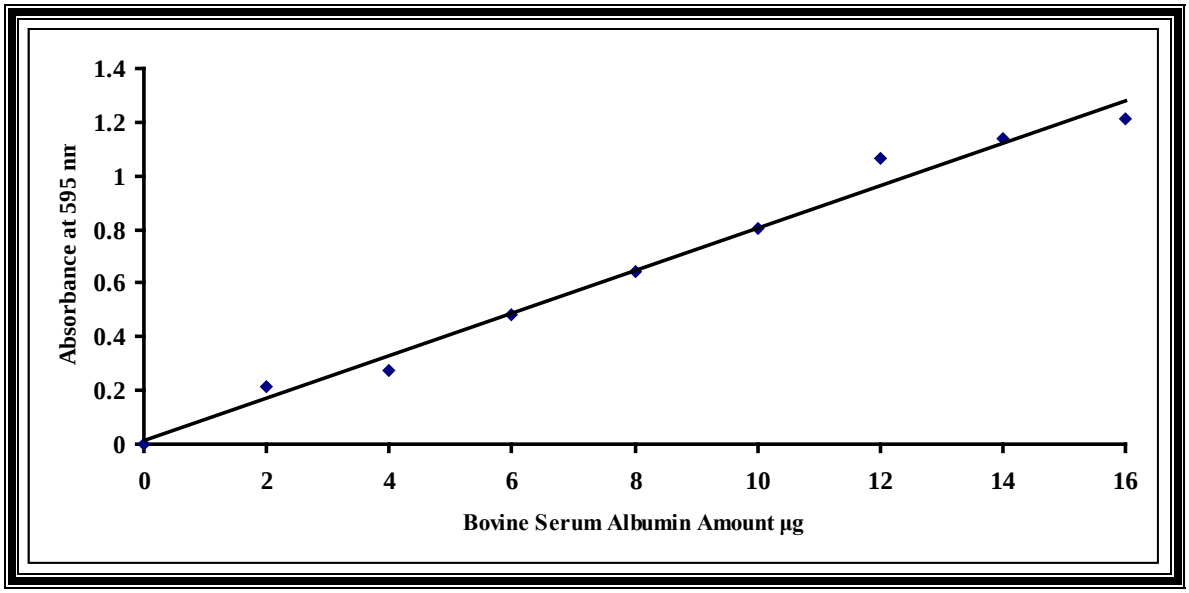
Quantitative Estimation of Protein

Protein concentration was estimated according to the method described by (Bradford, 1976) by using Commassie blue G-250 and Bovine serum albumin (BSA) to draw standard curve and estimate protein in concentrated filtrate.

Standard Curve of Bovine Serum Albumin

1. Bovine serum albumin (BSA) solution was prepared by dissolving 0.1 g of BSA in a quantity of D.W. and the volume was completed to 100 ml D.W.
2. Different concentrations of BSA (0,10, 20, 30, 40, 50, 60, 70 and 80 µg/ml) were prepared.
3. A portion of 20 µl from each concentration was transferred to sterile test tube, and then 50 µl of 1N NaOH was added.
4. A quantity of Commassie blue reagent 1 ml was added to these tubes. After well mixing, it was left at room temperature for 5 min followed by reading the absorption for each concentration at wavelength of 595 nm. Absorption was drawn against protein concentration.


Absorbance at 595 nm
1.4
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
16
Bovine Serum Albumin Amount µg

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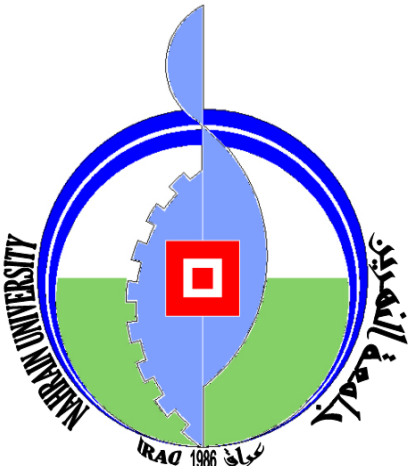

PRODUCTION AND CHARACTERIZATION OF CHOLESTEROL OXIDASE PRODUCED FROM LOCALLY ISOLATED *Pseudomonas aeruginosa* H48.

A Thesis

Submitted to the College of Science Al-Nahrain University as a Partial Fulfillment of the Requirements for the Degree of Master of Science in Biotechnology

By

Saddam Yahya Diwan Al-Jebouri

B.Sc. Biotechnology–Al-Nahrain University – 2004

November 2007

Dhul-Qa'da 1428

Summary

Ninety one samples were collected from different clinical and environmental sources to isolate cholesterol oxidase producing *Pseudomonas* sp. , from these samples, 110 isolate were obtained, sixty of them were belong to *Pseudomonas* sp. Since they have the ability to grow on citrimide agar medium. Results of screening of these isolates for cholesterol oxidase production showed that *Pseudomonas* sp. H48 was the most efficient among the tested isolates in enzyme production since the activity of crude enzyme in culture filtrate was 1.71 U/ml. Identification of this isolate using Api 20 E system indicated that it is *Pseudomonas aeruginosa.*

Optimum conditions for cholesterol oxidase production by *P. aeruginosa* H48 were studied using Lauria – bretani broth as a production medium. Results showed that the optimum conditions for enzyme production includes the use of glucose as a sole source for carbon and energy in a concentration of 2.5%, meat extract as a nitrogen source in a concentration of 0.5%, 0.018% of K_2HPO_4 and 0.006% of KH_2PO_4 as a phosphate source, while the optimum pH for production medium was 7.0, optimal conditions also includes the incubation at 32°C for 48hr. in a shaker incubator at 150 rpm. Under these conditions cholesterol oxidase specific activity was 3.84 U/mg.

Cholesterol oxidase was partially purified, first by precipitation with ammonium sulphate (70% saturation), followed by dialysis and purification by ion exchange chromatography using CM-cellulose column. Activity of cholesterol oxidase was appeared in the second peak of elution hence the enzyme activity was reaches to 4.4 U/ml. Optimum pH and temperature for enzyme activity was studied. Results showed that the optimum pH and

temperature for enzyme activity was pH 7.0 and 35°C respectively, while the optimum pH and temperature for enzyme stability was pH 6.5 and 40°C respectively.

Supervisor Certification

I certify that this thesis was prepared under my supervision at the Department of Biotechnology, College of Science, Al-Nahrain University as a partial requirement for the Degree of Science in Biotechnology.

Signature:
Supervisor: Dr. Hameed Majeed.

Degree:

In review of available recommendations, I forward this thesis for debate by Examining Committee.

Signature:
Dr. Prof. Kadhum Mohamed Ibraheem
Head of Biotechnology Department
College of Science
Al-Nahrain University

الإهداء....

الى نبي الأمه ونبراس الهدى ...ومعلم الخلق وأمام التقوى... الرسول الأعظم...

محمد صلى الله عليه وسلم

إلى ارض عظيمة أنجبتني ...

إلى من ارتقى مراتب الذرى ومرافئ الشمس ...

مناران أضاءا في ليلٍ طويل ... أبي وأمي ...

إلى انهار تنبع من قلبي وتصب فيه لتفيض حب وعذوبة في كل الفصول ...

أركان ، نهله ، نغم

إلى أرواح طاهرة تسكن في دمي ...

خالد الجبوري.

نوار الجبوري.

عدي الجبوري.

و إلى كل من كان سنداً لي ... إليهم جميعاً ... انثر جهدي المتواضع هذا حبات من اللؤلؤ ... لعلها يوماً تصاغ قلائد وفاءٍ لهم ...

صدام الجبوري.

الخلاصة:

جمعت ٩١ عينه من نماذج سريرية وبيئية مختلفة لعزل بكتيريا *Pseudomonas* sp. المنتجة للكوليستيرول أوكسيديز، وقد تم الحصول على ١١٥ عزلة من هذه العينات، تمكنت ٦٠ منها النمو على وسط citrimide المتصلب بمادة الأكار مما يعد دليلا على عائدية هذة العزلات لجنس *Pseudomonas* sp. وقد أشارت غربلة قابلية هذه العزلات على انتاج انزيم الكوليستيرول اوكسيديز تبين ان العزلة *Pseudomonas* sp.H48 كانت هي الأكفا في انتاج الأنزيم، إذ بلغت فعالية الأنزيم الخام في رائق مزرعتها البكتيرية ١,٧١ وحدة / مللتر، ولدى تشخيص هذه العزلة البكتيرية بأستخدام العدة التشخيصية Api 20 E تبين انها *Pseudomonas aeruginosa* .

درست الظروف المثلى لأنتاج الكوليستيرول اوكسيديز بواسطة العزلة ا لبكتيرية *P. aeruginosa* H48 بأستخدام وسط الأنتاج L.B. السائل. وقد أظهرت النتائج أن الظروف المثلى لأنتاج الأنزيم تضمنت استخدام الكلوكوز بتركيز ٢,٥ % مصدراً كاربونياً ومستخلص اللحم بتركيز ٠,٥ % مصدرا نيتروجينياً و٠,٠١٨% من K_2HPO_4 و ٠,٠٠٦ % من KH_2PO_4 مصدراً فوسفاتياً، وكان الرقم الهيدروجيني الأمثل لوسط الأنتاج ٧,٠ ، كما وتضمنت الظروف المثلى للأنتاج الحضن بدرجة ٣٢ °C لمدة ٤٨ ساعه في الحاضنة الهزازه بسرعة ١٥٠ دورة/ دقيقة. إذ بلغت الفعالية النوعية للكوليستيرول اوكسيديز الخام المنتج تحت هذه الظروف ٣,٤٨ وحدة/ ملغرام.

تم تنقية الأنزيم جزئياً وذلك بالترسيب أولاً بأستخدام كبريتات الأمونيوم بنسبة إشباع ٧٠% ثم الديلزة والتنقية بتقنية التبادل الأيوني بأستخدام المبادل الأيونيCM-Cellulose. أظهرت النتائج وجود ثلاثة قمم للبروتين الأولى ضمن الأجزاء الناتجة من الغسل وقمتين ضمن الأجزاء الناتجة من الأسترداد. وتبين ان فعالية الأنزيم المسترد من القمة الثالثة والناتجة من اجزاء الأسترداد حيث وصلت فعالية الأنزيم الى 4.4 وحدة / مللتر . تم دراسة الرقم الهيدروجيني ودرجة الحرارة المثلى لفعالية الأنزيم وثبات الفعالية الأنزيمية وأظهرت النتائج أن الرقم الهيدروجيني ودرجة الحرارة المثلى للفعالية الأنزيمية انها ٧,٠ و ٣٥ °C على التعاقب، بينما كان الرقم الهيدروجيني و درجة الحرارة المثلى لثبات الفعالية الأنزيمية ٦,٥ و ٤٠ °C على التعاقب.

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

وَاللّهُ أَخْرَجَكُم مِّن بُطُونِ أُمَّهَاتِكُمْ لاَ تَعْلَمُونَ شَيْئًا وَجَعَلَ لَكُمُ الْسَّمْعَ وَالأَبْصَارَ وَالأَفْئِدَةَ لَعَلَّكُمْ تَشْكُرُونَ.

صدق اللّهِ العظيم

سورة النحل الأيه ٧٨

جمهورية العراق
وزارة التعليم العالي والبحث العلمي
جامعة النهرين
كلية العلوم
قسم التقانة الأحيائية

تنقية و توصيف الكوليستيرول أوكسيديز المنتج من بكتيريا *Pseudomonas* sp. المعزولة محلياً

رسالة

مقدمة الى كلية العلوم - جامعة النهرين وهي جزء من متطلبات نيل درجة

ماجستير علوم في التقانة الأحيائية

مِنْ قِبَلْ

صـــدّام يـحيى ديـــوان الجبـــوري

بكالوريوس تقانة احيائية – جامعة النهرين –٢٠٠٤

تشرين الثاني ٢٠٠٧

ذو القعدة ١٤٢٨