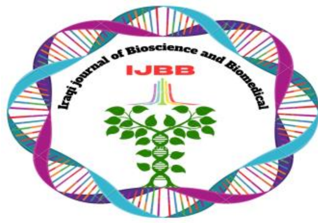

Iraqi journal of Bioscience and Biomedical
Published Online First: 30/ June /2026.
p-ISSN: 3007-5491 / e-ISSN:3007-5505

# Biochemical and Kinetic Characterization of L-Leucine Oxidase purified from Pseudomonas aeruginosa and Evaluation of Its Cytotoxic Activity

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**Received: 26/4/2026, Accepted: 5/6/2026, Published: 30/6/2026.**

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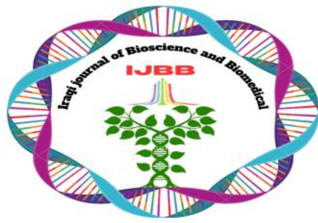
## Abstract

L-leucine oxidase is an amino acid oxidase with potential biotechnological importance. Its production and biochemical properties depend on the producing microorganism and culture conditions. This work aimed to evaluate the ability of *Pseudomonas aeruginosa* to produce L-leucine oxidase, optimize enzyme production, characterize the purified enzyme and assess its cytotoxic activity. Clinical isolates were identified by cultural and biochemical characteristics and confirmed by the Vitek 2 Compact system. L-leucine oxidase production was screened quantitatively and culture conditions were optimized by testing different carbon and nitrogen sources, pH values, temperatures and incubation periods. The enzyme was purified by DEAE-cellulose ion-exchange chromatography and Sephadex G-150 gel filtration chromatography. Characterization included the pH and temperature effects on enzyme activity and stability, effectors/inhibitors analysis and kinetic study. Finally, the cytotoxic activity of the purified L-leucine oxidase was evaluated against human hepatocellular carcinoma (HepG2) and normal human dermal fibroblasts neonatal (HdFn) cell lines using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit. Twenty isolates of *Pseudomonas aeruginosa* were recovered from 130 clinical samples. All isolates produced L-leucine oxidase, with isolate P13 showing the highest enzyme activity (3.125 U/mL) and specific activity (21.853 U/mg protein). Maximum enzyme production was obtained with glucose, yeast extract, pH 7 and 24 h incubation. After optimization, the crude enzyme extract from isolate P13 showed an activity of 5.6 U/mL and a specific activity of 28 U/mg protein and was subsequently used for purification. The purification procedure increased the specific activity of L-leucine oxidase from 28 to 111.1 U/mg, with a 3.9-fold purification and a 57.1% yield. Maximum activity was observed at pH 7 and 37ºC, while the enzyme remained most stable at pH 6-7 and 32-37ºC. $MnCl_2$ enhanced enzyme stability, whereas $CaCl_2$, $CuSO_4$ and EDTA reduced it. Kinetic analysis gave a km of 18.63 mM and a Vmax of 1.303 mM/min. The purified L-leucine oxidase showed concentration-dependent cytotoxic activity against HepG2 cells, with a half maximal inhibitory concentration of 112 µg/mL, while exhibited low cytotoxic activity against normal HdFn cells. *Pseudomonas aeruginosa* P13 is a promising producer






of L-leucine oxidase and the purified enzyme showed favorable biochemical, kinetic and preliminary cytotoxic properties that support its possible biotechnological and biomedical applications.

**Keywords:** Characterization, cytotoxicity, L-leucine oxidase, *Pseudomonas aeruginosa*, purification.

## Introduction

*Pseudomonas aeruginosa* (*P. aeruginosa*) is a heterotrophic, Gram-negative, non-spore-forming bacterium that exist either in biofilms or planktonic forms[1]. It can grow on simple and inexpensive culture media and is considered nutritionally versatile, which supports *P. aeruginosa* ability to thrive in diverse environments such as soil, plant surfaces, waste and surface water, inert surfaces and moist habitats as a result of its minimal nutritional requirements[2,3]. Although *P. aeruginosa* is a known pathogen, it also offers numerous biotechnological benefits for example the production of enzymes of medical and industrial importance, such as oxidases, lipases, and proteases[4]. In addition, other species of the genus Pseudomonas have been reported to produce L-amino acid oxidase with notable antibacterial and cytotoxic activities[5].

Microbial enzymes are important biocatalysts involved in a wide range of biochemical, biological, and industrial processes and are produced by microorganisms such as bacteria, fungi, and archaea, these enzymes are valued for their diverse catalytic properties[6]. Microbial enzyme synthesis is quicker, more affordable, scalable, and amenable to genetic manipulations, so they are generally preferred over plant- or animal-derived enzymes[7]. Therefore, screening microbial isolates for the production of valuable enzymes remains an important field of research.

L-leucine oxidase belongs to the broader group of L-Amino acid oxidases (LAAOs; EC 1.4.3.2), which are a flavin-dependent enzymes that convert most of proteinogenic L-amino acids into α-keto acids with the release of ammonia by the oxidative deamination reaction. In this reaction, molecular oxygen acts as an electron acceptor and hydrogen peroxide are generated as a by-product[8]. In contrast to the broader substrate range reported for some L-amino acid oxidases, L-leucine oxidase acts specifically on L-leucine as its substrate. This substrate specificity makes it an interesting enzyme for studies related to amino acid metabolism and microbial enzyme production.

L-amino acid oxidases have gained increasing interest because of their potential biomedical significance, especially their cytotoxic activity against tumor cells. During catalysis, LAAOs generate hydrogen peroxide reactive by-product that may contribute substantially to oxidative stress and subsequent cellular damage. Several studies have shown that L-amino acid oxidases exhibit antiproliferative and apoptosis-inducing effects in different cancer cell lines, supporting their possible value in anticancer research[9,10].

Previous studies have shown that members of the genus *Pseudomonas* have the ability to produce amino acid-oxidizing enzymes and L-amino acid oxidase activity has been reported in *P. aeruginosa* under defined cultivation conditions[11,12]. However, studies specifically addressing the isolation of efficient producer of L-leucine oxidase, screening their productivity, optimization of enzyme production and characterization remain limited.

Therefore, the present study aimed to isolate and identify bacterial isolates, screen their ability to produce L-leucine oxidase and select the most efficient producer. The selected isolate was then used for optimization of enzyme production, followed by purification and biochemical characterization and evaluation of its cytotoxic activity.

## Materials and Methods

### Ethical Approval

The Research Ethics Committee of Al-Nahrain University College of Biotechnology approved this research (Approval No. REC.COB/0505/19; May 5, 2026).

### Isolation, identification and screening of *Pseudomonas aeruginosa* for L-leucine oxidase production

A total of 130 clinical samples were obtained from patients suffering from burns, wounds, otitis media and UTIs in Iraq between October and December 2025. Collected samples were inoculated into Brain Heart Infusion (B.H.I) broth and incubated at 37 ℃ for 24 h to. After incubation, colony morphology was examined by aseptically streaking each sample onto selective and differential culture media. Bacterial isolates suspected to be *Pseudomonas aeruginosa* were subjected to biochemical testing for preliminary identification, followed by confirmation using the VITEK 2 Compact system.

For screening of L-leucine oxidase production, each confirmed *P. aeruginosa* isolate was cultured in Czapek Dox medium (prepared by dissolving 3.0 g of Sucrose, 2.0 g of $NaNO_3$, 0.5 g of KCl, 1.0 g of $K_2HPO_4$, 0.5 g of $MgSO_4{\cdot}7H_2O$, and 0.01 g of $FeSO_4{\cdot}7H_2O$ in 990 ml of distilled water) supplemented with 0.5 g of L-leucine incubated at 37°C for 24 hours. After incubation, the cultures were centrifuged at 6000 rpm for 30 minutes at 4°C using a cooling centrifuge. The resulting cell-free supernatant was collected in plain test tubes and used as the crude enzyme[13]. Protein concentration was determined according to Bradford (1976)[14]. Enzyme activity was assayed by the Nesslerization method to estimate the concentration of ammonia released during the oxidation of L-leucine[13,15]. Briefly, 500 µl of the crude enzyme extract was mixed with 500 µl of 20 mM L-leucine. The reaction mixture was pre-incubated for 30 minutes at 37°C. Following incubation, 100 µl of 1% TCA was added to terminate the enzyme activity and then it was centrifuged at 4000 rpm for 5 minutes. The resulting supernatant was transferred into a clean test tube. Subsequently, 500 µl of Nessler's reagent was added, mixed thoroughly with the supernatant using a vortex mixer and incubated at room temperature for 10 minutes. The absorbance was measured at 480 nm against a blank. The specific activity of the enzyme was determined using the following equation:

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

## Optimization of culture conditions for L-Leucine Oxidase production

The cultural conditions were optimized using a one-factor-at-a-time strategy to increase the production of L-Leucine oxidase enzyme. This strategy involves testing each condition (carbon source, nitrogen source, initial pH, incubation temperature, and incubation time) separately while keeping other parameters constant.

### Carbon sources

Five carbon sources (glucose, lactose, glycerol, sucrose and starch) were supplemented separately to the production medium at a final concentration of 1%. The medium was inoculated with *P. aeruginosa* P13 isolate and incubated at 37°C for 24 hours. After incubation, enzyme activity and protein concentration were measured.

### Nitrogen sources

Five nitrogen sources (yeast extract, ammonium sulfate, peptone, tryptophan and casein) were tested separately. Each of the five nitrogen sources was added to the production medium, it was added at a final concentration of 1%. The cultures were inoculated with *P. aeruginosa* P13 and incubated at 37°C for 24 hours. Following incubation, enzyme activity and protein concentration were measured.

### pH

pH of the production medium was adjusted to different values (5, 6, 7, 8, and 9). After inoculation and incubation, both L-Leucine oxidase activity and protein concentration were determined.

### Incubation temperature

Czapex Dox medium adjusted to the optimal pH, supplemented with the optimal carbon and nitrogen sources, was inoculated with *P. aeruginosa* P13 isolate and incubated for 24 h at different temperatures (27°C, 32°C, 37°C, and 42°C). The temperature with the highest L-Leucine oxidase enzyme production was identified as the optimal temperature for its production and used in subsequent experimental steps.

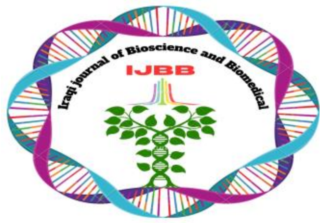
### Incubation time

Czapex Dox medium supplemented with optimal carbon and nitrogen sources, its pH adjusted to the optimal value was inoculated with *P. aeruginosa* P13 isolate and incubated at the determined optimal temperature for various periods (12, 24, 36 and 48 h).

## Purification of L-leucine oxidase

### Ammonium sulphate precipitation

Under ideal circumstances, L-Leucine Oxidase is exposed to ammonium sulfate at saturation ratios from 20% to 80%. A magnetic stirrer was used to gently mix the crude enzyme with ammonium sulfate at each saturation ratio for three h at 4°C. The next step included spinning it in a cooled centrifuge at 10,000




Iraqi journal of Bioscience and Biomedical
Published Online First: 30/ June /2026.
p-ISSN: 3007-5491 / e-ISSN:3007-5505

rpm for 15 min. The proteins that precipitated were dissolved in a suitable amount of a 0.05 M phosphate buffer at a pH of 7. L-Leucine Oxidase activity, protein concentration, and specific activity were determined. After precipitation, enzyme solution was dialyzed using a dialysis tube with a 3,500 Dalton MW cutoff. The dialysis was performed toward the same buffer for 24 h under cooling conditions (4°C), with stirring. The buffer was changed four times. Activity of enzymes, protein concentration, and specific activity were detected during this period, and the enzyme utilized for further steps of purification.

## Chromatographic purification of L-leucine oxidase

L-leucine oxidase was purified by ion-exchange chromatography on DEAE -cellulose according to Whitaker and Bernard (1972)[١٦] followed by gel filtration chromatography on Sephadex G-150. The enzyme solution was first loaded onto a DEAE-cellulose column (1.5 x 25 cm) equilibrated with 0.05 M potassium phosphate buffer (pH 7.0). After washing, bound proteins were eluted with increasing concentrations of sodium chloride and the active fractions were pooled and concentrated. The concentrated enzyme mixture was then applied to a Sephadex G-150 column (1.5 x 40 cm) equilibrated with the same buffer. Fractions were collected, monitored at 280 nm and those exhibiting L-leucine oxidase activity were combined for further analysis.

## Characterization of L-leucine oxidase

## Determinations of pH impacts on L-Leucine oxidase activity

To determine the impact of pH on L-Leucine Oxidase activity the substrate was prepared by dissolving it using buffer solutions with different pH values ranging from pH 5.0 to 9.0. The buffers included sodium acetate (pH 5.0 and 6.0), potassium phosphate buffer (pH 7.0) and Tris-base (pH 8.0 and 9.0).

## Determinations of pH impacts on L-Leucine oxidase stability

The purified enzyme was pre-incubated in buffer solutions covering a pH range of 5.0 to 9.0, for 30 minutes at 37 °C. After incubation, the tubes were immediately cooled in an ice bath. The residual enzyme activity (%) for L-Leucine oxidase was plotted against the pH value.

## Determinations of temperature impacts on L-Leucine oxidase activity

The activity of L-Leucine Oxidase was evaluated at different temperatures (27, 32, 37, 42 and 47°C) to determine the optimal temperature for its catalytic function. Enzyme activity at each temperature was measured and plotted against temperature.

## Determinations of temperature impacts on L-Leucine oxidase stability

Partially purified L-Leucine Oxidase was pre-incubated in a water bath at various temperatures (32, 37, 42, 47 and 52°C) for 30 minutes. After this, the enzyme was rapidly transferred to an ice bath to stop the effect of temperature. The remaining enzyme activity (%) was measured at each temperature and the activity was graphed.

## Estimation of enhancers and inhibitors impact on L-Leucine oxidase action

Different enhancers and inhibitors were used including $MgCl_2$, $MnCl_2$ EDTA, CuSO4 and ZnSO4. Each compound was prepared at three final concentrations: 1mM, 3 mM and 5 mM). For each compound solution, 250 µl of the compound solution was pre-incubated with 250 µl of the enzyme solution for 30 minutes. Following pre-incubation, the enzyme activity was measured. The L-Leucine Oxidase residual activity (%) was determined and plotted against each compound's concentration.

## Kinetic study of L-Leucine Oxidase enzyme

Different concentrations of L-leucine substrate were used to determine the kinetic parameters (Michaelis-Menten (Km) and maximum reaction velocity (Vmax)) of the purified enzyme.[17] The substrate solutions were freshly prepared at concentration of (0, 0.02, 0.04, 0.08, 0.16, 0.32, 0.64, 1.28 and 2.5 mM before use for more accurate results. Enzyme activity was measured under initial velocity conditions and al measurements were performed in triplicate.

The absorbance values obtained for each substrate concentration were used to calculate the initial reaction rates ($V_0$), which were plotted against substrate concentration to generate the Michaelis-Menten curve. The kinetic parameters Km and Vmax were primarily determined by nonlinear regression analysis using GraphPad Prism version 10 based on the Michaelis-Menten equation:

$$V_0 = \frac{V_{max}[S]}{K_m + [S]}$$

Where: $V_0$ = initial reaction velocity, $[S]$= substrate (L-leucine) concentration, $V_{\max}$= maximum reaction velocity and $K_m$= Michaelis-Menten constant, representing the substrate concentration at which $V_0 = \frac{1}{2}V_{\max}$. A Lineweaver-Burk plot was also constructed for visualization and comparison.

## *In vitro* MTT assay toward HepG2 and HdFn cell lines

The cytotoxic effect of L-leucine oxidase was examined via using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit (Korea). HepG2 and HdFn cells were seeded into 96-well micro-titer plates containing 200 µL of complete culture medium at a cell density of $1 \times 10^5$ cells/well. The plates were gently shaken, sealed with sterile parafilm and incubated at 37°C with 5% $CO_2$ for 24 h. After incubation, the medium was discarded and the cells were treated with 2-fold serial concentrations of L-leucine oxidase (25, 50, 100, 200, and 400 µg/mL) dissolved in phosphate buffer. Control cells received culture medium without L-leucine oxidase. Each concentration and the control were tested in triplicate. Following 24 h exposure, 10 µL of MTT solution was added to each well and the plates were incubated for an additional 4 h at 37°C with 5% $CO_2$. The medium was carefully removed, and 10 µL of solubilization solution was added to dissolve the formed formazan crystals. After 5 min incubation, the absorbance was measured at 575 nm using an ELISA reader. The half-maximal inhibitory concentration ($IC_{50}$) values for all cell lines were determined from the optical density data after statistical analysis using the equation:

$$Inhibition\ (\%) = \frac{OD\ of\ negative\ control - OD\ of\ treated\ cells}{OD\ of\ negative\ control} \times 100$$

## Statistical analysis

All experiments were performed in triplicate and the results were expressed as mean ± standard deviation (SD). Microsoft Office Professional Plus 2021–Excel was used to calculate the mean and SD. GraphPad Prism version 10 software (GraphPad Software Inc., La Jolla, CA, USA) was employed to draw graphs and calculate the IC50 value. The IC50 value for the MTT assay was calculated using nonlinear regression analysis based on the dose–response curve.

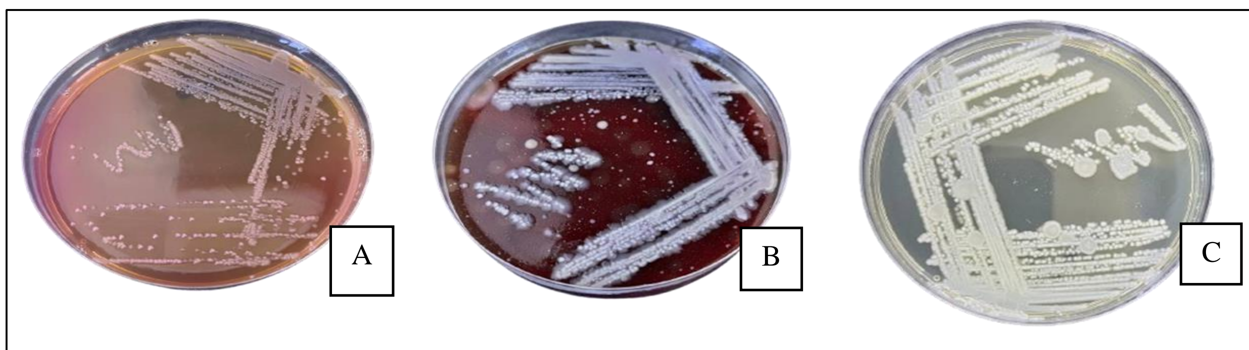
## Results and Discussion

Table 1 shows the distribution of collected clinical samples according to their sources. A total of 130 clinical samples were obtained from patients suffering from burns, wounds, otitis media and urinary tract infections (UTIs) (Table 1).

**Table 1. Distribution of clinical samples according to source**

| Source of samples | Number of clinical samples | Number of *P. aeruginosa* isolates |
|---|---|---|
| Burns | 21 (16.153%) | 5 |
| Wounds | 36 (27.692%) | 2 |
| Otitis media | 29 (22.307%) | 3 |
| Urine | 44 (33.846%) | 10 |

These isolates were first cultured on MacConkey agar and only 45 isolates were able to grow on it, they grew as tiny round convex colonies with a pale yellowish color (Fig. 1A).To support the cultural characterization of the presumptive of *P. aeruginosa* isolates, clinical isolates were streaked blood agar to evaluate their hemolytic activity, all isolates developed as big flat bacterial colonies with a grape-like odor and after 24 hours of incubation colonies exhibited β-hemolysis on blood agar (Fig. 1B). Finally, bacterial isolates were further identified by streaking onto Pseudomonas agar, only 20 isolates were able to grow on Pseudomonas agar with smooth circular colonies (Fig. 1C).



Iraqi journal of Bioscience and Biomedical
Published Online First: 30/ June /2026.
p-ISSN: 3007-5491 / e-ISSN:3007-5505

**Figure 1. The growth of bacterial isolates on: (A) MacConkey agar, (B) Blood agar and (C) Pseudomonas agar after incubation for 24 hours at 37°C.**

Results of the current study revealed that out of 130 only 20 (15%) isolates tested positive for *P. aeruginosa* infections, while the remaining 110 (85%) isolates tested negative. All of the 20 bacterial isolates were subjected to microscopic investigation and some biochemical tests and identification was confirmed by VITEK 2 compact system.

The present study confirmed that *Pseudomonas aeruginosa* can be isolated from different clinical infections and that its isolates differ in their ability to produce L-leucine oxidase, the recovery of the opportunistic *P. aeruginosa* from various clinical sources such as burns, wounds, urinary tract infections and ear infections in this study corroborates previous findings regarding its extensive pathogenic distribution and metabolic versatility in hospital-associated and community infections[18,19].

## Screening the ability of *P. aeruginosa* for L-leucine Oxidase production

The enzyme activity, protein concentration and specific activity of L- leucine oxidase produced by clinical isolates of *P. aeruginosa* was quantitatively screened, as shown in Table (2). All isolates were able to produce the enzyme, with enzyme activity ranging from 0.705 to 3.125 U/ml and specific activity ranging from 3.502 to 21.853 U/mg protein. The highest enzyme activity was recorded for isolate P13 (3.125 U/ml) which also exhibited the highest specific activity (21.853 U/mg protein). The most promising producer of the target enzyme was isolate P13, so it was selected to undergo further experimental procedures.

**Table 2. Enzymatic activity, protein concentration and specific activity of L-leucine oxidase produced by local isolates of P. aeruginosa after incubation at 37°C for 24h.**

| Isolate number | Enzyme activity (U/ml) | Protein concentration (mg/ml) | Specific activity (U/mg protein) | Isolate number | Enzyme activity (U/ml) | Protein concentration (mg/ml) | Specific activity (U/mg protein) |
|---|---|---|---|---|---|---|---|
| 1 | 2.456 | 0.214 | 11.476 | 11 | 2.170 | 0.122 | 17.786 |
| 2 | 1.321 | 0.123 | 10.830 | 12 | 2.067 | 0.123 | 16.804 |
| 3 | 2.617 | 0.156 | 16.775 | 13 | 3.125 | 0.143 | 21.853 |
| 4 | 1.236 | 0.161 | 7.677 | 14 | 2.039 | 0.162 | 12.586 |
| 5 | 2.596 | 0.423 | 6.137 | 15 | 2.389 | 0.119 | 20.075 |
| 6 | 3.024 | 0.223 | 13.560 | 16 | 2.653 | 0.171 | 15.514 |
| 7 | 1.838 | 0.185 | 9.935 | 17 | 1.895 | 0.182 | 10.412 |
| 8 | 0.739 | 0.211 | 3.502 | 18 | 2.167 | 0.164 | 13.213 |
| 9 | 0.705 | 0.119 | 5.924 | 19 | 2.530 | 0.128 | 19.765 |
| 10 | 2.758 | 0.141 | 19.560 | 20 | 2.072 | 0.132 | 15.696 |

The results demonstrated that all recovered isolates were able to produce the enzyme, with the highest enzyme activity was recorded for isolate P13, this difference implies that the productivity of an

enzyme can be strain dependent, even within the same bacterial species. Therefore, isolate P13 was selected to undergo further experimental. Abdel-Monsef et al. (2024)[20], reported that L-amino acid oxidase purified from the marine bacterium *Bacillus velezensis* had a total activity of 3957 U, total protein of 11.35 mg/ml and specific activity of 352.35 U/mg protein and noted that the enzyme showed substrate selectivity towards L-leucine.

## Optimization of L-leucine oxidase production conditions

L-leucine oxidase production by *Pseudomonas aeruginosa* P13 was markedly affected by culture conditions. Among the tested carbon sources (Fig. 2A), glucose (1%) gave the highest specific activity (23 U/mg protein), followed by lactose (20 U/mg protein) and glycerol (19 U/mg protein), whereas sucrose and starch resulted in lower activities of 13.5 and 11 U/mg protein, respectively. For nitrogen sources (Fig. 2B), yeast extract (1%) produced the highest specific activity (28 U/mg protein), followed by ammonium sulfate (26 U/mg protein), peptone (24 U/mg protein), tryptone (21 U/mg protein) and casein (15 U/mg protein). The highest enzyme production was obtained at pH 7 (Fig. 2C), with a specific activity of 28 U/mg protein, while lower activities were recorded at pH 5,6,8 and 9. Temperature (Fig. 2D) also influenced enzyme production, with the maximum specific activity observed at 37ºC (29 U/mg protein), compared with 7 U/mg protein at 27ºC and 22 U/mg protein at 42ºC. In addition, the incubation (Fig. 2E) period of 24 h was optimal, yielding the highest specific activity (28 U/mg protein), whereas the activity was lower at 12 h (14 U/mg protein), 36 h (18 U/mg protein) and 48 h (8 U/mg protein). Overall, the optimum conditions for L-leucine production were glucose as the carbon source, yeast extract as the nitrogen source, pH 7, 37ºC and 24 h incubation.

Iraqi journal of Bioscience and Biomedical
Published Online First: 30/ June /2026.
p-ISSN: 3007-5491 / e-ISSN:3007-5505

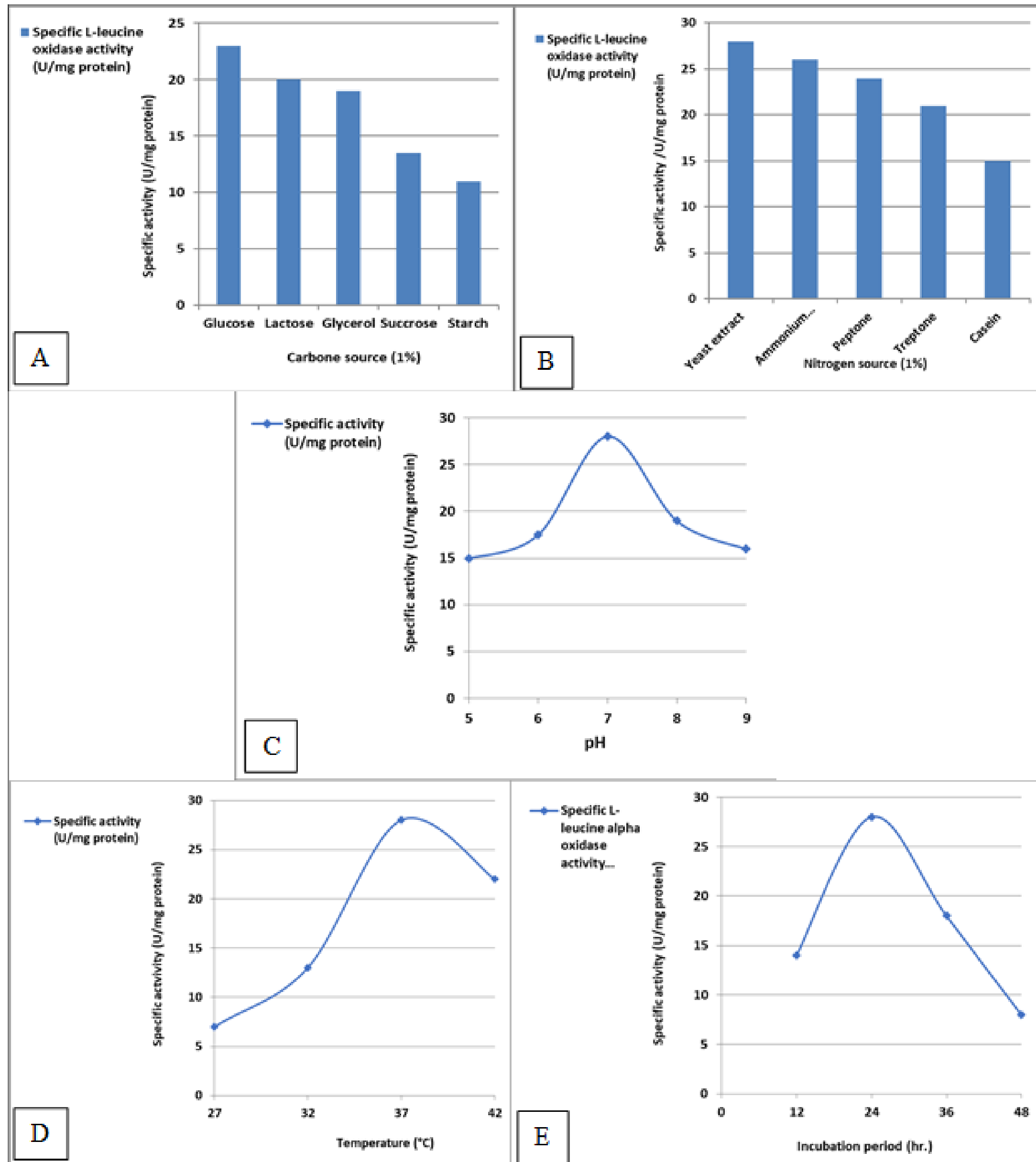



**Figure 2. L-leucine oxidase production under different optimum culture conditions: (A) carbon source (1%), (B) nitrogen source (1%), (C) pH, (D) temperature (ºC) and (E) incubation period.**

In order to maximize both the microbial growth and the yield of enzyme during cultivation, it is important to optimize the cultural conditions, since nutritional and physicochemical parameters such as a suitable carbon and nitrogen sources, temperature, pH and the incubation time, significantly influence microbial enzyme production[21]. In the present work, optimization experiments showed that the production of the enzymes was highly dependent upon the medium composition and incubation conditions. The most desirable carbon source was found to be glucose probably because it is easily metabolized by bacterial cells, promotes rapid growth and increase the metabolic rate to produce enzymes. As an example of optimizing

carbon source, Singh et al. (2009)[22] optimized the production of L-amino acids by Aspergillus fumigatus using different carbon sources; the results showed that the maximum enzyme production was obtained with 10 g/L glucose-containing medium.

Nitrogen sources also play an important role in the synthesis of amino acids, proteins, nucleotides, enzymes, and secondary metabolites involved in microbial growth and metabolism[23]. In the present study, the highest specific activity was achieved with yeast extract, which may be attributed to its content of easily assimilable nitrogenous compounds, such as amino acids, peptides, and vitamins, that support microbial growth and may enhance enzyme synthesis[24]. Temperature also plays an important role in microbial growth and enzyme production, activity, and stability. In the present study, the highest production was observed at 37°C, suggesting that this temperature provided the most suitable condition for bacterial growth and enzyme biosynthesis, whereas lower or higher temperatures were less favorable[25]. These results agree with previous reports on bacterial L-amino acid oxidases, which have demonstrated maximum enzyme specific activity at 37 ºC, followed by a decrease at higher temperatures due to reduced thermal stability[20].

Likewise, pH affects the growth of microbes and enzyme production by affecting nutrient solubility, ionization, enzyme activity and membrane transport[26]. The highest production at pH 7 indicates that neutral pH was the most suitable condition for L-leucine oxidase synthesis by the tested isolate. Similar findings were reported by Abed and Hussein (2025)[12], who reported that L-Lysine α-Oxidase produced by Pseudomonas aeruginosa exhibited maximum activity at pH 7, followed by gradual decrease at alkaline pH values. The incubation time can also be utilized to identify the time when the highest enzyme yield is obtained, as the growth and metabolic activity of microbes change during the fermentation process and a long incubation duration can lower the yield due to nutrient exhaustion and the accumulation of inhibitory compounds[27]. In the present study, the highest specific activity was recorded after 24 h of incubation, which may correspond to the active growth phase of the organism.

## Purification of L-leucine oxidase

As illustrated in Table 3, the crude enzyme extract exhibited a specific activity of 28 U/mg protein with an overall activity of 420 U. First, the crude extract was partially purified using ammonium sulfate precipitation, the maximum enzyme activity (12 U/ml) and specific activity (40 U/mg protein) were achieved at 70% saturation, corresponding to 1.4-fold purification with 71.4% of the total activity recovered as shown in Table 3, indicating that this concentration was the best for partial purification of the enzyme.

Iraqi journal of Bioscience and Biomedical
Published Online First: 30/ June /2026.
p-ISSN: 3007-5491 / e-ISSN:3007-5505

The partially purified enzyme was further purified by DEAE-cellulose ion-exchange chromatography. As illustrated in Fig. 3A, protein peaks were observed during both the washing and elution steps and the highest activity was detected in fraction 65 (18 U/ml), while the active enzyme was mainly distributed across fractions 62-69. Following this step, the specific activity rose to 73.3 U/mg protein, which corresponded to a 2.6-fold purification with a yield of 62.8%. The total activity after DEAE-cellulose chromatography was 264 U (Table 3). Sephadex G-150 gel filtration chromatography was utilized as a final purification. The elution of enzyme as a major active peak indicated further improvement in its purity (Fig. 3B). After this step, the specific activity of enzyme raised to 111.1 U/mg, resulting in an overall purification of 3.9-fold compared to the crude extract. The purified enzyme exhibited an activity of 10 U/mL, a total activity of 240 U and a total yield of 57.1% (Table 3).

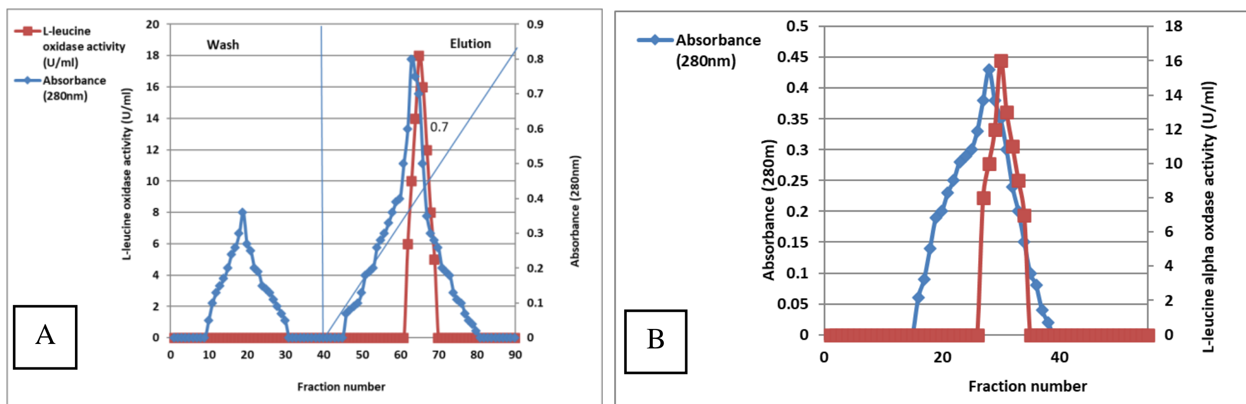



**Figure 3. L-leucine oxidase purification from *P. aeruginosa* by: (A) Ion exchange chromatography using DEAE-Cellulose column (1.5 x 25 cm) equilibrated with potassium phosphate buffer (0.05 M; pH 7), eluted with NaCl gradient (0.1-1 M) in flow rate 30 ml/h. (B) Gel filtration chromatography using Sephadex G-150 column (1.5 x 40 cm) equilibrated with potassium phosphate buffer (0.05 M; pH 7), eluted with the same buffer in flow rate 30 ml/h for each fraction.**

**Table 3: Steps of L-leucine oxidase from purification from *P. aeruginosa* P13**

| Purification step | Volume (ml) | Enzyme activity (U/ml) | Protein concentration (mg/ml) | Specific activity (U/mg) | Total activity (U) | Purification (folds) | Yield (%) |
|---|---|---|---|---|---|---|---|
| **Crude enzyme** | 75 | 5.6 | 0.2 | 28 | 420 | 1 | 100 |
| **Ammonium sulphate precipitation 70%** | 25 | 12 | 0.3 | 40 | 300 | 1.4 | 71.4 |
| **DEAE-cellulose** | 24 | 11 | 0.15 | 73.3 | 264 | 2.6 | 62.8 |
| **Sephadex G150** | 24 | 10 | 0.09 | 111.1 | 240 | 3.9 | 57.1 |

The purification results demonstrated that L-leucine oxidase from P. aeruginosa was progressively enriched through sequential ammonium sulfate precipitation, DEAE-cellulose ion-exchange chromatography and Sephadex G-150 gel filtration. Among the tested ammonium sulfate saturation levels, 70% was the most effective for partial purification, giving the highest activity and specific activity, which suggests selective precipitation of the target enzyme at this concentration. Subsequent purification on DEAE-cellulose markedly increased the specific activity, indicating effective separation according to charge properties, while gel filtration further improved enzyme purity by removing residual contaminating proteins based on molecular size. Although the total activity and yield gradually decreased during purification, this is a common feature of multistep enzyme purification procedures due to unavoidable enzyme loss during handling and fractionation. A previous study purified an L-amino acid oxidase-type enzyme from *Streptomyces* using a sequential multi-step protocol. First, the crude enzyme was precipitated at 60% saturation with ammonium sulfate precipitation and the specific activity was increased to 8.25 U/mg (1.8-fold; 20.8% yield). After precipitation, the enzyme solution was dialyzed and applied to DEAE-cellulose column, which led to a rise in specific activity to 25 U/mg (5.2-fold; 12.7% yield). Finally, gel filtration on Sephadex G-200 was performed as a polishing step, producing the highest purity and increasing the specific activity to 56 U/mg with an overall purification of 11.7-fold, although with a reduced final yield (4%)[28].

## Characterization of L-leucine oxidase

### pH effect on the activity and stability of L-leucine oxidase

The enzyme activity increased gradually from 8 U/mL at pH 5 to 9 U/mL at pH 6, reaching maximum value of 10 U/mL at pH 7. However, increasing pH resulted in a noticeable decrease in the enzyme activity to 7 U/mL at pH 8 and 6 U/mL at pH 9 (Fig. 5A). The effect of pH on enzyme stability is presented in Fig. 5B. The purified L-leucine oxidase retained 100% of its activity at pH 6 and pH 7, indicating maximum stability within this range. In contrast, the remaining activity decreased to 80% at pH 5, 75% at pH 8 and 65% at pH 9.

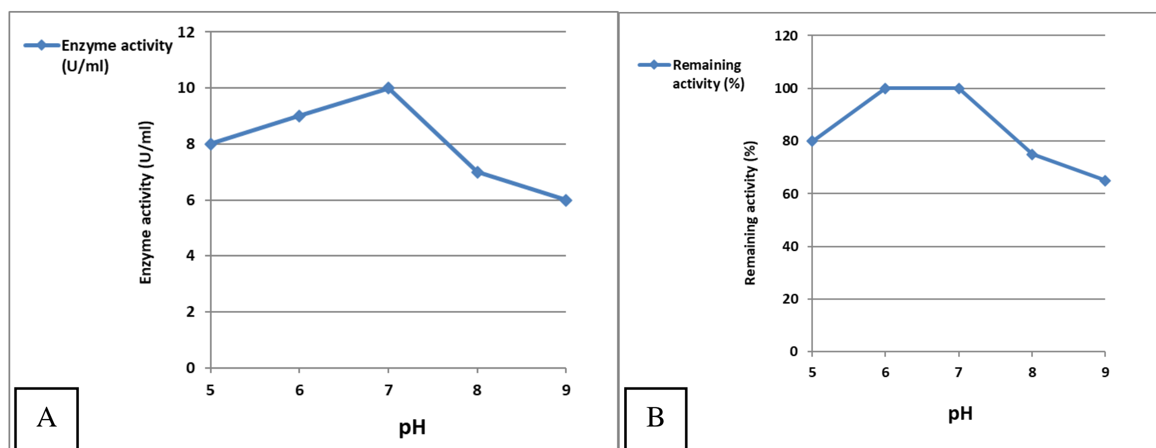



**Figure 5. Effect of different pH values on the (A) activity and (B) stability of L-leucine oxidase purified from *Pseudomonas aeruginosa*.**

## Temperature effect on the activity and stability of L-leucine oxidase

Enzyme activity increased progressively with increasing temperature and reached its maximum value (10 U/mL) at 37°C (Fig. 6A). However, the activity began to decrease when the temperature was increased and decreased above and below (37°C), with the lowest value of L-leucine oxidase activity (5 U/ml) was reported at (27°C). In terms of enzyme stability, L-leucine oxidase was fully stable (100%) at both 32°C and 37°C (Fig. 6B). Moreover, stability started to decline beyond 37°C, with 85%, 70% and 60% residual activity at 42°C, 47°C and 52°C, respectively.

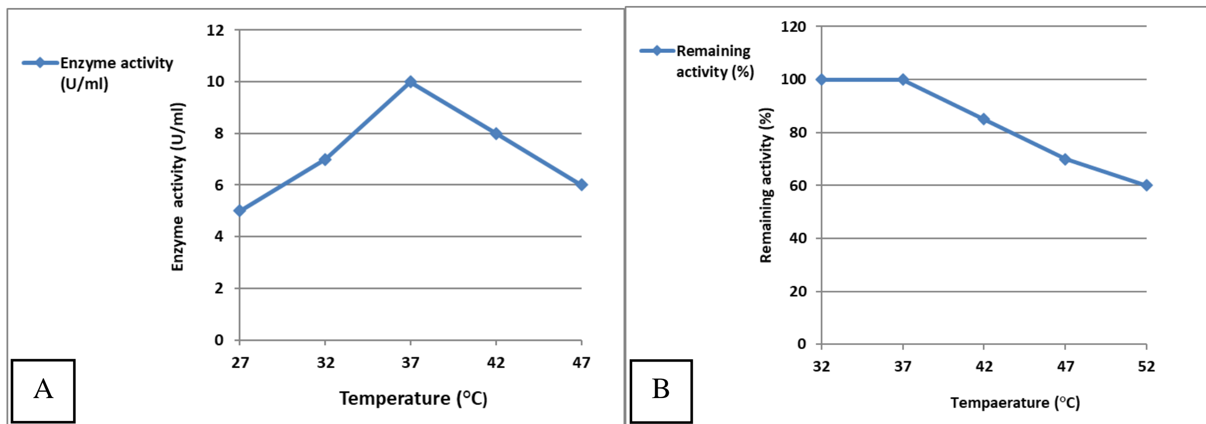


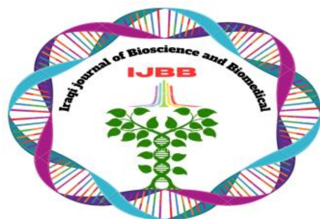

**Figure 6. Effect of temperature on the (A) activity and (B) stability of L-leucine oxidase purified from *Pseudomonas aeruginosa*.**

## Effect of activators and inhibitors on L-leucine oxidase stability

Results presented in Table 4, indicates that $CaCl_2$ markedly decreased enzyme stability, with residual activity decreasing from 29% at 1 mM to 5% at 3 mM and a complete inactivation at 5 mM. $CuSO_4$ also strongly inhibited the enzyme, with only 6% remaining activity at 1 mM and slight recovery at higher concentrations (20% and 25%), while EDTA reduced the stability of L-leucine oxidase in a concentration-dependent manner, with 27%, 9% and 2% remaining activity at 1, 3 and 5 mM, respectively. In contrast, the stability of the enzyme was greatly increased when $MnCl_2$ was added. With 1 mM, the activity was 90%, but with 3 mM, the activity was raised to 96% and at 5 mM, the remaining activity increased dramatically to 133%. Finally, the addition of $ZnSO_4$ showed moderate effect on L-leucine oxidase stability, with residual activities of 27% at 1 mM, 30% at 3 mM and 69% at 5 mM.

**Table 4. Effect of activators and inhibitors on the stability of L-leucine oxidase**

| Effectors | Concentration | Remaining activity % |
|---|---|---|
| **Control** | - | 100 |
| | 1mM | 29 |
| **$CaCl_2$** | 3mM | 5 |



Iraqi journal of Bioscience and Biomedical
Published Online First: 30/ June /2026.
p-ISSN: 3007-5491 / e-ISSN:3007-5505

| | | |
|---|---|---|
| | 5mM | 0 |
| | 1mM | 90 |
| **$MnCl_2$** | 3mM | 96 |
| | 5mM | 133 |
| | 1mM | 27 |
| **EDTA** | 3mM | 9 |
| | 5mM | 2 |
| | 1mM | 27 |
| **$ZnSO_4$** | 3mM | 30 |
| | 5mM | 69 |
| | 1mM | 6 |
| **$CuSO_4$** | 3mM | 20 |
| | 5mM | 25 |

Characterization of the purified enzyme revealed that maximum activity occurred at pH 7, while the highest stability was retained at pH 6–7. This suggests that the catalytic conformation of the enzyme is best maintained under neutral or slightly acidic-neutral conditions. Changes in pH can alter the ionization status of amino-acid residues involved in binding and catalysis and also because change in ionization can be coupled to conformational changes that affect protein stability[29]. Temperature characterization observed a similar pattern, where L-leucine oxidase showed maximal activity at 37°C and remained fully stable at 32–37°C, followed by a decline at higher temperatures. This indicates that the enzyme behaves as a mesophilic enzyme, which is consistent with its production by a clinical isolate of *P. aeruginosa*. The decrease in activity and stability at higher temperatures is most likely due to thermal disruption of the enzyme structure, whereas lower temperatures may reduce catalytic efficiency by decreasing molecular motion and enzyme-substrate interactions[30]. A recent study measured the optimum activity of the purified LAAO at different temperatures (20-60 °C) and found that the highest activity was achieved at 40°C[20].

The stability of L-leucine oxidase was markedly affected by both type and concentration of the tested effector. $CaCl_2$ and $CuSO_4$ led to significant decrease in enzyme stability, this indicates that $Ca^{2+}$ does not stabilize the structure of L-leucine oxidase and may interfere with its active conformation. Whereas the addition of $CuSO_4$ caused strong inhibition, this may be related to the ability of $Cu^{2+}$ to interact with thiol/sulfhydryl (-SH) groups of cysteine residues and promote thiol oxidation or abnormal disulfide bond formation, which can alter protein conformation and consequently reduce enzymatic activity[31,32]. Metal ions may affect the structure and function of the enzyme by binding to their active site and impair the enzyme's

capacity to catalyze amino acid oxidation[33]. EDTA also reduced enzyme stability in a concentration-dependent manner, the inhibitory effect of EDTA might be attributed to its high potency in chelating divalent metal ions that may remove ions required for maintaining structural stability or catalytic activity of the enzyme[34]. In contrast, the stability of the enzyme was greatly increased when $MnCl_2$ was added, suggesting a possible stabilizing or activating effect of $Mn^{2+}$ on L-leucine oxidase enzyme. The present study agreed with the El Hakim et al. (2015)[35] results, who reported that $Mn^{2+}$ increased the activity of L-amino acid oxidase purified from Egyptian *Cerastes cerastes* venom, possibly by stabilizing the structural integrity of the enzyme. $ZnSO_4$ showed moderate effect on L-leucine oxidase stability and partial recovery of remaining activity with increased concentrations of $Zn^{2+}$ indicates that zinc ions might have a role in structural stabilization or influence the conformational state of L-leucine oxidase enzyme.

## Kinetic study of L-leucine oxidase

The reaction velocity (V) was calculated at different concentrations of L-leucine using kinetic readings and the data was fitted to the Michaelis-Menten model by plotting enzyme velocity versus substrate concentration. Nonlinear regression analysis yielded a Km value of 18.63 mM and a Vmax of 1.303 mM/min. The value of Km (18.63 mM) indicates a moderate affinity of the enzyme to L-leucine, while the Vmax value (1.303 mM/min) indicates the maximum rate of catalysis when the enzyme active sites are fully saturated with the substrate (Fig. 7A). For comparison, a Lineweaver–Burk double-reciprocal plot was also constructed, generating kinetic values of Vmax = 1.34 mM/min, Km = 19.83 mM and an $R^2$ value of 0.9964 (Fig. 7B).

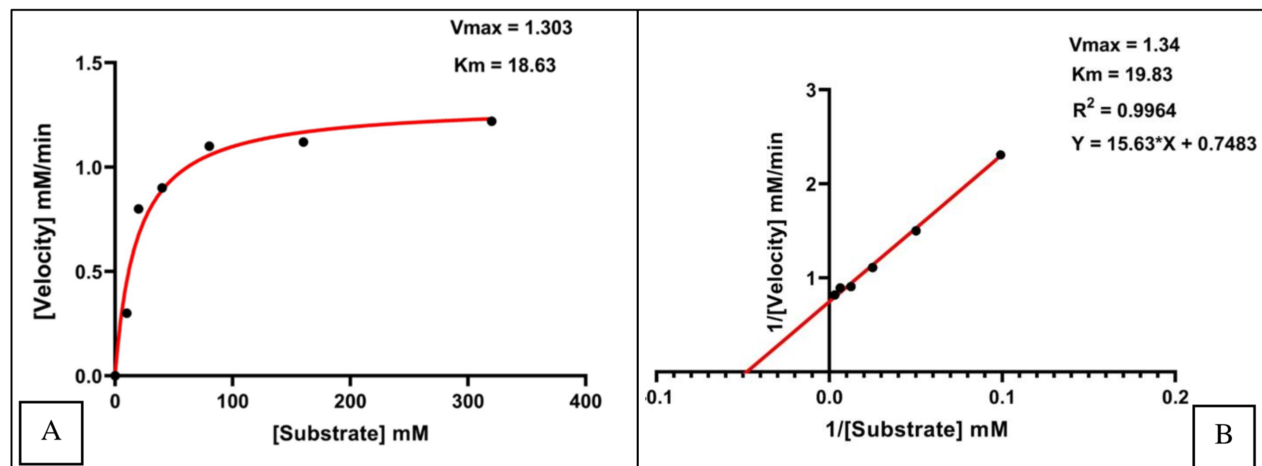



**Figure 7. (A) Michaelis-Menten plot for L-leucine oxidase at different concentrations of L-leucine as the substrate, (B) Lineweaver–Burk plot for L-leucine acid oxidase at different concentrations of L-leucine as the substrate.**

The close similarity between the kinetic constants obtained from the nonlinear Michaelis–Menten and Lineweaver–Burk plots support the reliability of the estimated parameters. Although nonlinear regression and the Lineweaver-Burk plot support the reliability of the estimated parameters. However, nonlinear regression was considered the primary method for determining Km and Vmax because it directly fits the untransformed velocity-substrate data.[36] Overall, the results suggest that L-leucine oxidase followed classical Michaelis–Menten kinetics under the tested conditions.

Iraqi journal of Bioscience and Biomedical
Published Online First: 30/ June /2026.
p-ISSN: 3007-5491 / e-ISSN:3007-5505

## Cytotoxic effect of L-leucine oxidase purified from *Pseudomonas aeruginosa* on HepG2 and HdFn cell lines

As illustrated in Table 5 and Fig. 8, treating HepG2 cell lines with L-leucine oxidase caused a concentration-dependent reduction in cell viability. The viability showed a gradual decrease from 94.48 ± 0.57% at 25 µg/mL to 50.46 ± 2.95% at 400 µg/mL, demonstrating a clear cytotoxic effect. The obtained IC50 was 112 µg/mL, which validates the inhibitory effect of L-leucine oxidase on HepG2 cell growth. Conversely, the normal HdFn cells showed only a slight reduction in viability across the tested concentrations, decreasing from 96.99 ± 0.34% at 25 µg/mL to 87.88 ± 1.77% at 400 µg/mL.

**Table 5. The cytotoxic effect of L-leucine oxidase on HepG2 and HdFn cell lines.**

| Concentration µg $mL^{-1}$ | Mean viability (%) ± SD | |
|---|---|---|
| | HdFn | HepG2 |
| **400** | 87.88 ± 1.77 | 50.46 ± 2.95 |
| **200** | 93.59 ± 0.53 | 62.03 ± 1.06 |
| **100** | 94.56 ± 0.2 | 74.26 ±1.03 |
| **50** | 96.06 ± 0.3 | 82.63 ± 2.92 |
| **25** | 96.99 ± 0.34 | 94.48 ± 0.57 |

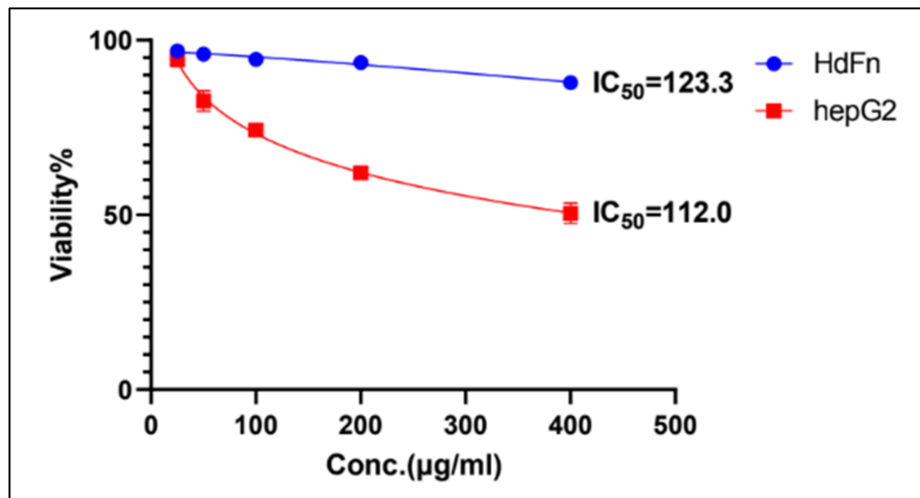
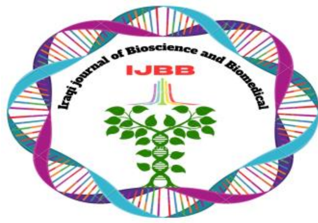



**Figure 8. Dose-dependent cytotoxic effect of L-leucine oxidase on HepG2 and HdFn cell lines.**

The effect of purified L-leucine oxidase on HepG2 cells may be attributed to the enzymatic activity of L-amino acid oxidases, which produces hydrogen peroxide during oxidative deamination of amino acid.





Hydrogen peroxide is known to induce oxidative stress, which can damage cellular components and activate cell death pathways in cancer cells[5,37]. The response of HepG2 cells in the current study is in agreement with previous reports showing that LAAOs can suppress the growth of hepatic cancer cells and induce apoptosis through oxidative mechanisms[38,39]. The relatively limited effect on HdFn normal cells may suggest some selectivity of the enzyme towards malignant cells. This may be explained by the fact that cancer cells usually exist under higher oxidative stress than normal cells and are therefore more sensitive to further increase in reactive oxygen species[40,41].

## Conclusions

The findings indicate that *P. aeruginosa* P13 is a promising local source of L-leucine oxidase. The purified enzyme showed favorable biochemical and kinetic properties, as well as preliminary concentration-dependent cytotoxic activity against HepG2 cells. These results support the potential biotechnological and biomedical relevance of the enzyme and provide a basis for complementary mechanistic investigations.

## Conflict of insert

The authors declare that there is no conflict of interest regarding the publication of this paper.

## Acknowledgments

The authors are grateful to the College of Biotechnology, Al-Nahrain University, for academic support.

## Author's Declaration

- We hereby declare that all tables, figures, images, culture plate photographs, and datasets included in this manuscript are original and were prepared or generated by the authors. No material has been reproduced, adapted, or copied from previously published sources, and therefore no permission was required.
- The study was conducted at Al-Nahrain University, College of Biotechnology. Written informed consent was obtained from all participants after they were informed about the purpose and procedures of the study. The authors confirm that the research was conducted in accordance with ethical research standards and with respect for participants' rights, privacy, and well-being.

## Funding information

The authors received no funding for this research.

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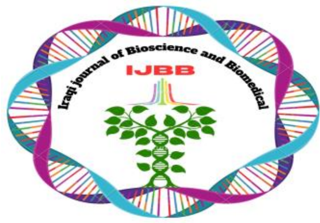



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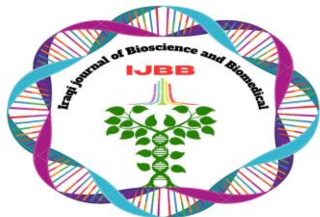



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