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Authors' Affiliation:
Biotechnology Department, College of Science, Baghdad University - Iraq

***Corresponding Author:**
Shahad Abdullah Shwan
Email: shahadabdullahhhhhhh@gmail.com

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Production and purification of Nattokinase from *Pseudomonas aeruginosa* P49

Shahad Abdullah Shwan*, Ali Jabbar Reshak

Abstract

Background: Nattokinase (NK) is a profibrinolytic serine protease enzyme produced by many bacterial strains, such as *Pseudomonas aeruginosa*. Therefore, this study aimed to produce NK from local isolate of *P. aeruginosa* P49 and optimize its conditions for the production of enzyme.

Methods: 150 samples were obtained from clinical sources, during the period from August to November 2022, from different hospitals. All samples were subjected to different examinations, in addition to VITEK2 system, to confirm that these isolates were *P. aeruginosa*.

Result: A total of one hundred *P. aeruginosa* isolates were screened to choose the best NK-producing isolates using skim milk agar, then broth media, whereas *P. aeruginosa* P49 gave the highest enzymatic activity (337.9 U/mg protein). Optimal conditions for the formation of NK were estimated, and the results showed that the maximum production of NK was gained using peptone-yeast medium containing sucrose, peptone, and CaSO4.2H2O at pH 7.5 and 37°C for 24 hours of incubation, whereas the activity of NK increased to reach a yield of 1603 U/mg protein. The NK was purified from *P. aeruginosa* P49 utilizing ion exchange chromatography (IEC) after precipitation by ammonium sulfate (0-75%). The results for enzyme purification gave 96% of NK enzyme with a purification fold of 3.6, and the specific activity was 2190.7 U/mg protein.

Conclusion: This result suggests *P. aeruginosa* P49 is a good source of NK production.

Introduction

Nattokinase is a serine protease with potent profibrinolytic activity and can degrade fibrin very effectively. Nattokinase has been manufactured using various host strains and possesses the benefits of increased stability, low cost, and minimal adverse effects. Unlike the other fibrinolytic enzymes, including streptokinase, tissue plasminogen activator (t-PA), and urokinase, NK demonstrates potential as a therapeutic drug against cardiovascular diseases as well as a healthy food supplement [1].

Recently, Nattokinase (NK) has drawn increasing attention owing to its wide range of potential applications [2]. By lowering plasma fibrinogen and blood viscosity levels, NK aids in the prevention and management of thrombotic diseases. Research points out its benefits in the areas of food safety, low cost, and extended biological activity [3,4]. Unlike other fibrinolytic drugs, this enzyme possesses special advantages such as suitability for oral administration, extended preventive effects, and tolerance in the digestive system. Also, NK is absorbed via the gastrointestinal system and then facilitates the lysis of fibrin later on [5]. In addition to its thrombolytic effect, NK shows various other effects such as the prevention of atherosclerosis, enhanced microcirculation, blood pressure regulation, inhibition of abnormal retinal vasculature proliferation, anticancer activity, and ability to counteract inflammation and oxidative stress. [6].

A significant amount of NK is also found to be produced by many marine organisms besides Bacillus and Pseudomonas species [1]. *Pseudomonas aeruginosa* are abundant organisms found in both human-made and natural environments, including those involving plants and animals [7]. Pseudomonads are opportunistic pathogenic bacterial species that can cause infectious diseases in the eyes, skin, and lungs of people with burns, abrasions, cystic fibrosis (CF), and HIV/AIDS [8]. Although some species of *P. aeruginosa* are pathogenic, these bacteria also produce different beneficial enzymes [9], like NK [1].

In general, NK is formed via submerged fermentation (SF) and solid-state fermentation (SSF) processes, but the latter has been utilized to a lower extent for production of these enzymes [10]. The culture of microorganisms in a liquid nutrient broth that is high in oxygen concentration is referred to as SF [11].

Despite the fact that the microbial synthesis of NK has been the subject of much research, the yields, activities, and stabilities of NK remain the most important factors limiting its applicability in industrial settings [12]. Many parameters affecting enzyme production from *P. aeruginosa* have been investigated, including the best substrate, the pH of the medium, the incubation time, salts, and the incubation temperature. All enzymes are grown in culture media that contain carbon sources, nitrogen sources, and other components that assist the growth of the enzymes. Many studies show that *P. aeruginosa* grows in response to carbon sources, with each carbon source having a unique impact on NK synthesis [13]. Also, NK is a neutral or weak alkaline enzyme since it is stable within the pH ranges of 6.0 and 12.0 and is quickly inactivated below pH 5 [12]. The optimum temperature for microbial growth is not necessarily the same as the optimum temperature for enzyme production, even between strains of the same species, due to the fact that temperature affects the cell's metabolic activities. Incubation temperature has a major effect on the metabolic activities of bacteria [14].

Hence, this study aimed to produce NK from local isolate of *P. aeruginosa*, optimize its production conditions, and purify it.

Methods

Collection of samples

A total of 150 samples were obtained from various clinical sources, including wounds and burns, during the period from August 2022 and November 2022, from patients attained Al-Kindi Hospital, Al-Yarmouk Hospital, Teaching Hospital of Baghdad Medical city, and other specific laboratories.

Identification of samples

All samples were subjected to different examinations, including microscopic examinations (gram stain) [15], cultural features (cetrimide agars and MacConkey agars, nutrient agars, brain heart agar, and blood agar base) [16], biochemical tests (Simmons citrate, catalase, and oxidase) [17] and growth at 4°C and 42°C, in order to isolate *P. aeruginosa* as well as VITEK 2 system was utilized [18] to confirm that these isolates were *P. aeruginosa*.

Screening the *Pseudomonas aeruginosa* isolate for NK production

Semi-quantitative screening (primary screening)

All *Pseudomonas aeruginosa* isolates were screened to choose the best isolates for NK production utilizing a plate assay via skim milk agar medium. A volume of one hundred fifty microliters of inoculum of bacteria consisting of 2.5×10^7 cells/milliliter was transferred into a skim milk agar plate and then placed in an incubator at 37°C for 24 hrs. The presence of NK secretion might be seen as a colorless zone of hydrolysis around the well [19].

Quantitative screening (secondary screening) for enzyme production using SF medium

Five isolates of NK-produced *Pseudomonas aeruginosa* were screened to choose the best isolates for NK production. SF medium, composed of 2.5 g yeast extract, 5.0 g of glucose, 2.5 g of peptone, 0.5 mg KH_2PO_4, 0.1 mg $MgSO_4$, and 2.5 g $NaCO_3$. This medium was poured into a moistened Erlenmeyer flask measuring 250 milliliters. The flask was tightened with cotton after adjusting the pH to neutral, and contents of the flask were mixed thoroughly. Then the flask was placed in an autoclave at 121°C temperature and 15 psi pressure for 15 minutes. After sterilization, the flasks were cooled at room temperature [20]. SF was used for enzyme production, where the five isolates with maximal productivities according to casein hydrolysis were chosen and cultured on fermentation medium. Five bacterial suspensions (2.5×10^7 cells/ml) were prepared from 24-hour cultures, each used to inoculate the fermentation broth with 0.75 microliter aliquots. The flasks were put in an incubator shaker for 24 hours at 37°C.

Extraction of NK enzyme

After an incubation period of twenty-four hours, enzyme from each flask was extracted by centrifuging the flasks for thirty minutes at 10,000 rpm. The supernatant was then filtered using filter papers of Whatman No. 1. It was determined that the supernatant may be used as a crude enzyme.

Determination of NK activities

The activity of NK enzyme was determined according to Senior (1999) method as shown below: 1.8 milliliter of casein solution was placed in a bath of water for 5 min at 37°C, then 0.2 milliliter of the crude enzyme was firstly put into the solution of substrates and then in an incubator for 30 min at 37°C [21]. After adding three millilitres of a TCA solution with a concentration of ten percent, the reaction was stopped, and the pellet was separated by centrifuging the mixture at ten thousand revolutions per minute for ten minutes. A blank was prepared by following the identical protocols as the test sample, with the exception that a 10% TCA solution was mixed with the solutions of caseins before the addition of 0.2 millilitres of enzyme. A UV-VIS spectrophotometer was used in order to determine the absorbances of the supernatants at 280 nm. The enzyme activities and concentrations of proteins were each measured twice, and the findings were reported based on the average of the two readings. According to the formula that follows, a unit of activity of the enzyme was the amount needed to raise the absorbance ratio at 280 nm by 0.01 per minute in the experimental conditions:

$$\text{Enzyme activity}\left(\frac{\text{U}}{\text{milliliter}}\right)=\frac{\text{Absorbance at 280 nm}}{0.01\times30\times0.2}$$

Where, 0.01 = Constant, 30 = Reactive time in minutes, and 0.2 = Volume of enzyme

Determinations of concentrations of proteins

Method of Bradford (1976) was utilized for measuring concentration of proteins, in accordance with Al-Sa'ady (2020) [22,23].

Determinations of specific activities

As per the following equation, the specific activity of the enzyme was calculated [24]:

$$\text{Specific activity}\left(\frac{\text{U}}{\text{mg}}\text{protein}\right)=\frac{\text{Enzyme activity}\left(\frac{\text{U}}{\text{milliliter}}\right)}{\text{Protein concentration}\left(\frac{\text{mg}}{\text{milliliter}}\right)}$$

Optimal conditions for the production of NK

Optimal culture medium

Six different media were examined to see which ones had the most impact on NK production. These substrates included:

1. Peptone 1 g/100 ml, yeast extract (0.25 g/100 ml), NaCl (1 g/100 ml).
2. Peptone (1 g/100 ml), yeast extract (0.25 g/100 ml), Glucose (1 g/100 ml).
3. Glucose (2 g/100 ml), soy bean (2 g/100 ml), NaCl 0.5 g/100 ml, KH_2PO_4 (0.1 g/100 ml), K_2HPO_4 (0.4 g/100 ml), $MgSO_4$ (0.05 g/100 ml).
4. Shrimp shell 1 g/100 ml, KH_2PO_4 0.1 g/100 ml, $MgSO_4$ 0.05 g/100 ml
5. Nutrient broth (1 g/100 ml), shrimp shell (1 g/100 ml).
6. Glucose (0.5 g/50 ml), peptone (0.25 g/50 ml), yeast extract (0.25 g/50 ml), KH_2PO_4 (0.05 g/50 ml), $MgSO_4$ (0.01 g/50 ml), and $NaCO_3$ (0.25 g/50 ml).

In each 250 milliliter flask, 100 milliliters of distilled water was added to each agricultural waste and sterilized using an autoclave for 15 min at 121°C. The chosen bacterial inoculum of 1 ml and having a bacterial concentration of 2.5×10^7cells/milliliter, was used to inoculate each flask. These flasks were then incubated at 37°C for 24 hours, and the enzyme activity and protein concentration were estimated.

Effect of carbon source

To determine the best carbon source for NK production from *P. aeruginosa* P49 in selected medium, different carbon sources were used including glucose, sucrose, maltose, fructose, starch, lactose and cellulose, whereas 0.5% of each source was added into 100 milliliter of selected medium and the medium flask was sterilized using autoclave at 121°C for 15 min. Then, each flask was inoculated with 1.0 milliliter of selected bacterial inoculum (2.5×10^7cell/milliliter) and placed in an incubator for 24 hours at 37°C [25]. Specific activity of enzyme was also estimated.

Effect of nitrogen source

To determine the best nitrogen source for NK production from *P. aeruginosa* P49 in selected medium, different nitrogen sources were employed, including peptone, yeast extract, and meat extract. For this purpose, the following procedure was carried out: firstly, each nitrogen source was used separately at concentration 20 g/L then half amount of two-nitrogen sources together and finally equal amount of three different nitrogen sources were employed, as follows: 20 g/L of peptone, 20 g/L of yeast extract, 20 g/L of meat extract, mixture of 10 g/L of peptone and 10 g/L of yeast extract, mixture of 10 g/L of peptone and 10 g/L of meat extract, mixture of 10 g/L of meat extract and 10 g/L of yeast extract and mixture of 6.6 g/L of peptone, 6.6 g/L of yeast extract and 6.6 g/L of meat extract. All flasks were placed in an autoclave and inoculated with 1.0 milliliter of chosen inoculum of bacterial species (2.5×10^7cell/milliliter) and put in an incubator for 24 hrs at 37°C. Specific activity of enzyme was estimated.

Effect of salts

To determine the salt effect on NK production from *P. aeruginosa* P49 in the selected medium, different mineral salt solutions (MS) were used, including $FeSO4.7H_2O$, $CuSO_4.5H_2O$, $MgSO_4.7H_2O$, $MnSO_4.7H_2O$, $CaSO_4.2H_2O$, and $ZnSO_4.7H_2O$. For this purpose, the following procedure was carried out: firstly, each salt was used separately at a weight of 0.1 g/100 milliliter, and half amount of two salts was utilized together. Besides the estimation of enzyme's specific activity, all flasks were inoculated as per previously described method and incubated for 24 hours at 37°C [26].

Initial pH

The pH of the chosen medium was varied throughout a wide range, from 3 to 9, including 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8, 8.5, and 9. Similar steps of bacterial inoculation of the flasks and incubation, along with the estimation of specific enzyme activity, were repeated as mentioned in the previous section after autoclaving [26].

Optimum temperature

To find the ideal temperature for the production of NK, the selected medium was subjected to different temperatures of 25, 30, 35, 37, and 40°C. Each flask was inoculated after autoclaving as previously explained with bacterial inoculum, incubated, and specific enzyme activity was calculated [26].

Optimum incubation period

The determination of optimum incubation duration for the production of NK was estimated by inoculating each medium as previously described, incubating along with the estimation of specific enzyme activity, but this time the incubator was operated at different periods of time, that is, 12, 24, 36, and 48 hours [26].

Purification of NK

Through the use of ammonium sulphate precipitation and ion exchange chromatography, NK was extracted from a local isolate of *P. aeruginosa* P49 and then purified.

Ammonium sulfate precipitation and ion exchange chromatography

At a temperature of 4°C, solid ammonium sulphate ranging from 0 to 75% was progressively added to the crude enzyme. The ingredients were then mixed carefully for 2 hours and 45 minutes. After that, the mixture was centrifuged at a speed of 10,000 revolutions per minute for twenty minutes, and the supernatant was thrown away. The precipitate was put in 10 millilitres of a buffer that contained 0.2 M potassium phosphate. It was possible to determine both the enzyme's activity and the concentration of proteins.

Based on the method suggested by Schütte (1985), Ion exchange chromatography (DEAE-Cellulose) was utilized for NK purification [27]. During the stage that included ammonium sulphate precipitation and by utilizing a clean Pasteur pipette that moved in circular motions on the wall of column, a total of 13 millilitres of the concentrated enzyme was placed onto a DEAE-Cellulose column that measured 23 by 1.7 centimeters. The column was cleaned with an equilibrating solution of Tris-HCl with a pH of 8, a concentration of 0.2 M, and a flow rate of 30 milliliters/hr, with 3 milliliters being used for each fraction till the optical density at 280 nm read zero. The elution was carried out at a flow rate of 30 milliliters/hr using the gradient concentrations of NaCl ranging from 0.0 M to 1.0 M that were formed in a Tris-HCl buffer.

The protein fractions of both the washed and eluted fractions were determined at a wavelength of 280 nm. The portions of the protein peaks that had enzymatic activity were collected, and the sections of the protein peaks that did not include enzymatic activity were tested for NK activity. It was possible to determine both the enzyme activity and the concentration of the protein.

Results

Isolations and Identifications of *P. aeruginosa*

All samples were subjected to various examinations, including microscopic examinations, cultural characteristics, and biochemical, in order to isolate *P. aeruginosa*. All the essential tests and examinations for initial diagnoses of the well-grown bacterial isolates were performed as displayed in Table 1.

Examination	Characteristics	*P. aeruginosa*
Microscopic	Shape of cells	Rods
	Gram staining	Negative
Cultural Characteristics	Cetrimide agar	Growing with green, fluorescent light, large colonies, and a grape-like odor
	MacConkey agar	Elevated colonies of light non-lactose Fermenters
	Nutrient agar/ Brain hart agar	Develop with a green, fluorescent light, large colonies, and a grape-like odor
	Hemolysis	Positive (+ve) - Beta
Biochemical	Simmons citrate	Positive (+ve)
	Catalase	Positive (+ve)
	Oxidase	Positive (+ve)
	Growth at 42°C	Positive (+ve)

Table 1: Results of examinations for identification of *P. aeruginosa* isolates from various clinical specimens.

Screening Program for NK (NK) Production

The findings of primary screening displayed that only five isolates of *P. aeruginosa* from 150 isolates were the better isolates for NK production compared with the other isolates, as shown in Figure 1A.

Secondary Screening

The five *P. aeruginosa* isolates (P11, P23, P42, P49, and P104) with the highest hydrolysis zone in the main screening were screened once again for their enzymatic activities utilizing the technique of digesting caseins. This was done for the purpose of further detection and the careful selection of an effective bacterial isolate to generate NK. *P. aeruginosa* P49 had the greatest enzymatic activity out of the five isolates tested. Its NK-specific activity in crude supernatant was 337.9 U/mg protein, while for supplementary strains, it varied from 222.9 to 314.1 U/mg protein, as displayed in Figure 1B.

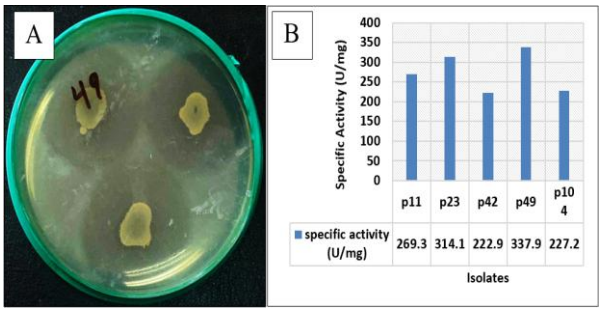

Figure 1: A) Proteolytic activity of *P. aeruginosa* P49 on skim milk agar; **B)** Quantitative screening of *P. aeruginosa* isolates for NK production utilizing SF medium.

Optimum Conditions for NK Production

The various bioprocess parameters that impact NK synthesis by *P. aeruginosa* P49 under SF were optimized for maximal production of enzymes, and the findings that were obtained were among the most fascinating ones. These results are explained in more detail below:

Optimum Culture Media

Six types of culture media were examined for their efficiency in NK production for *P. aeruginosa* P49. Of all media, the first culture medium of *P. aeruginosa* P49 showed the most enhanced NK production with a NK specific activity value of 521.12 U/mg protein, whereas for second culture media this value was 105.40 U/mg, for third culture media it was 50.25 U/mg, for the fourth culture media it was 302.80 U/mg, for the fifth culture media it was 111.75 U/mg, and for the sixth culture media it was 227.20 U/mg, as shown in Figure 2A.

Optimum Carbon Source

A variety of carbon sources, including lactose, starch, fructose, maltose, sucrose, glucose, and cellulose, were examined at a concentration of 0.5% to see how well they contribute to the production of NK. The results for NK production from *P. aeruginosa* P49 showed that sucrose was the best carbon source with high specific activity (759.88 U/mg protein) compared with other sources, as shown in Figure 2B.

Optimum Nitrogen Source

Numerous kinds of nitrogen sources were examined for their efficiency in NK production. The result demonstrated that the peptone was the best source for NK production from *P. aeruginosa* P49. The specific activity of NK using the peptone was 1023.7 U/mg, while the specific activity of the enzyme was lower when using the nitrogen sources, as shown in Figure 2C.

Effect of salts

Numerous kinds of salts were examined for their efficiency in NK production by *P. aeruginosa* P49. These mineral salt solutions (MS) included: ($FeSO_4.7H_2O$, $CuSO_4.5H_2O$, $MgSO_4.7H_2O$, $MnSO_4.7H_2O$, $CaSO_4.2H_2O$, and $ZnSO_4.7H_2O$). These salts were added separately to the selected medium. The optimum salts for NK production were $CaSO_4.2H_2O$, which gave a high specific activity of NK (1424 U/mg), as observed in Figure 2D.

Effect of Initial pH

To study the impacts of the initial pH on NK production by *P. aeruginosa* P49, the selected medium was adjusted to different pH values. The optimum pH for NK production was pH 7.5 with high specific activity of NK (1579U/mg protein), while pH 3, 3.5, 4, and 9 didn't give any result for NK production, as shown in Figure 2E.

Purification Steps	Volume (milliliter)	enzyme Activity (U/milliliter)	Protein Concentration (Mg/milliliter)	Specific activity (U/mg)	Total activity (U)	Purification Fold	Yield (%)
Crude enzyme	100	11.7	0.007	1598.8	1117	1	100
Ammonium sulfate precipitation (0-75%)	13	68.16	0.024	2840	954.2	1.78	86
Ion exchange chromatography (DEAE-Cellulose)	64	34.23	0.006	5705	2190.7	3.6	96

Table 2: The purification step of NK from *P. aeruginosa* P49 isolate.

Optimum temperature

Different temperatures were examined to determine the appropriate temperature for NK production by *P. aeruginosa* P49. These temperatures were 25, 30, 35, 37, and 40°C. The optimum temperature for NK production was 37°C, with a higher specific activity of NK (1580U/mg protein) as compared with other temperatures, and mentioned in Figure 2F.

Optimum Incubation Period

After 24 hours of incubation, the specific activity reached a maximum value of 1603 U/mg protein. The findings of the experiment were shown in Figure 2G, which showed that the specific activity was 514.5 U/mg after 12 hours. In contrast, the specific activity was 1058 U/mg after 36 hours of incubation, but it had dropped to 1010 U/mg at 48 hours of incubation, as shown in Figure 2G.

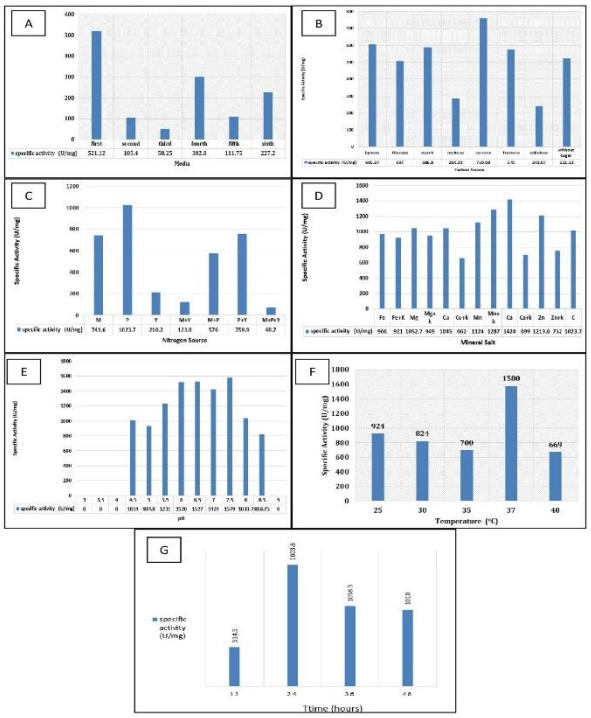

Figure 2: Effect of different **A)** media; **B)** Carbon sources; **C)** Nitrogen sources (M: Meat; P: Peptone; Y: Yeast); **D:** Salts (C: Control); **E:** pH; **F:** Temperature; **G:** Incubation period on NK production from *P. aeruginosa* P49 under the optimum conditions.

Purification of NK

The crude extract was purified by precipitation with ammonium sulfate. For the purpose of this investigation, the ammonium sulfate saturation ratios ranging from 0 to 75% were used for the NK precipitation. After that, the second step for NK purification, which was ion exchange chromatography technique (DEAE-cellulose), was utilized after precipitation by ammonium sulfate (0-70%). The findings in Figure 3 showed one peak of protein in the wash stage. These results suggested that NK carried a positive charge comparable to the charge of the resin under the experimental circumstances, and then the fractions that had activity were collected. As indicated in Table 2, the yield reached 96% when this step's specific activity was 2190.7U/mg protein with a purification fold of 3.6. In contrast, the elution step shown in Figure 3 displayed five peaks of protein when it was eluted by gradient salt; nonetheless, there was no NK activity present. All steps of purification are illustrated in Table 2.

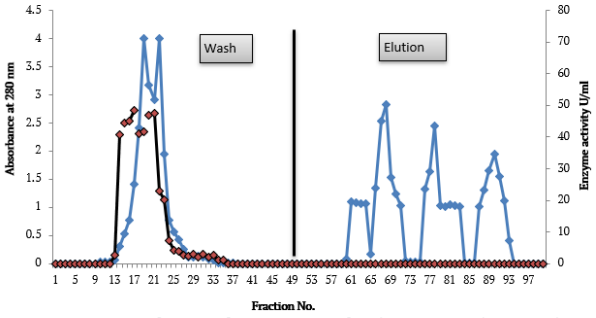

Figure 3: Ion exchange chromatography for NK purification from *P. aeruginosa* P49 by using DEAE-Cellulose column (23×1.7 cm) equilibrated with Tris-HCl buffer (0.02 M, pH 8.0), eluted with Tris-HCl buffer and NaCl gradient 0 M-1.0 M at a flow rate of 30 milliliter/hour.

Discussion

After all these examinations, 150 isolates of *P. aeruginosa* were obtained. All isolates of *P. aeruginosa* were subjected to VITEK2 system to confirm their species.

Pseudomonas aeruginosa is a leading cause of illness and death in hospitalized patients owing to its high level of antibiotic resistance [28]. This study asserts the same evidence as MKK (2019), who indicated the isolation frequency of the most common bacterium in diverse clinical specimens to be *P. aeruginosa* [29]. Similarly, 35.1% (40 out of 114) of patient samples proved to have *P. aeruginosa* through the documentation of Shatti (2022) [30]. In the same way, Shilba (2015) mentioned the infection of 37.9% of the patients admitted to Karbala hospitals with this

microbe [31]. A considerable difference in protease yields between strains existed. This difference may result from the strain-specific ability of *P. aeruginosa* to produce varied protease enzymes, e.g., Nattokinase (NK) [32]. Genetic diversity [33] and varied sources of isolation, yet from the same species, may also account for such differences [34].

Choubey (2016) conducted research and revealed 18 different bacterial strains with the ability to produce NK through the formation of haloes in skim milk agar plates [35]. Chandrasekaran *et al.* (2015) showed in their work that strains of *Pseudomonas aeruginosa* grown on corresponding media showed very high enzyme production ability [36]. Such variation in the activity of NK in different strains of *P. aeruginosa* can be the result of the intrinsic variation in the origin and type of bacteria and genetic content [37]. Based on these experimental results, a nutrient-rich medium combining peptone and yeast extract was selected to optimize NK yield. Among these components, peptone proved most effective for enzyme production, owing to its rich composition of proteins, essential vitamins, and other bioactive compounds that support microbial metabolism [38].

Experimental evidence showed that supplementation with sucrose provided maximum enzyme biosynthesis, and the said carbohydrate was used specifically based on its ability to activate NK production. Earlier work has shown that composition of the growth medium significantly affects exoenzyme secretion and carbon and nitrogen substrates are especially important regulatory elements in this respect [25,39].

A study performed by Ibrahim (2015) proved that different organic compounds of nitrogen, such as beef extract, casamino acids, peptone, and skim milk, could promote the synthesis of protease in different species of bacteria with varying efficacy depending upon the microbe strain [40]. In addition to this, peptone is also a highly nutrient-dense supplement with rich amounts of essential micronutrients (vital elements and vitamins) and protein derivatives (amino acids and peptides) important for proper microbe growth and metabolism [41].

As compared to the other studied cations, calcium ions exhibited the most noteworthy enhancement in the synthesis of protease. The majority of the other ions that were examined resulted in a considerable decrease in the enzyme production. It has been shown that calcium ions may stimulate the synthesis of proteases in a variety of species. In addition, the presence of Ba ions increased the amount of protease that *Bacillus* sp. produced. It has been found that the active shape of the enzyme was maintained at high temperature despite the presence of these metal ions, which prevented the enzyme from being denatured by heat. *Bacillus sp.* had their protease production severely inhibited when the medium was supplemented with zinc, copper, iron, and cobalt, and this was most noticeable at high cation concentrations [41]. The addition of $MgSO_4$ and $CaCl_2$ led to an increase in the amount of NK produced by *B. subtilis* MX-6 [42].

In general, the impact of pH on enzyme production may be related to the fact that it plays a role in the solubility of the medium's nutritious elements, has an influence on the ionization of the substrate, and makes the substrate available to the microbe. In addition, pH has an influence on the stability of the enzyme. On the other hand, the fermentation process and the pace of enzyme production are often affected by the pH of the culture medium. The pH value of the medium influences cellular physiology to a significant extent via alteration of plasma membrane conformation. These conformation changes then modify membrane-associated ribosomes and their attendant protein biosynthesis activities [43].

Chandrasekaran *et al.* (2015) documented the ideal condition being pH 7 at 1514 U/ml and 2.8 mg/ml for the highest NK activity of *P. aeruginosa*. Equally, Ju *et al.* (2019) also discovered the ideal condition to be pH 7.0 when they registered the highest NK production of 3284 ± 58 IU/milliliter from their *P. aeruginosa* P49 strain [36,44].

Temperature is a vital factor that needs to be properly controlled in biological processes since its optimum range usually differs greatly between different organisms [45]. For example, Chandrasekaran *et al.* (2015) recorded the highest NK activity from *P. aeruginosa* at 25°C with 1514 U/ml and 2.8 mg/ml. Against this background, Ju *et al.* (2019) documented *Bacillus subtilis* producing its highest NK production of 3284 ± 58 U/mg at 30°C [36,44]. High temperatures are also recognized to disrupt or destroy the structure of proteins and reduce their protease activity [46]. Aside from its direct effect on enzymes, temperature also affects the availability of oxygen and the mass transfer rate in the fermentation broth and the metabolic activity rates in microorganisms [46].

The variations in yield of NK according to the temperature may be due to changes in the culture conditions that occur during these intervals, such as a decrease in oxygen and nutrients and an accumulation of toxic metabolites that prevent the development of bacteria. According to the findings of certain investigations, the manufacturing of the enzyme began in the earliest stages of the development of the microorganism [45].

In agreement with the results of this study, previous studies indicated that the optimal incubation period is 24 hours for the production of NK from *Bacillus* spp. and *P. aeruginosa* [36,44].

The simplicity of the charge differences-dependent separation principle, potential of reactivation for repetitive utilization, large capacity, simple handling, high power of resolution, and good separation are only a few of the numerous benefits offered by DEAE-Cellulose resin [47].

Author Contributions

Shahad Abdullah Shwan directed the research work, collection of data, and preliminary drafting of the manuscript.

Ali Jabbar Reshak supervised the research, helped with the interpretation of data, and critically reviewed the manuscript.

The final version of the manuscript was read and approved by both authors.

Competing Interest

The authors declare no conflict of interest concerning the publication of this manuscript.

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