Optimization and purification of L-methioninase enzyme purified from clinical Samples of *Pseudomonas* spp.

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ABSTRACT

Background: This study focused on *Pseudomonas aeruginosa*, which secrete important enzymes in medical and pharmaceutical applications and are isolated from clinical samples. One of these enzymes is L-methioninase, the most desirable enzyme in the medical and industrial fields today since it converted L-methionine into methanethiol, ammonia, and alpha-ketobutyrate. **Objective**: Evaluated the optimum conditions of L-methioninase production and then purified it. **Methodology:** Pseudomonas samples were identified by microscopic and biochemical tests and confirmed with the VITEK2 system. After the L-methioninase production was examined, the optimal conditions for the production of L-methioninase (MGL) were established using clinical samples of *P. aeruginosa*. Additionally, MGL was purified from the supernatant of *P.aeruginosa* using ammonium sulfate precipitation, DEAE-cellulose, and Sephadex G150 accordingly. **Results**: There were 33 isolates of *P.aeruginosa* according to microscopic characteristics and biochemical tests, followed by the VITEK2 system to ensure identification. The optimization conditions were achieved when supplement media with sucrose 1% w\v, casein 1% w\v, pH 7, temperature 37oC, and incubation for 48hrs within the specific activity of 1.6, 3.3, 3.4, 3.4, and 3.5U\mg protein, respectively, and 4.6 fold the amount of the purified enzyme as compared to the crude enzyme. A single 55 kDa band on the SDS-PAGE indicates the enzyme is purified. **Conclusion:** *P.aeruginosa* can be an efficient source of L-methioninase, and this enzyme had better and higher specific activity after applying optimum conditions for it and purifying it.

Keywords: L-methioninase, Optimization, Purification.
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1-INTRODUCTION

Pseudomonas is one of the most metabolically diverse and widely distributed genera of bacteria, with over 200 species that are utilized in environmental protection, biotechnology, and medicine. Some species of this genus can cause opportunistic disease in animals, plants, and humans or exhibit intrinsic resistance to antimicrobials, while other species are distinguished by their capacity to successfully adapt to a wide range of habits, metabolic flexibility, and genetic variability (1). Despite this knowledge, many of the genetic bases for Pseudomonas' adaptation and specialization to various lifestyles and many of its genes and metabolic pathways remain unknown (2).

Previous studies have focused on the microbial L-methioninase (MGL) produced by various microorganisms, implicating yeast, bacteria, and filamentous fungus (3,4). MGL (EC4.4.1.11) is produced by

adding L-methionine to the media, which causes the bacteria to convert their L-methionine to methanethiol, ammonia, and alpha-ketobutyrate. L-methioninase is extensively distributed in bacteria, especially in Pseudomonas spp. L-methioninase crystal structures have been declared from Pseudomonas putida (5). The growth medium's nutrients are known to significantly impact MGL generation. It has been noted that adding supplements like nitrogen, carbon sources, and trace elements causes microorganisms to produce more MGL (6). Copper Cu, cobalt Co, magnesium Mg, manganese Mn, and other metal salts have been seen to boost production slightly.

Maximizing microbial growth and enzyme synthesis by microorganisms requires optimizing the culture conditions. For the generation of microbial enzymes, the ideal temperature, pH range, and carbon and nitrogen supplies are the most crucial physical and chemical factors (7). It is done by changing one factor at a time (OFAT) while keeping the others constant, which is the traditional approach for optimization (8). For optimum L-methioninase production, various bacterial and fungal species share a lot of the same ideal growing conditions. However, there appear to be some variances between the species regarding required nutrients in the growing medium (9).

Enzyme purification is crucial for understanding structural and functional characteristics as well as predicting applications. The final level of purity of a specific enzyme depends on its intended usage. While to obtain the largest yield of the required enzyme with the highest catalytic activity and highest purity is the goal behind choosing the purifying technique. The choice of the right techniques that maximize yield and purity with the fewest possible stages is essential for successful and effective purification strategies (10).

Several methods exist for determining the purity of the final enzyme preparation. Analytical gel filtration, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and mass spectrometry are the most often employed techniques (11).

Objectives of the study

To demonstrate the optimum conditions for L-methioninase production and then purification of this enzyme by three stages after being isolated from clinical samples of *P.aeruginosa.*

2-Material and Method

2.1 Subjects

150 clinical samples were collected from patients of different sources, such as urinary tract infections (UTIs), burns, sputum, wounds, and ear from otitis media patients who attended AL-Kadhmiya Teaching Hospital and Medical City Hospital in Baghdad /Iraq from September to November 2022.

2.2 Isolation and Identification of *P. aeruginosa*

Every sample was cultured in a brain heart infusion broth medium and kept at 37 ^{o}C for 24 hours to encourage bacterial growth. After that, the colonies were re-cultured on MacConkey agar plates to ensure their purity. The acquired colonies were streaked onto Cetrimide agar to examine the production of fluorescein and pyocyanin dyes, and the plates were cultured for an additional 24 hours at 37°C (12). The morphological (size, margin, color, Shape, and consistency colonies), microscopical characteristics, biochemical tests, and the use of the VITEK2 system in the detection of bacterial growth were employed to identify the positive growth.

2.3 Preparation of inoculum

The 33 isolates of *P.aeruginosa* were cultured on modified M9 media according to (13), and only 15 of them were able to produce the enzyme. Imada *et al.* (1973) used the Nesslerization method to examine the activity of microbial L-methioninase, and the best isolate was chosen for the following stage (14).

2.4 Optimum Conditions for L-methioninase Production

Enzyme production was optimized by changing one factor at a time. In (5 ml) of the sterile modified M9 medium, 2% of the bacterial seed medium was added to the medium as an inoculant.

Multiple variables were successively examined as part of the optimization process, and the best option from the first test was selected for the following stage. Five carbon sources—glucose, maltose, xylose, galactose, and five nitrogen sources—yeast extract, casein, NH_4CL, peptone, and tryptone—were separately added to the M9 medium as a first step. These sources were all included in the medium at a final concentration of 1% (w/v) and incubated for 48 hours at 37°C. The optimum carbon and nitrogen sources were then investigated at various pH ranges (pH 5-9). The effect of the incubation period on the selected isolate's L-methioninase production was examined by incubating the production medium at various times (8, 12, 18, 24, 48, and 72 hours) after observing the incubation temperature (27, 32, 37, 42, and 47 °C) (8).

According to Imada *et al*. (1973), the bacteria's MGL production was measured using a spectrophotometric assay based on the amount of ammonia released using Nessler reagent, and enzyme yield was expressed as an UmL^{-1} medium. Using enzyme and substrate blanks as controls, the spectrophotometer was used to measure the absorbance at 480 nm (14).

2.5 Purification of L-methioninase enzyme

According to Selim *et al.* (2015), the purification of *P.aeruginosa's* L-methioninase required three steps: $(NH_4)_2SO_4$ precipitation, ion exchange chromatography, and gel filtration chromatography (15). Ammonium sulfate was gradually added to the crude enzyme while being continuously mixed on ice at a saturation ratio of 70%, and then the mixture was centrifuged at 6,000 rpm for 20 min at 4°C to precipitate ammonium sulfate. The precipitate in each concentration was dissolved in the appropriate quantities of phosphate buffer solution (pH 7), while the supernatant was discarded. The optimal saturation ratio was determined by measuring the enzyme's activity. The previous step's ammonium sulfate precipitate was dialyzed for 24 hours at a cooling (4°C) temperature in a dialysis tube with a 3500 Mw cutoff against phosphate buffer (pH 7) (16).

Enzyme solution after dialysis was then added to DEAE-cellulose column. Following that, an equivalent volume of the same phosphate buffer (pH7) was used to wash the column, and successively increasing sodium chloride concentrations (0.1–1M) were used to elute the associated proteins. A spectrophotometer was used to test each fraction's absorbance at 280 nm at a flow rate of 3 ml per minute. For the following purification processes, fractions with L-methioninase activity were combined and stored (17).

A purified L-methioninase obtained from the ion exchange step was applied to the Sephadex G-150 matrix of the gel filtration column. Then, using phosphate buffer with a pH of 7, elution was accomplished at a flow rate of 3 ml/fraction. The absorbance of each fraction was then measured at 280 after that. Peak fractions' L-methioninase activity was assessed (18).

2.6 Determination of Molecular Weight

The purified L-methioninase homogeneity was examined by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and was performed as described by Bollag *et al.*, 1996 using 12.5% polyacrylamide. Proteins were estimated by Coomassie Brilliant Blue R250 staining (19).

3-Results

3.1 Isolation and Identification of *P. aeruginosa*

On MacConky agar, 62 isolates from 150 samples could grow; the isolates were light-colored, non-lactose fermenting, and formed yellow colonies. Of these, only 33 isolates developed to grow on cetrimide agar and showed characteristics of *P. aeruginosa*, including smooth, mucoid colonies with flat edges, an elevated center, green or creamy-colored colonies, and a fruity odor. The other samples included different species of *Pseudomonas*, and these results agreed with 93.9% of the traits of *P. aeruginosa*.

All isolates performed biochemical testing, indicating they were positive for characteristics of the *P. aeruginosa* species (Table 1).

Table (1): Biochemical and cultural characteristics of *Pseudomonas* isolates

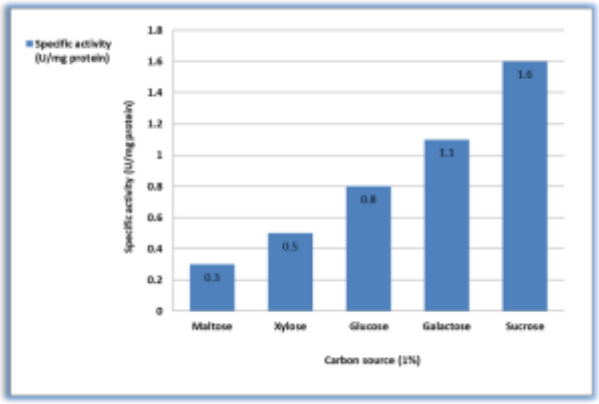
Cultural and Biochemical tests	Result	Cultural and Biochemical tests	Result
Growth under aerobic condition	+	Gram stain	-
Catalase	+	Shape	Rodes
Oxidase	+	Citrate	+
Indole	-	Growth on cetrimide	+
Urease	-	Lactose fermentation	-

3.2 Optimal conditions for L-methioninase production

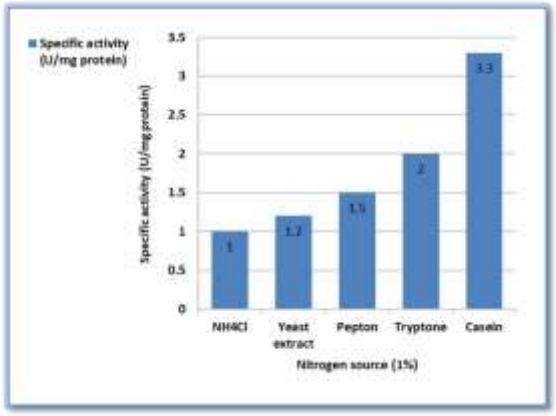
Various carbon sources were the primary influencing components (glucose, sucrose, maltose, xylose, and galactose) and were utilized as the sole source of energy and carbon to identify the best carbon source for *P.aeruginosa's* L-methioninase synthesis. The minimum medium was supplemented with each of these carbon sources individually at a concentration of 1% w/v. When exposed to maltose, the specific activity of the enzyme was low at 0.3 U/mg protein, thereafter increased with additional carbon sources until it reached the highest specific activity of 1.6 U/mg protein with sucrose; as a result, it was utilized as the main source of carbon in subsequent tests (Figure 1).

The optimum nitrogen source was investigated using yeast extract, peptone, NH4Cl, casein, and tryptone. It was separately added in a 1% w/v concentration to the production media. When casein was added, the maximum amount of L-methioninase was produced, and the specific activity increased to 3.3 U/mg proteins (Figure 2). The optimum pH value for the production of MGL was 7.0, and the specific activity for generating MGL at this pH was 3.4 U/mg proteins (Figure 3).

Among the various incubation temperatures, 37^{o}C produced the greatest amount of 3.4 U/mg protein-specific activities, as shown in (Figure 4), and that specific activity reached 4 U/mg over a 48-hour incubation period. However, after 48 hours, the activity progressively decreased until it reached 2 U/mg proteins after 72 hours (Figure 5).



Figure(1): Optimal carbon source for L-methioninase production from *Pseudomonas aeruginosa* after Incubation at 37°C for 48 hr.



Figure(2): Optimal nitrogen source for L-methioninase production from *Pseudomonas aeruginosa* after Incubation at 37oC for 48 hr.

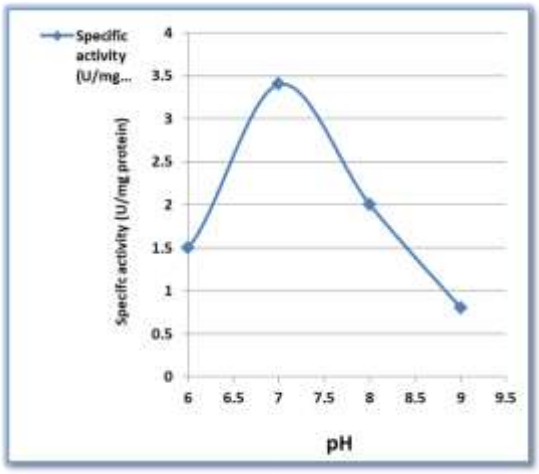


Figure (3): Optimal pH for L-methioninase production From *Pseudomonas aeruginosa* after incubation at 37°C for 48 hr.

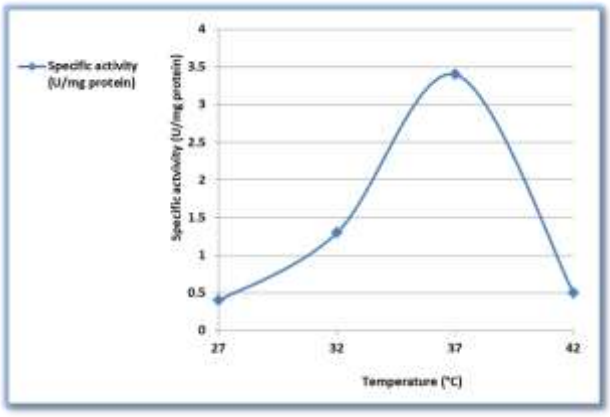


Figure (4): Optimal incubation temperature effected on L-methioninase production from *Pseudomonas aeruginosa* after incubation at 37°C for 48 hr.

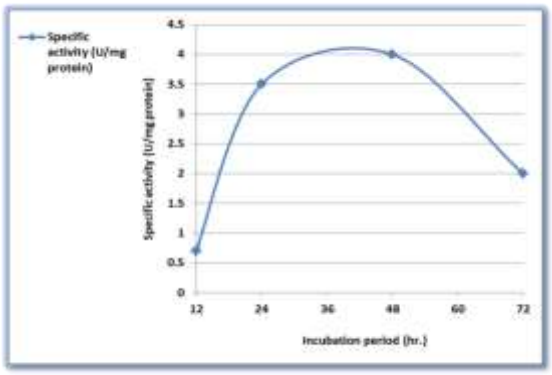


Figure (5): Optimal incubation period for *P.aeruginosa* producing L-methioninase at 37 °C.

3.3 L-methioninase purification

MGL produced from *P. aeruginosa* was purified using ammonium sulfate precipitation followed by ion exchange chromatography and gel filtration. Among the various fractions, the 70% protein fraction precipitated by ammonium sulfate exhibited 9.1 U/mg of L-methioninase activity with 2.2-fold purification. The protein was concentrated, dialyzed, and then further purified using a DEAE-cellulose ion exchange column. Anion exchange chromatography produced a specific activity of 10.7 U/mg for the enzyme; the active fractions were then mixed and added to a gel filtration column (figure 6). The protein was purified using a gel filtration column, and Phosphate buffer (pH7) was used to elute the enzyme from the Sephadex G-150 column, which resulted in a 4.6-fold improvement in purity and a 44.8% recovery rate (figure 7). Table 1 displays the summary of the purification stages.

Table (2): Purification steps for L-methioninase produced by *Pseudomonas*

Purification step	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg protein)	Total activity (U)	Purification (folds)	Yield (%)
Crude enzyme	**75**	**2**	**0.5**	**4**	**150**	**1**	**100**
Ammonium sulphate precipitation (70%)	**15**	**5.5**	**0.6**	**9.1**	**82.5**	**2.2**	**55**
DEAE-cellulose	**24**	**3**	**0.28**	**10.7**	**72**	**2.6**	**48**
Sephadex-G150	**24**	**2.8**	**0.15**	**18.6**	**67.2**	**4.6**	**44.8**

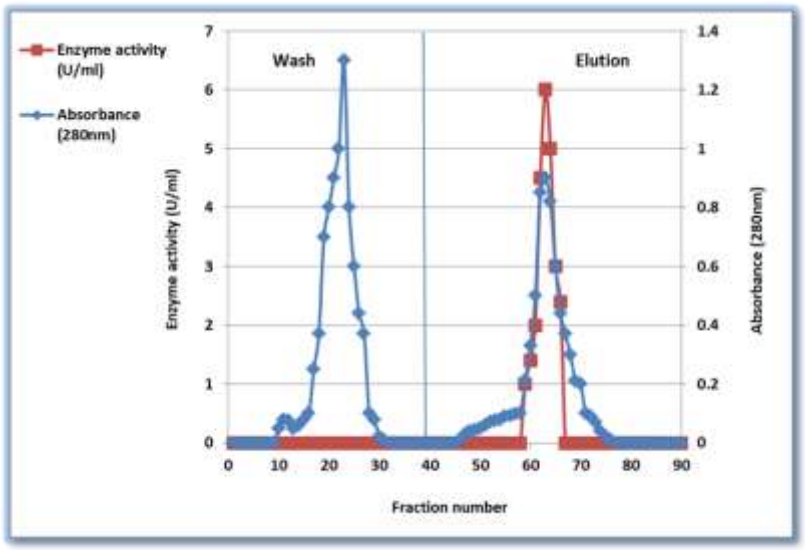


Figure (6): Ion exchange chromatography for L-methioninase ion exchange chromatography produced from *P. aeruginosa* A6 equilibrated via buffer pH7 with a 30ml/h flow rate.

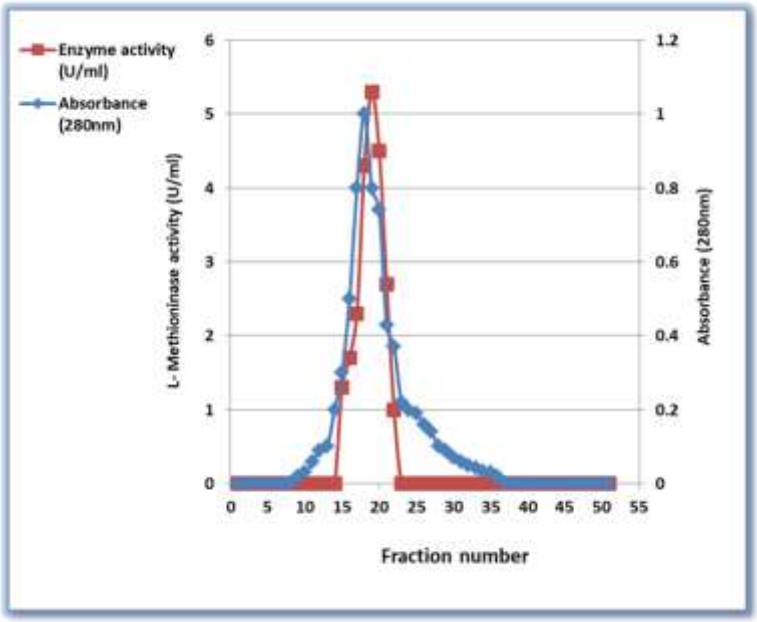


Figure (7): Gel filtration chromatography of L-methioninase production from *P. aeruginosa* A6 with the use of Sephadex G-150 column equilibrated by 0.05M sodium phosphate buffer pH 7 at 30 ml/h flow rate.

3.5 Determination of the Purity Degrees of L-methioninase Molecular Weight Assay

The purity of L-methioninase was assessed using polyacrylamide gel electrophoresis. Following the gel filtration phase, the purified L-methioninase from *P. aeruginosa* revealed a single band with a molecular weight of around 55 kDa, which interpolation in Figure (8).

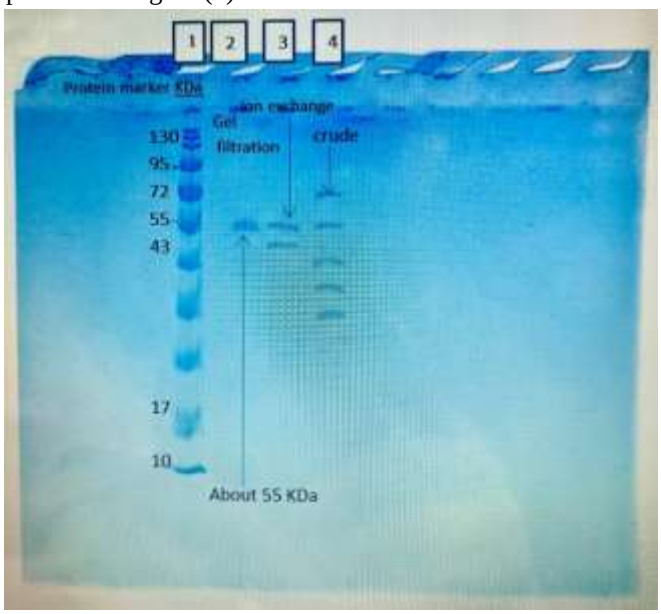


Figure (8): SDS Polyacrylamide gel electrophoresis of crude and purified L methioninase production from *P. aeruginosa*. (1): Proteins marker (2): Protein band after gel filtration step. (3): Protein band after ion exchange step.(4) Proteins bands in crude filtrate.

4-Discussion

Pseudomonas represents one of the most widespread and metabolically diverse bacterial genera. The results agreed with Jasim (2021), who showed that *P. aeruginosa* is considered among the most destructive clinical organisms and poses significant concerns regarding public health after collecting various *P.aeruginosa* from various sources. The variation in isolation rates across sample sources could be attributed to various factors such as the quality of medical treatment provided, the approach adopted to treat burn patients, the patient's prompt hospital admission, the fact that the patient had taken antibiotics beforehand, or the length of the patient's hospital stay (20). This proportion could rise in less than 72 hours if someone contracts an infection from a hospital through patient interaction or contaminated equipment that spreads the bacteria (21).

All isolates were subjected to biochemical tests. Leboffe and Pierce (2011) demonstrated that *P. aeruginosa* were all positive for the catalase and oxidase test and negative for the indole and urease test (22), while Shehab (2019) stated that the Simmon citrate test was positive for *P. aeruginosa* (23).

The highest production of L-methioninase in the medium was reached in the following conditions: sucrose (1.6 U/mg protein), casein (3.3 U/mg protein) in pH 7, and at 37^{o} C after 48 h incubation as the supplements. Carbon sources and other operational parameters have been shown to affect the ability of microorganisms to synthesize enzymes (24). Enzyme production and bacterial growth were greatly reduced without any carbon source. This suggests that, in order for *Trichoderma harzianum* to produce L-methioninase, a carbon source must be used as a co-dissimulator; much like *Aspergillus flavipes* does (4).

This study's results agreed with Abu-Tahon and Isaac (2016), who mentioned that sucrose produced outstanding results as the best carbon source for *P.aeruginosa's* L-methioninase production (25). However, *Hafnia alvei* has demonstrated that the maximum MGL production (49 U/m L) was obtained when galactose was its sole carbon source (8).

As previously observed by Selim *et al*. for *Geotrichum candidum* and *Candida tropicalis*, the production of L-methioninase was enhanced by several nitrogen sources, including yeast extract and peptone in the media (15). The pH of the culture medium changes when the concentration of hydrogen ions (H+) increases or decreases. Because H+ and/or OH compete with hydrogen bonds and ionic bonds in an enzyme, causing enzyme denaturation could result in significant alterations to the three-dimensional structure of proteins (26). El-Sayed (2011) reported a comparable result, stating that the neutral pH was the optimal range for L-methioninase activity produced by *Aspergillus fumigatus* (27); the optimum pH for *Methylobacterium* sp. to produce L-methioninase was 7.0 as well (28).

Any enzymatic process will experience a significant reduction in rate at temperatures above or below its optimal range. This could be because the enzyme has lost its three-dimensional structure or has become denatured. Breaking hydrogen bonds and other non-covalent bonds causes a protein to become denatured (26). Additionally, after 48 hours of incubation, the amount of L-methioninase was at its greatest; thereafter, the enzyme's production declined that, agreed with Alshehri (2020) (8), who reported after 48 hours of incubation, *H. alvei* produced the maximum yield of MGL enzyme. The decrease may be caused by product inhabitation or substrate restrictions (29).

Ionizable molecules can be separated using Ion-Exchange Chromatography (IEC) based on changes in their charge characteristics. The optical density (OD) of the resulting fractions at 280 nm was measured using a spectrophotometer. L-methioninase from *Aspergillus fumigatus* was purified using a DEAE-cellulose exchanger; the purified enzyme's specific activity was 23.74 U/mg, and its purification fold was 1.76 and 81.2% of recovery yield (30). In another study, the enzyme was purified from *Streptomyces* DMMMH60; the purification fold reached 2.16, and the specific activity 178.8 U/mg (31). Then purified with gel filtration in a previous study, discovered that using Sephadex G-100 gel filtration column, the specific activity of the L-methioninase recovered from *Alcaligenes aquatilis* was (7.66U/mg) and the degree of purity reached 7.15 (32). Purified *Streptomyces* DMMMH60 L-methioninase was shown by Abdelraof *et al*. (2019) to display a single band of 55 kDa subunits on

the SDS-PAGE (31). However, the L-methioninase produced by *Trichoderma harzianum* had a single band with an observed molecular weight of 48 kDa (4).

5-Conclusion

The present investigation's results indicate that *P. aeruginosa* is capable of producing L-methioninase, and the optimum conditions to accomplish this were examined by supplemented minimal salt M9 media with sucrose 1%, casein 1% in pH 7, and incubation temperature 37^{o}C for 48hr. L-methioninase was purified from *P. aeruginosa* using ammonium sulphate precipitation (70%), DEAE cellulose, and Sephadex G-150, with 4.6-fold with 44.8% recovery. According to SDS-PAGE electrophoresis, a single band at 55 kDa indicates that the purification process was successful.

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تحسين و تنقية انزيم المثيونينز المنقى من العزلات المرضية لبكتريا *Pseudomonas aeruginosa*

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الخلاصة

خلفية البحث: ركزت هذه الدراسة على بكتيريا *Pseudomonas aeruginosa* التي تفرز إنزيمات مهمة في التطبيقات الطبية والصيدلانية و التي تم عزلها من العينات السريرية. احد هذه الانزيمات هو إنزيم L-methioninase الذي يعد اكثر الإنزيمات المرغوبة المستخدمة في المجالات الطبية والصناعية اليوم حيث يحول L-methionine إلى ميثانيثيول وأمونيا وألفا كيتوبوتيرات. **الأهداف**: تقييم الظروف المثلى لإنتاج إنزيم L-methioninase ومن ثم تنقيته. **الطرق ومواد العمل**: تحديد عينات *Pseudomonas* عن طريق الاختبارات المجهرية والكيميائية الحيوية، بالإضافة إلى تأكيد التحديد بنظام VITEK2. بعد فحص إنتاج l-methioninase، تم تحديد الظروف المثالية لإنتاج L-methioninase (MGL) من خلال العينات السريرية لبكتيريا *Pseudomonas aeruginosa*. بالإضافة إلى ذلك، تمت تنقية MGL من راشح *P. aeruginosa* باستخدام ترسيب كبريتات الأمونيوم، وDEAE-السليلوز، وSephadex G150. **النتائج**: تم الحصول على 33 عزلة من بكتيريا *P.aeruginosa* حسب الصفات المجهرية والاختبارات الكيموحيوية، وأتبعها نظام VITEK2 للتأكد من التشخيص. تم تحقيق ظروف التحسين عند استخدام الوسائط التكميلية مع السكروز 1% وزن\حجم، الكازين 1% وزن\حجم، الرقم الهيدروجيني 7، درجة الحرارة 37 درجة مئوية والحضانة لمدة 48 ساعة ضمن النشاط المحدد 1.6، 3.3، 3.4، 3.4، و 3.5 وحدة\ ملغ. البروتين، على التوالي، و4.6 أضعاف كمية الإنزيم المنقى مقارنة بالإنزيم الخام. يشير النطاق الواحد 55 كيلو دالتون الموجود على SDS-PAGE إلى أن الإنزيم قد تمت تنقيته. **الاستنتاجات**: يمكن أن تكون *P.aeruginosa* مصدراً فعالاً لإنتاج إنزيم l-methioninase، وكان لهذا الإنزيم نشاط نوعي أفضل وأعلى بعد تطبيق الظروف المثلى له وتنقيته.

الكلمات المفتاحية: L-ميثيونيناز، التحسين، التنقية.