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# Production, Extracellular Purification, Characterization, and Cytotoxic Activity of L-Asparaginase Isolated from Locally Bacillus SPP

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**Annotation:** Ninety soil samples were obtained from diverse areas in Baghdad, and the examination revealed that 45 samples contained Bacillus spp. bacteria, and only 5 out of 45 showed the highest productivity in producing the L-asparaginase enzyme. The samples were named B1, B2, B3, B4, and B5, respectively. B4 produced the best enzyme activity (2.87 U/mg) after crude filtrate. This sample was also chosen to determine the optimal conditions to produce the best possible activity of the L-asparaginase after purification processes: the first step was L-asparaginase precipitated with 80% saturation ammonium sulfate, second purification step was used ion exchange chromatography employing) DEAE Cellulose( and the last step by gel filtration chromatography with Sephadex G-200. The enzyme specific activity was increased to 83.6 U /mg and 88.7 % enzyme recovery. After characterization and examination of the enzyme, It was revealed that the molecular weight that found for L-asparaginase from Bacillus sp. B4 was about 47,000 Dalton, The optimal pH for enzyme

activity and stability ranged from pH8 to pH7 respectively. Optimum enzyme activity was obtained with 40 ºC temperature and L-asparaginase was stable at temperature from 40 to 70 ºC. also fond L-asparaginase activity was increased by add (1, 10 and 20mM) $MnCl_2$ and was inhibited by (1, 10 and 20Mm) $CaCl_2$,(4mM) cysteine and (4mM) EDTA, but it was not influenced by(1, 10 and 20mM) NaCl and KCl. The isolated L-asparginase had cytotoxic activity against the CCL-119 cell line while exhibiting reduced toxicity against normal cells, indicating its potential for further pharmaceutical use as an anticancer option.

## 1. Introduction

Microbial enzymes have widely applied in different industries in most countries, including the food, pharmaceutical industries **[1].** The transformation of L-asparagine to (L-aspartic acid, ammonia) was used as a Chemo therapeutic agent **[2].** This enzyme's therapeutic activity is due to the decrease of L-asparagine tumor cell incapable of synthesizing this amino acid are preferentially destroyed by L-asparagine deficiency. This also used as candidate for (acute lymphoblastic leukemia), lymphosarcoma, and many other clinicals investigations involving tumor treatment in conjunction with Chemo therapy **[3-7].** L-asparaginase is also utilized as a processing aid in food manufacturing to convert asparagines to aspartic acid in order to decrease cancer-causing acrylamide generation. As new uses are developed, the need for L-asparaginase may rise several folds in the future **[8].** Extracellular L-asparaginase enzyme is more favorable than intercellular because it may be generated extensively in culture broth under normal conditions and filtered cheaply **[9].**

## 2. Materials and Method

### 2.1. Isolation and identification of *Bacillus spp.*

All 90 soil samples were collected and processed as follows: 1 gram from each sample has been taken then suspended in 9 ml of sterile distilled water and putted in a water bath shaker at 37 degrees Celsius, Serial of dilutions for all samples were cultivation on Nutrient agar plates and incubated at 37°C for 24 hours. The colony size, shape, color, and odor of bacterial isolates were investigated. Suspected isolates with characteristics similar to Bacillus were chosen for biochemical test identification.

### 2.2. Screening for L-asparaginase production and Activity Assay

The isolated Bacillus was been examined for L-asparaginase production using an agar plate. The isolated bacterial was grown on modified culture media and incubated at 37 Cº for 1day.A pink-colored microbial culture indicated L-asparaginase production, and colonies were selected for the direct Nesslerizations method to determine the L-asparaginase activity according to **[10]** as follows: Enzymatic solution (0.5ml) was combined with 1ml of tris buffer and 0.5ml of (0.1 M) L-

asparagine. Test tubes had incubated in a water bath at 37 degrees Celsius for 30 minuet. The reaction was halted by add 0.5 cc of 5% TCA. The solution was separated by centrifuge at 6,000 rpm for 30 min. The ammonium concentration was then calculated by combining 4.4 ml of D.W, 0.5 mL of ( Nessler reagent ), and 0.1 ml of enzyme in a vortex and measuring the absorbance at 436 nm.

### 2.3. L-asparaginase Purification

The first purification stage employs ((NH4)2SO4), which was introduced into the crude enzyme with saturation ratios ranged from 30 % to 80 %. The liquid was stirred using a magnetic stirrer at 4 degrees Celsius for 20 minuet. Then Samples were centrifuged at (6,000 rpm for 20 min at 4 degrees Celsius). The proteins which precipitated have been dissolved in 0.05 M buffer composed of Tris-HCl (pH 8). The enzyme activity and protein concentrations were measured. The resulting ammonium sulfate precipitates were dialyzed in a dialysis tube with a 10000 MW cut-off against 0.05M Tris-HCl buffer pH 8 for 3 hours, followed by dialysis with the same buffer overnight. The resulting L-asparaginase was then loaded in an ion exchange chromatography containing DEAE-Cellulose that was already equilibrated with ( 0.05M ) buffered Tris-HCL at pH 8. The column was rinsed subsequently with equal volume of the same buffer, and the associated proteins were gradually eluted with increasing sodium chloride concentration (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, and 0.7 M). The column flow rate was 3ml/fraction, and the absorbance for each fraction was measured whit (UV-VIS spectrophotometer at 280nm). Fractions exhibiting L-asparaginase activity were refrigerated and added to the Gel Filtration matrix, which had previously been equilibrated with 0.01M buffer (Tris-HCl) pH 8. Elution was carried out at(3 ml / fraction) flow rate , and the same buffer solution used for equilibration. The absorbency of each component had measured at (280 nm), and the enzyme activity determined for all fraction.

### 2.4. Measurement Purity and Molecular Weight of L-Asparaginase

The L-asparaginase purity was determined by polyacrylamide gel Solutions were prepared according to the method described by Hames [11].

### 2.5. L-asparaginase Characterization

#### 2.5.1. Impact of pH on L-asparaginase Activity

The purified enzyme had treated with ((0.1mM) L-asparagine, (0.05 M) buffer) with pH varying from (4 to 9). The buffers used are sodium acetate (4, 5 pH), phosphate buffer (5, 6 pH), and tris buffer (7, 8, 9 pH). Then enzymatic activity was assessed, and the relationship between pH values was displayed.

#### 2.5.2. Impact of pH on L-asparaginase Stability

The enzyme had been pre-incubated in a buffer solation with a pH ranging from (4–9) for 30 minutes at 37°C. The tubes were next placed in a cold water bath. The enzyme activity was calculated, and the residual activity then displayed against the pH value.

#### 2.5.3. Impact of Temperature on L-asparaginase Activity

Enzyme activity had assessed at various temp (20, 30, 40, 50, 60, 70, 80) Cº. Then, L-asparaginase activity was displayed versus temperature.

#### 2.5.4. Impact of Temperature on L-asparaginase Stability

Pre-incubation of (L-asparaginase) in a water bath at (20, 30, 40, 50, 60, 70, 80) Cº for 30 minutes. Then moved to ice bath and activity of the enzyme was measured. The residual activity was displayed vs temp.

#### 2.5.5. Impact of Various Ions and Inhibitors on L-asparaginase Activity

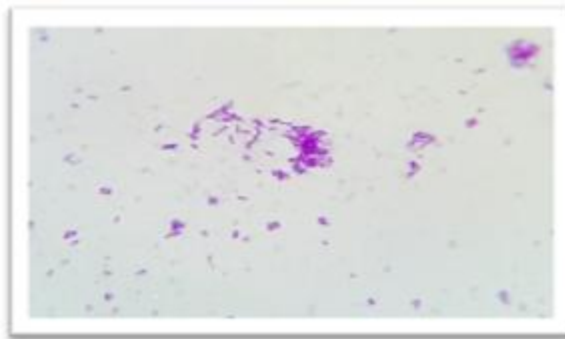
Pre-incubation with different salts (NaCl, $CaCl_2$, KCl, $MnCl_2$) was used to study inorganic ions effect of on enzyme activity. produced at the concentrations (1, 10 and 20mM) for 1 hour at 37Cº.

Inhibitors and chelating agents (Cystein and EDTA) were tested at varying doses (0.5, 1.2, and 4Mm) and incubated with the enzyme at 37°C for 1 hour.

## 3. Results and Dissection

### Isolation, Morphological and Microscopical Identification of Bacillus spp.

Only 45 of the 90 soil samples contained Bacillus spp. These 45 isolates were tested for their capacity to manufacture L-asparaginase and only five isolates have the highest productivity on the culture media, Then the morphological test has done and conforms after forming colonies on nutrient agar and had unacceptable odor with rough surface. That result was agreed with **[12-13]** and the isolated Bacteria suspected of being Bacillus spp. were gram-positive, spore-forming, and often formed short chains or single cells (**Figure** 1).



**(Figure 1): Microscopic field to *Bacillus spp.***

### 3.1. Biochemical Identification

The (table 1) showed the Results of five *Bacillus spp.* as they tested in different Biochemical

| Biochemical test | Isolates | | | | |
|---|---|---|---|---|---|
| | **B1** | **B2** | **B3** | **B4** | **B5** |
| Catalase | - | - | - | - | - |
| Indole production | - | - | - | - | - |
| Citrate utilization | + | + | + | + | + |
| Degradation of urease | + | + | + | + | + |
| Liquefaction of gelatin | + | + | + | + | + |
| Growth under aerobic condition | + | + | + | + | + |
| Starch hydrolysis | + | + | + | + | + |

**(Table 1) bio chemical test for *Bacillus spp* identification.**

### 3.2. Screening the ability of Bacillus spp. For L-asparaginase production

To produce L-asparaginase, isolates were cultured in L-asparaginase broth medium at 37°C for 24 hours and enzyme specific activity was measured in the culture filtrate.

**(Table 2): specific activity of L-asparaginase that isolated from *Bacillus sp.***

| Isolate number | Specific activity (U/mg) |
|---|---|
| B1 | 1.734 |
| B2 | 2.371 |
| B3 | 1.448 |
| B4 | 2.87 |
| B5 | 1.539 |

The specific activity of L-asparaginase in Bacillus culture is ranging between 1.448 and 2.87 Unit/mg, (B4) had produce the best enzyme with a specific activity of 2.87 U/mg in crude filtrate.

According to these findings, the B4 isolate was chosen for further research on L-asparaginase synthesis, purification, and application.

### 3.3. L-asparaginase Purification

B4 L-asparaginase crude enzyme was purified under optimal conditions through several purification steps, first ammonium sulfate(($(NH4)_2SO4$) precipitation with 80% saturation, then ion exchange chromatography (DEAE-Cellulose), and the last step gel filtration chromatography with (Sephadex G-200). The enzyme activity was enhanced to (6 U/ml), after (20.1) fold purification and (88.7) percent enzyme recovery, The results proved an increase in the activity of the purified enzyme and showed in (Table 3).

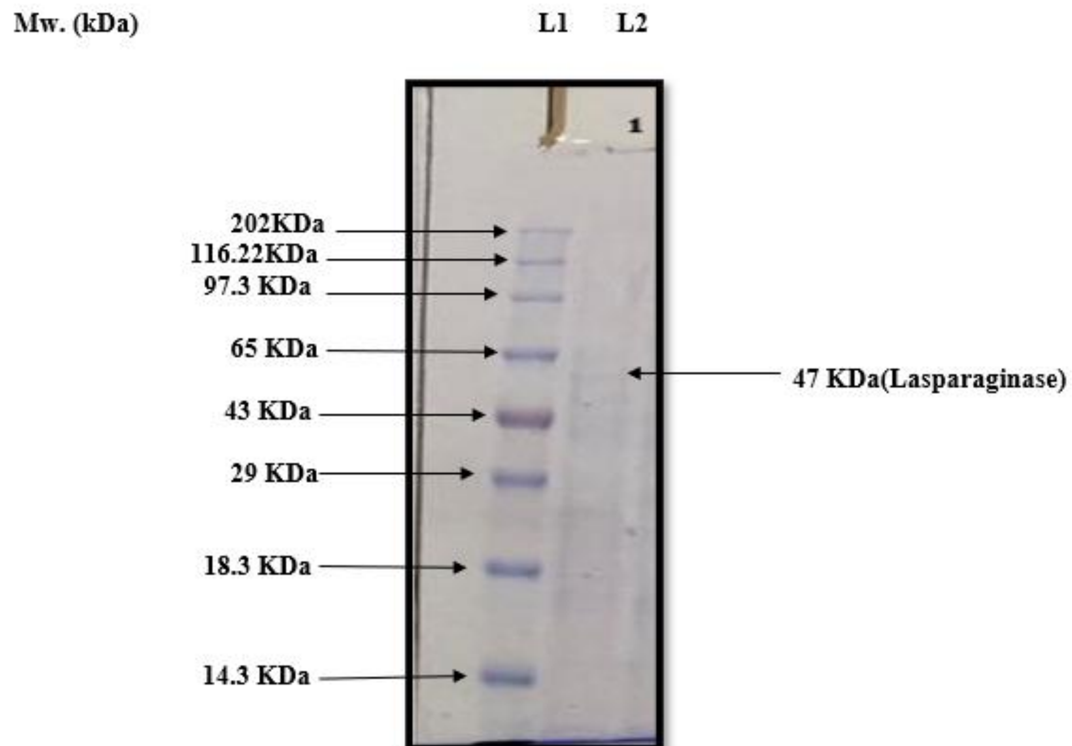
**(Table 3) steps of purification for L-asparaginase produced from soil isolated *Bacillus* B4.**

| purification step | volume(ml) | Enzyme activity (U/ml) | specific activity (U/mg) | Protein concentration (mg/ml) | purification(folds) | total activity(U) | yeild (%) |
|---|---|---|---|---|---|---|---|
| Crude enzyme | 100 | 0.83 | 2.87 | 0.2 | 1 | 85 | 100 |
| Ammonium sulfate precipitation (80%) | 25 | 2.4 | 5.35 | 0.4 | 1.3 | 54.5 | 67.6 |
| DEAE-cellulose | 15 | 4.5 | 45 | 0.1 | 10.3 | 67.4 | 79.4 |
| Sephadex G-200 | 13 | 6 | 83.6 | 0.07 | 20.1 | 78 | 88.7 |

### 3.4. Characterization of an isolated enzyme (L-asparaginase).

#### 3.4.1. Determination of L-asparaginase Molecular Weight

The purity of L-asparaginase was determined using electrophoresis on polyacrylamide gels. Following gel filtration, the protein profile of pure L-asparaginase from Bacillus sp revealed one protein band with a molecular weight of 47 kD showed **in (figure 2)**




**(Figure 2) Electrophoresis with Polyacrylamide gel for purified (L-asparaginase) produced by *Bacillus* B4**

**L1: Standard Proteins markers**

**L2: Purified L-asparaginase enzyme**

### 3.4.2. Effect of Ions and Inhibitors on L-asparaginase Activity

The purified enzyme activity was tested against various ions (NaCl, $CaCl_2$, KCl, and $MnCl_2$) and inhibitors (EDTA and cystein). Table 1-4 shows that whereas NaCl and KCl concentrations (1, 10, and 20 mM)had no significant effect on enzyme activity, $CaCl_2$ concentrations inhibited enzyme activity by 80 percent and 70 percent, respectively. $MnCl_2$ increases enzyme activity at 1mM concentration. At 4 mM Cystine and EDTA concentrations, the enzyme demonstrated a consistent loss of approximately 60 percent and 70 percent respectively (table 5).

**(Table 4) the effect of Ions on L-asparaginase activity produced by *Bacillus sp.* B4**

| Ions | Concentration (mM) | Remaining activity (%) |
|---|---|---|
| NaCl | 1 | 100 |
| | 10 | 98.9 |
| | 20 | 97.55 |
| KCl | 1 | 95 |
| | 10 | 94.6 |
| | 20 | 94 |
| $CaCl_2$ | 1 | 80 |
| | 10 | 70 |
| | 20 | 70 |
| $MnCl_2$ | 1 | 165 |
| | 10 | 100 |
| | 20 | 100 |

**(Table 5) effect of Inhibitors on L-asparaginase activity produced by *Bacillus sp.* B4**

| Agent | Concentration (mM) | Remaining activity (%) |
|---|---|---|
| Cystein | 0.5 | 100 |
| | 1.2 | 97 |
| | 4 | 75 |
| EDTA | 0.5 | 100 |
| | 1.2 | 95 |
| | 4 | 60 |

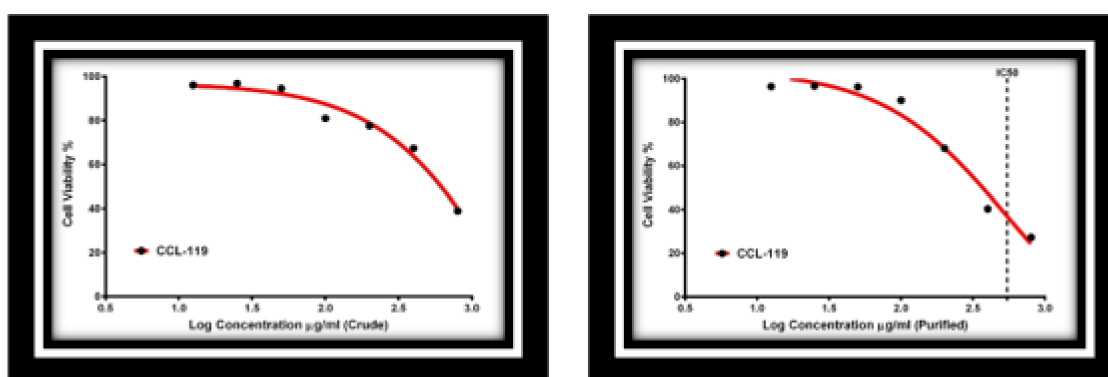
### 3.4.3. Cytotoxic Activity of L-asparaginase Using MTT Assay

The cytotoxic impact of pure L-asparaginase on tumor cell lines was established by assessing its influence on the proliferation of the CCl-119 cell line at various L-asparaginase concentration levels. The MTT test was used to examine the cytotoxic impact of crude and purified L-asparaginase on an acute lymphoblastic leukemia cell line (CCL-119). The cytotoxic impact was reported as a percentage of inhibition growth rate (I.R), which examines the cytotoxicity of L-asparaginase. The cytotoxic impact of refined L-asparaginase on the CCL-199 cell line was much greater than the crude enzyme, as indicated in (table 6).

**(Table 6) the effect of Inhibitor different concentrations ratio at crude and purified L-asparaginase**

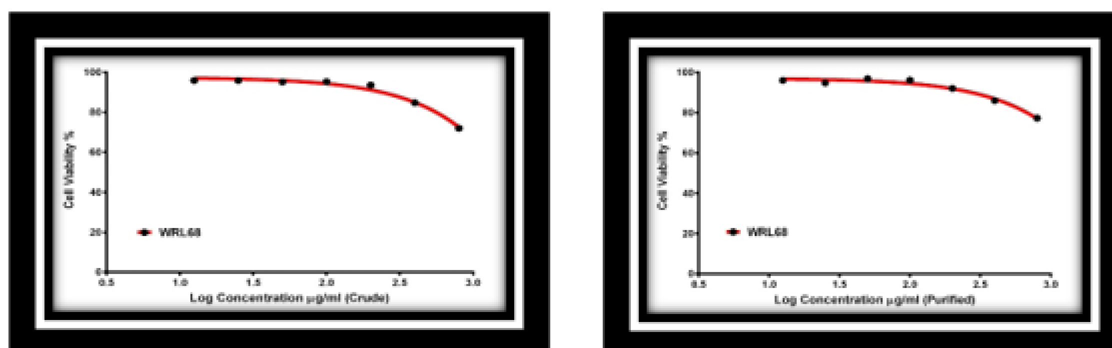
| Concentration(µg/mg) | IR(%) of crude enzyme | IR(%) of purified enzyme |
|---|---|---|
| 800 | 38.93 ± 2.483 | 27.31± 1.315 |
| 400 | 67.28± 1.900 | 40.32± 1.937 |
| 200 | 77.62± 1.556 | 68.02± 2.239 |
| 100 | 80.97± 1.142 | 90.05± 1.400 |
| 50 | 94.56± 1.620 | 96.22±0.3916 |
| 25 | 96.88± 0.5711 | 96.64± 0.7878 |
| 12.5 | 96.10± 0.9849 | 96.37± 0.4693 |

The half of the maximal inhibitory concentration of cell growth (IC 50) for crude L-asparaginase was 1994 µg/ml, while IC 50 of 546.4 µg/ ml was obtained from purified L-asparaginase against CCL-119 cell line




**(Figure 3) Cytotoxic effect of crude and purified L-asparaginase produced from *Bacillus sp*. B4 on CCL-119 cell line by using MTT assay after 24 hrs. at 37 C°.**

Crude and purified L-asparaginase appeared to be dose dependent, resulting in a decrease in cell viability at higher concentrations shown in (figure 1-3), however L-asparaginase from *Bacillus* B4 show a slight toxicity on normal cell line tested WRL-68 (figure 4)




**(Figure 4) Anticancer activity of crude and purified L-asparaginase produced from soil isolate Bacillus B4 on the WRL-68 normal cell line using MTT assay after 24 hours.**

L-asparaginase is use in clinical for treatment of acute lymphoblastic leukemia [3,5]. Also, [14] L-Asparaginase is reported to be a promising enzyme for the treatment of many malignancies.

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