



Optimization of Production of the Anticancer Agent L-methioninase by *Pseudomonas extremaustralis*

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ABSTRACT

The present study aim optimization *Pseudomonas extremaustralis* production of L-methioninase. The bacterial isolate ability for L-methioninase production was tested on a modified mineral salt M9 agar medium, using phenol red as the pH indicator. Result of optimization L-methioninase production, the cultural and nutrition parameters revealed to the maximum amount of L-methioninase produced by *P. extremaustralis* were 0.192U/min/ml, obtained after 48h at 35°C of incubation in shaking incubator (150rpm) in culture supplemented with L-methionine mixed with L-glutamine (0.15%), in the presence of glycerol (0.3%) as carbon source at pH6.

Key Words: L-methioninase, methionine, antitumor agent, anti-cancer, *Pseudomonas extremaustralis*.

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INTRODUCTION

MGL production involves one-step degradation of L-methionine by versatile enzyme L-methionine g-lyase. A pyridoxal -phosphate-dependent enzyme catalyzing α, γ - elimination of L-methionine to α -ketobutyrate, methanethiol and ammonia and that were existed in many bacteria such as *Pseudomonas putida* [1]. Tumor freed from efficient methionine synthase and external methionine comes from the diet [2]. L-methioninase is a therapeutic agent against tumors [3, 4]. Methionine reduction has a broad spectrum of antitumor activities [5]. L-methioninase found in most organisms except mammals. Moreover, found in the cell-free extract [6,7] and many bacteria species as intracellular enzyme [8], in bacteria, intracellular L-methioninolytic enzyme have extra limiting step during the scale production while in fungi was both intracellular and extracellular [9].

Aim:

Bacteria screening of L-methioninase in the Saudi marine environment, the enzyme production was estimated by the

Nesslerization method, the highest enzyme production was obtained from a seashell sample, then the optimization of the production conditions for maximum L-methioninase production.

MATERIALS AND METHODS

Bacterial isolate:

Pseudomonas extremaustralis obtained from other studies by Al-Zahrani and Bukhari, (10).

Optimize L-methioninase production by *Pseudomonas extremaustralis*

Effect of incubation conditions:

A loop full of a 24h culture of the selected bacterial isolate was inoculated with 30ml of a sterile seed medium in a test tube and incubated at $35 \pm 2^\circ\text{C}$ in a constant incubator for 24h. The optimization of enzyme production was evaluated by varying one factor at a time.

For the best incubation conditions for L-methioninase production by *Pseudomonas extremaustralis*, a modified

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M9 medium; $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ 6, KH_2PO_4 , NaCl 0.5, L-methionine 1, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.25, CaCl_2 0.014, Glucose 2 g/L was used as the fermentation medium. The bacterial cultures incubated at $35 \pm 2^\circ\text{C}$. L-methioninase in the cell-free filtrate was assayed after 72h of incubation.

Best incubation temperature and fermentation period were studied, cultures were incubated at different temperatures ($25\text{--}40 \pm 2^\circ\text{C}$). L-methioninase in cell-free filtrate CFF was assayed after 24, 48 and 72h. Different pH (4-8) were adjusted to determine the best pH value on L-methioninase production.

Determine the carbon and nitrogen nutrition to optimize L-methioninase production

Optimizing L-methioninase production, the most suitable carbon and nitrogen sources and concentrations were studied. For the best carbon source, different carbon at 2g/L was added to the fermentation medium. The optimal concentration of carbon for L-methioninase production was determined, different concentrations (1-9g/L) of the best carbon source were added. The optimal concentration of L-methionine was determined by using different concentrations (0.5–1.5g/L) of L-methionine.

For the best nitrogen source, different amino acid and organic sources (1.25g/L) were added to the fermentation medium.

Mixed of L-methionine with different organic sources (tryptone, peptone and yeast extract) and different of amino acids (L-form), (asparagine and glutamine) with L-methionine mixed with different nitrogen sources (1.25g/L) were used.

Mixed of L-glutamine and peptone (0.75-1.75g/L) were added to the fermentation medium, in the presence and absence of glycerol (3ml).

The influence of different inorganic nitrogen sources was studied. Inorganic sources as KNO_3 , NaNO_3 and $(\text{NH}_4)_2\text{SO}_4$ were substituted individually in place of L-methionine.

The optimal concentration of the inorganic nitrogen source for L-methioninase production was determined. Different concentrations (1–1.45g/L) of the best inorganic nitrogen source were tested. The cultures were prepared in duplicates and were incubated at the propriety temperature, the CFF culture was assayed by Nessler assay for L-methioninase after different incubation periods in hours. The culture without L-methionine (substrate) was set as control.

L-methioninase assay by Nesslerization method

L-methioninase activity was determined by the Nesslerization method [11] with modifications. The standard reaction was 1 ml of 1% L-methionine in 0.5M phosphate buffer (pH 7.0), 0.1ml of pyridoxal phosphate, and 1ml of crude enzyme and incubated at 30°C for 1 h. and stopped activity by adding 0.5ml of 1.5mol/L

trichloroacetic acid. And centrifuged at 5,000rpm for 5min to remove precipitated protein and added to 3.7ml of distilled water where released ammonia was determined using 0.2ml of Nessler reagent. L-methioninase was defined as liberates ammonia at $1\mu\text{mol}/\text{min}$ under optimal assay conditions. Specific activity for L-methioninase expressed as the enzyme in terms of units per milligram of protein [9].

RESULTS:

Effect of incubation periods and different temperatures on L-methioninase production

The highest amount of the enzyme was accumulated when the culture incubated at $35 \pm 2^\circ\text{C}$ 0.05U/min/ml by *Pseudomonas extremaustralis* after 48h of incubation, but higher or lower of this degree, the production of the enzyme was decreased, results in Figures (1).

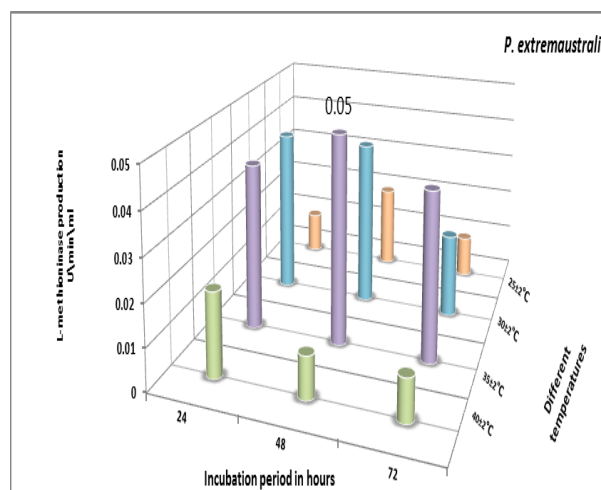


Fig. 1: Effect of incubation periods and temperatures on L-methioninase production

Different carbon sources effect on L-methioninase production

Different carbon sources (Glucose, Mannitol, Sucrose, Xylose, Maltose, Lactose, Galactose, Fructose, and Glycerol) Effect on L-methioninase production by the selected isolate of bacteria were studied.

From Data, Figure (2) obviously, the glycerol was the best carbon source for the highest amount of L-methioninase by *Pseudomonas extremaustralis* 0.06U/min/ml followed by xylose 0.04U/min/ml and mannitol, galactose and fructose 0.03U/min/ml, compared to control 0.009U/min/ml.

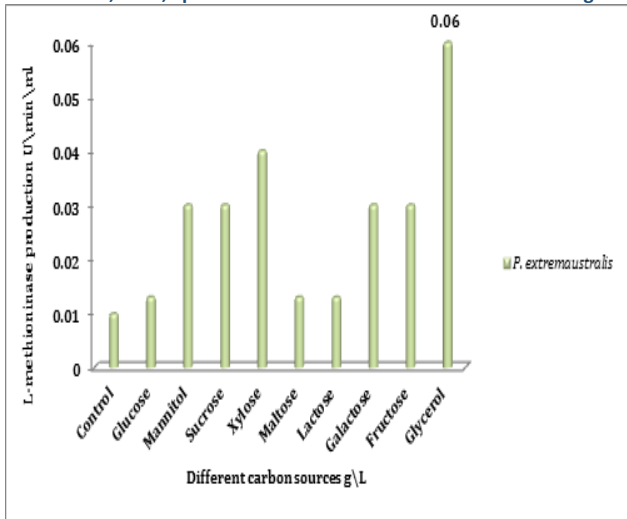


Fig. 2: Different carbon sources effects of on L-methioninase production

Effect of carbon source concentrations on L-methioninase production

The maximum amount of L-methioninase production 0.08 U/min/ml was obtained when 3 ml of glycerol was added to the fermentation medium Figure (3).

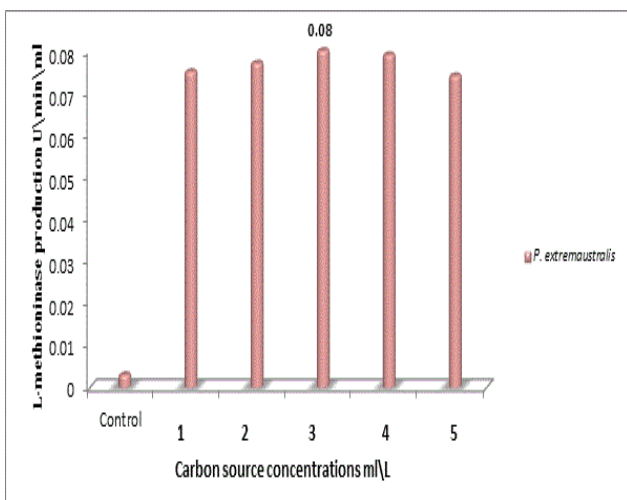


Fig. 3: Effect of carbon source concentrations on L-methioninase production

Effect of pH on L-methioninase production

Result in Figure (4) revealed the maximum productivity of L-methioninase by *Pseudomonas extremaustralis* by decreasing the pH value of the medium to pH 6, it was 0.09 U/min/ml. These amounts decreased at lower or above pH 6 of the fermentation medium.

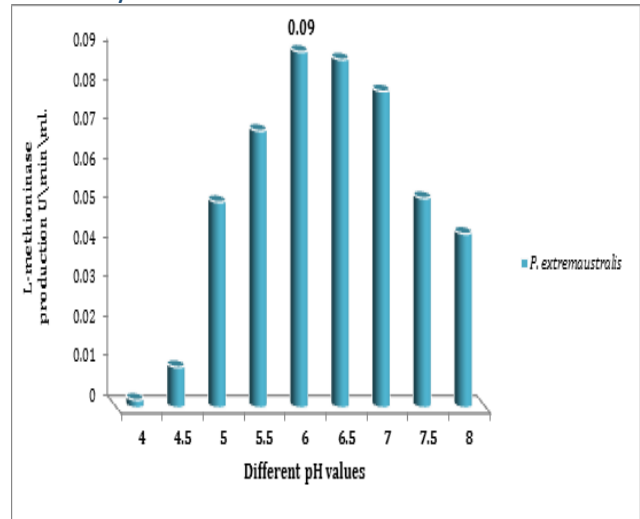


Fig. 4: The effect of pH on L-methioninase production

Effect of different L-methionine concentrations on the production of L-methioninase

Results in Figure (5) show that the highest L-methioninase was 0.09 U/min/ml was produced by *Pseudomonas extremaustralis* when 1.25 g/L of L-methionine was added to the medium. The enzyme production was reduced by increasing of L-methionine concentration.

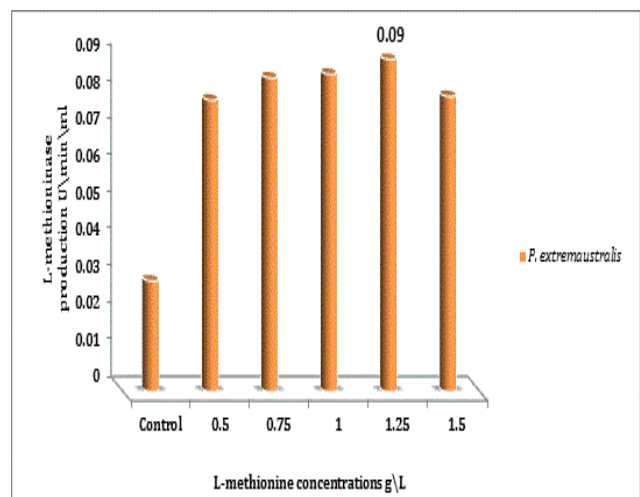


Fig. 5: Effect of L-methionine concentrations on L-methioninase production

Effect of Magnesium Sulfate concentrations on L-methioninase production

Result in Figure (6) showed *Pseudomonas extremaustralis* produced the highest amounts of L-methioninase 0.098 U/min/ml when 0.45 g/L $MgSO_4 \cdot 7H_2O$ concentration was added in the fermentation medium after 48h of incubation, increasing both incubation period and magnesium sulfate above these concentration in the fermentation medium decreased the production of the enzyme.

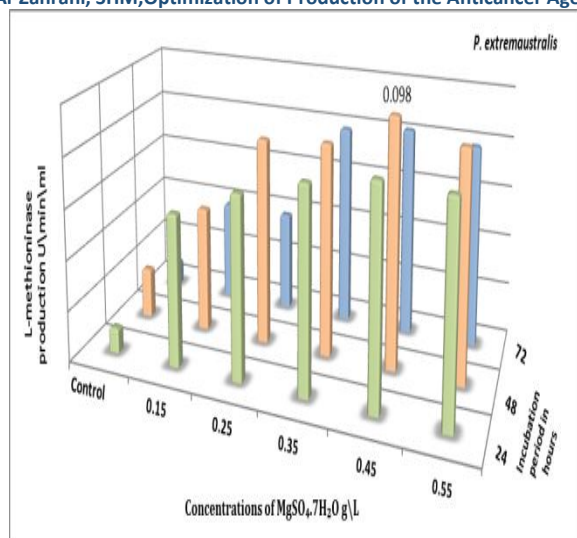


Fig. 6: The effect of Magnesium Sulfate concentration on L-methioninase production

Effect of mixed of L-methionine with different L-glutamine concentrations on L-methioninase production

Data in Figure (7) offer knowledge that *Pseudomonas extremaustralis* produced the highest amounts of L-methioninase also when L-glutamine was used as nitrogen source 0.14U/min/ml, followed by L-asparagine, peptone, L-methionine and yeast extract 0.115, 0.106, 0.105 and 0.1U/ min/ml, while using tryptone as nitrogen source the production of L-methioninase was 0.095U/ min/ ml by this isolate compared to control.

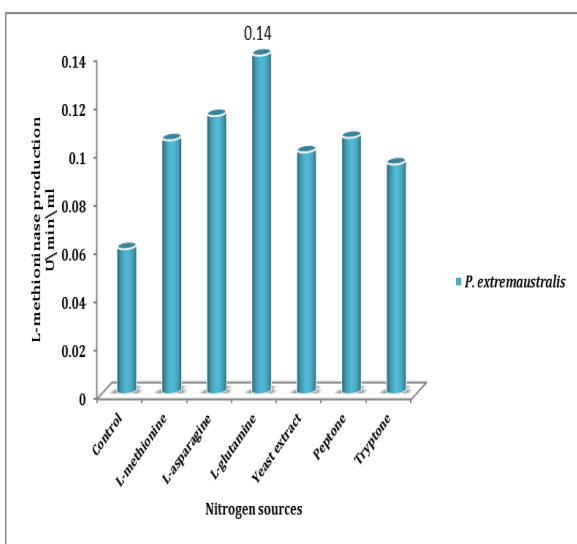


Fig. 7: Effect of nitrogen sources on L-methioninase production

Effect of mixed L-methionine with different nitrogen sources on the production of L- methioninase

The goal of this test enhances the production of L-methioninase by the selected bacterial isolate, using mixed of L-methionine with different nitrogen sources.

The highest level of L-methioninase 0.181U/min/ml was produced by *Pseudomonas extremaustralis* when the

medium supplemented with L-methionine and L-glutamine as nitrogen sources and with glycerol was added as carbon source after 72h of incubation at $35 \pm 2^\circ C$, followed by peptone and yeast extract 0.18 and 0.160U/ min/ ml, result in Figures (8).

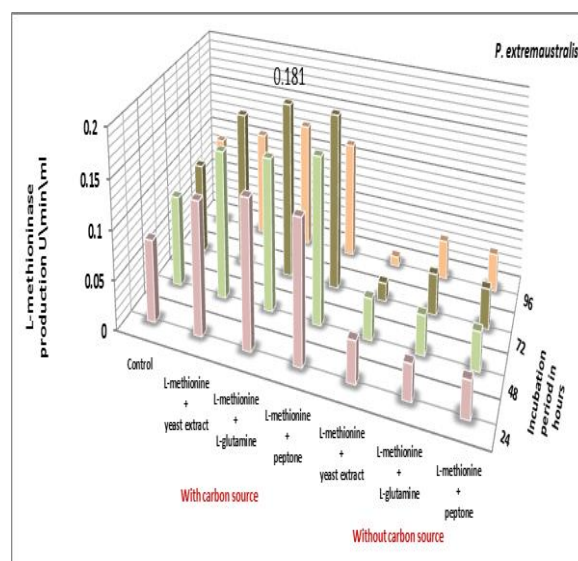


Fig. 8: Effect of mixed of L-methionine with nitrogen sources on the production of L- methioninase

Effect of mixed of L-methionine with different concentrations of L-glutamine on L-methioninase production

The previous results showed that L-glutamine was the best nitrogen source for the highest ability of L-methioninase production by *Pseudomonas extremaustralis*.

Figure(9) showed increasing L-glutamine concentrations in fermentation medium in the presence of L-methionine maximized L-methioninase production to 0.192U/min/ml when 1.5g/L of L-glutamine was added after 72h of incubation at $35 \pm 2^\circ C$ compared with control. While adding different concentrations of peptone showed that 1g/L of peptone with L-methionine was the best concentration for the highest amount of L-methioninase 0.137U/min/ml after 48h of incubation at $35 \pm 2^\circ C$ compared with control.

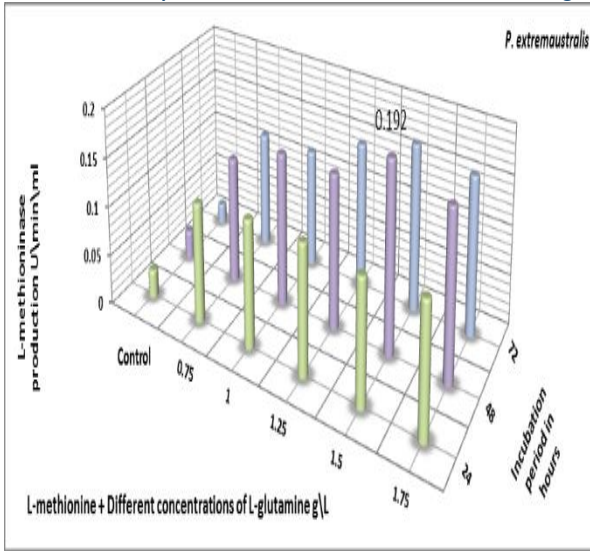


Fig. 9: Effect of mixed of L-methionine with different concentrations of L-glutamine on L-methioninase production

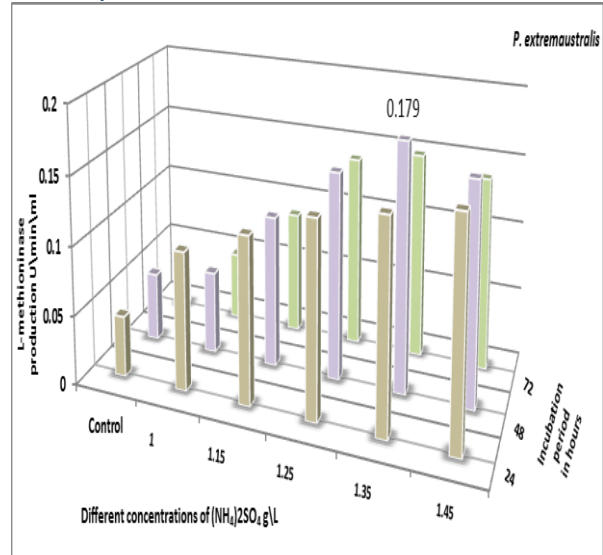


Fig. 11: Effect of inorganic nitrogen source concentrations on L-methioninase production

Effect of inorganic nitrogen sources on L-methioninase production

In this study, the best inorganic nitrogen source was ammonium sulfate for the highest L-methioninase production was 0.112U/min/ml, Figure (10). Result in Figure (11) offering data, *P. extremaustralis* was accumulated 0.179U/ min/ ml by using 1.35g\ L of (NH₄)₂SO₄ as nitrogen source above and lower this concentration decreased the production of the enzyme by this isolate.

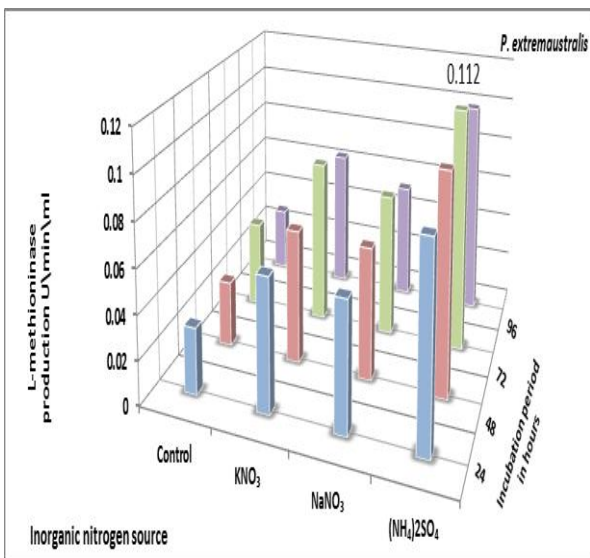


Fig. 10: The effect of inorganic nitrogen source on L-methioninase production by *Pseudomonas extremaustralis*.

DISCUSSION:

The fermentation is a metabolic process in many microorganisms involves oxide-reduction reactions resulting in the breakdown of complex organic compounds into simpler by-products and energy; the microbes carry out this metabolic action by the release of 27 extracellular enzymes [12]. Culture broth on L-methionine glucose media have shown good L-methioninase production under submerged conditions [9]. This media gives the necessary minimal nutrition required and provides L-methionine as carbon source, which is used by bacteria, as the growth substrate.

This media allows the growth of even those bacteria which can use glucose therefore to further screen the obligate L-methionine degraders, 100% L-methionine basal agar where L-methionine is the only sole source of carbon and nitrogen [13]. In cultures contain L-methionine as nitrogen source and glucose as carbon source and incubated in shaking incubator thirty-two bacterial isolates found to be positive.

Shaking velocity is a parameter affecting enzyme productivity and the highest amount produced of the enzyme in shaking cultures, of the static cultures which could be as mechanical forces lead to vacillation of older hyphal compartments and that weakened hyphae and/or accelerating hyphal fragmentation while in fungi Paul *et al.*, (14) found physiologically, SSF has enzyme manufacturing potential. Inductivity variation on the production of alkaline L-methioninase *Aspergillus flavipes* could be related to the fluctuations in the chemical composition [9].

Sharma *et al.*, (15) revealed that high volumetric concentration products, less affluent generation and simple requirement.

In this study, the result confirmed the presence of the enzyme when L-methionine was used as the sole organic

source, the result also showed that the enzyme production was enhanced in the presence of carbon source maybe because the carbon source act as growth-supporting its ability for L-methioninase producing, this result agree with that found by [6, 9, 13, 16]

Tunga *et al.*, (17) explained that fermentation time is a very important physical variable for SmF and SSF. In this study the highest amount of L-methioninase was after 48h of incubation, production of enzyme decreased after passing 48 h. The maximum output of enzymes could only be achieved after a certain period of incubation allowing the culture to develop in a stable state [18], and later activity will be reduced. the reduction could be related to substrate limitation or product inhibition [19].

Gradually reduction in L-methioninase with increasing temperature, which may be due to the denaturation of microbial strain at higher temperatures. The optimum temperature of this isolate for the maximum amount of the enzyme was at $35\pm 2^{\circ}\text{C}$, the result agrees with *Pseudomonas putida* and *Aeromonas* sp. that found by [16, 20, 21]. Similar ranges for many bacteria sp. as *Brevibacterium Linens* [22] and *Citrobacter intermedius* [23]. Besides, (24) found optimum temperature for L-methioninase production was at $35\pm 2^{\circ}\text{C}$. Furthermore, the enteric protozoans such as *Entamoeba histolytica* and *Treponema denticola* [25, 26]. Moreover, the maximum production of L-methioninase from *Streptomyces variabilis* and *A. flavipes* was at $30\pm 2^{\circ}\text{C}$ [27, 28] was the optimum temperature at $45\pm 2^{\circ}\text{C}$.

Growth and metabolism, along with enzyme production were governed by a significant factor pH. The essential microorganisms characteristic was its strong dependence on pH and producing enzymes [17].

The maximum productivity of L-methioninase by *P. extremaustralis* was at pH 6, this maybe can be explained as reported by [29] attributed to its metabolic versatility and ubiquity in nature.

Carbon source in medium was considered as key factors for growth as well as metabolites production by microorganisms. Glycerol was the best carbon source by *P. extremaustralis*, this result supported by [6, 8] who found that glycerol was the favored carbon source for L-methioninase production by *P. ovalis* and *Trichomonas vaginalis*.

This indicates the requirement of a carbon source as a co-dissimulator for L-methioninase production by *P. extremaustralis* similar to *T. Harzianum* [30] and *A. Flavipes* [31].

The significant reduction in enzyme manufacturing in the lack of carbon source could be ascribed to *P. extremaustralis* capacity to use L-methionine to start the growth, but it could not utilize its catabolized products to continue as a reported by [16]. This was comparable to the consequence discovered by [30]. Ruiz-Herrera and

Starkey, (32) and Khalaf and El-Sayed, (31) found glucose is favoured carbon source for L-methioninase production.

L-methionine using as a nitrogen source in the medium for L-methioninase production by many microorganisms [31]; on yeast [33] and bacteria [34, 35].

The results showed that *P. extremaustralis* produced L-methioninase in the presence of L-methionine, this result agrees with that found by many types of research, but the maximum amount of the enzyme was produced by *P. extremaustralis* in media L-methionine independent, this result disagrees with that found by [9, 27] who were reported that among the various nitrogen sources supplemented, L-methionine promoted maximal L-methioninase production by *A. flavipes*, *S. variabilis* 3MA2016, *Achromobacter starkeyi*, *S. marcescens*, *Candida tropicalis* and *Yarrowia lipolytica* [28, 35-37].

Khalaf and El-Sayed (31) have reported that 0.8% L-methionine was found to be the best nitrogen supplement for maximum L-methioninase production by *A. flavipes* under submerged conditions, and the maximum L-methioninase production by *A. ustus* AUMC 10151 with the addition of L-methionine [16].

Decrease enzyme productive with increasing L-methionine concentrations could be the downregulation of GATA gene transcription and blocked gene expression of L-methioninase [38, 39], L-methionine catabolic suppression, or the trans inhibition phenomenon [40].

Lockwood and Coombs, (6) reported that several microorganisms attack L-methionine while do not grow on it, related to its inability to metabolize the deaminated and demethylated residues of L-methionine. Bacteria rehabilitation on L-methionine growth could be partially throughout using growth-supporting organic compounds such as glucose.

Khalaf and El-Sayed, (31) reported, L-methioninase formation depended on containing medium and [41] on *Y. lipolytica*. Besides, the use of peptone and yeast extract in the medium for the production of L-methioninase was reported by [42] from *G. candidum* and *C. tropicalis* by [33].

CONCLUSION

L-methioninase production by *P. extremaustralis* was isolated from seashell and cheese samples. Optimization of media was carried out using SmF has higher of L-methioninase production and was able to enhance the L-methioninase production by *P. extremaustralis*. L-methioninase could potential candidate as an anticancer enzyme.

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