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TRIAGEM, PURIFICAÇÃO E CARACTERIZAÇÃO DA L-ASPARAGINASE PRODUZIDA POR *LACTOBACILLUS CURVATUS*

SCREENING, PURIFICATION, AND CHARACTERIZATION OF L-ASPARAGINASE PRODUCED FROM *LACTOBACILLUS CURVATUS*

أنزيم الأسباراجينيز المنتج من بكتريا *Lactobacillus Curvatus* فحص وتنقية وتوصيف

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RESUMO

Introdução: A L-asparaginase, uma enzima utilizada nas indústrias alimentícia e de tratamento do câncer, decompõe a L-asparagina em L-aspartato e amônia. Ela preserva a qualidade dos alimentos e previne a formação de acrilamida. Microrganismos benéficos em probióticos fornecem benefícios biológicos, como a remoção de substâncias mutagênicas e cancerígenas. Probióticos são comumente definidos como bactérias benéficas, como as espécies de *Lactobacillus* e *Bifidobacterium*. **Objetivo:** Este estudo visa rastrear, purificar e caracterizar a L-Asparaginase de isolados de *Lactobacillus curvatus*. **Métodos:** O estudo analisou isolados de *Lactobacillus curvatus* quanto à sua capacidade de produzir a maior atividade de L-ASNase. A enzima foi extraída e seu peso molecular foi estimado usando SDS-PAGE. O efeito do pH, temperatura, íons metálicos e detergentes na estabilidade da L-ASNase também foi examinado. O estudo descobriu que a atividade da L-ASNase foi influenciada por vários fatores, incluindo pH, temperatura, íons metálicos e detergentes. **Resultados:** Entre os 11 isolados bacterianos analisados, 7 demonstraram capacidade de produzir L-Asparaginase formando uma zona rosa distinta (17,5-20 mm), com o isolado de *Lactobacillus curvatus* nº 11 exibindo a maior atividade específica de 4,86 U/mg. A purificação da enzima foi otimizada usando precipitação com sulfato de amônio 70%, resultando em fator de purificação de 6,6 e rendimento total de 86,6%. A purificação via cromatografia de troca iônica e cromatografia de filtração em gel aumentou a atividade específica da enzima para 6 U/mg, alcançando fator de purificação de 12 com rendimento total de 72%. O SDS-PAGE determinou o peso molecular da enzima como 37 kDa. A enzima exibiu atividade máxima em pH 7 e 40 °C, com estabilidade na faixa de pH 6-7 e temperaturas entre 35-40°C. A atividade enzimática foi significativamente aumentada pelos íons Mg^{2+} e P^{5+} (111%). Mercaptoetanol, EDTA e Tween-80 aumentaram a atividade enzimática para 109%, 103% e 100%, respectivamente. **Discussão:** O estudo descobriu que apenas 7 dos 11 isolados bacterianos desenvolveram um diâmetro de zona rosa grande (17,5-20 mm), sendo o isolado Nº 11 o maior produtor de L-ASNase. A atividade específica dos isolados produtores ativos de L-ASNase variou, com a maior atividade específica medida em 4,86 µ/mg. A enzima purificada tinha peso molecular de 37 KDa, atividade ótima em pH 7, estabilidade em pH 6, 6,5 e 7, e temperatura de 40 °C. A atividade enzimática aumentou significativamente com íons metálicos, atingindo seu nível mais alto em 111% quando Mg e P estavam presentes. **Conclusões:** O estudo identifica *Lactobacillus curvatus* produtor de L-Asparaginase de fontes anteriores, que foi purificado e apresentou atividade específica de 6 U/mg com fator de purificação de 12.

Palavras-chave: *Lactobacillus curvatus, atividade enzimática, enzima purificada, atividade específica.*

ABSTRACT

Background: L-asparaginase, an enzyme employed in the food and cancer treatment industries, breaks down L-asparagine into L-aspartate and ammonia. It preserves food quality and prevents the formation of acrylamide. Beneficial microorganisms in probiotics offer biological benefits, including the removal of mutagenic and

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carcinogenic substances. Probiotics are commonly defined as helpful bacteria such as *Lactobacillus* and *Bifidobacterium* species. **Aim:** This study aims to screen, purify, and characterize L-Asparaginase from *Lactobacillus curvatus* isolates. **Methods:** The study screened *Lactobacillus curvatus* isolates for their ability to produce the highest L-ASNase activity. The enzyme was extracted, and its molecular weight was estimated using SDS-PAGE. The effect of pH, temperature, metal ions, and detergents on L-ASNase stability was also examined. The study found that L-ASNase activity was influenced by various factors, including pH, temperature, metal ions, and detergents. **Results:** Among the 11 bacterial isolates screened, 7 demonstrated the ability to produce L-asparaginase, forming a distinct pink zone (17.5–20 mm) with Lactobacillus curvatus isolate no. 11 exhibiting the highest specific activity of 4.86 µg/mg. Enzyme purification was optimized using ammonium sulfate precipitation at 70%, yielding a 6.6-fold purification and an 86.6% total yield. Purification via ion exchange chromatography and gel filtration chromatography enhanced the enzyme's specific activity to 6 µM/mg, achieving a 12-fold purification with a 72% overall yield. SDS-PAGE analysis determined the molecular weight of the enzyme to be 37 kDa. The enzyme exhibited maximum activity at pH 7 and 40 °C, with stability in the pH range of 6–7 and temperatures between 35 °C and 40 °C. Enzyme activity was significantly enhanced by Mg^{2+} and P^{5+} ions (111%). Mercaptoethanol, EDTA, and Tween-80 increased enzyme activity to 109%, 103%, and 100%, respectively. **Discussion:** The study found that only 7 out of 11 bacterial isolates developed a large pink zone diameter of (17.5-20) mm, with isolate No.11 being the highest producer of L-ASNase. The specific activity of active L-ASNase-producing isolates varied, with the highest specific activity measured at 4.86 U/mg. The purified enzyme had a molecular weight of 37 kDa, with optimum activity at pH 7, stability at pH 6, 6.5, and 7, and a temperature of 40 °C. Enzyme activity increased significantly with metal ions, reaching its highest level at 111% when Mg and P were present. **Conclusions:** The study identifies L-Asparaginase-producing *Lactobacillus curvatus* from previous sources, which was purified and found to have a specific activity of 6 µg/mg with a 12-fold purification.

Keywords: *Lactobacillus curvatus, enzyme activity, purified enzyme, specific activity.*

المستخلص

الخلفية L-Asparaginase **:**هو إنزيم يستخدم في الصناعات الغذائية وعلاج السرطان، حيث يقوم بتكسير L-asparagine إلى L-aspartate والأمونيا. يحافظ على جودة الغذاء ويمنع تكون مادة الأكريلاميد. توفر الكائنات الحية الدقيقة المفيدة الموجودة في البروبيوتيك فوائد بيولوجية مثل إزالة المواد المسببة للطفرات والمسرطنة. يتم تعريف البروبيوتيك بشكل شائع على أنها بكتيريا مفيدة مثل أنواع Lactobacillus و .Bifidobacterium **الهدف:** تهدف هذه الدراسة إلى فحص وتنقية وتوصيف إنزيم L-Asparaginase من عزلات *Lactobacillus curvatus*. **الطرق:** قامت الدراسة بفحص عزلات *Lactobacillus curvatus*لقدرتها على إنتاج أعلى نشاط لـ .L-ASNase تم استخلاص الإنزيم وتقدير وزنه الجزيئي باستخدام .SDS-PAGE تم أيضًا فحص تأثير الرقم الهيدروجيني ودرجة الحرارة والأيونات المعدنية والمنظفات على ثبات .L-ASNase ووجدت الدراسة أن نشاط L-ASNase يتأثر بعوامل مختلفة، بما في ذلك الرقم الهيدروجيني ودرجة الحرارة والأيونات المعدنية والمنظفات. **المناقشة:** وجدت الدراسة أن 7 عزلات بكتيرية فقط من أصل 11 عزلة تطورت بقطر كبير للمنطقة الوردية يتراوح بين (17.5-20) ملم، وكانت العزلة رقم 11 هي الأعلى إنتاجاً لـ .L-ASNase تنوع النشاط النوعي للعزلات النشطة المنتجة لـL-ASNase ، حيث تم قياس أعلى نشاط محدد عند 4.86 وحدة / ملغ. كان للإنزيم المنقى وزن جزيئي قدره 37 كيلو دالتون، والنشاط الأمثل عند درجة الحموضة 7، والاستقرار عند درجة الحموضة 6، 6.5، 7، ودرجة الحرارة 40 درجة مئوية. زاد نشاط الإنزيم بشكل ملحوظ مع أيونات المعادن، حيث وصل إلى أعلى مستوى له عند 111% عند وجود الماغنيسيوم والفوسفور. **الاستنتاج:** حددت الدراسة بكتيريا *Lactobacillus curvatus* المنتجة للأنزيم L-Asparaginase من المصادر السابقة، والتي تم تنقيتها ووجد أن نشاطها النوعي هو 6 وحدات/مجم مع 12 طية تنقية.

الكلمات المفتاحية: *Lactobacillus curvatus*، فعالية الانزيم، الانزيم المنقى ، الفعالية النوعية

1. INTRODUCTION:

Lactobacillus curvatus is a Gram-positive bacterium with curved, bean-shaped rods. It is a non-spore-forming, facultative heterofermentative bacterium that can grow at temperatures ranging from 30°C to 37°C (Rainey *et al*., 2015). It is commonly isolated from fermented meat, dairy products, and plant-based products (Lucquin *et al*., 2012; Chaillou *et al*., 2015).

L. curvatus produces class II bacteriocins, which can inhibit pathogenic and spoilage bacteria (Castellano *et al*., 2008; Castro *et al*., 2011). It is considered a bioprotective agent in fermented meat products and has been found in the feces of animals and humans (de Souza *et al*., 2015; Chen *et al*., 2020). The bacterium has also been studied for its potential health benefits in treating obesity, hyperlipidemia (Yoo *et al*., 2013; Park *et al*., 2013; Ahn *et al*., 2015) and dextran sodium sulfate-induced colitis (Katsuki *et al*., 2019).

Enzymes are biocatalytic molecules widely utilized in various industries to produce essential products. L-Asparaginase is an enzyme that breaks down L-Asparagine into L-Aspartate and ammonia, which are essential for protein synthesis and nutrition (Chand *et al*., 2020). It has been used in several industries, including food, medicine, and cancer treatment (Qeshmi *et al*., 2018).

L-Asparaginase (L-ASNase) is an effective enzyme in preventing tumor cell proliferation by depriving the cells of nutrients, causing them to starve and die, with minimal effects on normal cells due to their ability to produce L-asparagine synthetase (Moguel *et al*., 2020). L-ASNase has the potential to act as an antitumor agent, making it a suitable treatment option for lymphomas, acute lymphoblastic leukemia, and various types of cancer (Ghasemi *et al*., 2017). By removing L-asparagine, an amino acid essential for tumor cell growth and proliferation, L-ASNase acts as an anticancer agent.

The enzyme plays a significant role in treating lymphoma and contributes to the therapeutic enzymes used in cancer treatment. L-ASNase induces the death of cancer cells by depleting L-asparagine, a crucial nutrient for cell growth. Consequently, it is effective in treating leukemia and cancer, especially in children and adults. L-ASNase can be used for the treatment of various diseases, such as acute myelomonocytic leukemia, acute myelocytic leukemia, lymphosarcoma, reticulosarcoma, melanosarcoma, and Hodgkin's disease.

In foods treated with heat, L-ASNase can be used to reduce the formation of carcinogenic acrylamide (Qeshmi *et al*., 2018). The most commonly used microorganisms for isolating and purifying L-ASNase include Streptomyces albidoflavus, Escherichia coli, Bacillus sp, and actinomycetes from the rhizosphere of medicinal plants (El-Naggar and El-Shweihy, 2020). Obtaining and purifying enzymes from animals and plants is challenging, which is why microbes are often used. Therefore, screening for novel microorganisms that can produce L-ASNase in high quantities is essential (Moguel *et al*., 218).

While many bacterial species can produce L-ASNase, the most commercially approved forms used as medication are recombinant L-ASNase from Escherichia coli and Erwinia chrysanthemi (Muneer *et al*., 2020). Lactic acid bacteria, including Lactococci, Streptococci, and Lactobacilli, have been widely studied for their enzymes, as they are generally recognized as safe (GRAS) (Aishwarya *et al*., 2019).

This work aims to screen, purify, and characterize the L-Asparaginase enzyme from *Lactobacillus curvatus* to investigate its potential properties in future applications.

2. MATERIALS AND METHODS:

2.1. Materials

In this study, the following materials were used:

Ammonium sulfate BDH(England), Bovine serum albumin (BSA) BDH (England), Sodium dodecyl Sulphate (SDS), Phosphate Buffer Saline (PBS) pH 7, M9 medium, EDTA, Urea, Tween-80, and sodium azide.

2.2. Methods

2.2.1 Sample Collection and Identification

Eleven *Lactobacillus curvatus* isolates were obtained from previous sources (Mahdi *et al*., 2019) and screened for their ability to produce the highest L-ASNase activity. The ability of L-ASNase-producing *L. curvatus* isolates to create a pink zone around the colonies on the agar surface of the M9 medium provided the basis for the initial rapid plate test method (semi-quantitative screening) (Gulati *et al*., 1997). *L. curvatus* isolates were then cultured in 100 ml of M9 broth (containing 1% L-asparagine), incubated anaerobically for 48 hours at 37°C, and then centrifuged. The supernatant was plated on the agar surface of the M9 medium (containing 1% L-asparagine) (Alrumman *et al*., 2019). Bradford assay was used to determine the protein concentration, using bovine serum albumin (BSA) as a standard. The precipitate obtained was dialyzed and then loaded onto the DEAE-Cellulose ion exchange column (Whitaker and Bernhard, 1972).

2.2.2 Isolation and primary screening of L-asparaginase-producing bacteria (semi-qualitative assay)

All isolates were screened for L-asparaginase activity using the modified M9 medium (1 l: 3.0 g KH_2PO_4; 6.0 g $Na_2HPO_4 \cdot 2H_2O$; 0.5 g NaCl; 5.0 g L-asparagine; 0.5 g $MgSO_4 \cdot 7H_2O$; 0.014 g $CaCl_2 \cdot 2H_2O$; 2.0% (w/v) glucose; and 15.0 g agar) combined with a pH indicator (phenol red), supplemented with 0.005% phenol red dye. Plates were inoculated with test organisms using one loopful of bacterial culture and incubated at 37°C for 48 hours. The plates contained L-asparagine as the sole source of nitrogen. L-asparaginase hydrolyzes L-asparagine into L-aspartic acid and ammonia, which can be easily detected by the change in the medium's pH due to ammonia production. The color change of the medium (from yellow to

pink) indicates positive L-asparaginase production. The L-asparaginase activity of each bacterium was recorded by measuring the pink zone on the plate. The enzyme index was determined using Equation 1:

Hydrolysis zone = diameter of the zone/diameter of the bacterial colony. (Eq. 1)

2.2.3 Secondary screening of L-asparagine-producing bacteria (quantitative assay)

Microorganisms isolated from the previous screening were cultured in liquid media with modified asparagine broth at pH 6.5. All experiments for quantitative screening were performed in triplicate after incubation at 37°C for 48 hours. The culture broth was centrifuged at 10,000 rpm for 10 minutes, and the supernatants were collected. Enzyme activity was then measured using the Nesslerization method (Imada *et al*., 1973).

2.2.4 *Purification of L-Asparaginase*

2.2.4.1 Crude Extraction

To extract the L-asparaginase enzyme, L. curvatus isolate no. 11 was cultured in 100 mL of M9 broth medium and incubated in a shaker incubator at 37°C for 48 hours under anaerobic conditions. Afterward, the culture was centrifuged at 6,000 rpm for 20 minutes at 4°C to obtain the cell-free extract (crude). Enzyme activity in the crude supernatant was then measured. The enzyme activity was 0.1 U/ml, and the specific activity was 0.5 U/mg protein, as demonstrated in Table 3.

2.2.4.2 Ammonium Sulfate Precipitation

Ammonium sulfate precipitation is one of the most widely used methods for purifying and concentrating enzymes. This method relies on precipitating the enzyme by adding ammonium sulfate salts (salting out). Different saturation ratios of ammonium sulfate (50%, 60%, 70%, 80%) were added to the crude enzyme (supernatant). To determine the optimum saturation ratio, ammonium sulfate was gradually added to the crude enzyme solution in an ice bath, and the mixture was gently stirred for 1 hour using a magnetic hot plate stirrer to minimize foaming. The resulting mixture was left overnight before being centrifuged at 10,000 rpm for 20 minutes. The supernatant was separated and mixed with ammonium sulfate to obtain the next saturation ratio. Enzyme activity, specific activity, and protein concentration were measured (Fathi *et al*., 2021).

2.2.5: Preparation of Ion Exchange Column

Sephadex–G100 column (2×40cm) was prepared and packed according to the instructions of the manufacturing company (Pharmacia Sweden). Phosphate buffer (pH-7.0; 0.05M) was used for column equilibration. The molecular weight and purity of L-ASNase were determined by SDS-PAGE, which was performed according to Laemmli's method (Whitaker and Bernhard, 1972).

2.2.6 Characterizing the purified enzyme

2.2.6.1 Molecular Weight Determination

The molecular weight of L-Asparaginase was determined using the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) technique. The molecular weight of L-ASNase was calculated based on the standard curve of protein markers, using the relationship between Rm and the logarithm of the molecular weight of standard proteins (Garfine, 1991).

2.2.6.2: Effect of pH

The effect of pH on the activity of L-ASNase was examined at 37°C using different buffers at various pH values (3, 4, 5, 6, 7, 8, 9, and 10). The stability of L-ASNase at different pH levels was tested by adding equal volumes of the purified enzyme to test tubes containing buffers at different pH values (3, 4, 5, 6, 7, 8, 9, and 10).

2.2.6.3 Effect of Temperature

The temperature profile of the purified enzyme was studied by measuring its activity at different temperatures (25, 30, 35, 40, 45, 50, 55, and 60°C). Equal volumes of purified L-ASNase were incubated in a water bath at these temperatures for 20 minutes. The relationship between the remaining activity (%) and temperature was plotted to determine the optimum temperature for L-ASNase stability (Yadav and Sarkar, 2014).

2.2.6.4: Effect of metal Ions

To determine the effect of various metal ions on L-ASNase activity, one volume of the purified L-ASNase was mixed with an equal volume of a 5 mM solution of each metal ion

(Co, Hg, Mg, Mn, Fe, Zn, Ca, Cu, Na, and K). The mixtures were then incubated at 37°C for 30 minutes (Darnal *et al*., 2023).

2.2.6.5: Effect of surfactants

The effect of various non-ionic and ionic detergents, EDTA, urea, Tween-80, and sodium azide (each at 1.0% w/v) on L-ASNase stability was determined. One volume of the purified L-ASNase was mixed with an equal volume of each detergent solution (Darnal *et al*., 2023).

3. RESULTS AND DISCUSSION:

3.1. Results

3.1.1 Screening of L-Asparaginase

In this study, only 7 out of 11 bacterial isolates developed an identifiable pink zone, with diameters ranging from 17.5 to 20 mm (Figure 1). Compared to the other isolates, *L. curvatus* isolate no. 11 produced a distinct pink zone and was identified as the highest producer of L-ASNase (Table 1). Another study using the same semi-quantitative method for screening Bacillus sp. for L-Asparaginase production found that, out of 9 isolates examined, *Bacillus sp*. produced the largest pink zone (3.6 cm). Furthermore, *Bacillus sp*. formed a larger zone (3.2 cm) compared to the zone (1.9 cm) produced by the standard strain *Pectobacterium carotovorum* MTCC 1428.

3.1.2 Quantitative screening of L-asparagine production

To select the most productive isolate of the L-Asparaginase enzyme, *L. curvatus* isolates were screened for their enzyme production levels. The enzyme produced by *L. curvatus* exhibited a specific activity of up to 5 µ/mg protein. The specific activity (S.A.) of the active L-Asparaginase-producing isolates ranged from 2.33 to 4.86 U/mg. The most productive isolates exhibited specific activities ranging from 4.05 to 4.86 U/mg, resulting in the L-asparagine broth medium turning light pink over 24 hours. *L. curvatus* isolate no. 11 exhibited the highest specific activity at 4.86 U/mg, while isolate no. 5 had the lowest at 2.33 U/mg (Table 2). Based on these results, *L. curvatus* isolate no. 11 was selected for further optimization to enhance its L-asparaginase production capacity.

3.1.3 Purification of L-Asparaginase

3.1.3.1 Ammonium Sulfate Precipitation

The results demonstrated that when ammonium sulfate was used at different saturation rates (50%, 60%, 70%, and 80%), the optimum enzyme precipitation was achieved at a saturation ratio of 70%. The enzyme's specific activity was determined to be 3.3 U/mg protein, with a purification fold of 6.6 and an overall yield of 86.6%, as shown in Table 3.

3.1.3.2 Ion Exchange Chromatography

According to the results shown in Figure 2, two protein peaks were observed when each fraction's absorbance was measured at 280 nm, with one peak in the washed fractions. Tubes 44–52 exhibited the highest level of L-Asparaginase activity, with only one peak in the eluted fractions at NaCl gradients of 0.5 and 0.7 M. Given that it bonded with the cationic ion exchange DEAE-cellulose, these results suggest that L-Asparaginase produced by *L. curvatus* has a negative net charge. As indicated in Table 3, the fractions were combined and examined for L-Asparaginase activity, which was found to be 0.3 U/ml, with a specific activity of 3.75 U/mg (protein) and a purification fold of 7.5, resulting in an overall yield of 84%.

3.1.3.3 Gel filtration chromatography

The results show that a single protein peak was observed when the absorbance of each fraction was measured at 280 nm (Figure 3). Tubes 20–24 exhibited the highest level of L-Asparaginase activity. As indicated in Table 3, the fractions were combined and examined for L-Asparaginase activity, which was determined to be 0.3 U/ml, with a specific activity of 6 U/mg (protein) and a purification fold of 12, resulting in an overall yield of 72%.

3.1.4 Characterization of L-Asparaginase

3.1.4.1 Molecular Weight of L-Asparaginase

This study showed that L-Asparaginase has a molecular weight of 37 kDa. The presence of a single protein band, as shown in Figure 5, indicates the high purity of the L-Asparaginase purified from *L. curvatus* using SDS-PAGE.

3.1.4.2 The Effect of pH on L-Asparaginase Activity

The purified L-Asparaginase was active

over a wide pH range (3–10), reaching a maximum activity of 63.5 U/mL at pH 7, as shown in Figure 6. Although the enzyme lost activity at higher pH values, it retained 22.17% activity at pH 8.

3.1.4.3 The Effect of Temperature on L-Asparaginase Activity

The purified L-Asparaginase exhibited activity over a broad temperature range (25–60°C), with a maximum activity of 65.2 U/mL at 40°C. However, L-ASNase activity decreased at higher temperatures, as shown in Figure 7.

3.1.4.4 The Effect of pH on L-Asparaginase Stability

The purified L-asparaginase was found to be stable at pH levels of 6, 6.5, and 7, as shown in Figure 8. After 30 minutes of incubation, the enzyme exhibited maximum remaining activity (100%) at these pH values. It retained approximately 93% of its activity at pH 5.5 and 32% at pH 8, while at pH 9, the remaining activity dropped to 13%. As the pH increased, the enzyme's activity declined, indicating its sensitivity to alkaline conditions.

3.1.4.5 The Effect of Temperature on- L-L-Asparaginase Stability

The stability of L-Asparaginase at different temperatures was examined. Maximum stability was observed at 35°C and 40°C, as shown in Figure 9, where the enzyme retained 100% of its activity after 20 minutes of incubation. However, prolonged incubation and exposure to higher temperatures (50°C and 60°C) for 90 minutes resulted in a rapid decline in L-Asparaginase activity, with remaining activity of 53.9% and 25.8%, respectively.

3.1.4.6 The Effect of Metal Ions on L-Asparaginase Activity

The results shown in Figure 10 demonstrate the impact of various inorganic ions on L-Asparaginase activity. Enzyme activity increased significantly in the presence of Mg^{2+} (111%), P^{5+} (111%), Co^{2+} (106%), and Ni^{2+} (102%), reaching its highest level when Mg^{2+} and P^{5+} were present (111%). However, a slight decrease in enzyme activity was observed in the presence of Cu^{2+} and Zn^{2+}, with L-Asparaginase retaining approximately 59% and 47% of its activity, respectively.

3.1.4.7 The Effect of Surfactants and Chelating Agents on L-Asparaginase Activity

The results shown in Figure 11 indicate that in the presence of SDS, sodium azide, and urea, the remaining activity of purified L-Asparaginase was 81%, 63%, and 53%, respectively. Conversely, the enzyme activity increased to 109%, 103%, and 100% in the presence of mercaptoethanol, EDTA, and Tween-80, respectively.

3.2. Discussion

The isolates were identified based on morphological and cultural characteristics to confirm the presence of *Lactobacillus curvatus* isolates. In this study, the agar plate method was used for the semi-quantitative screening of L-Asparaginase production. This plate test is the quickest and most accurate method for identifying L-Asparaginase-producing strains (Gulati *et al*., 1997). The screening was performed by assessing each colony's ability to produce a pink zone when cultivated on L-asparagine medium containing phenol red. The intensity of the pink color was proportional to the amount of enzyme produced. According to the results, only 7 out of 11 bacterial isolates developed an identifiable pink zone, with diameters ranging from 17.5 to 20 mm. Among these, *L. curvatus* isolate no. 11 produced the most distinct pink zone and was identified as the highest L-ASNase producer. A similar study utilized the same semi-quantitative method to screen *Bacillus sp*. for L-Asparaginase production. They found that out of 9 isolates examined, *Bacillus sp*. exhibited the largest pink zone (3.6 cm). Additionally, they observed that *Bacillus sp*. formed a larger zone (3.2 cm) compared to the standard strain *Pectobacterium carotovorum* MTCC 1428, which produced a zone of 1.9 cm (Pradhan *et al*., 2013).

Genetic mutations in the genes responsible for producing this enzyme may explain the differences in the ability of the isolates to produce it.

The results of the current study showed that the optimal range for enzyme precipitation was at 70% saturation. The enzyme activity was found to be 3.3 units/mg of protein. These results are consistent with another study, which found a 70% ammonium sulfate saturation for L-Asparaginase precipitation from P. aeruginosa, yielding an enzyme-specific activity of 2.87 U/mg protein with a purification fold of 11. Another study

reported that purification with an 80% ammonium sulfate saturation ratio increased L-asparaginase specific activity to 1.35 U/mg, resulting in a 2.7-fold increase and raising enzyme activity to 27.96 U/ml (Darnal *et al*., 2023). The resulting ammonium sulfate precipitate was then placed in a dialysis tube for dialysis after being dissolved in an appropriate volume of 0.05 M phosphate buffer at pH 7.0 and refrigerated at 4°C for further purification.

Following ammonium sulfate precipitation, as shown in Figure 2, the enzyme and low-molecular-weight impurities became more concentrated. Dialysis was performed using a phosphate buffer to remove impurities, and then the dialysate was passed through the ion exchange column (DEAE-Cellulose). A 0.05 M potassium phosphate buffer solution was used to obtain the L-Asparaginase, and the absorbance of the eluted fractions was measured at 280 nm. Once the absorbance reached the baseline, gradual concentrations of sodium chloride (0.1–1 M) were prepared using the same buffer.

A previous study obtained negatively charged L-Asparaginase extracted from *Streptomyces rochei* using an anion exchange chromatography method, yielding highly pure L-Asparaginase with a specific activity of 119.51 U/mg (protein) and a purification fold of 16.18 (El-Naggar and El-Shweihy, 2020). Another study reported a purification fold of 3.84 using DEAE-cellulose ion exchange chromatography to extract L-Asparaginase from *Bacillus cereus* (El-Fakharany *et al*., 2022).

Following ammonium sulfate precipitation and ion exchange chromatography with DEAE-cellulose, Sephadex G-100 chromatography was employed as the third step in the purification process for further purification and characterization of L-Asparaginase. For example, L-Asparaginase produced from *Pseudomonas aeruginosa* was purified using this technique, exhibiting a specific activity of 3.66 U/mg and a purification fold of 6.6, with a yield of 1.5% (Amany *et al*., 2021). Similarly, L-Asparaginase produced from *Fusarium equiseti* was purified using Sephadex G-200, achieving a specific activity of 48.81 U/mg and a purification fold of 2.67, with 48% enzyme recovery (El-Gendya *et al*., 2021).

The current investigation yielded results similar to those of a study that found the molecular weight of isolated L-Asparaginase from *Pseudomonas* to be 37.0 kDa (Darnal *et al*., 2023). Another study reported that the molecular weight of pure L-Asparaginase produced from *Vibrio cholerae*, with the gene being a recombinant acquired through overexpression in *E. coli*, was 36.6 kDa (Radha *et al*., 2018). A different study found that the purification of L-Asparaginase from *Thermococcus* resulted in a band of nearly 72 kDa (Hong *et al*., 2014). Another study found that the L-asparaginase produced by the marine *Aspergillus terreus* had a molecular weight of 85 kDa (Hassan *et al*., 2018).

In most enzymatic reactions, the reaction pH is very important. Several studies have been conducted on the activity of enzymes within the normal pH range. L-Asparaginase from *Lactobacillus casei* exhibited maximal activity at pH 6 (Aishwarya *et al*., 2019). However, another study showed that the maximal activity of L-asparaginase, extracted from Pseudomonas aeruginosa, occurred at a pH of 8 (Fatima *et al*., 2019). According to a separate study, pH 8.5 was found to be the optimal pH for the activity of L-Asparaginase from *Pseudomonas aeruginosa* (Darnal *et al*., 2023). Additionally, a study found that pH 8.5 was the optimum pH for the maximum activity of L-Asparaginase produced from *Bacillus amyloliquefaciens* (Yim and Kim, 2019).

A study showed that L-ASNase generated by *Lactobacillus casei* functions best at 40°C (Aishwarya *et al*., 2019). Another study recorded similar results for L-ASNase isolated from *Streptomyces gulbargensis*, with maximum activity at 40°C (Amena *et al*., 2010). As reported by Kushwaha *et al*. (2012), L-ASNase activity increased at 37°C. The highest activity of L-ASNase isolated from *Aspergillus terreus* was found to occur at 37°C (Siddalingeshwara *et al*., 2011). Due to this characteristic, the enzyme is most suited for completely removing L-asparagine from the bodies of patients undergoing L-asparaginase treatment for tumors.

In this study, the enzyme's activity began to decline simultaneously, indicating that it was sensitive to alkaline pH. The ionic state of the enzyme's active site is impacted by pH (Sawheney, 2008), suggesting that pH affects the purified enzyme. Unfortunately, due to its effects on the enzyme's tertiary and secondary structures, which cause the enzyme to lose activity in buffer solutions that are far from the optimum pH, as well as its impact on the enzyme's structure, which alters the ionic state of the active site or denatures the enzyme molecule, enzymatic activity

decreases at different pH values. A study reported that enzymes can become permanently denatured in highly acidic or basic conditions (Mahdi *et al*., 2019). The isolated L-asparaginase from Burkholderia pseudomallei demonstrated pH stability, retaining 95.1% of its activity at a pH of 8 (Darwesh *et al*., 2022). According to a study examining the activity of L-asparaginase purified from Escherichia coli, the enzyme exhibited pH stability in the pH range of 7.5–8 (Shahnazari *et al*., 2022). Another study on pH stability revealed that, within the pH range of 7–10, pure Streptomyces gulbargensis L-asparaginase retained 80% of its activity (Amena *et al*., 2010). Additionally, it has been demonstrated that all L-asparaginases extracted from microbial sources are stable in the pH range of 6–10 (Ghasemi *et al*., 2017).

In the present study, the maximum stability of L-Asparaginase was observed at 35°C and 40°C, where the enzyme retained 100% of its activity after 20 minutes of incubation. The results of the current study are consistent with another study, which reported that purified L-ASNase from *Pseudomonas sp*. demonstrated stability, maintaining 100% of its activity after 200 minutes of incubation at 37°C and retaining 90% of its activity for 70 minutes at 50°C (Darnal *et al*., 2023). However, the enzyme lost 90% of its activity at 70°C and 60°C after 5 and 10 minutes of incubation, respectively. After 20 minutes of incubation at 50°C, another study found that the L-Asparaginase produced from *Streptomyces fradiae* retained 86% of its original activity (El-Naggar and El-Shweihy, 2020). Nevertheless, after 90 minutes of incubation at 50°C, 72% of the L-Asparaginase activity was lost; in contrast, a significant decrease in enzyme activity of 16% was noted after 90 minutes of incubation at 80°C. According to a study, L-asparaginase derived from pathogenic E. coli exhibited 100% activity after a 15-minute incubation period at temperatures ranging from 20 °C to 37°C. However, when incubated at 60°C, 70% of its activity was lost (Hazim *et al*., 2010).

According to a study, these metal ions can act as cofactors to activate enzymatic reactions (Knape *et al*., 2015). It was initially believed that Mg^{2+} was the activating metal capable of activating the substrate and attaching directly to the enzyme-substrate complex. The enzyme-substrate complex is stabilized by Mg^{2+}, which also facilitates the rapid release of reaction products. Metal ions are essential for maintaining enzymes in their active conformation, which increases enzyme activity, stabilizes its structure, and protects it against heat-induced denaturation (Ayodeji *et al*., 2019). Another study suggested that the chelation of L-ASNase sulfhydryl groups with metal ions may be the reason divalent ions significantly reduced enzyme activity (Darnal *et al*., 2023). Metal ions, such as Cu^{2+}, also significantly decreased enzyme activity. The effects of Ni^{2+}, Ba^{2+}, Ca^{2+}, Na^{+}, Co^{2+}, and Mg^{2+} on partially purified L-Asparaginase activity were reported at a final concentration of 0.5 mM (Kumar *et al*., 2022). Only Ni^{2+} increased the activity of L-Asparaginase by approximately 16%, while other metal ions inhibited enzyme activity, with Na^{+} causing the highest inhibition (22%) (Ahmed *et al*., 2015).

By removing inhibitors or metal ions from the aqueous environment, chelating compounds like mercaptoethanol and EDTA (ethylene diamine tetraacetic acid) can stimulate the activity of several enzymes, particularly L-ASNases (Miyano *et al*., 1985). The beneficial impact of chelating agents on L-ASNase activity is demonstrated by the availability of the enzyme's active site for substrate reaction when they form a complex with metals in the reaction environment (Oviedo and Rodriguez, 2003; Karnchanatat *et al*., 2008). Tween-80 increased the enzyme activity, achieving 100% relative activity, which was consistent with the findings of a study (Moubasher *et al*., 2022), where the formation of disulfide bonds was identified as the primary mechanism stabilizing the native protein structure (Qin *et al*., 2015). Surfactants like Tween-80 tend to lower the surface tension of aqueous systems, which can alter a liquid's emulsification, solubilization, detergency, and other characteristics (Hsieh *et al*., 2015). A decrease in enzyme activity was observed in the presence of sodium dodecyl sulfate (SDS). These results were consistent with another study, which found that SDS disrupts the structure of proteins by changing their folded structure into a linear model and coating them with uniformly negative charges, thus masking the charges on the proteins (Thakur *et al*., 2013). Ionic detergents, such as SDS, can increase enzyme activity at low concentrations (1-2 mM) but have the opposite effect at higher concentrations (20 mM) (Debeche *et al*., 2000). The enzyme's three-dimensional structure is altered by SDS interference in its hydrophobic areas, suggesting

that these concentrations may be crucial and lead to enzyme denaturation (Nelson *et al*., 2011).

4. CONCLUSIONS:

This study reports the screening of L-Asparaginase-producing *Lactobacillus curvatus* from previous sources. L-Asparaginase was concentrated and purified using ammonium sulfate precipitation, ion exchange chromatography, and gel filtration, yielding a specific activity of 6 U/mg with a 12-fold purification and an approximate molecular weight of 37 kDa. The enzyme exhibited optimal activity at pH 7.0 and 40 °C, with maximum stability at pH levels ranging from 6 to 7.5, as well as at temperatures of 35 °C and 40 °C. Enzyme activity increased significantly in the presence of Mg, P, Co, and Ni. Additionally, the highest activity of purified L-ASNase was observed in the presence of mercaptoethanol, EDTA, and Tween-80. Further research on the thorough purification and characterization of L-Asparaginase is recommended to evaluate its in vivo toxicity.

5. DECLARATIONS

5.1. Study Limitations

While this study successfully screened, purified and characterized L-asparaginase from *Lactobacillus curvatus*, several limitations must be acknowledged. These limitations highlight areas for further research and optimization to enhance the applicability and understanding of L-asparaginase from *Lactobacillus curvatus* in various practical applications.

1. Strain/Isolate Specificity:

The findings are based solely on *Lactobacillus curvatus* isolates. These results may not be generalizable to other bacterial species or strains capable of producing L-asparaginase.

2. Scale and Yield:

The purification process was conducted on a small laboratory scale, yielding relatively limited quantities of the purified enzyme. This poses challenges for scaling up production for industrial or therapeutic applications.

3. In Vitro Characterization:

The enzyme's properties, such as pH and temperature optima, were studied only in vitro. Further investigations are required to assess its stability, efficacy, and therapeutic potential (e.g., anticancer properties) *in vivo*.

4. Statistical Analysis:

While the experimental methods and results were detailed, the statistical methods used to analyze the data were not explicitly described, which could affect the robustness of the findings.

5. Comparative Analysis:

The study did not include direct comparisons of the purified L-asparaginase with commercially available or other microbial enzyme preparations in terms of activity, specificity, etc.

6. Reproducibility:

The reproducibility of the findings across different isolates, environmental conditions, or other research settings was not assessed.

7. Environmental Factors:

The effects of environmental conditions, such as oxygen levels and medium composition, on L-asparaginase production have not been extensively evaluated.

8. Resource Availability:

The study required specialized equipment, such as chromatography systems, which may not be available in all research settings. This could limit the reproducibility of the enzyme purification process in settings with limited resources.

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5.4. Competing Interests

The authors declare no conflicts of interest.

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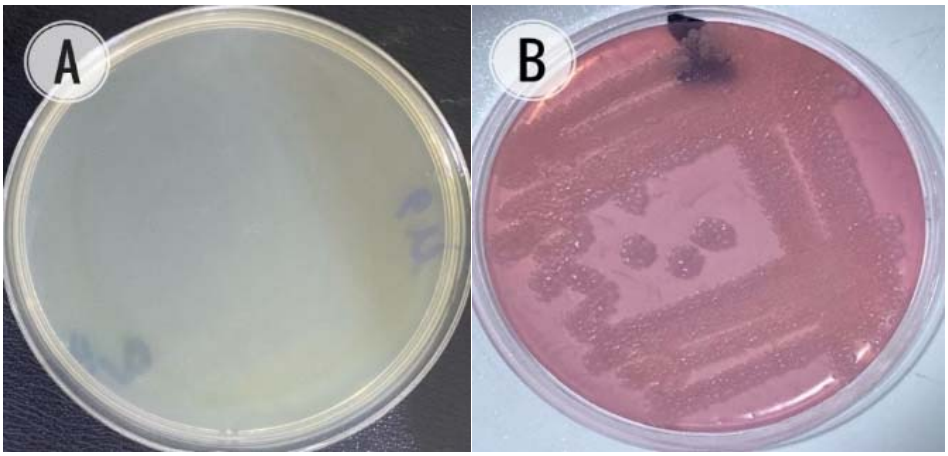

Figure 1. *L-Asparaginase production on M9 agar medium after 48 hrs of incubation under anaerobic conditions at 37 º C. A: Control negative (yellow, no color), B: Strong producing isolate (change to pink color).*

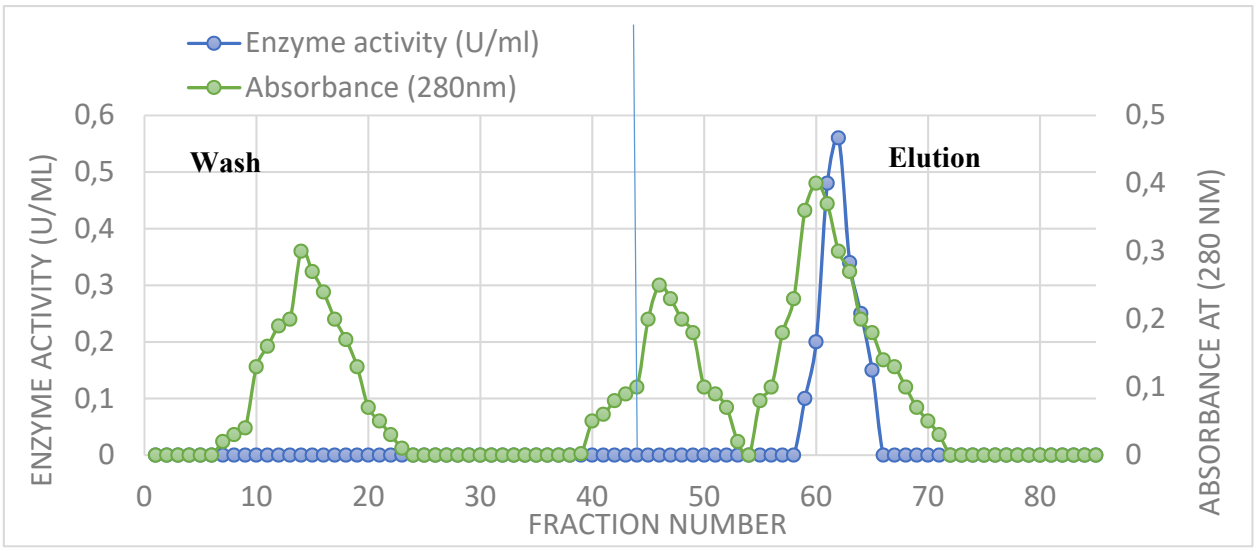

Figure 2. *Ion exchange chromatography of L-Asparaginase produced by L. curvatus, using a DEAE-Cellulose column (2.5×20cm).*

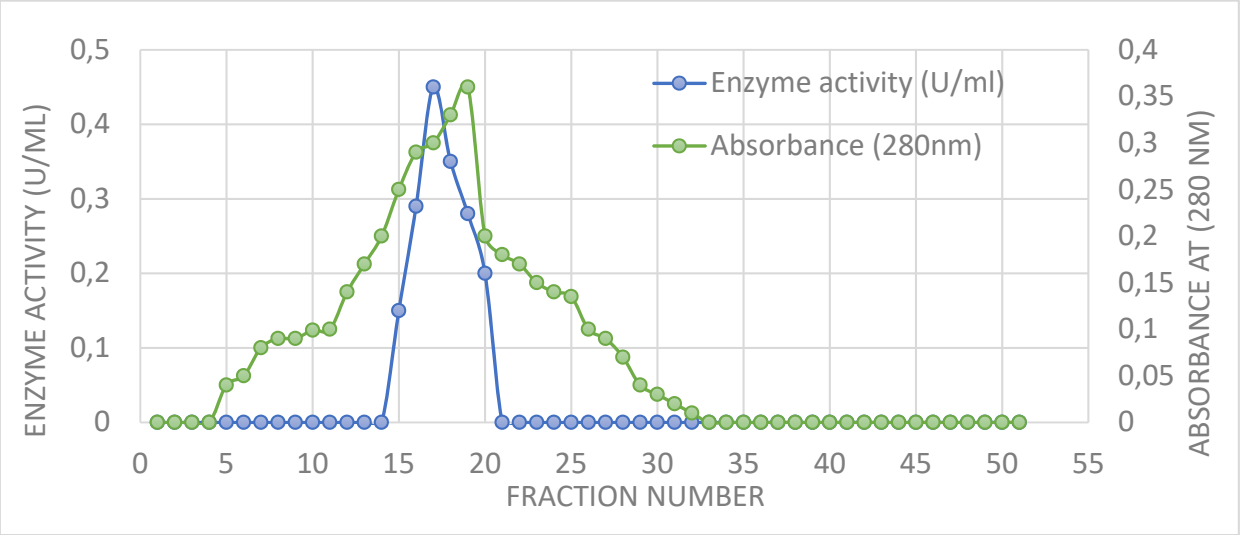

Figure 3. *Gel filtration chromatography of L-Asparaginase produced by L. curvatus using SephadexG-100 column (2×40cm).*

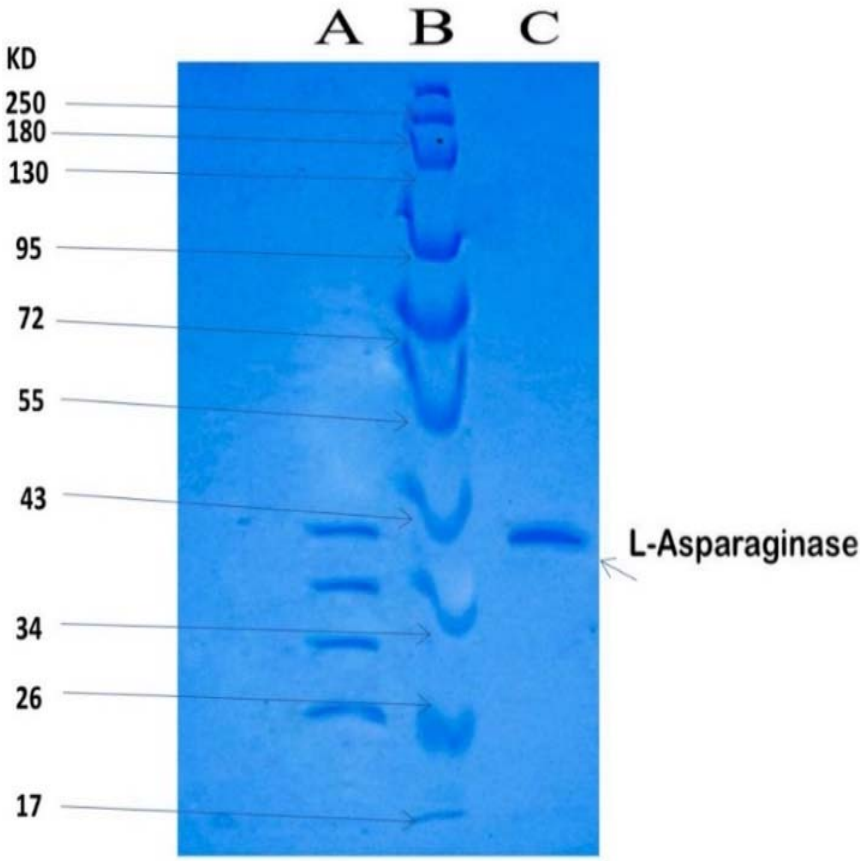

Figure 4. *SDS-PAGE of purified L-ASNase produced by L. curvatus . A: Protein bands after ion exchange step. B: Protein marker. C: Protein bands after gel filtration step.*

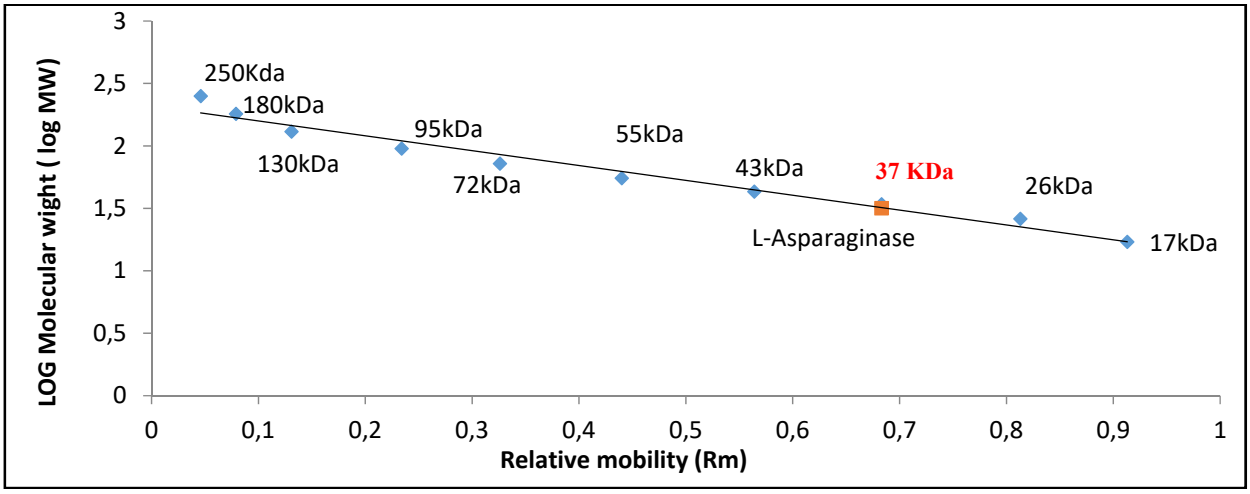

Figure 5. *Log molecular weight of L-Asparaginase produced by Lactobacillus curvatus.*

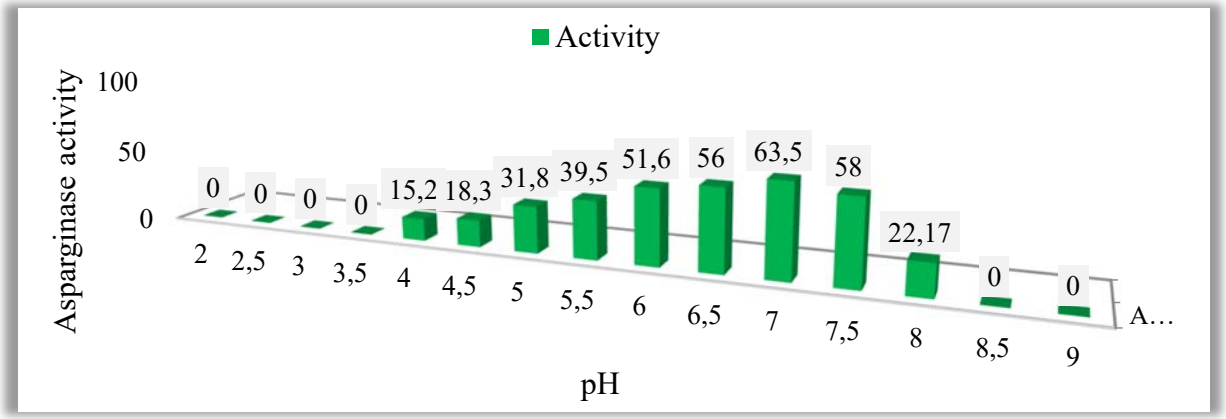

Figure 6. *The activity of L-Asparaginase at different pH values.*

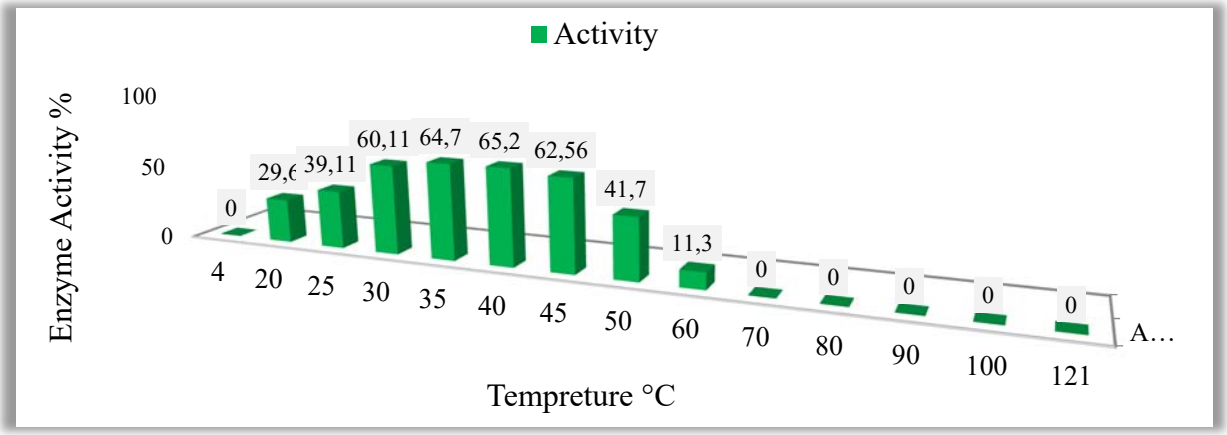

Figure 7. *The activity of purified L-ASNase at different temperatures.*

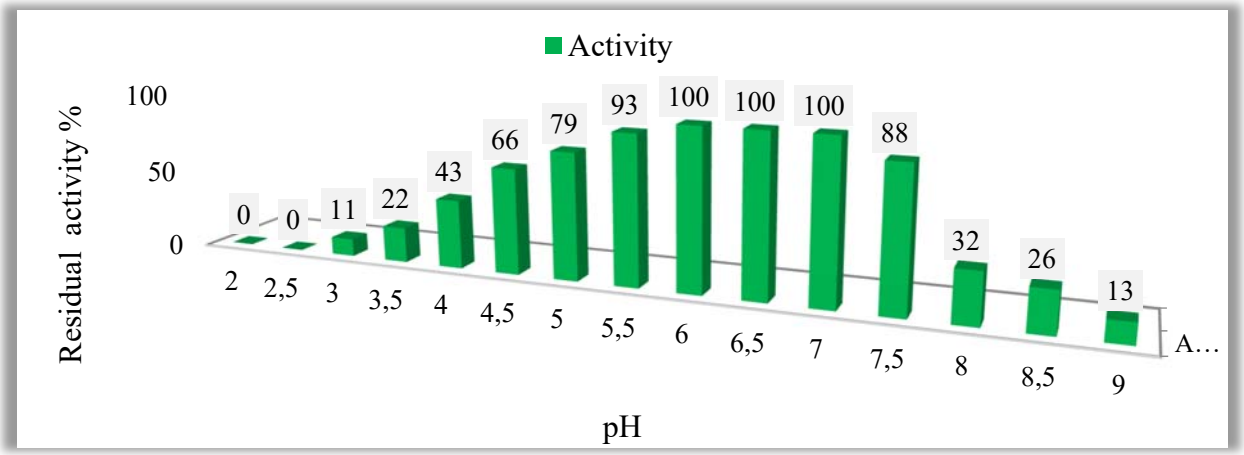

Figure 8. *pH stability of L-Asparaginase at different pH values.*

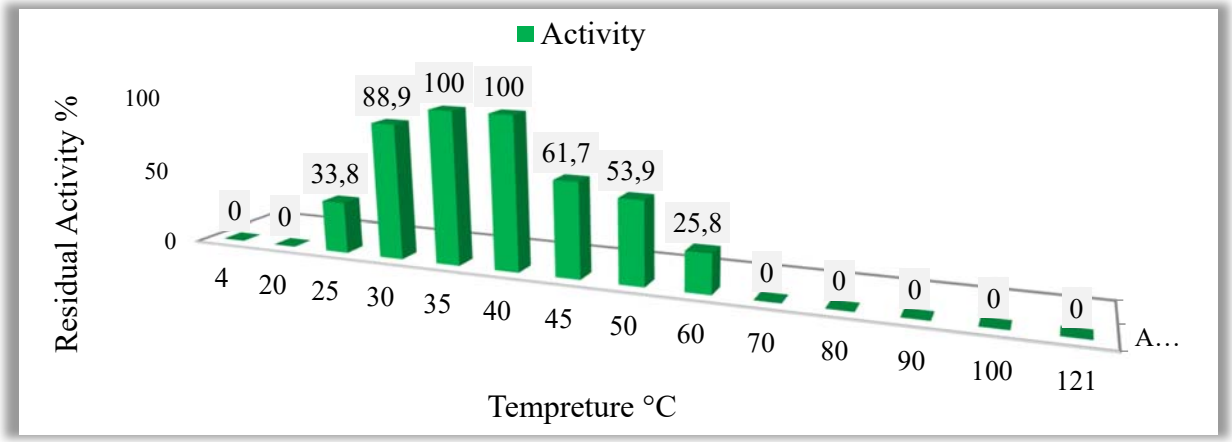

Figure 9. *Thermal stability of purified L-ASNase at different temperatures.*

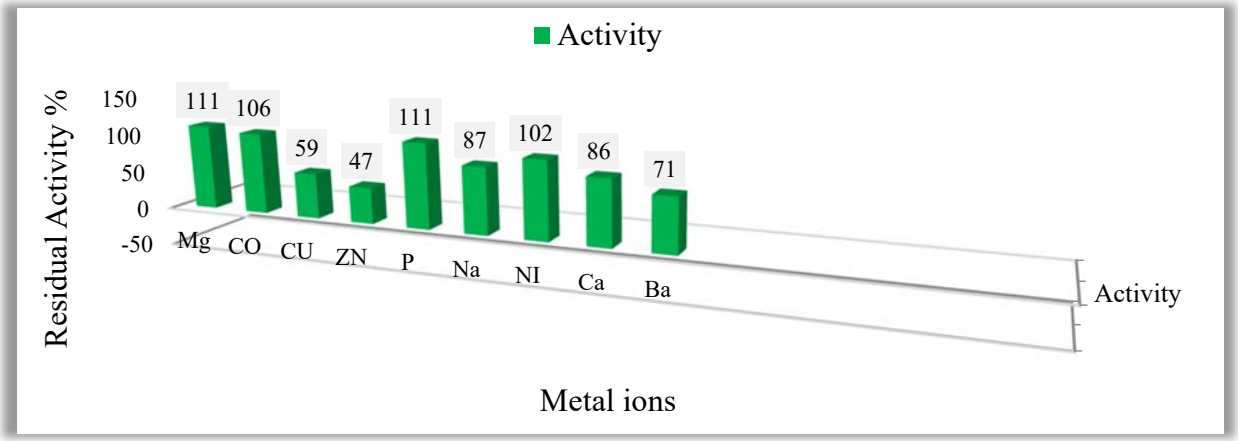

Figure 10. *The effect of metal ions on L-Asparaginase activity.*

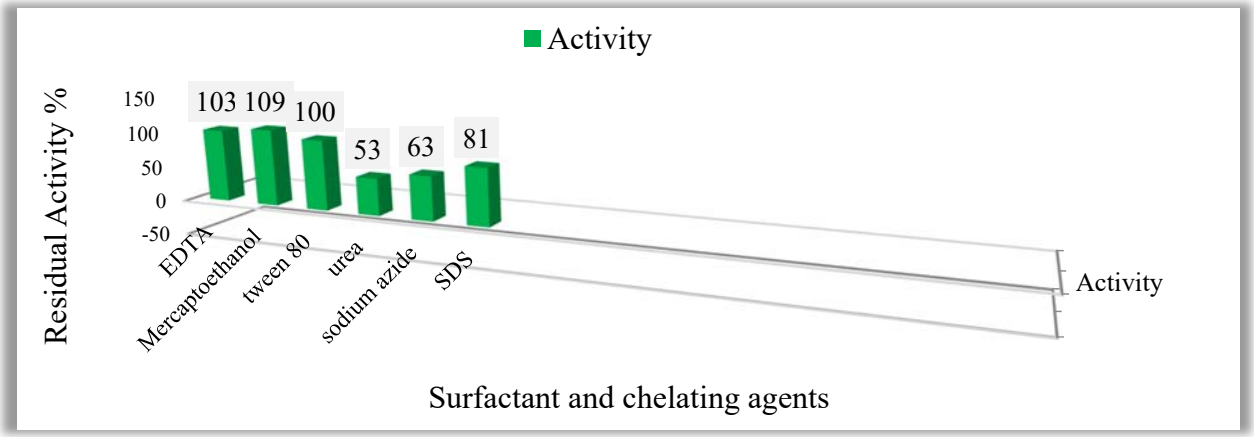

Figure 11. *The effect of surfactant and chelating agents on the stability of purified L-Asparaginase.*

Table 1. *Semi-Quantitative Screening for L-Asparaginase Productivity.*

***L. curvatus* isolates**	**Pink zone (mm)**
***L. curvatus* no.1**	19
***L. curvatus* no.2**	18
***L. curvatus* no.3**	12
***L. curvatus* no.4**	18.4
***L. curvatus* no.5**	11
***L. curvatus* no.6**	18.5
***L. curvatus* no.7**	13
***L. curvatus* no.8**	6
***L. curvatus* no.9**	17.5
***L. curvatus* no.10**	18.5
***L. curvatus* no.11**	20

Table 2. *The Specific Activity of L-Asparaginase.* +++: strong pink, ++: weak pink, +: pale pink.

***L. curvatus* isolates**	**Specific activity U/mg**	**Intensity of the color**
***L. curvatus* no.1**	4.20	+++
***L. curvatus* no.2**	4.65	+++
***L. curvatus* no.3**	2.88	++
***L. curvatus* no.4**	4.37	+++
***L. curvatus* no.5**	2.33	++
***L. curvatus* no.6**	4.05	+++
***L. curvatus* no.7**	2.70	++
***L. curvatus* no.8**	2.43	++
***L. curvatus* no.9**	4.21	+++
***L. curvatus* no.10**	4.33	+++
***L. curvatus* no.11**	4.86	+++

Table 3. *The purification steps of L-Asparaginase.*

Purification step	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification Fold	Yield (%)
Crude enzyme	75	0.1	0.2	0.5	7.5	1	100
Ammonium sulphate precipitation 70%	13	0.5	0.15	3.3	6.5	6.6	86.6
DEAE-cellulose	21	0.3	0.08	3.75	6.3	7.5	84
Sephadex-G150	18	0.3	0.05	6	5.4	12	72