Screening and Optimization of L-Methioninase Production by Gram-Negative Bacteria from Various Hospitals in Baghdad City

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Abstract

L-methioninase, a pyridoxal 5′-phosphate dependent enzyme that catalyze degradation of L-methionine to ammonia, methanethiol and α-ketobutyrate. L-methioninase had received a lot of interest for its importance, so to improve the production of L-methioninase, media optimization was done. In this study, clinical isolates of gram-negative bacteria have been collected from several hospitals located in Baghdad city, these isolates were *Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Acinetobacter baumannii* and *Serratia marcescens.* The screening for detecting L-methioninase production was performed by semi-quantitative and quantitative analysis, the results revealed that out of all obtained isolates, 31(41.33%) were L-methioninase producers from semi-quantitative screening while by quantitative method only 16 isolates out of these 31 isolates revealed specific activity ranged from 0.19 to 1.15 U/mg and the maximum specific activity was for *E. coli U8*, which was chosen as best producer isolates. The L-methioninase activity reached its maximum level when *E. coli U8* was cultivated with the best conditions, which is consisted of using modified mineral salt M9 broth medium supplemented with L-methionine mixed with galactose (2 g/L) as carbon source and L-glutamine (1.5 g/L) and incubated at 37°C for 48 hours at pH 7.

1. Introduction

L-methioninase [EC 4.4.1.11], also known as L-Methionine γ-lyase (MGL), methionase, L-methionine-γ-demethiolase, L-methionine methanethiol-lyase (deaminating) and L-methionine-α-deamino-γ-mercaptomethane-lyase, is a pyridoxal phosphate (PLP)-dependent enzyme that catalyze the conversion of L-methionine to ammonia, methanethiol and α-ketobutyrate through α,γ-elimination reaction [1].

L-methioninase is stimulated by the addition of L-methionine to the medium and is regarded as a key enzyme in catabolism methionine. A-ketobutyrate, a main product of L-methionine catabolism [2]. L-methioninase is an intracellular enzyme in utmost of the bacterial isolate from different origins. Gram-negative and gram-positive bacteria are both L-methioninase enzyme producers, gram-negative bacteria acquired further prominence as contrasted to gram-positive. L-methioninase is considered as the essential enzyme for L-methionine amino acid metabolism in bacteria [3].

L-methioninase was found in different sources such as bacteria, protozoans, fungal, archaeon andplants except mammals (human) [4]. This enzyme has been demonstrated to be present in various bacteria, such as *Pseudomonas* spp. [5], *Serratia marcescens, Bacillus subtilis, E. coli, Klebsiella oxytoca, Klebsiella pneumonia, Citrobacter intermedius, Citrobacter freundii, Aeromonas* sp. [6], *Brevibacterium linens* [7], *Clostridium sporogenes* [8], and the plant *Arabidopsis thaliana* [9]. Fungus like, *Aspergillus flavipes, Penicillium notatum, Fusarium solani*. Also, in the protozoan *Trichomonas vaginalis* [10] and *Entamoeba histolytica* [11]. The aim of this research was to investigate the L-methioninase activity in some gram-negative bacteria, in addition to optimizing production conditions.

2. Materials and Methods

2.1. Ethical Statement
The current study was conducted in accordance with ethical guidelines provided by the College of Science ethic committee of Mustansiriyah University for *in vitro* research(Ref: BCSMU/1221/00032M) did not involve direct experimentation on human or animal subjects. The clinical bacterial isolates used in this research were obtained from previously collected clinical samples in collaboration with Baghdad hospital, Alkadhmiya teaching hospital, Alkarama teaching hospital, Imam Ali hospital, Al-Kindy teaching hospital, and Al-Yarmuk hospital, following ethical protocols to ensure patient anonymity and sample integrity. Permission was obtained from the patients prior to sample collection, and participants were fully informed about the purpose and procedures of the study, and participation was voluntary, with the right to withdraw at any time without any repercussions.

2.2. Collection and Identification of Bacterial Isolates
Seventy-five clinical isolates for different gram-negative bacteria (*Escherichia coli, Pseudomonas aeruginosa, Klebsiella pneumonia*, *Acinetobacter baumannii*, *Serratia marcescens*) were collected from hospitals from different clinical sources, including: urine, blood, sputum, pus, burn, wound & ear swabs and diagnosed depending on the microscopic characteristics, cultural characteristics [12,13], biochemical tests [14], and then examined by VITEK 2 compact system.

2.3. Screening Methods for L-Methioninase Production
2.3.1. Semi-Quantitative Analysis
All bacterial isolates were tested for their ability of L-methioninase production by a rapid plate assay procedure using a modified M9 agar medium, L-methionine 5 g/L, $MgSO_4. 7H_2O$ 0.25 g/L, NaCl 0.5 g/L, $CaCl_2$ 0.014 g/L, $Na_2HPO_4.2H_2O$ 6 g/L, KH_2PO_4 3 g/L, agar 20 g/L, Glucose 2 g/L., pH 7±0.2 and just before pouring the plates, phenol red was added to the medium as an indicator at a final concentration of 0.009%. The plates were incubated in an inverted position at 37°C for 48 hours. All bacterial colonies that produced L-methioninase were chosen on the basis of creating a yellow zone surrounding the colonies. Well, isolated colonies purified, and subcultures from each plate were chosen for further studies [15].

2.3.2. Quantitative Analysis
The selected isolates were inoculated in modified M9 broth medium and incubated at 37 ℃ for 24 h 10 ml of activated bacteria was inoculated in 100 ml of modified M9 broth medium and incubated at 37℃ for 48 h, the cells were precipitated by cooling centrifuge at 4000 rpm for 30 min., the supernatant discarded and the pellet washed twice with 20mM potassium phosphate buffer at pH 7.2. The cells were resuspended in Potassium phosphate (20mM, at pH 7.2)-EDTA-PMSF-2-mercaptoethanol buffer and the L-methioninase was extracted from bacterial cells by rupturing with glass beads, then the activity of L-methioninase was determined [16].

2.4. L-Methioninase Assay and Protein Quantification
The L-methioninase activity was measured by using a partially modified 3-methyl-2-benzothiazolinone (MBTH) stopped spectrophotometric rate determination method [17, 3]. 0.02 ml of L-methioninase enzyme was added to tube containing 2 ml of potassium phosphate buffer (100 mM at pH 7) containing 25 mM L-methionine and 0.01 mM pyridoxal 5-phosphate which equilibrate to 37 ℃, mixed and incubated at 37 ℃ for exactly 10 min. The reaction was stopped by adding 0.25 mL of 50% TCA. One ml of the test reaction was transferred to other new tube contained 2 ml of sodium acetate buffer (1 M at pH 5) then 0.8 ml of (MBTH) was added with mixing and incubated at 50 ℃ for exactly 30 min then incubated at 25 ℃ for 30 min. The amount of α-ketobutyrate produced was determined spectrophotometrically based on the increase in absorbance at 320 nm from the blank.

Protein quantification in the test isolates was carried out by Bradford protein assay method [18] with bovine serum albumin as standard. L-methioninase's specific activity was determined in units per milligram of protein [19].

2.5. Determination the Optimal Conditions for L-Methioninase Production

L-methioninaseproduction from the selected isolate was optimized by changing one factor at a time in Erlenmeyer flasks (250 ml) with 100 ml of the modified M9 broth medium. The medium was inoculated with 10 ml of the bacterial seed medium. In the procedure of optimization, different factors were tested subsequently, and the best alternative of the first test was chosen for the next step. In this study the process parameters optimized and included incubation temperature (4-45°C), pH (4-9), incubation period (24-96 h), inoculum size (1-10 %), carbon sources (fructose, galactose, glycerol, pomegranate juice wastes, whey and dates at 2g/L), best carbon source concentration (0.5, 1, 1.5, 2 and 2.5 g/L), nitrogen sources with and without L-methionine (yeast extract, peptone, potassium nitrate, sodium nitrate, L-glutamine and L-asparagine), and best nitrogen source concentration (0.5, 1, 1.5, 2 and 2.5 g/L).

3. Results and Discussion
3.1. Collection and Identification of Bacterial Isolates
The bacterial isolates examined depended on its cultural, microscopic and biochemical characteristics. All gram-negative bacteria (21*Escherichia coli,* 21 *Pseudomonas aeruginosa, 18 Klebsiella pneumonia*, 10 *Acinetobacter baumannii*, *5 Serratia marcescens*) grew on MacConkey agar. On MacConkey agar, *E. coli* colonies due to lactose fermentation were large bright pink colonies, raised low convex with an entire edge, circular. *Klebsiella pneumoniae* colonies were pink, mucoid and lactose ferment. *Acinetobacter baumannii* were small, pale color due to non-lactose fermentation. *Serratia marcescens* colonies were smooth, raised, circular and pale in color due to non-lactose fermentation. While *Pseudomonas aeruginosa* was small, pale-yellow colonies due to non-lactose fermentation. These features were mentioned by [12, 13, 20].

According to biochemical tests all bacterial isolates gave catalase positive and oxidase negative except *Pseudomonas aeruginosa* that was oxidase positive [14, 21].

3.2. Screening for L-Methioninase Production
In this study, the bacterial isolates were screened for L-methioninase production in two methods; Semi-quantitative and quantitative analysis

3.2.1. Semi-Quantitative Analysis
In semi-quantitative screening modified M9 agar medium was used for production of L-methioninase by all bacterial isolates. Depending on Table (1), only 31 isolates possess the ability to produce L-methioninase. Most L-methioninase producers from *E. coli* followed by *pseudomonas aeruginosa* and *K. pneumonia*.

Table (1): Numbers and Ranges of diameter zone for L-methioninase producers and non-producers' bacterial isolates.

Bacterial isolates	No. of isolates	No. of non-producers isolates	No. of producers isolates	Range of diameter zone (mm)for producers isolates
E. coli	21	5	16	11-22
P. aeruginosa	21	11	10	9-20
K. pneumoniae	18	13	5	7-21
A. baumannii	10	10	-	-
S. marcescens	5	5	-	-

According to the results there were weak, moderate and strong producers for L-methioninase that were surrounded with visible yellow zone in various levels as in Figure (1A 1, 2, & 3). In contrast the negative

isolates had not shown hydrolysis yellow zone as revealed in Figure (1A 4). *E. coli U8* was the best producer for L-methioninase with highest diameter (22 mm) and intensity yellow zone as in Figure (1B).
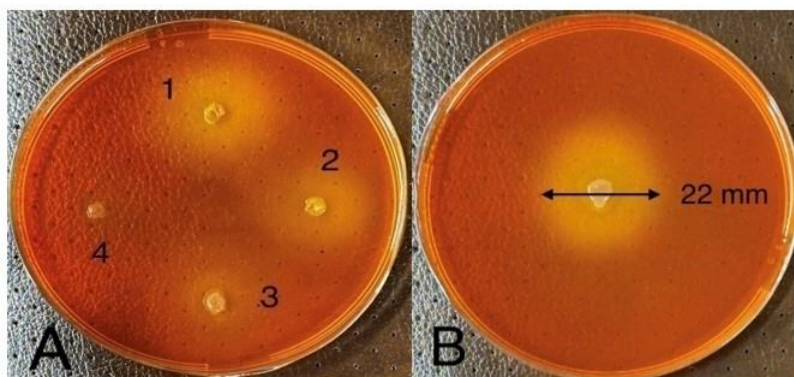

Figure (1): (A) (1) strong producer, (2) moderate producer, (3) weak producer, and (4) non producer. **(B)** *E. coli U8* producer isolate on Modified M9 agar medium.

The basic pH change is shown by phenol red, which turns yellow in acidic conditions and red in alkaline condition [22]. Similarly, this technique is used to study the dissimilation of L-methioninase for L-methionine. Therefore, on the modified M9 medium, the isolates showing the yellow zone were selected for primary detection. Because of the production of α-keto butyric acid, this causes a pH decrease, which causes the color to change to yellow [23, 24, 25].

3.2.2. Quantitative Analysis
In the quantitative method we selected only 16 isolates out of these 31 isolates that gave a bigger hydrolysis yellow zone. These 16 isolates revealed various levels in production of L-methioninase with specific activities ranged from 0.19 to1.15 U/mg with maximum L-methioninase activity 1.74 U/ml and 1.15 U/mg of specific activity by *E. coli* U8 as shown in Figure (2).
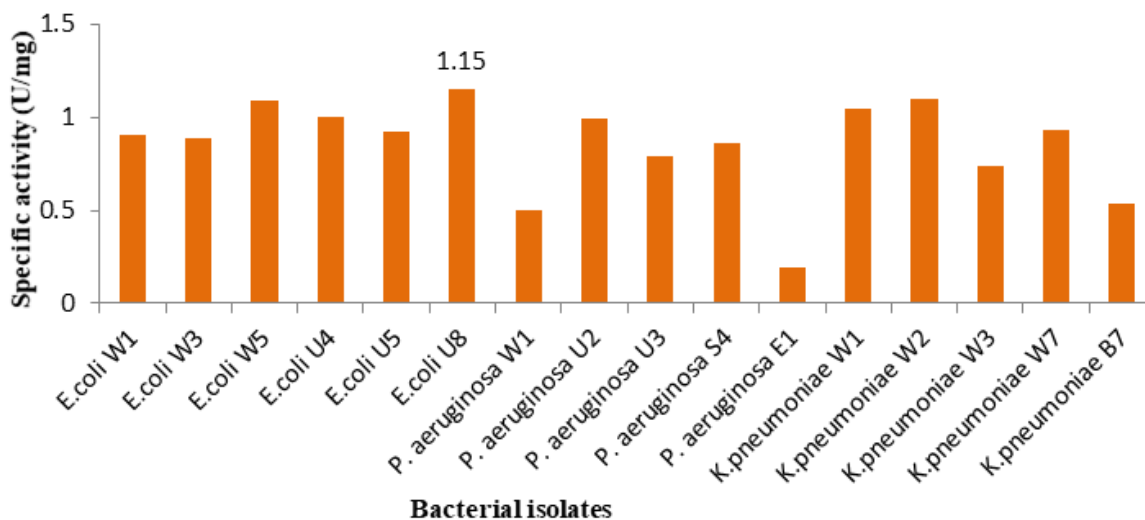
Figure (2): Specific activities for selected isolates in quantitative method by using Modified M9 broth.

The amount of α -ketobutyrate produced was determined spectrophotometrically with the help of MBTH (3-methyl-2-benzothiazoline hydrazone). MBTH reacts with the end product α-ketobutyrate to form colored products [26].

Bopaiah*et al*., (2020) Considered that L-methioninase from *Bacillus haynesii* which isolated from different locations, that produced a larger yield of L-methioninase and it chosen based on hydrolytic zone formation around the colonies and highest activity level of L-methioninase was recorded with 7.38 U/ml and 9.22 U/mg of specific activity [27].

3.3. Optimization of Environmental Conditions for Maximum L-Methioninase Production
3.3.1. Effect of Incubation Temperature
Figure (3) reveals the effect of various incubation temperatures (4, 25, 30, 37, 40 and 45 °C) on L-methioninase production by *E. coli U8*. Maximum L-methioninase production was obtained at 37℃. At this temperature the specific activity was 1.16 U/mg protein.

Researchers mentioned that during low temperature L-methioninase yield was reduced because of the cell membrane decreased ability to transfer their substrate and mesophilic temperature led to optimum L-methioninase yield since L-methioninase production stated with increasing of the reaction rate and increasing kinetic energy of reacting molecules. In contrast, the higher temperatures induced denaturation of metabolic pathways which increased the energy needed for cellular growth maintenance, which resulted in lower production of metabolites [28].

Alshehri, (2020) found that the optimum temperature for L-methioninase production from *Hafnia alvei* and *Pseudomonas putida* was 35°C and 37°C respectively [29]. In contrast, Selim *et al.,* (2015b) reported maximum L-methioninase production by *Candida tropicalis* is observed at temperature 45°C [30].
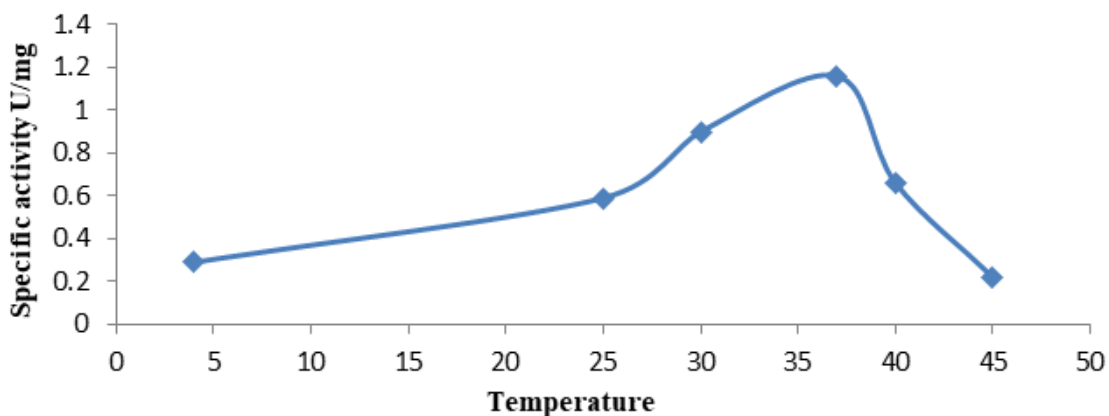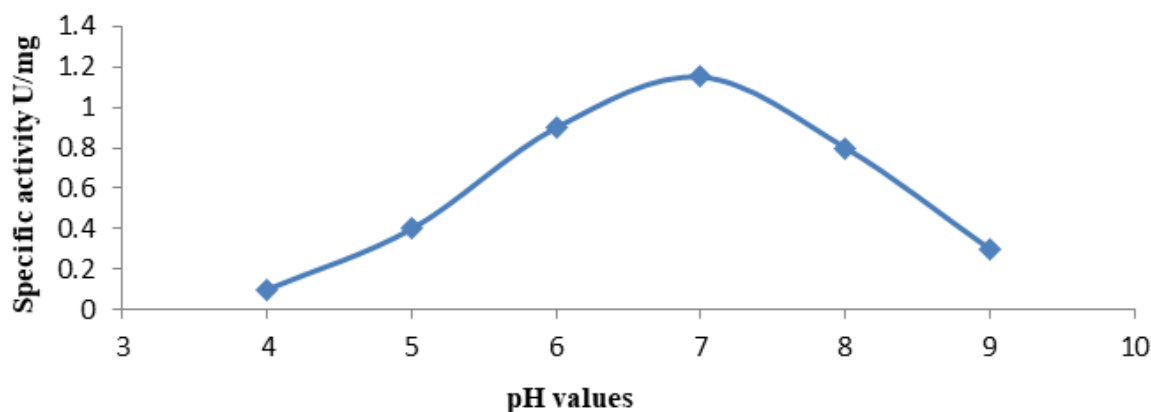

Figure (3): Effect of incubation temperature on L-methioninase production.

3.3.2. Effect of pH
The optimal pH for L-methioninase production by *E. coli* U8 was 7 with L-methioninase specific activity 1.15 U/ mg than other pH values, Figure (4).

Changes in the acidity or pH of the environment can alter or totally inhibit the enzyme from catalyzing a reaction. This change in pH will affect the non-polar and polar intramolecular attractive and repulsive forces and alter the enzyme shape and the active site as well to the point where the substrate molecule could no longer fit, and the chemical change would be inhibited from taking place as efficiently or not at all [31, 32]. Selim *et al.,* (2015b) noticed that the maximum production of L-methioninase from *Pseudomonas ovalis* when the medium of production was adjusted to pH 7.2 [30].

Figure (4): L-methioninase production by *E. coli* U8 at various pHs.

3.3.3. Effect of Incubation Period

Results revealed that the incubation time led to increase the L-methioninase specific activity and reached the maximum value (1.16 U/mg) after 48 h of incubation, Figure (5). Al-Zahrani *et al.,* (2019) recorded that the highest amount of enzyme from *Pseudomonas extremaustralis* was after 48h of incubation. The maximum yield of enzymes could only be achieved after a certain time of incubation allowing the culture to develop in a stable state, and later activity will be reduced. The reduction could be related to product inhibition or substrate limitation [28].
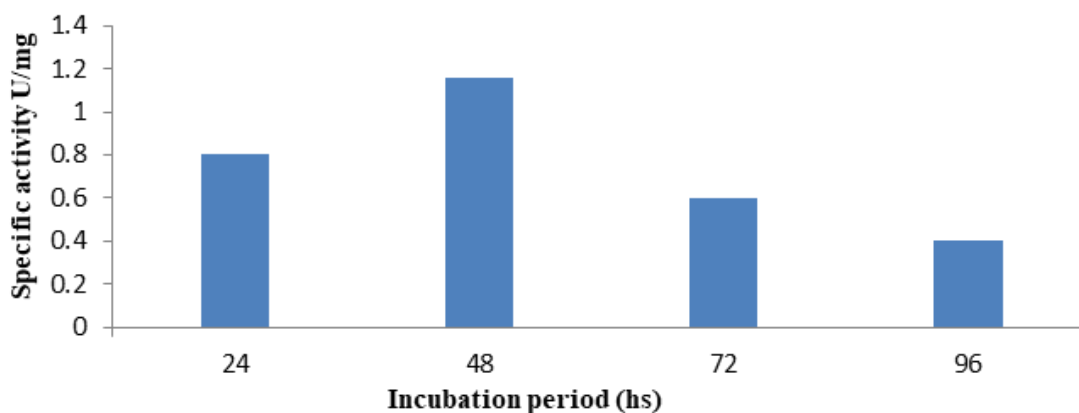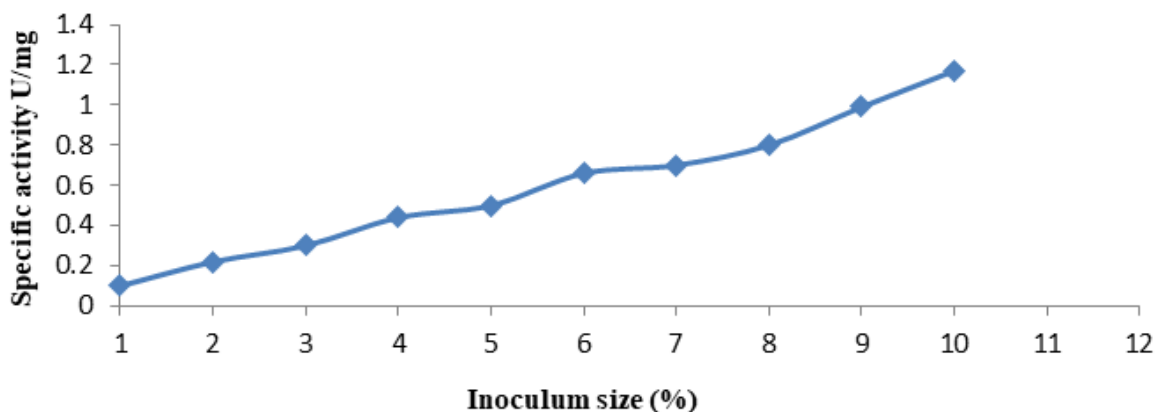

Figure (5): L-methioninaseproduction by *E. coli* U8 incubation in different periods.

3.3.4. Effect of Inoculum Size

Figure (6) revealed that production of L-methioninase increased with increasing the inoculum size and reached the highest point of production at 10%, with (1.17 U/mg) specific activity.

Lower level of inoculum may not be sufficient for the growth and enzyme synthesis on different substrates as increased number of cells ensures enzyme synthesis and a rapid proliferation of biomass [33].

Alshehri (2020) revealed that the maximum L-methioninase activity was at inoculum size 10% of *Hafnia alvei* [29], while Sharma *et al.,* (2018) showed that maximum L-methioninase activity was showed at 2% inoculum size from *Klebsiella oxytoca* [34].

Figure (6): L-methioninase production by *E. coli* U8 at different inoculum sizes.

3.3.5. Effect of Carbon Sources

The result in Figure (7) showed that among six different carbon sources, the galactose was the best carbon source and provided higher L-methioninase specific activity 1.20 U/mg. Zhang *et al.,* (2019) observed that galactose and lactose were the best carbon sources for a bacterium *Lactobacillus plantarum* to enhance L-methioninase production in comparison with other saccharides [35].
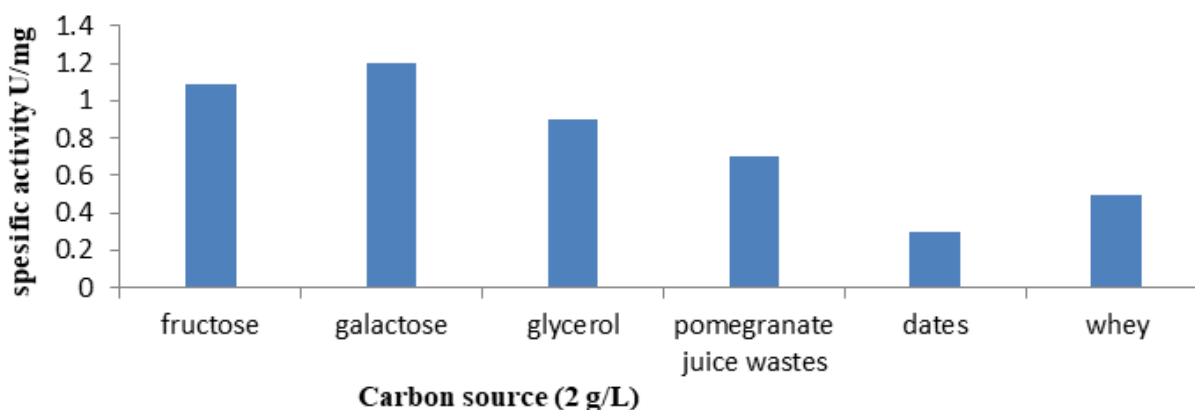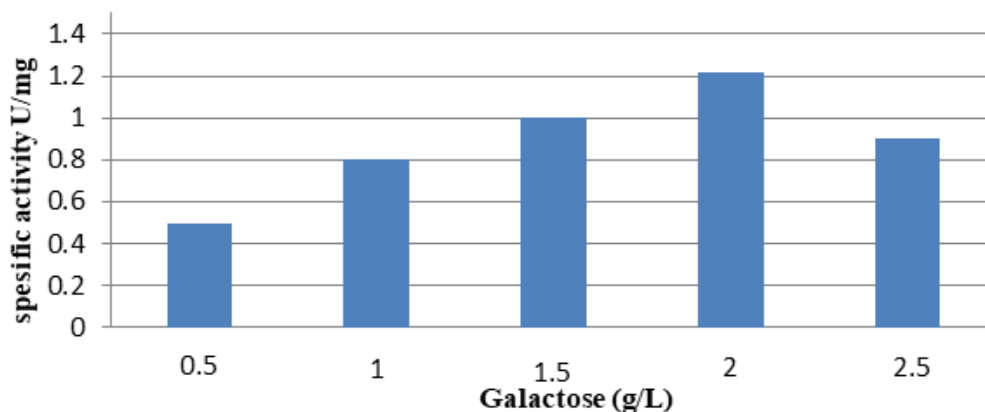

Figure (7): L-methioninase produced by *E. coli* U8 at different carbon sources.

3.3.6. Effect of Best Carbon Source Concentration

Figure (8) demonstrates the effect of carbon concentrations on the L-methioninase production and found that 2g/L of galactose was the most suitable for L-methioninase production and the specific activity was 1.22 U/mg protein at this concentration.

Environmental conditions and nutrition significantly affect the composition and microbial synthesis. Nitrogen and carbon sources generally play a significant role, because these nutrients are directly linked to the metabolite biosynthesis and cell proliferation. Also, can regulate the secondary metabolism by phenomena such as catabolic repression [36].
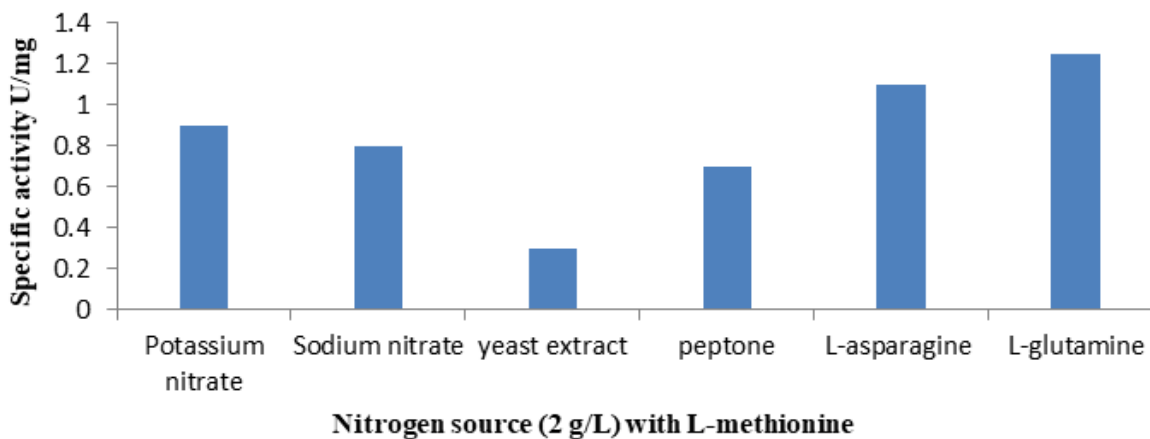
Figure (8): L-methioninase production by *E. coli* U8 at different galactose concentrations.

3.3.7. Effect of Nitrogen Sources with and without L-Methionine

The results showed that L-glutamine with L-methionine demonstrated to be the best for L-methioninase production from *E. coli* U8 with 1.25 U/mg protein of specific activity, Figure (9).

Nitrogen source is the principal nutrient after carbon that is crucial for the growth of microorganisms in larger amounts. Nitrogen is an essential part in enzymes, nucleotides, protein and a cofactor which plays a main role in the metabolism [37]. Abu-Tahon and Isaac (2016) found the same results that the L-glutamine yielded the highest L-methioninase activity [24]. On the other hand, Alshehri (2020) revealed that L-asparagine was the best one as the sole nitrogen source with best L-methioninase activity in comparison to other nitrogen sources [29].
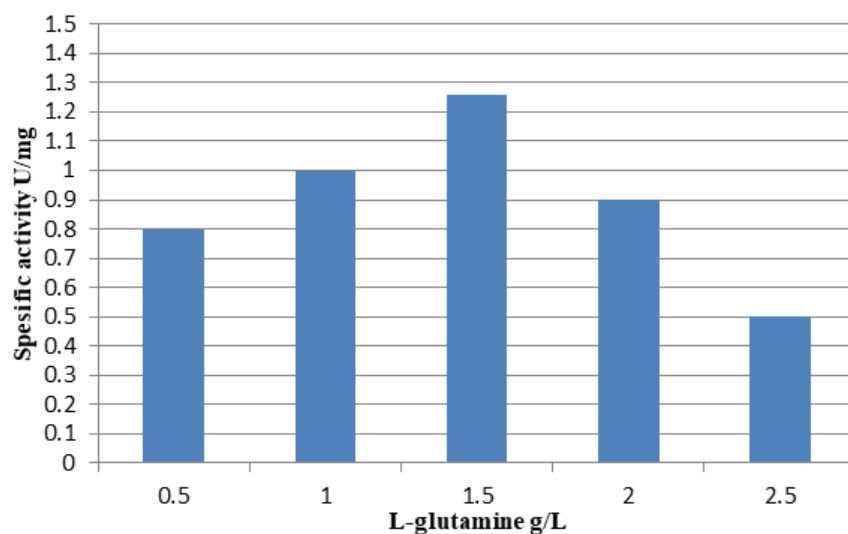

Figure (9): L-methioninase production by *E. coli* U8 at different nitrogen sources with L-methionine.

3.3.8. Effect of Best Nitrogen Sources Concentration

The results obtained in Figure (10) showed that the best productivity was at 1.5 g/L of concentration with 1.26 U/mg of specific activity.

Figure (10): L-methioninase production by *E. coli* U8 at different L-glutamine concentrations.

4. Conclusions

Some gram-negative bacteria were collected from different clinical sources and tested for L-methioninase production. Most of L-methioninase producers were *E. coli* then *pseudomonas aeruginosa* and *K. pneumonia*. In contrast, *Acinetobacter baumannii* and *Serratia marcescens* had not ability to L-methioninase production. Optimization of media was carried out to enhance the L-methioninase production by *E. coli*, by using modified mineral salt M9 broth medium with L-methionine mixed with galactose (2 g/L) as carbon source and L-glutamine (1.5 g/L) and incubated at 37°C for 48 hours at pH 7.

Conflict of Interest: The authors declare that there are no conflicts of interest associated with this research project. We have no financial or personal relationships that could potentially bias our work or influence the interpretation of the results.

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