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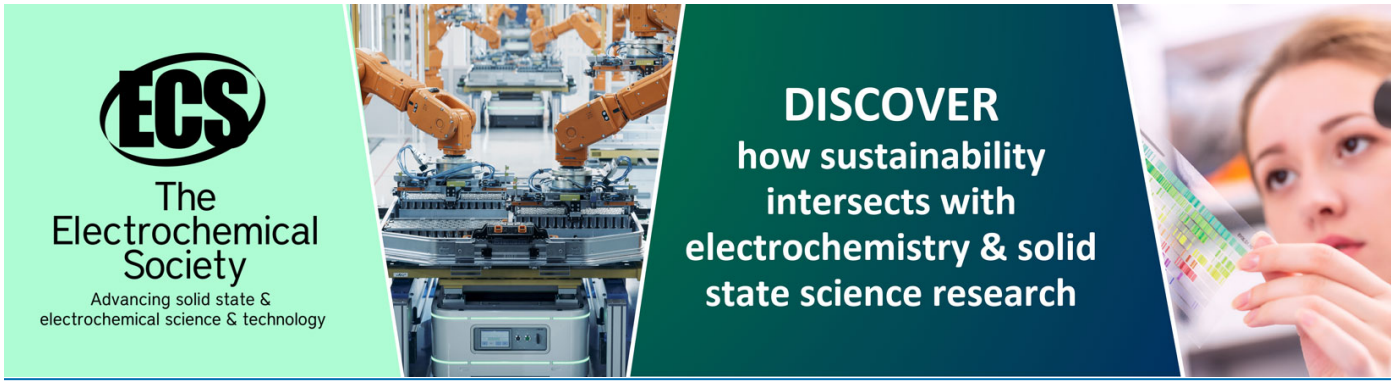

Biochemical study of alkaline phosphatase in *Escherichia coli*

Ibtihal Idrees Kanaan[1*] and Najwa Khaleel Ibrahim[2]

[1]College of Environmental Sciences and Technology, Al-Mosul University, Iraq.
[2]Department of Biology, College of Education for Pure Sciences, Al-Mosul University, Iraq.

*E-mail: kanaan84@uomosul.edu.iq

Abstract. The present research aimed to isolate and purify Alkaline phosphatase enzyme from crude protein extract (Lysate supernatant) of *Escherichia coli*, by using different biotechnologies. To proceed, the following steps were taken: Firstly, The verification of the existence of enzyme in bacteria, the bacteria were diagnosed by using the API 20 stripe that consists of (20) items. the enzyme was isolated to ensure its availability in bacteria within the logarithmic phase and this was done through proliferating them for 18 hours in a nutrient agar. It had been detected that the enzyme was intracellular because of the occurrence of enzyme activity in the lysate supernatant without occurring it in the cell free culture supernatant. Secondly, Enzyme purification, the enzyme had been purified through three stages: precipitation of protein by ammonium sulphate, dialysis and finally, the protein extract was passed through column chromatography by using Sephadex G-100 gel, the estimated enzyme activity after this step was 22.0 in comparison with its activity before the purification processes (crude protein extract). The approximate molecular weight of alkaline phosphatase was 81.000 Dalton estimated by using gel filtration technique.

-Keywords. Alkaline phosphatase, *Escherichia coli*, Molecular weight.

1. Introduction

Escherichia coli is a bacillus bacterium negative for a gram stain, located within the family of Enterobacteriaceae, negative for oxidase, facultative anaerobic, animated by peripheral flagella, capable of fermentation for various carbohydrates with gas and acid production [1] (Colonies of bacteria are circles, small, slightly convex, pale, and on the MacConkey agar appear to be pink because of its fermentation of lactose sugar. *E. coli* bacteria widespread in nature and in the intestines of humans and animals. It has the potential to cause many opportunistic infection such as Urinary infection, Cystitis and Pyelonephritis pelvic inflammatory [2]. Alkaline phosphatase enzyme is referred to as ALP (EC 3.1.3.1), [3, 4, 5], which was approved by the International Association of Enzymes. The Alkaline phosphatase enzyme is also called Orthophosphate monoester-phosphohydrolases [6, 7], a hydrolases enzyme [8], and metalloenzyme [9], to contain its active positions on four zinc ions Zn^{+2} and ioni magnesium Mg^{+2} [10, 11,12], as many researchers pointed out that the wide-ranging peculiarity of the enzyme of the foundation material [12] led to the study of its role in the processes of: analysis of both phosphoester phosphate compounds [5], and phosphate

endings of DNA and in the transfer of phosphates to their receptors Trans phosphorylation, especially when there are high concentrations of phosphate receptors such as Tris-buffer and Ethanol amine. The Alkaline phosphatase enzyme is positioned in bacteria within the periplasmic space [13]. While it is associated with the plasma membrane in the leubes [7, 14]. This explains his role in the latter with cellular transport mechanics [5]. The synthesis of the enzyme begins during the logarithmation and under the conditions of phosphate limitation [15] in the bacterial cell cytoplasm. It is characterized by a molecular weight of (110,000-67,000) Dalton [4] and its lack of both carbohydrates and fatty acids within its composition. As well as its stability towards temperatures up to (80) °C [16], it has optimum pH at 8 [17]. The alkaline phosphatase enzyme in the mamliam is characterized as sugar and protein [18] contains between 12 [19] to 15% [20] carbohydrates from the total weight of protein. There is no evidence of the participation of carbohydrate units in stimulation [21]. The enzyme is associated with the plasma membrane in most animal cells [4, 22], which releases from the membrane to the bloodstream in the case of inflammation and when regeneration, redivision of cells [23], and in body fluids, cancer cells. Therefore it is used to track the development of malignant diseases [24]. The enzyme has a molecular weight of (130,000-170,000) Dalton [25], and plays a defensive role in its removal of phosphate from the exocellular ATP molecule, which is considered as Pro-inflammatory. It has a role during acute inflammatory response by removing endogenous dephosphorylating when it is in great need [9].

2. Materials and Methods

2.1. Bacteria used and sources of access

Escherichia coli bacteria, obtained from microbiology laboratories / Al-Salam Hospital in, Mosul, which was diagnosed using the tape (API 20) (Analytical profile Insera, Biomeriuex France).

2.2. Isolation of the alkaline phosphatase enzyme

2.2.1. Detection the logarethmetic phase of bacteria

This phase was determined by the measurement of the bacterial turbidity in the nutrient broth after every two hours of incubation in a rocking incubator of the type (Wink Hamp, England) at a temperature (37) °C in succession for 24hours, using spectrophotometer (Sartorius BL 210S, Labomed, Inc. sepec setrosc), at (600) nanometer [26].

2.2.2. Preparation bacterial suspension

Prepare the nutrient broth media in a glass sized (1000 cm3) and sterilized by autoclave under temperature (121) °C and pressure (1) atmosphere for (20) minutes. Then leave to cool to a (45-50) °C and culture with *E.coli* for (18) hours at temperature (37) °C in a vibrator incubator (180 cycles / minutes).

2.2.3. Isolation of alkaline phosphatase

Measured the bacterial growth at the wavelength (600) nm, the bacterial cells precipitated using a centrifuge Cooling ultracentrifuge (Heraeus chrest Gm bH, Cryofuge 6-4) at speed (19470xg), temperature (4) °C for 30 minutes, cell-free broth preservation at (4) °C, washed sedimentary cells (3) times using Tris - HCL buffer 8.0 mM [16] (Kumar et al., 2008). The walls of bacterial cells were destroyed using the Ultrasonic MSE, SEP. No PG 1545) by exposing it to (20,000) pulse / seconds, for (30) seconds and repeated the process (3) times with stops of (30) seconds at a temperature (4) °C. Separate the sediment from the precipitated cells using a refrigerated centrifuge, at the same

conditions mentioned above, preservation of broken cells supernatant (lysate supernatant) at a temperature (4) °C [27].

2.3. Purification of alkaline phosphatase

2.3.1. Precipitation of protein by ammonium sulphate

Ammonium sulfate has been added in its solid state to the lysate supernatant and with full saturation to (75%). Then separate the mixture with the cooled centrifuge for (30) minutes (19470Xg), and quickly after which it was obtained on the deposit and melted in the 100 mMTris HCL buffer pH 9.8 [16, 27].

2.3.2. Dialysis

It was carried out by placing the protein solution in the plastic tying cellophane bags. Then plunged into a volume container containing the 100 mM Tris HCL buffer 9.8 [16]. The screening process was conducted at a temperature of (4) °C with movement using the magnetic motor)

2.3.3. Gel Filtration Chromatography

2.3.3.1. Gel preparation

Use Sephadex-G100 gel [28, 29], which separates protein compounds with molecular weights that fall within range (150,000-4000) Dalton [30]. Swelling of the gel and then degassing gas exhalation was performed under a sieve using the Aspirator A-2S Eyela of Japanese origin [27].

2.3.3.2. Packing of the Column

Use the column with the (1.7×75) cm dimenostraion to separate the enzyme that was filled with gel quickly on the walls of the column [27].

2.3.3.3. Add standard materials

Recovery volumes were estimated for five standard proteins known as molecular weight (blue dextran, cow serum albums, egg albums, a pepsin enzyme, insulin).

2.3.3.4. Add the sample

(2.5) cm^3 of protein solution resulting from dialysis and standard materials were injected circularly on the walls of the column. By the addition of (2,5) cm^3 of distilled water, which was used as an Elution solution for the purpose of pushing the model inside the column. Then the protein material was recovered manually and followed up with its protein content by reading the absorption at 280 nm using a UV-Visible spectrophotometer from APEL company. In addition, the effectiveness of the alkaline phosphatase enzyme was followed up in each of the separated parts. When the unknown protein solution was injected into the separation column for the purpose of follow-up the enzyme during the separation process, that's the combination of protein parts that are in enzyme-effective. A linear relationship was then drawn between standard protein recovery volumes. It is known as molecular weights for use in estimating the approximate molecular weight of the Alkaline phosphatase enzyme [27].

2.4. Cooling Drying Technology (Lyophilization)

Lyophilization of the protein pack produced by the gel filtration technique was dried using a lyophilization device after freezing at a temperature (-20) °C to obtain a concentrated protein solution (Crude protein).

2.5. Estimation of alkaline phosphatase activity

The activity of the enzyme was estimated in both lysate supernatant and cell-free broth. After each step of purification by rapid detection of the phenol liberated from the reaction using the 4-amino antipyrine reagent and Potassium ferric amide. The reaction was stopped by adding Sodium arsenate using Ready packaging) alkaline phosphatase –kite (manufactured by Biomerieu xsa).

2.6. Estimation of total protein

The amount of protein was estimated in both lysate supernatant and cell-free broth. After each step of purification using the Folin-Lowry method [31] and modified by researchers [32]. Bovine Serum Albumin (BSA) was used as a standard solution to prepare the standard protein curve with optical absorption coefficient (Extinction coefficient) equal to 0.67 [33]. Figure 1 shows the typical standard curve of protein.

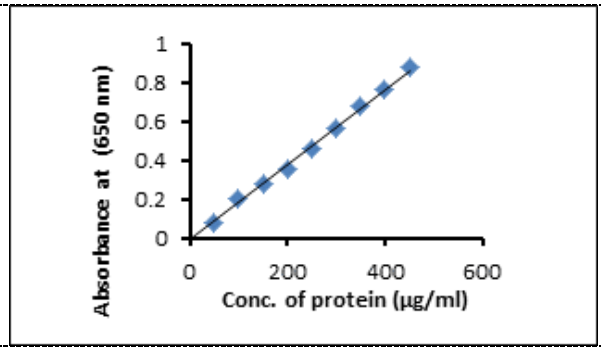

Figure 1. Illustrates a typical standard curve for a protein.

3. Results

3.1. Diagnosis of Escherichia coli bacteria

This Based on the biochemical tests and tests found in the API20 strip, *E.coli* bacteria are diagnosed in Figure 2.

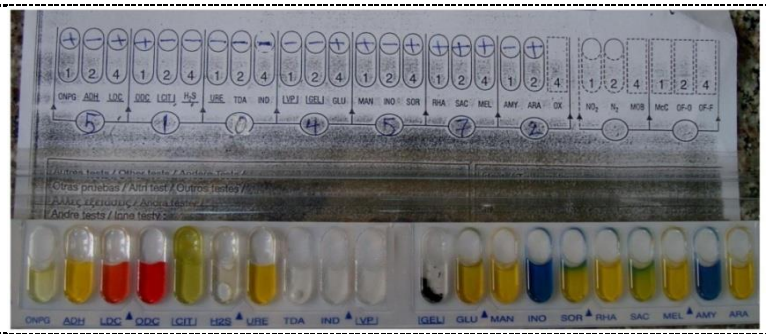

Figure 2. Results of the API20E diagnostic System.

3.2. Alkaline phosphatase enzyme

3.2.1. Determine the Logarithmic growth phase

The logarithmic growth phase of *E. coli* bacteria was determined, and due to the fact that the bacteria in this phase are almost all of their cells are alive and the speed of growth is constant. So the Spectrophotometer was used to calculate the bacterial growth in the nutrient broth media used under the study, after every two hours of incubation for a period of 24 hour (Figure 3). It was found that this phase starts approximately from the hour (4) to the hour (20) (Figure 3), and accordingly, the bacteria were adopted at an age of (18) hours, which is the logarithmic growth phase, for use in subsequent experiments.

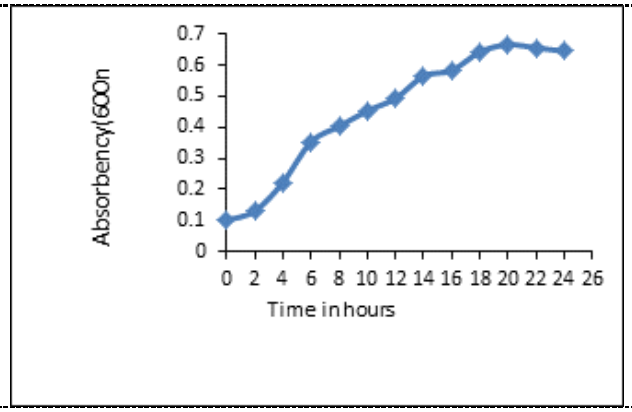

Figure 3. Growth curve and logarithmic phase determination of *Escherichia coli*.

3.2.2. Growth curve and logarithmic phase determination of Escherichia coli

The location of the alkaline phosphatase enzyme was determined by measuring its effectiveness according to paragraph (Isolation of alkaline phosphatase) described in the materials and methods of work. After the growing of bacteria in the nutrient broth for 18 hours, and after depositing their cells using a refrigerated centrifuge. The results showed lack of effectiveness of the enzyme in the cell-free filtrate and its presence in the layers of broken cells by the ultrasound device. The total activity reached (1.52) units (Table 1), and the total protein amount (47.48) mg (Figure 4). This confirms that the alkaline phosphatase enzyme is an internal enzyme. It is located inside the cells and not outside it. Therefore, the suspension of broken cells was approved as a raw extract of the enzyme in experiments related to its purification.

3.3. Purification of the alkaline phosphatase enzyme

Use lysate supernatant as a pure extract of the alkaline phosphatase enzyme, which has been purified and according to the following sequential steps:

3.3.1. Protein precipitation using ammonium sulphate

Proteins found in the raw extract were deposited by adding ammonium sulfate gradually and saturation (75%). And after obtaining the protein deposit dissolved in the 100 ml mollur tris-HCl pH9.8. Then estimated the enzymatic activity, where the specific activity of each protein extract was observed to increase by (2.0). Once compared to the raw extract as shown in Table (1), the amount of protein in it (according to the Lowry modified method) where a decrease in the amount of the total protein is about in the raw extract and as shown in Figure (4).

3.3.2. Dialysis

The results showed an increase in the specific activity of the alkaline phosphatase enzyme , which amounted to (0.10) Unit /amalgam Table (1), with low protein content compared to raw protein extract as shown in Figure (4).

3.3.3. Gel filtration chromatography

After the treatment of the lysate supernatant (crude protein) by ammonium sulfate and dialysis was separated the protein compounds by passing the separation column (containing the gel Sephadex G-100). It gave two protein packs (A and B), and by measuring the effectiveness of the alkaline phosphatase enzyme in all parts collected separately, it was found that the effectiveness is concentrated in the protein pack (B), as shown in Figure (5). A percentage increase in specific activity of (22.0) times was observed compared to its effectiveness in the crude protein, as shown in Tables (1) which confirms the purification of the enzyme by this technique. As well as a decrease in the total protein intake during the purification stages of the enzyme as shown in the Figure (4).

Table 1. Steps of purification alkaline phosphatase from *Escherichia coli*.

Steps of purification	volume (cm^3)	Total enzymatic activity unit (U)	Specific activity** Unit/mg	Percentage increase in specific activity%	Percentage of restoration %
Crude extract	20	1.52	0.03	1.0	100.0
Precipitate solution of ammonium sulfate	4	1.37	0.06	2.0	90.0
dialysis	7	1.32	0.10	3.3	86.8
Gill filteration by Sephadex G-100 Peak (B)	75.5	1.15	0.66	22.0	75.7

**Specific activity: the number of enzyme units [the enzyme unit measured in (Kind and King U / dl)] / mg protein. Kind and King: It expresses the amount of enzyme needed to release (1) mg of phenol in 15 minutes at a temperature of (37)° C.

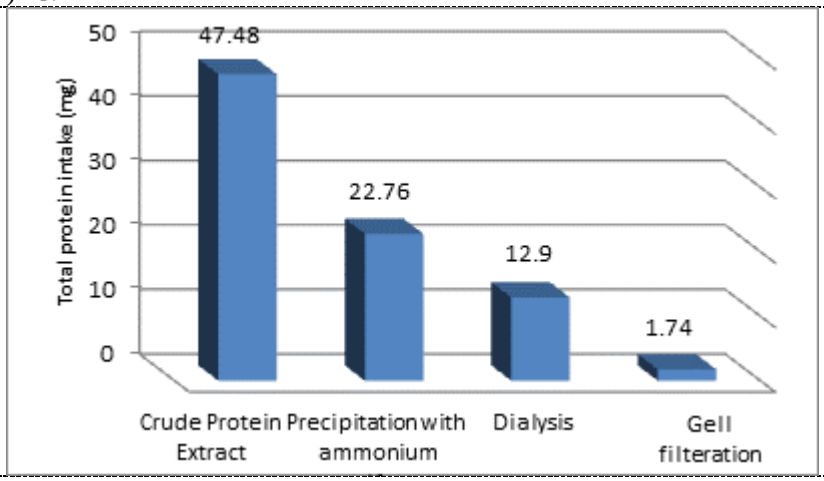

Figure 4. Total protein intake during the purification phases of alkalinel phosphatase for *Escherichia coli*.

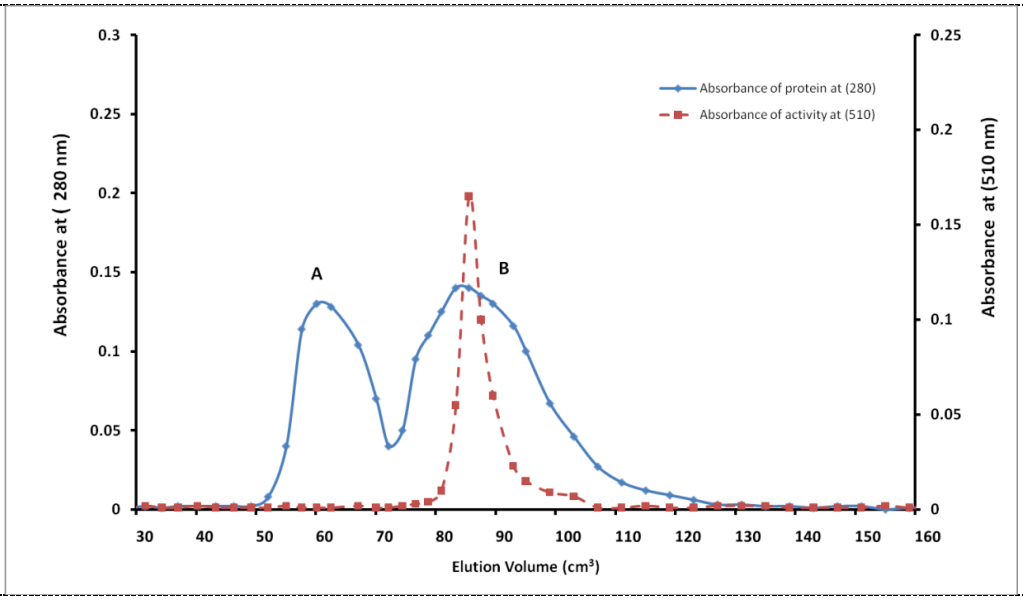

Figure 5. Profile of *Escherichia coli* proteolytic extract profile using gel filtration technique by using a separation column with dimensions (1.7 × 75 cm^3) containing the gel material (Sephadex G-100) at a height of 70 cm) at a flow velocity of 36 cm 3 / hour, volume Each segment is (3) cm^3**.**

3.4. Estimating the molecular weight of the alkaline phosphatase enzyme using the technique gel filtration

The approximate molecular weight of the protein pack (B) was estimated for *E. coli* bacteria containing the alkaline phosphatase enzyme by the researcher method (Andrews, 1965) using the gel filtration technique. The number of compounds known as molecular weight were passed by its molecular weights range from (2,000,000-204) Dalton on the separation column for the purpose of setting the characteristics of the column in terms of internal size (Vi). As well as the empty size of granules (Vo). The standard curve was then drawn Figure (6), based on the size of the organ for each substance versus the molecular weight of the lugarium. The approximate molecular weight (81,000) Dalton was determined based on the sizes of the Proteins bundle (83.3) cm^3.

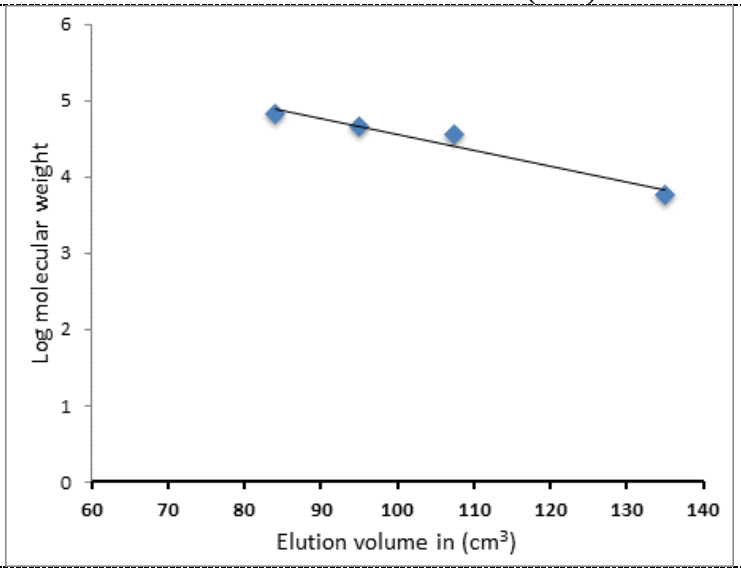

Figure 6. Standard curve for determination of molecular weight by gel filtration technology using sephadex G-100.

4. Discussion

The current study was based on the isolation and purification of the alkaline phospatase enzyme (EC 3.1.3.1) from the pathogenic bacteria *Escherichia coli*. It is based for its diagnosis on the shape of its cells after staining it with a gram stain and on the biochemical tests in the API20 strip. It is one of the rapid diagnostic methods used worldwide as well as the easy and accuracy of diagnosis. The logarithmic phase is one of the most important phases that bacteria go through during their growth, because the cells in it are highly metabolically effective [34] and have distinct physiological and chemical properties. Therefore, bacteria at this stage are suitable for use in physiological and biochemical studies [35]. Because the formation and production of the alkaline phosphatase enzyme begins during this phase in the cytoplasm. Then it is exported through the plasma membrane to the area surrounding the plasma [15]. The effectiveness of the enzyme increases only after folding, which occurs only after it is transferred to the space around the plasma. Then the formation of the dimethyl sulfur [16, 36] which is considered the important part of in bacteria because they contain most of the enzymes necessary to obtain phosphate and carbon sources [37]. So this phase of bacteria under study has been identified and is approximately 20-4 hours long. Previous studies have identified this phase within the time period (15-6) hours [38], and within the period (16-6) hours in bacteria *E. coli* [39]. The relative disparity observed with the results of this study, may be due to the size of the inoculum and the type of nutrient media, noting that increasing the size of the inoculum and adding a new nutritional medium shortens the printing period to a few hours. As well as the source of bacteria isolation and the extent of their virulence, play an important role in determining the period of the phases of the bacterial growth curve [40]. The alkaline phosphatase enzyme was isolated from the bacteria under study after it was grown in the appropriate nutrient medium incubated for about 18 hours. The study revealed that the alkaline phosphatase enzyme is one of the endogenous enzymes [41]. In terms of the absence of any activity of enzyme in the cell free culture supernatant. Its specific activity was recorded in the extract of broken cells (Lysate supernatant) (0.03) because the outer membrane of the gram-negative bacteria acts as a barrier that prevents the enzyme from escaping into the medium surrounding the bacteria [42]. The phospholipid layer present within the plasma membrane in the Gram-negative bacteria also acts to prevent the secretion of the enzyme externally. The enzyme is located within the space surrounding the plasma and it is one of the proteins that are never secreted outside the bacterial cells into the growing culture medium in it. [43] So the total enzyme units were obtained from the extract of broken cells and not from the full and Intact cells [41]. As the researcher [44, 45] pointed out the presence of the enzyme is also present in the expanse surrounding the plasma of a bacterium *Pseudomonas aerogenosa*. The enzyme was purified after obtaining the crude protein from the broken cell of the ultrasonic frequency device through the following steps; First: Protein percipitation with ammonium sulfate. This step is one of the first stages of purification during which proteins found in the crude extract are deposited depending on the degree of saturation of Ammonium sulfate [27]. By gradually adding it to the protein extract, because it is a salt that is characterized by its ability to dissolve at high speed, and its non-effect on enzymes, and depending on the type of competition between ammonium sulfate and proteins to dissolve and combine with water molecules, protein molecules tend to combine aggregation with each other in high concentrations of salt which is due to strong protein-protein interferences that overcome protein-solvent overlaps [46]. So they were used in the current study of alkaline phosphatase enzyme deposition and saturation (75%). It was shown that the results compared to the raw protein extract increase dissertated in quality efficacy and correspond to the results obtained by many researchers who used ammonium sulfate at different saturation rates [47, 48]. Second: dialysis, which is one of the methods widely used during the purification stages to get rid of salts and materials with low molecular weights in enzymatic solutions. [27] such as proteins with molecular weights that are smaller than 14,000 Dalton and some carbohydrates. Although from the reduction of the extract as a result of this process, an increase in qualitative effectiveness was observed about three times that of the raw extract and in comparison to the increase referred to by [16]. Third: The technique of gel filtration

chromatography. This technique is one of the methods used globally to separate compounds depending on the difference in the size of the rogane Elution volume. Large particles pass through a column separation of gel granules while small particles take longer due to their entry into their granules. Therefore, this technique has been used to separate protein compounds that were made and obtained from sedimentation with ammonium sulfate (after the process of dialysis and reduction of its volume by lyophilization method) using Sephadex G-100 gel. This is because it separates protein compounds with weights of molecular that falls within range (150,000-4,000) Dalton[29]. A protein pack (B) was obtained and as shown in Figure (5) and when measuring the enzymatic effectiveness of the package, it was found that the protein pack (B) is highly enzymatic. This confirms the presence of one type of alkaline phosphatase enzyme in *E. coli* bacteria [49]. The results also showed an increase in the specific effectiveness of the enzyme by about 22.0 times it in the raw protein extract, which confirms that the enzyme purification using this technique [50]. Also, the decrease in the total protein amount along the purification stages indicates that the enzyme has been purified. This is consistent with the results of many researchers in studies related to this aspect [41, 47, 48, 50]. Also, using gel filtration technology, the approximate molecular weight of the molecularly purified alkaline phosphatase enzyme was determined, as it was found that its molecular weight in *E. coli* (81,000) Dalton was similar to what was found by [5]. [5] indicated that the molecular weight of the enzyme in these bacteria is located within the run (90,000-85,000) Dalton.

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