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Purification and characterization of L-asparaginase produced from *Bacillus* sp.

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Abstract

The objective of this study was to isolate and identify the asparaginase-producing bacteria, then purify and characterize the enzyme in order to investigate their properties in the future. Fifteen local bacterial isolates were isolated from various sites in the city of Baghdad, identified by conventional morphological and biochemical procedures, and confirmed using vitek 2 methods, and submitted to primary screening processes for asparaginase production. For secondary screening, eight isolates with the greatest yellow zone ability on a specific solid medium were chosen. *Bacillus* sp. was reported to have the highest enzyme production (7.5 U/mg proteins). After 24 hours of incubation, submerged fermentation yielded optimal conditions for the production of L-asparaginase (L-ASNase) by the chosen isolate, with medium (2) serving as the optimal medium for production and fructose serving as the optimal source of carbon. In pH 6 at 40°C, Sephadex G-150 gel filtration chromatography was used to purify the enzyme. The final purification folds were increased by 2.5 times, resulting in an enzyme yield of 93.7%. It also showed the highest purified enzyme activity and stability was at 37°C. Also it revealed the highest activity and stability at pH 7.0 and pH 8.0 respectively. Enzyme lost activity when exposed to several metallic ions at concentrations of 1, 5, and 10 mM.

Keywords: Asparaginase, Bacteria, Optimum conditions, Purification, Characterization.

Introduction

Enzymes are biological catalytic molecules that are widely exploited in industry to produce different essential products. Enzyme technology has enabled scientists to utilize, modify, and improve the efficacy of enzymes in order to achieve maximal functionality such as food industry, microbiology, and in medicine[1]. As a potential oncological agent, microbial L-asparaginase (E.C.3.5.1.1) has been extensively investigated and utilized[2]. Asparagine's amide group is hydrolyzed by this enzyme, producing ammonia and aspartate[3]. L-asparaginase (L-ASNase), an amidohydrolase, is often used as a cancer treatment. L-asparagine, which is present on the majority of cancer cell surfaces, is converted by the enzyme into ammonia and L-aspartate, which causes cell death since L-asparagine is required for cell proliferation and activation[4]. L-ASNase contributes significantly to the medical enzymes that are commonly utilized as anti-lymphoma agents[5]. In the presence of L-ASNase, cancer cells lack a vital growth factor and therefore cannot survive. Thus, this enzyme may be applied to treat everyone (particularly children) with leukaemia and cancer [6]. Hodgkin's disease, melanosarcoma, reticulosarcoma, lymphosarcoma, lymphocytic leukaemia, acute myelomonocytic leukaemia, and acute myelocytic leukaemia are only a few of the diseases that may be treated with L-ASNase [7]. L-asparaginase production is very sensitive to factors such as the composition of the fermentation medium used and culturing parameters including temperature, pH, inoculum size, incubation period, and agitation rate [8]. L-

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asparagine is produced by normal cells using L-asparagine synthetase. The most common organisms used to isolate and purify L-asparaginase include *Streptomyces albidoflavus, Escherichia coli, Bacillus* sp., and actinomycetes from the rhizosphere of medicinal plants [9]. As a result, it is critical to screen novel organisms capable of producing new and high-productivity L-ASNase. The goal of this work was to isolate L-asparaginase producing bacteria from waste active sludge and soil, then optimize for maximum enzyme activity before L-ASNase purifying and characterization to study its properties in the future.

Materials and Methods

Chemicals

The reagents and chemicals utilized in this study were procured from Hi-Media and Sigma-Aldrich, India. The list of materials includes asparagine, EDTA, agar-agar, $HgCl_2$, Tris-HCl, yeast extract, cysteine, nutrients agar and several others.

Collection of samples and bacterial isolation

Ten bacterial isolates were isolated from the waste active sludge (WAS) sample collected from the dry tank of Al-Rustumiah wastewater treatment plant in south Baghdad, Iraq, and five bacterial isolates were isolated from the soil of different sites of University of Baghdad, Iraq. These isolates were identified using morphological, and biochemical assays and a Vitek 2 test after cultivation on Brain heart broth(Merck), followed by brain heart agar (Merck)[10]. The isolates were then grown on nutrient agar medium and stored in the refrigerator at 4ºC for the experiment of screening. Following that, L-asparaginase production was assessed using the method reported by Hasan and Hussein [11].

Screening of isolates for the production of L-asparaginase

Primary (qualitative) screening

With few modifications, qualitative screening for L-asparaginase production was carried out in accordance with Sharma and Husain [12]. Asparagine solid medium (10g asparagine, 2g glucose, 1.52 g KH_2PO_4, 0.52 g KCl, 0.52 g $MgSO_4$, 0.03 g $FeSO_4$, 0.03 g Zn SO_4, 0.05g $CuSO_4$, and 15 g agar-agar), was used to screen 15 isolates for the highest asparaginase producers. The substance was dissolved in one liter of distilled water, thoroughly mixed, and the pH was adjusted to 7.0 autoclaved, then 0.09 g/L phenol red added after filtering through a Millipore filter 0.22µ in sterilized asparagine medium, then poured onto plates to select the best ones that produced enzyme. Following that, wells are aseptically poked on an agar surface plate using a sterile cork borer. Bacterial isolates were plated out on asparagine agar at a concentration of 100 µL per well and incubated at 37 ºC for 24 hours. Asparaginase secretion was assumed to be occurring when a yellowish color appeared around the colony wells.

Secondary (Quantitative) screening

From the primary screening, eight isolates with the highest yield were chosen and cultivated on a liquid asparagine medium. Fifty mL of modified submerged medium (10 g asparagine, 2 g glucose, 1.52 g KH2PO4, 0.52 g KCl, 0.52 g MgSO4, 0.03 g FeSO4, 0.03 g Zn SO4, and 0.05 g CuSO4) is placed in a 250 mL flask along with 1 L of distilled water that has a pH of 7.0. After being autoclaved, one mL of overnight-culturing isolates containing 3 x 10^{11} cells/mL was inoculated. The flasks were incubated in a 37 ºC, 150 rpm rotary shaker incubator. Each flask's culture was centrifuged for 10 minutes at 10,000 rpm after 24 hours in the incubator. The asparaginase activity and protein concentration in the crude enzyme were measured using the supernatant. The enzyme's activity and protein concentration were both repeatedly evaluated [12].

Assay for L-asparaginase

The Nesslerization method, which has an absorbance limit of 625 nm and depends on the breakdown of L-asparagine to L-aspartate and ammonia, was modified slightly, according to Al-Dulimi [13]. One mL of crude L-ASNase was added to the tube, and it was then incubated at 37°C for 30 minutes with one mL of 200 mM L-asparagine in 0.05 M potassium phosphate buffer (pH 8.0). After incubation, the reaction was stopped by adding 1 mL of 1.5 M trichloroacetic acid to the solution mixture. The mixture was centrifuged at 10000 rpm for 10 minutes. For each sample, the direct Nesslerization method was used to determine the L-ASNase activity in the supernatant by mixing 1 mL of Nessler's reagent with 1 mL of supernatant of the enzyme with vigorous agitation. The mixture was then kept at 37°C in an incubator for 30 minutes. At 625 nm, the

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optical density was determined. To prepare the blank, 1 mL of Nessler's reagent was added to 1 mL of the above reaction mixture, except that 1 mL of trichloroacetic acid was added before adding 1 mL of crude enzyme. The amount of enzyme that, under experimental conditions, releases 1 µmole of ammonia per minute was designated as one unit of L-ASNase. The concentration of proteins was measured using the Bradford procedure [14].

Optimization of the production of L-ASNase from selected isolate

The influence of varied culture requirements on L- ASNase production was investigated. Numerous regulating factors were assessed, including composition of the fermentation medium, the carbon source, pH value, temperature, and incubation time.

The influence of fermentation medium

To determine the best medium for L-ASNase production, six different media were tested.: (1) asparagine 10 g/L, glucose 2g/L, KH_2PO_4 1.52 g/L, KCl 0.52 g/L, $MgSO_4$ 0.52 g/L, $FeSO_4$ 0.03 g/L, $ZnSO_4$ 0.03 g/L and $CuSO_4$ 0.05 g/L [12]. (2) Glucose 2g/L, KH_2PO_4 1.52 g/L, KCl 0.52 g/L, $MgSO_4$ 0.52 g/L, $FeSO_4$ 0.03 g/L, Zn SO_4 0.03 g/L and $CuSO_4$ 0.05 g/L [12] with modification. (3) Peptone 1 g/L, glucose 1 g/L, yeast extract 0.3 g/L, asparagine 1 g/L, and meat extract 0.3 g/L [15]. (4) Peptone 1g/L, glucose 1 g/L, yeast extract 0.3 g/L, and meat extract 0.3 g/L [15] with modification. (5) Asparagine 1 g/L, glucose 0.1 g/L, K_2HPO_4 0.1 g/L, yeast extract 0.05 g/L, trypton 0.05 g/L [16]. (6) Glucose 0.1 g/L, K_2HPO_4 0.1 g/L, yeast extract 0.05 g/L, trypton 0.05 g/L [16] with modification. Autoclaving duplicate 250 mL Erlenmeyer flasks with 50 mL of each test medium. Then, 1.0 mL of an overnight culture with 3 x 10^{11} cells/mL of the chosen isolate was added to these flasks, and then were set in a 150 rpm shaker incubator at 37°C for 24 hours. After incubation, each flask's culture was centrifuged, and the filtrate's enzyme activity, protein content, and specific activity were all assessed. [13,14].

Best sources of carbon

To choose an ideal carbon source, in 250mL flasks, the pH of the 50 mL of optimal medium was adjusted to 7.0, autoclaving for 15 minutes at 121 ºC. After sterilization with a 0.22µ millipore filter unit, 2 g/L of glucose, sucrose, fructose, lactose, and starch as carbon source solutions were added to each flask individually (in duplicate). Before incubating the flasks at 37 ºC (150 rpm) for 24 hours, a 2% (cells/mL) overnight culture of a *Bacillus* sp. isolate was added. To determine enzyme activity, concentration of protein, and specific activity[13,14] , the supernatants were collected after centrifuging the cultures at 10,000 rpm for 10 minutes.

The pH influences

The initial pH value of the medium has been determined for evaluating enzyme production after the optimal carbon source was determined. Before autoclaving, the pH was adjusted to 5, 6, 7, and 8 in 250 mL Erlenmeyer flasks containing 50 mL of optimized medium. After inoculating the medium with a bacterial culture isolate at a concentration of 3 x 10^{11} cells per mL, the medium was incubated for 24 hours at 150 rpm and 37°C in a shaker incubator. Thereafter, measurements were made of the activity of enzyme, protein amount, and specific activity [13, 14].

The influence of temperature

Temperatures of 28, 30, 37, and 40 ºC were tested to determine the best temperature for ASNase production. The chosen medium was inoculated with 2% (3 x 10^{11} cells/mL) of a culture of a bacterial isolate grown overnight, and incubated for 24 hours at different temperatures in a shaker incubator set to 150 rpm. After centrifugation, the specific activity was determined according to Al-Dulimi et al. and Bradford[13,14].

Optimal period for incubation

Several incubation periods were tested to determine the production efficacy of asparaginase. A 2% inoculum of the overnight-selected isolate (3x10^{11} cells/mL) was added to the optimum medium and incubated at 40°C for 24, 48, and 72 hours. The assessment of specific activity was performed according to Al-Dulimi et al. and Bradford [13,14].

L-asparaginase purification

Bacillus sp. was grown under optimum conditions for ASNase production, and the enzyme was extracted by centrifugation at 10000 rpm for 10 minutes. The crude L-ASNase was purified using (Sephadex - G150) gel filtration to remove unwanted proteins and other components: as follows:

The column was attended to and packaged in accordance with the manufacturer's (Pharmacia-Sweden) instructions. A crude enzyme sample was subjected to column chromatography using a Sephadex G-150 column with dimensions (1.5 x 21

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cm), and the elution phase was carried out with 0.05M Tris-HCl, pH 8, at a flow rate of 20 mL/h, with 3mL of each fraction. After measuring the protein fractions at 280 nm, these fractions' enzyme activity was measured. The activity and concentration of protein were estimated after determining the volume of effective fractions. The volume has been reduced with sucrose and placed in individual containers before being frozen[17].

Characterization of partial purified ASNase

Temperature impact on partial purified enzyme activity

The activity of partially purified enzyme was assessed at four distinct temperatures: 37, 45, 50, and 55°C. In order to determine the optimal temperature for ASNase activity[18], the correlation between enzyme activity and temperature was then investigated.

Temperature's influence on L-ASNase stability

L-ASNase that had been partially purified was placed in a water bath at varying temperatures (37, 45, 50, and 55°C) for 15 minutes before being transferred to an iced bath. At the optimal temperature for enzyme activity, the activity was then measured. The remaining activity was subsequently calculated.

The influence of pH on partially purified L-ASNase activity

The asparagine has been prepared as a substrate in a range of buffer solutions, such as 0.05M acetate buffer (pH 5, 6), 0.05M potassium phosphate buffer (pH 7, 8), and 0.05M tris-base buffer (pH 9), to test the impact of pH on the partially purified enzyme activity. By measuring the activity and showing the correlation between enzyme activities and pH values, the ideal pH for L-ASNase activity was determined [18].

Influence of various pH values on the stability of partially purified enzyme

At different pH ranges (5-9), partially purified enzyme and buffers were mixed in the same amount (1:1). The solution was placed in a 37°C water bath for 15 minutes. Immediately the samples were transported to an ice bath. The enzyme activity was measured, and the remaining enzymatic activity has been estimated and plotted for each value of pH to establish the optimal pH for L-ASNase stability.

Influence of certain chemical compounds on the activity of L-ASNase

The effect of chemical compounds ($ZnSO_4$, KCl, $HgCl_2$, NaCl, EDTA, and cysteine) on the activity of partially purified L-ASNase was investigated. The 1 mM and 5 mM solutions were prepared by dissolving each substance in a phosphate buffer of 0.05 M concentration at 37 ^oC for 15 minutes. The enzyme solution and metal ion solution were mixed in a 1:1 (v/v) ratio. The activity of the enzyme was then measured and compared to the control (enzyme without treatment). The remaining percentage of activity was then evaluated.

Results and Discussion

Screening bacterial isolates for L-asparaginase production

Primary (qualitative) screening

Fifteen bacterial isolates were tested qualitatively using an asparaginase plate agar medium, and the appearance of yellow color surrounding the colony wells was considered indicative of asparaginase secretion. According to the intensity of the created yellow color, eight bacterial isolates were asparaginase producers (Fig. 1). The production of a yellow color due to the breakdown of asparagine in a solid medium including phenol red as a pH indicator demonstrates the bacteria's ability to alter the culture's pH from acidic to alkaline. This is a result of L-asparaginase breaking down amide bonds in L-asparagine and the accumulation of ammonia in the medium. There was a correlation between L- ASNase activity and the formation and diameters of yellow zones surrounding wells. The yellow zone's diameter varied from 20 to 45 millimeters. These isolates were chosen to undergo secondary screening. L-ASNase activity zone for soil bacterial isolates ranged from 0.8 to 13mm[19]. According to Wakil and Adelegan [20], the yellow color zone of L-ASNase isolated from Ibadan soil bacteria in varied from 3 to 5 cm.

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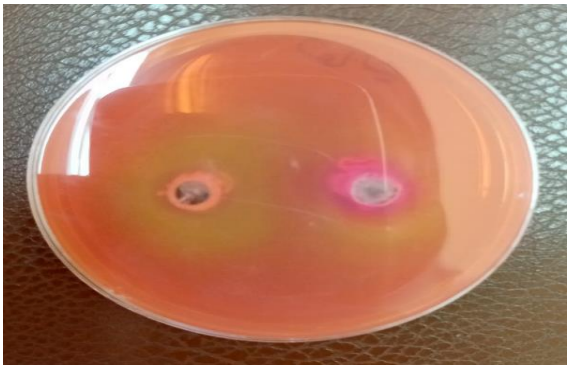

Figure 1. L-asparaginase activity by bacterial isolate on solid medium at 37ºC for 24 hr.

Secondary (Quantitative) screening

Submerged fermentation was used to test the enzymatic activity of the eight bacterial isolates that produced the highest yellow coloring during the primary screening. The Nesslerization process, which degrades asparagine to ammonia and aspartate and has an absorbance of 625nm, has been used[13]. Among eight isolates, the *Bacillus* sp. isolate exhibited the highest enzyme activity, with 7.5 U/mg protein specific activity of a crude enzyme (Fig. 2). The *Bacillus* sp. isolate with the highest specific activity was selected for the next study despite the fact that the asparaginase specific activity of the other isolates ranged from 0.3 to 6.8 U/mg. It may be possible to explain why different species members have different abilities for producing asparaginase. By considering the isolates' origin, type, and genetic diversity as well as culture parameters including aeration, stirring, pH, temperature, and media components that support the isolates of *Bacillus* sp.'s ability to synthesize the enzyme in liquid medium[21]. Poongothai and his colleagues[22] reported that *Bacillus* S8 sp. can produce asparaginase via submerged fermentation, with 1.08 U/mg of specific activity.

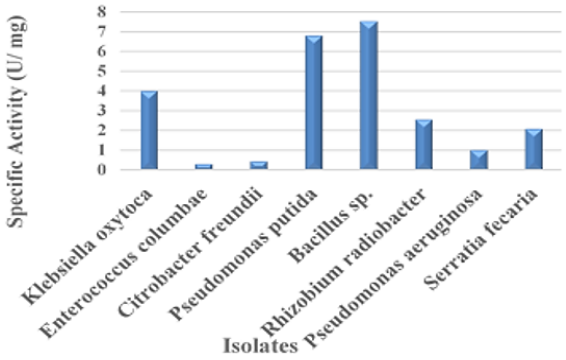

Figure 2. Production of asparaginase by different bacterial isolates in shaker incubator 150 rpm at 37°C for 24 hrs.

Optimal asparaginase production conditions

The influence of the fermentation medium

Bacillus sp. was cultivated in six different media to evaluate the influence of the medium on asparaginase production. Among the six media investigated, medium (2) was found to be the best medium for asparaginase production based on specific activities. The maximum specific activity of asparaginase found was 52 U/mg, however a specific activity of asparaginase utilizing media 1, 3, 4, and 5 was reduced to 31.7, 24.2, 20.2, and 51.4 U/mg protein respectively (Fig. 3). The other fermentation parameters' activity was maximized by using this medium. The production of L-ASNase was greatly influenced by the composition of the medium and the culture conditions (such as pH, temperature, and nutrients). There is no specific medium among variable microorganisms that maximizes L-ASNase production. In order for each microorganism to achieve its maximum L-ASNase production capacity, it must satisfy certain conditions, it is necessary to optimize the medium components and environmental conditions. Different environmental conditions and medium components are necessary for the growth of microorganisms and enzyme production, as the nature of the microorganism greatly affects the culture conditions necessary for enzyme formation [23].

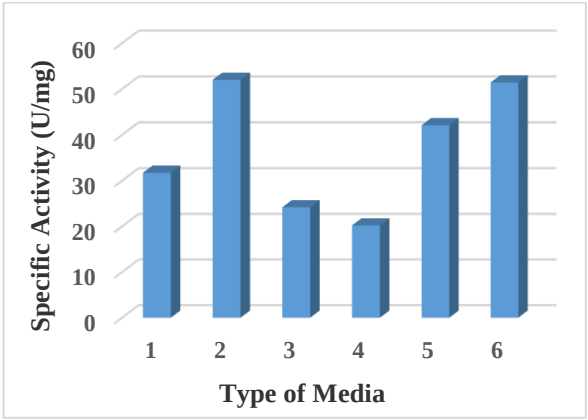

Figure 3. *Bacillus* sp. asparaginase production in a shaker incubator at 150 rpm, 37°C, for 24 hours, using a variety of liquid media.

Optimal sources of carbon

Asparaginase production was studied using several types of carbon sources included in the medium at a concentration of 2 g/L. Among five distinct carbon sources, fructose was discovered to enhance L- asparaginase production (Fig. 4). Asparaginase's specific activity rose to 58 U/mg in comparison to 13.4 U/mg. in a medium containing

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sucrose. For organism growth, carbon is a necessary element, and the majority of microorganisms choose it as a source of energy. As a consequence, In order to support microbial growth and the production of metabolites, it is usually employed as a substantial carbon source[24]. Moawad and his colleagues[25] found that asparaginase may be produced from *Aspergillus flavus* by submerged fermentation, with fructose serving as the best carbon source and having the maximum enzymatic activity (52.4 U/mL).

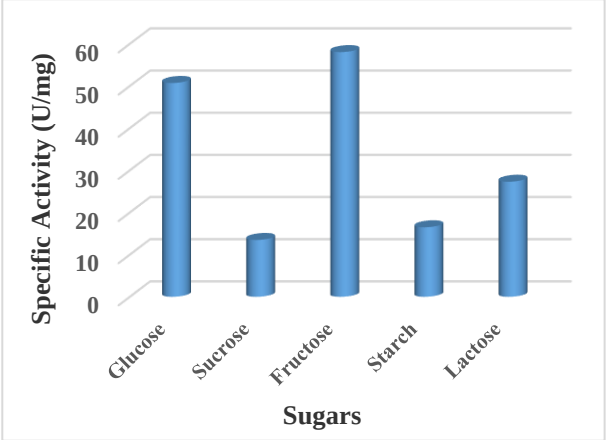

Figure 4. Influence of carbon sources on asparaginase produced by *Bacillus* sp. in submerged culture at 37 °C and pH 7.0 for 24hrs

The pH effect

The pH of the production medium was varied to evaluate the effect of the initial pH on asparaginase production (5, 6, 7, and 8). As indicated in Fig. 5, the greatest asparaginase specific activity (67.1) U/mg was acquired at pH 6.0; however, increasing or decreasing the pH value above or below 6.0 reduced enzyme activity. *Bacillus* sp. asparaginase was shown to have the maximum activity around neutral pH. According to Wakil and Adelegan[20], the optimal pH for *Bacillus circulans* L-asparaginase production is 6. Any decrease or increase in the concentration of hydrogen ions (H^+) induces pH variations in the reaction mixture, which may cause significant changes in the three-dimensional structure of the protein, leading to enzyme denaturation[26]. Fermentation course and enzyme production rate are commonly influenced by the pH of the culture medium because the medium impacts the plasma membrane's conformation and consequently, the ribosomes that adhere to the membrane and synthesize proteins [27].

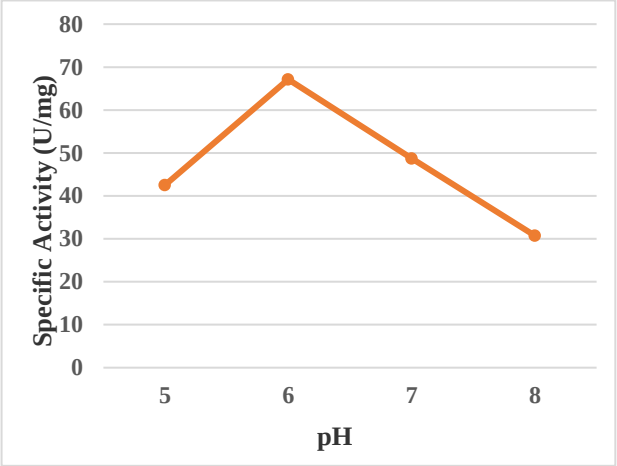

Figure 5. Impact of pH on *Bacillus* sp. asparaginase synthesis in a shaker incubator at 150rmp for 24 hours at 37ºC

Temperature effects on the production of L-asparaginase

The results in Fig. 6 examine how well an isolate of *Bacillus* sp. grows and releases L-ASNase throughout a wide temperature range (28, 30, 37, and 40°C). At 40 °C, asparaginase may be produced at its highest levels, with a specific activity of 75.3 U/mg. Lower yields (44.7 U/mg proteins) were achieved at a lower temperature of 28°C. Any microbe's growth and metabolism depend heavily on temperature. Temperature has an impact on the synthesis of enzymes by affecting the solubility of oxygen in the medium, the rate of enzyme reaction, and the vibration energy of cell molecules [28]. These effects may be either positive or negative.

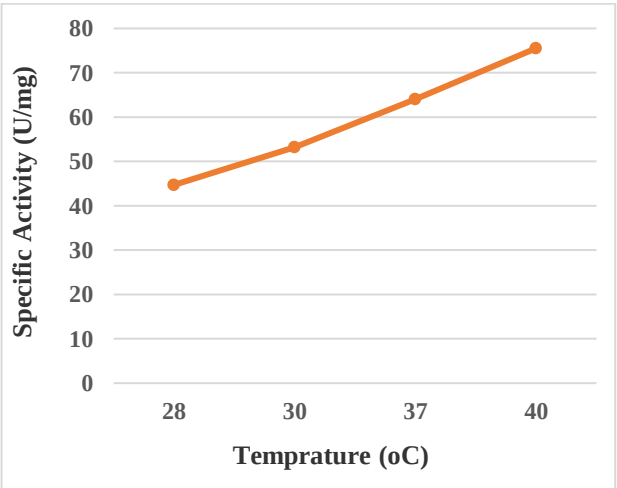

Figure 6. The temperature effect on ASNase production from *Bacillus* sp. in a shaker incubator at 150 rpm for 24 hours

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Optimal incubation time

The optimal incubation period was determined by testing the growth and enzyme synthesis phases. The specific activity of asparaginase produced by *Bacillus* sp. reached 75.5 U/mg of protein after 24 hours of incubation, as illustrated in Fig. 7. The specific activity decreased as the incubation period increased due to alterations in the culture conditions, including accumulation of toxic metabolites, depletion of oxygen, and nutrient consumption, which inhibit the bacterial growth [29].

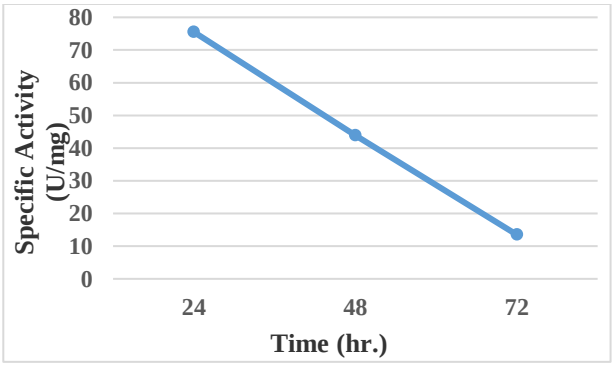

Figure 7. The influence of incubation time on asparaginase production by *Bacillus* sp. in a shaker incubator at 150 rpm and 40°C.

L-asparaginase purification by gel filtration chromatography

The results revealed that in the eluted fractions, one protein peak separated from the column, as well as one peak of asparaginase activity (15-29) fractions (Fig. 8). With 2.5 fold of purification, the specific activity was 204.3 U/mg proteins, and the yield was 93.7% (table 1). L-ASNase was isolated from *Streptomyces albidoflavus* by Narayana et. al.[30] and purified using Sephadex G-100 and CM-Sephadex C-50, the specific activity of L-ASNase increased to 101 and 437 U/mg, respectively. Additionally, they noticed that utilizing CM-Sephadex C-50 increased L-asparaginase purity to 99.3-fold purity with a 40% recovery. Dhevagi and Poorani [31]observed that after 83-fold purification, the L-asparaginase isolated from marine actinomycetes using Sephadex G-200 gel filtration revealed specific activity with 64.07 U/mg protein and a final yield of 2.18%.

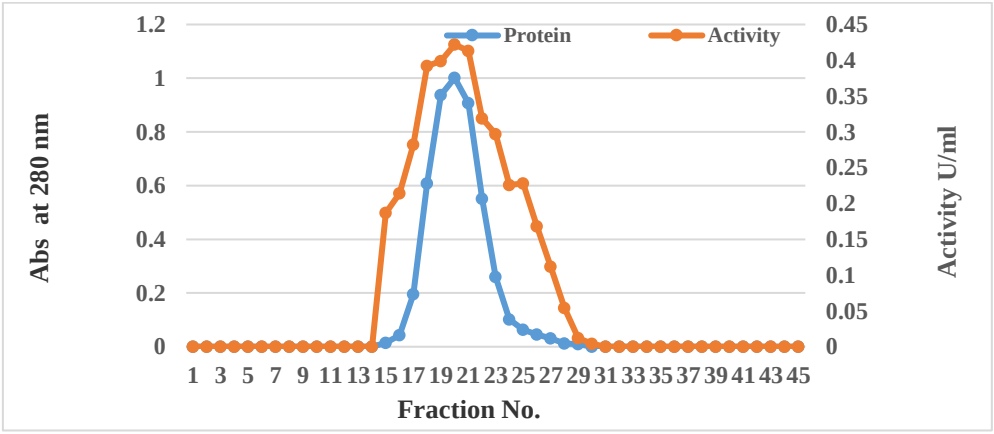

Figure 8. Gel Filtration chromatography for L. ASNase purification from *Bacillus* sp. Utilizing Sephadex G150 column (1.5x21) cm equilibrated and eluted with Tris-HCl buffer(pH 8, 0.05 M), in rate of flow 20 ml/hr.3 ml for every Fraction.

Table 1. Purification steps of L-asparaginase from *Bacillus* sp.

Sample	Volume (ml)	Activity (U/mg)	Protein (mg/ml)	Specific Activity (U/mg)	Total Activity(U)	Purification Fold	Yield (%)
Crude	50	0.48	0.006	80	24	1	100
Sucrose	20	0.71	0.0075	94.7	14.2	1.2	59.2
Gel filtration using Sephadex G-150	45	0.5	0.0025	200	22.5	2.5	93.7

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Characterization of purified enzyme

The influence of temperature on L-ASNase activity

Temperature ranges (37°-55°C) were used to assess the activity of partially purified L-ASNase. Fig. 9 shows that activity increased to 0.51 U/mL at 37°C, then decreased as temperature increased to 50°C and reaching at 55°C a minimum of 0.11 U/mL. The results revealed that the reaction speed increased until it reached 37°C, then started to fall beyond 45°C owing to an increase in the impact between the enzyme molecules participating in the reaction and the substrate due to the increased movement energy of the molecules. Temperatures over 50°C cause a decrease in enzymatic activity due to denaturation of enzyme activity[32]. 37ºC was found to be the best temperature for purified L-asparaginase activity from *Aspergillus terreus* KLS2 [33].

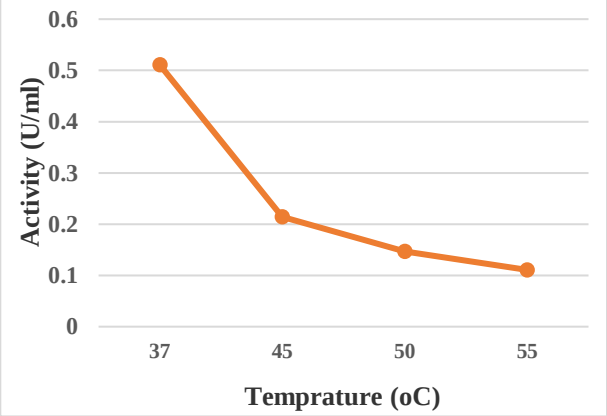

Figure 9. The effect of temperature variations on the activity of partially purified L-ASNase from *Bacillus* sp.

Temperature's influence on L-ASNase stability

The L-ASNase temperature stability was determined by incubating the enzyme for 15 minutes at temperatures varying from 37 to 55°C. The percentage of remaining activity is then determined. In the present study, the activity of L-ASNase was maintained at 37°C (Fig.10), whereas the activity begins to decline between (45 and 55°C). Due to its sensitivity to high temperatures, L-asparaginase loses activity over 37°C; typically, temperature below or above the optimal temperature for an enzymatic process will considerably slow down the rate of reaction. This may be a result of the enzyme being denaturized or losing its three-dimensional structural properties. Hydrogen bonds and other non-covalent bonds are broken while a protein is denaturing[26]. L-ASNase purified from *Fusarium culmorum* ASP-87 was subjected to thermal stability tests, which showed that this enzyme is very stable between 30 and 40 ºC for 120 minutes, but deactivates beyond that temperature while retaining 50% activity for an hour at 60 ºC [34].

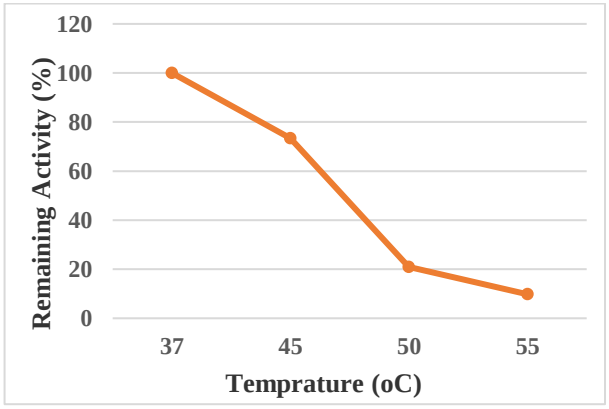

Figure 10. The influence of temperature variations on the stability of partially purified L-ASNase from *Bacillus* sp.

The effect of pH on partially purified L-ASNase activity

As shown in Fig. 11, the pH effect on partially purified L-ASNase from *Bacillus* sp. was evaluated over a pH range of 5.0 to 9.0. Maximum enzyme activity was detected at a pH of 7, it reached 0.68 U/mL, however the optimum range was between 7 and 8, whereas in acidic pH (5 and 6) it was 0.36 U/mL and 0.42 U/mL, respectively, and in alkaline pH (9) the activity decreased to 0.38 U/mL. The variability in the ideal pH is primarily attributed to its impact on enzyme activity through several mechanisms, such as its effect on the ionization of groups in the enzyme's active site, its impact on the ionization of groups in the substrate, or the ability to alter the conformation of either the enzyme or the substrate [35]. Moorthy and colleagues discovered that the optimal pH for L-asparaginase activity from a soil isolate of *Bacillus* sp. was 7 [36].

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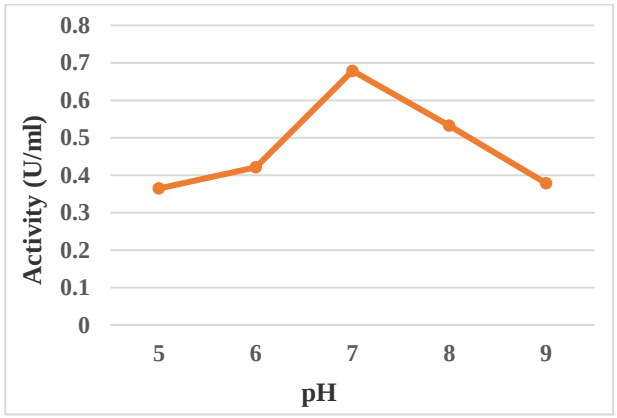

Figure 11. The impacts of different pH levels (5.0-9.0) on the activity of partially purified L-asparaginase from *Bacillus* sp.

The effect of different pH levels on the stability of partially purified L-ASNase

The findings in Fig. 12 show that pH 8.0 is optimal for the stability of L-ASNase, as the enzyme retained approximately 100% of its activity at pH 8.0, but only 26.7% at pH 7.0. The enzymatic activity decreased outside of the ideal pH range. The remaining enzyme activity was 13.3% at a pH of 5.0, decreased at a nearly acidic pH, and reached 18.9% at pH 6.0. Also, enzyme activity decreased as pH became more alkaline, by 93.3% at pH 9.0. Hassan and Hussein [37] determined that the pH of the L-asparaginase stability of *Staphylococcus aureus* was 8. The decrease in enzymatic activity observed at pH values outside the optimal range can be attributed to the effect of pH on enzyme structure, which results in enzyme denaturation or changes in the ionic state of the active site. In addition, pH can affect the secondary and tertiary structure of the enzyme, resulting in a loss of activity in buffer solutions considerably outside the optimal pH [38].

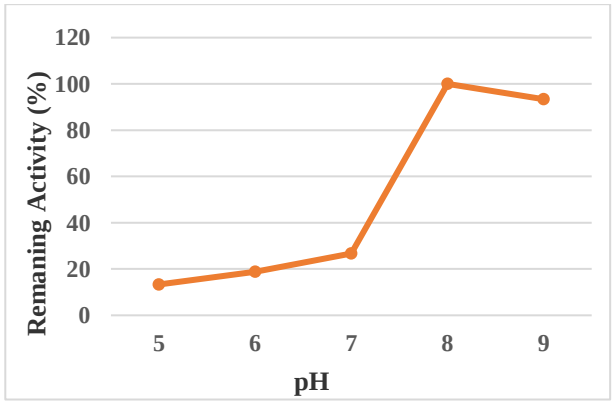

Figure 12. The impact of changing the pH of partially purified enztme from *Bacillus* sp. from 5.0 to 9.0.

Influence of certain chemical compounds on the activity of L-asparaginase

Purified L-asparaginase from *Bacillus* sp. was treated with chemical compounds. The results in Fig. 13 demonstrate that, with a few minor exceptions, all of these compounds reduced the L-asparaginase enzyme. Depending on the enzyme origin, metal ions have different effects on L-ASNase activity, although it has been shown that at concentrations of 1 and 5 mM, $ZnSO_4$, NaCl, $HgCl_2$, KCl, EDTA, and cysteine all suppress enzyme activity to lower levels than their original activity (control value). At concentrations of 1 mM and 5 mM, $HgCl_2$ inhibited L-ASNase with 50 and 32% residual activity, respectively. At doses of 1 and 5 mM, EDTA suppresses enzyme activity, with residual activity equivalent to 60.8 and 48.3%, respectively (Fig. 13). Also, using 1 and 5 Mm of $ZnSO_4$, NaCl, KCl, and cysteine decreased activity to 23, 14.6, 17.5, and 78.3% respectively at 1mM and 37, 74.8, 37.8 and 72% respectively at 5mM. Recent studies have indicated that enzyme function is influenced by different types and concentrations of ions, with a tendency for the effects to increase as the concentration increases. The formation of a complex with the enzyme impedes its ability to bind to the substrate and catalyze the production of the desired product, resulting in a reduction in enzymatic activity [39]. The enzyme was observed to be inhibited by $HgCl_2$ at concentrations of 1 and 5mM, which indicates that $HgCl_2$ oxidized SH groups in the enzyme's active site. Furthermore, $HgCl_2$ forms a complex with the enzyme that prevents it from binding to the substrate to generate the product, resulting in a decrease in activity [40]. The inhibitor's impact on L-ASNase activity was tested using EDTA. This substance displayed decreasing enzyme activity with increasing concentration of the inhibitor, as indicated by the remaining enzyme activities. Furthermore, the findings demonstrated that the enzyme was a metalloenzymatic and that divalent ions were critical to the increased activity when the enzyme was incubated with EDTA; hence, EDTA was employed to investigate the influence of inhibitors on the activity of L-ASNase. When an enzyme is treated with cysteine, the disulfide connections within its structure and conformation weaken, resulting in the protein fragmenting into its component components and a consequent decrease in activity. The findings of the study suggest that the enzyme under investigation was found to possess disulfide bonds [41].

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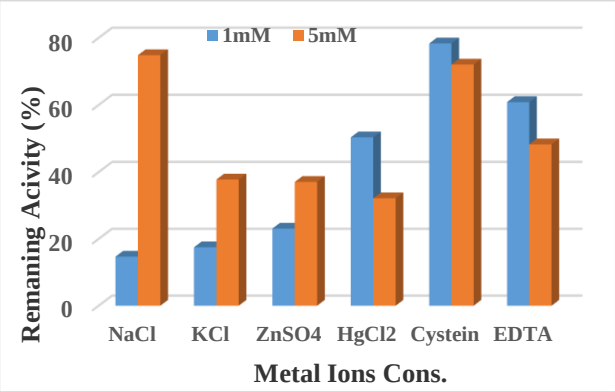

Figure 13. The effect of certain chemical and ions on the enzyme activity of *Bacillus* species at 37 ºC

Conclusion

Local *Bacillus* sp. isolates could produce L-ASNase on solid media or in submerged culture. The ideal conditions for the production of ASNase by the chosen isolate were determined, using medium (2) acting as the best production medium and fructose serving as the best carbon source at 40 °C and pH 6 after 24 hours of incubation. Using Sephadex G-150 gel filtration chromatography to purify the crude enzyme. The optimal temperature for L- ASNase activity and stability was 37°C for 15 minutes, while the optimal pH for L- ASNase activity and stability was found to be 7.0 and 8.0 respectively. However, the activity of L- ASNase was inhibited by certain metallic and heavy metallic ions.

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Authors' Declaration

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Ethical Clearance: The project was approved by the local ethical committee in University of Baghdad.

Authors' Contribution Statement

S.I.H. conceived, designed, acquired the data and drafted the manuscript. While Z.A.H Interpret the results of the study, revision, paraphrasing and proofreading of the manuscript.

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تنقيه وتوصيف الاسبراجينيز المنتج من *Bacillus* sp.

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الخلاصة

هدفت الدراسة الى عزل وتشخيص البكتيريا المنتجة للأسباراجينيز ، ثم تنقية الإنزيم وتوصيفه من أجل تحديد خصائصه في المستقبل. تم عزل 15 عزلة بكتيرية محلية من مواقع مختلفة في مدينة بغداد، وتم تشخيصها بوساطة الاختبارات المظهريه والكيميائية واختبارات الفيتيك، خضعت العزلات لعمليات الفحص الأولي لإنتاج الأسباراجينيز؛ حيث تم اختيار ثماني عزلات للفحص الثانوي ذات القابليه الاعلى في انتاج الهاله الصفراء في الوسط الصلب . بينت النتائج أن البكتريا العصويه من جنس *Bacillus* sp. لديها اعلى إنتاجية إنزيمية (7.5 وحدة / ملغم بروتين) بعد 24 ساعة من الحضانة ، حدتت إلى الظروف المثلى لإنتاج الانزيم بواسطة العزلة المختارة عن طريق التخمير المغمور ، حيث اختير الوسط (2) كأفضل وسيط للإنتاج وبوجود الفركتوز كأفضل مصدر للكربون عند درجة حموضة 6 ودرجه حرارة 40 درجة مئوية. تم تنقيه الانزيم بوساطه كروماتوغرافيا الترشيح الهلامي باستخدام جل السيفادكس –ج 150 ، حيث ازدادت عدد مرات التنقية النهائية بمقدار 2.5 مرة ، مما أدى إلى إنتاج إنزيم بنسبة 93.7٪. كما أظهر الانزيم المنقى أعلى فعاليه وثباتيه عند درجه حرارة 37 م ، و اعطى اعلى فعاليه عند الرقم الهيدروجيني 7.0 واعلى ثباتيه عند الرقم الهيدروجيني 8.0. و فقد الإنزيم نشاطه عند تعرضه لعدة أيونات معدنية بتركيزات 1 ، 5 ملي مولر.

الكلمات المفتاحية: الأسباراجينيز، البكتيريا، الظروف المثلى، التنقية، توصيف.