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## Research Article

# Impact of Physical and Chemical Factors on L-asparaginase Enzyme Purified from Soil Bacteria

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## Abstract

**Background and Objective:** L-Asparaginase is one of the amidohydrolases enzymes which catalyses the deamination of asparagine to aspartic acid. Our study aimed to produce and purify L-asparaginase enzyme from soil bacteria and investigate the physical and chemical factors that effect on the amount of produced L-asparaginase enzymes. **Materials and Methods:** Fifty soil samples were collected from soil surface, the soil suspension was then inoculated on selective nutrient agar medium, Isolated bacterial colonies were identified by biochemical tests to select the most active bacterial strains for production of L-asparaginase enzyme. The L-asparaginase enzyme was purified by Sephadex G-100 column. The effects of temperature, pH, different nutritional factors were tested on the amount of produced L-asparaginase enzyme. **Results:** *Erwinia carotovora* has the highest L-asparaginase enzyme activity with 14 mm of intense pink zone, followed by *E. coli* (13 mm) while *Streptococcus pyogenes* has (12 mm). Optimal conditions for L-asparaginase enzyme production from *Erwinia carotovora* were pH 7.0 at 37°C. The best carbon source was glucose with activity (2.7) and best concentration of glucose was 10 g L<sup>-1</sup>. The best nitrogen source was L-asparagine by concentration of 5 g L<sup>-1</sup> which gave activity (4.7). Finally this study indicated that the maximum L-asparaginase activity was obtained after incubation for 10 min at 37°C, pH 8.0 with adding 1.0 mL of magnesium chloride (MgCl<sub>2</sub>) as activator. **Conclusion:** Our study concluded that the soil can be used for obtaining L-asparaginase produced bacteria and the *Erwinia carotovora* is consider as the most active bacteria for L-asparaginase production.

**Key words:** L-asparaginase, *Erwinia carotovora*, production, purification, enzyme activity, physical properties, chemical properties

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**Competing Interest:** The authors have declared that no competing interest exists.

**Data Availability:** All relevant data are within the paper and its supporting information files.

## INTRODUCTION

L-asparaginase belongs to a group of homologous amidohydrolases family, which catalyses the hydrolysis of amino acid L-asparagine (amide of 2-aminosuccinic acid), to aspartic acid and ammonia<sup>1</sup>. They are naturally occurring enzymes expressed and produced via animal tissues, bacteria, plants and in the serum of certain rodents, however it is absent in human<sup>2</sup>. Large quantity of microorganisms that consist of *Erwinia carotovora*, *Pseudomonas stutzeri*, *Pseudomonas aeruginosa* and *E. coli* has been recognized to produce L-asparaginase<sup>3</sup>.

The presence of asparagine is indispensable for a regular path of the cell cycle. In most human cells the deficit of the endogenous asparagine may additionally be compensated by way of alternative synthesis from aspartic and glutamic acid involving the asparagine syntheses. L-asparaginase enzyme is absent in sure neoplastic cells, particularly in lymphoblasts, for this reason the depletion of asparagine from the plasma leads to DNA, RNA and protein synthesis inhibition and in consequence to blast cell apoptosis<sup>1</sup>.

Different sorts of asparaginase can be used for pharmaceutical enterprise as an alternative for treatment of exclusive cancers such as acute lymphoblastic leukemia (ALL), malignant ailments of the lymphoid system and Hodgkin's lymphomas<sup>4</sup>.

Cancer cells, often lymphatic cells, require high quantity of asparagine for fast and malignant growth. In this way, cancer cells require the amino acid from diet (blood serum) as well as amino acids produced by way of themselves. However, leukemic lymphoblasts and some others tumor cells do no longer have or existing low volume of L-asparagine synthetase used for L-asparagine synthesis. Thus, these malignant cells are dependent of asparagine from blood serum for their proliferation and survival<sup>5</sup>. L-asparaginase hydrolyzes asparagine from blood serum, leading tumor cells to dying by means of lacking of critical thing for protein synthetases (p53-dependent apoptosis). However, healthful cells are now not affected, because they are capable to produce asparagine, the use of L-asparagine synthetase existing in enough quantities<sup>6</sup>. The soil metagenome derived L-asparaginase with enhanced activities could be a potential candidate to develop as a drug in ALL therapy<sup>7</sup>. Also, El-naggar *et al.*<sup>8</sup>, concluded that the L-asparaginase enzyme can be used for development of chemotherapeutic drug. L-Asparaginase (L-asparagine aminohydrolase, E.C. 3.5.1.1) has been proven to be competent in treating ALL, which is widely observed in pediatric and adult groups<sup>9</sup>.

Also, this enzyme is used in food industry to prevent the acrylamide formation when ingredients are processed in excessive temperatures<sup>10</sup>. Several researches show that L-asparagine is the primary amino acid responsible for acrylamide production in fried and baked meals when reducing sugars are condensed with a carbonyl source. This phenomenon does now not manifest in boiled food<sup>11</sup>. Many research confirmed that the use of L-asparaginase enzyme earlier than frying or baking food manner should decrease greater than 99% acrylamide degree in the processed final product. This is due to the fact the enzyme reduces extra than 88% of the L-asparagine concentration from the initial feedstock<sup>12</sup>. This use is essential because acrylamide is a neurotoxin categorized as potentially carcinogenic to humans<sup>13</sup>.

L-asparaginases are enzymes derived mainly from bacteria and the three enzymes that have been used are derived from *E. coli* (*E. coli*-asparaginase), a pegylated form of native *E. coli*-asparaginase (PEG-asparaginase) and an *Erwinia chrysanthemi*-derived asparaginase (*Erwinia*-asparaginase)<sup>14</sup>.

The purpose it is preferred for the reason is it is biodegradable, non-toxic and can be administered at the nearby site quite easily. Other sellers are observed pretty painful when administered to the patient and additionally these are pretty costly. Current clinical studies point out that this enzyme is additionally a promising agent in treating some varieties of neoplastic cell diseases in human<sup>15</sup>. Industrial L-asparaginase production offers some challenges, such as the search for new microorganisms in a position to produce it with much less unfavorable effects. Nowadays, industrial manufacturing is carried out using bacteria such as *E. coli* and *Erwinia chrysanthemi*, fungi and yeast. Currently, new studies have been carried out aiming to increase manufacturing process and set up new approaches for enzyme synthesis<sup>6</sup>. This study aimed to detect and purify L-asparaginase enzymes from soil bacteria and study the effect of different physical and chemical factors on the enzyme activity.

## MATERIALS AND METHODS

**Study area:** This study was carried out in Gastroenterology Surgical Center, Mansoura University, Egypt during the period February, 2017 till August, 2019.

**Collection and preparation of soil samples:** Fifty soil samples were collected from soil surface and depth of approximately 20 cm (around the plant roots) of cultivated potato field. The

samples were taken in sterilized screw capped cup using sterilized spatula different soil samples were passed through a sieve (1.7 mm mesh) to remove large pieces of debris and vegetation. One gram of the soil samples were added to 10 mL of water to make soil suspension<sup>16</sup>.

**Inoculation:** The soil suspension was then inoculated on selective nutrient agar medium supplemented with antifungal agents (Fluconazole 75, Ketoconazole 75  $\mu\text{g mL}^{-1}$ ) to control the fungal contamination. The inoculated agar plates were incubated at 37°C for 24 h.

**Bacterial Identification and selection:** Isolated bacterial colonies were identified by biochemical tests (coagulase, starch hydrolysis, oxidase, urease, catalase and indole). To select the most active bacterial strains for production of L-asparaginase enzyme the identified and purified isolated bacteria were point inoculated on agar-based modified medium (composition ( $\text{g L}^{-1}$ ):  $\text{KH}_2\text{PO}_4$  2.0, L-Asparaginase 6.0,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  1.0,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  1.0 and Glucose 3.0) supplemented with 0.004% phenol red dye (prepared in ethanol) and pH was adjusted at 6.1. The inoculated plates were incubated at 37°C for overnight. Diameter zone and appearance the pink color indicated that L-asparaginase production<sup>16</sup>.

**L-asparaginase purification:** The ammonium sulphate precipitate was applied to a Sephadex G-100 column. Seven grams of Sephadex G-100 were equilibrated with 0.05 M borate buffer (pH 7.5) in water bath at 60°C for 3 h. The gel was then cooled to room temperature. A glass column 1.0×38 cm was packed with swollen gel and equilibrated with the same buffer. Fractions after ammonium sulfate precipitation were subjected to gel filtration column. The protein elution was done with the same buffer at a flow rate of 2 mL every 10 min. The fractions were collected using a fraction collector (LKB Ultorace) at 40°C. It was assayed for protein content at 595 nm as well as for enzyme activity. Preparation of crude enzyme solution by inoculated a loop full of each isolated bacteria in 100 mL of broth-based modified medium. All flasks were incubated under shaking condition (150 rpm) for 2 days at 30°C. After incubation period, the cultures were filtered, then the filtrates were centrifuged at 5000 rpm for 10 min the clarified filtrate represented crude enzyme of L-asparaginase<sup>17</sup>.

**Characterization of L-asparaginase enzyme:** Effect of temperature on production of L-asparaginase enzyme was determined by incubation the flasks at different temperatures (25, 30, 37, 40 and 50°C) for 2 days<sup>18</sup>. The influence of pH on

the enzyme activity was done by using 0.1 M of sodium acetate and 0.1 M of phosphate buffer at different pH levels (5, 6, 7, 8 and 9). The inoculated flasks were incubated for 2 days at 30°C and shaken at 150 rpm<sup>19</sup>. The effect of nutritional factors on L-asparaginase production, was studied by adding different carbon sources (Glucose, maltose, fructose, lactose, galactose and raffinose) and different nitrogen source (L-asparagine, urea, ammonium sulphate, ammonium chloride, ammonium nitrate and sodium nitrate) to flasks, then incubated for 2 days at 30°C and shaken at 150 rpm<sup>20</sup>.

The impact of different concentrations of glucose as carbon source on enzyme production was determined by preparing different concentrations from glucose (2, 4, 6, 8 and 10  $\text{g L}^{-1}$ ) as well as, preparation of different concentrations of L-asparagine as nitrogen source (2.5, 5, 7.5 and 10  $\text{g L}^{-1}$ ), then flasks were incubated for 2 days at 30°C and shaken at 150 rpm<sup>20</sup>.

The effect of different incubation periods on L-asparaginase enzyme production was analyzed by preparing mixture consisting of 0.5 mL of 0.08 M of L-asparagine, 1 mL of 0.05 M of borate buffer and 0.5 mL of purified enzyme, then incubated at 37°C for different period times (10, 20, 30 and 40 min).

The impact of different temperatures and pH on enzyme production was studied by incubated the enzyme mixture at 25, 30, 37, 40 and 50°C for 10 min and different pH (5, 6, 7, 8 and 9) at 37°C<sup>19</sup>. The effect of the amounts of  $\text{MgCl}_2$  as activator on enzyme activity was tested by preparing several amounts (0.2, 0.4, 0.6, 0.8 and 1.0 mL) of activator, then incubated at 37°C for 10 min<sup>21</sup>.

## RESULTS

The enzyme production was determined by measuring the diameter zone of L-asparaginase producing bacteria. *Erwinia carotovora* has the highest (14 mm) intense pink zone, followed by isolate *E. coli* (13 mm) while isolate *Streptococcus pyogenes* has (12 mm) pink zone as shown in Table 1.

All the L-asparaginase-positive isolated bacteria were subjected to quantitative screening using CARY 60 spectrophotometer. The results in Table 1 and 2 revealed that *Erwinia carotovora* showed the highest activity for L-asparaginase production, so we selected for further study.

The results of environmental and nutritional factors affecting on production of L-asparaginase from *Erwinia carotovora* show in Fig. 1. The first environmental factor is incubation temp. This indicated that the maximum enzyme activity (150  $\text{U mL}^{-1}$ ) and specific activity (2.7  $\text{U mg}^{-1}$ ) occur at

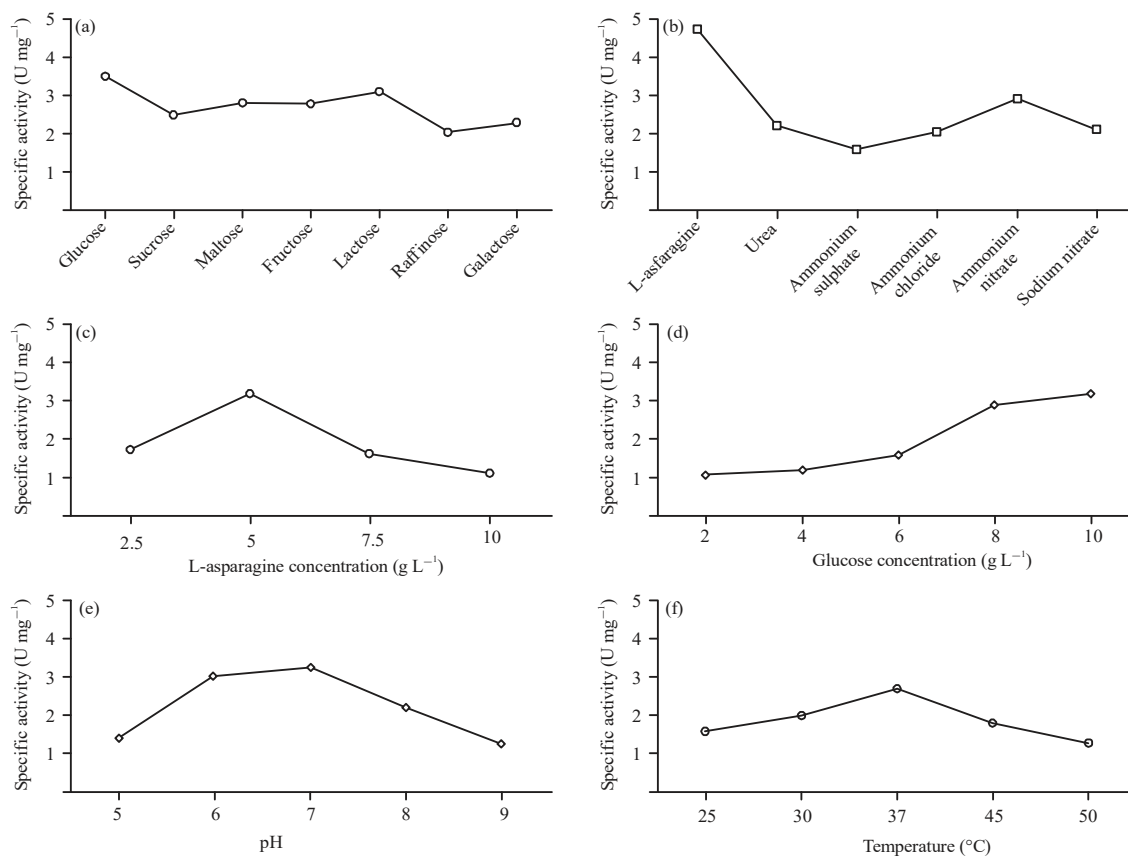


Fig. 1 (a-f): Specific enzyme activities were measured at different nutrients, (a) Different carbon sources, (b) Different nitrogenous sources, (c) L-asparagine concentrations, (d) Glucose concentrations, (e) pH and (f) Temperatures

Table1: Diameter zone of L-asparaginase producing bacteria

Isolated bacteria	Diameter zone (mm)	Density of pink colour
<i>Erwinia carotovora</i>	14	+++
<i>Escherichia coli</i>	13	++
<i>Staphylococcus epidermidis</i>	11	++
<i>Streptococcus pyogenes</i>	12	+
<i>Proteus vulgaris</i>	9	+

Table 2: Quantitative screening for L-asparaginase activity of isolated bacteria in liquid medium

Isolated bacteria	Activity (U mL <sup>-1</sup> )	Protein (mg mL <sup>-1</sup> )	Specific activity (U mg <sup>-1</sup> )
<i>Erwinia carotovora</i>	176	49	3.5
<i>Escherichia coli</i>	164	50	3.2
<i>Staphylococcus epidermidis</i>	129	55	2.3
<i>Streptococcus pyogenes</i>	117	96	1.2
<i>Proteus vulgaris</i>	99	65	1.5

37°C. Regarding to the second environmental factor it was initial pH value, which showed that the maximum enzyme activity (206 U mL<sup>-1</sup>) and specific activity (3.25 U mg<sup>-1</sup>) occur at pH 7. The first nutritional factor is carbon source, a goal of this experiment is study the effect of changing the carbon source on L-asparaginase production. It was found that glucose as the best carbon source for L-asparaginase

production with enzyme activity (200 U mL<sup>-1</sup>) and specific activity (3.5 U mg<sup>-1</sup>). The second nutritional factor is nitrogen sources, a goal of this experiment is to study the effect of changing the nitrogen source on L-asparaginase production. It was found that L-asparagine is the best nitrogen source for L-asparaginase production with enzyme activity (127 U mL<sup>-1</sup>) and specific activity (4.7 U mg<sup>-1</sup>). The optimum glucose conc. was 10 g L<sup>-1</sup>, which have the highest enzyme activity (169.5 U mL<sup>-1</sup>) and specific activity (3.2 U mg<sup>-1</sup>). However, the optimum nitrogen conc. was 5.0 g L<sup>-1</sup>, which have the highest enzyme activity (170 U mL<sup>-1</sup>) and specific activity (3.19 U mg<sup>-1</sup>).

Figure 2 shown that the optimum incubation time, temp. and pH of partially purified activities and the rate of substrate hydrolysis was investigated by incubating the enzyme for with the corresponding substrate at optimum conditions. The maximum enzyme activity and specific activity were obtained after incubation for 10 min at 37°C and pH 8. Enzyme activity at different activator concentrations was investigated, the optimum enzyme activity and specific activity were obtained by addition of 1 mL magnesium chloride (MgCl<sub>2</sub>).

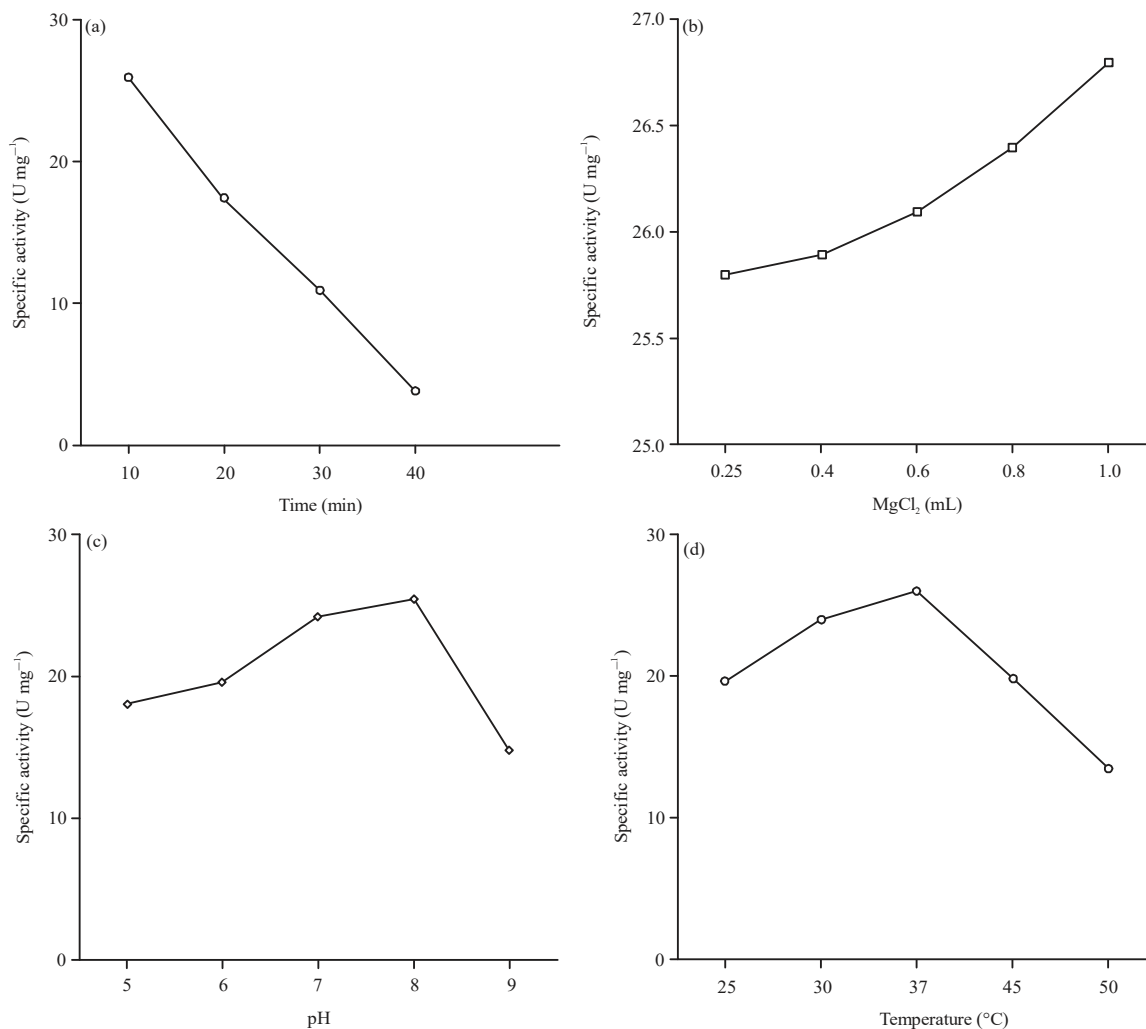


Fig. 2(a-d): Specific enzyme activities were measured at (a) Different times, (b) Magnesium chloride concentrations, (c) pH and (d) Temperatures

## DISCUSSION

The present investigation included selection of the most potent bacterial isolate that capable of producing L-asparaginase enzyme. Then, increase productivity of L-asparaginase enzyme by studying the best factors affecting L-asparaginase production. In addition, attempts for purification and characterization of L-asparaginase were also investigated.

Five asparaginase producing bacteria were isolated from fifty soil samples collected from cultivated potato field. This may be attributed to the fact that soil is a rich source for potential enzyme producing organisms.

In this study, the result of screening test revealed that L-asparaginase producing bacteria were able to hydrolyze L-asparagine since they utilized L-asparagine as their substrate and breakdown asparagine to L-aspartate and ammonia

which further reacts with water to produce  $\text{NH}_4\text{OH}$ , hence the pH of the medium is basic which subsequently changes the medium from yellow to pink, hence the pinkish zone observed around the colony of asparaginase producing bacteria. This is in accordance with the work of Gulati *et al.*<sup>22</sup> who proved that color transformation was due to L-asparaginase production. The bacterial isolates with highest L-asparaginase activity were identified as *Erwinia carotovora*, *E. coli* and *Streptococcus pyogenes*.

In this study the diameter zones of L-asparaginase enzyme activity were determined, in which the isolate *Erwinia carotovora* has the highest intense pink zone (14 mm), followed by *E. coli* (13 mm), then *Streptococcus pyogenes* (12 mm). This is also similar to the report of Devi and Ramanjaneyulu<sup>23</sup>, who reported that strain exhibiting diameter zone above 9 mm are referred as good L-asparaginase producers, those strains with diameter zone

6-9 mm and those having below 6 mm diameter zone may be referred to as moderate and poor L-asparaginase producers, respectively.

*Erwinia carotovora* was subjected to several physiological studies to improve its enzyme production. Effect of incubation temperature of L-asparaginase enzyme production was determined in shake flasks using basal medium. The maximum specific activity of L-asparaginase ( $2.7 \text{ U mg}^{-1}$ ) was obtained at  $37^\circ\text{C}$ . This indicates that the enzyme production was reduced gradually with further increase in incubation temperature. This result was similar to the study of Abbas and Askar<sup>24</sup> and Maladkar *et al.*<sup>25</sup>, who reported that optimum temperature is  $37^\circ\text{C}$  of L-asparaginase enzyme activity by *Erwinia* spp., from *Staphylococcus* spp. by Prakasham *et al.*<sup>26</sup> and from *Pseudomonas stutzeri* by Manna *et al.*<sup>27</sup>.

Our results disagreed with Wakil and Adelegan<sup>3</sup>, who indicated that the optimum incubation temperature at which *Bacillus firmus*, *Streptococcus* spp. and *Bacillus circulans* showed highest asparaginase yield was  $45^\circ\text{C}$ . Borkotaky and Bezbaruah<sup>28</sup> that reported optimum incubation temperature for L-asparaginase activity by *Erwinia* spp. was  $35^\circ\text{C}$ . Narayana *et al.*<sup>29</sup>, who indicated that the optimum incubation temperature for *Streptomyces albidoflavus*, *Bacillus polymyxa* and *Paenibacillus validus* had their highest production at incubation temperature of  $35^\circ\text{C}$ . Ghosh *et al.*<sup>30</sup>, who proved that the highest production of L-asparaginase from *Serratia narcerens* at  $30^\circ\text{C}$ .

Among the physical parameters, pH of the growth medium plays an important role by inducing morphological changes in the microorganisms and in enzyme secretion. The result of the effect of pH showed that maximum L-asparaginase yield by *Erwinia carotovora* was at pH 7.0. These results were agreed with Ghosh *et al.*<sup>30</sup>, who indicated that the maximum L-asparaginase production from *Serratia narcerens* was at pH 7.0. However *Streptomyces albidoflavus* was recorded most active at pH 7.5 by Narayana *et al.*<sup>29</sup>. This result disagreed with Wakil *et al.*<sup>3</sup>, who proved that the highest yields of L-asparaginase enzyme from *Streptococcus* spp., *Streptococcus* spp. and *Bacillus polymyxa* was at pH 8.0. Dhevagi and Poorani<sup>31</sup>, who reported that the maximum L-asparaginase by *Streptomyces* spp. was at pH 8.0-8.5. However, Deshpande *et al.*<sup>32</sup> and Akilandeswari *et al.*<sup>33</sup> proved that *Streptomyces ginsengisoli* produced the highest yields of L-asparaginase enzyme at pH 8.0 and 9.0, respectively. Abdel-Fattah and Olama<sup>34</sup> indicated that *Streptomyces albidoflavus* was recorded most active at pH 8.0.

The induction of *Erwinia carotovora* was studied using media with L-asparagine and other various carbon

sources. The data obtained indicated that the maximum L-asparaginase activity ( $3.5 \text{ U mg}^{-1}$ ) was recorded in presence of glucose as carbon source. These findings were comparable to that described by Prakasham *et al.*<sup>26</sup> who reported that glucose was the best carbon source under aerobic conditions for the synthesis of L-asparaginase by *Staphylococcus* spp. Venil *et al.*<sup>35</sup> has reported decrease in L-asparaginase production when supplemented with fructose. Prakasham *et al.*<sup>26</sup> reported that *Staphylococcus* spp. which was recorded most active when supplemented with maltose and Akilandeswari *et al.*<sup>33</sup> used proline as the best carbon source for L-asparaginase production. Wakil *et al.*<sup>3</sup>, who indicated that mannitol, sucrose and maltose were the best carbon sources for *Bacillus firmus*, *Streptococcus* spp. however, Kenari *et al.*<sup>36</sup> reported that lactose was the best carbon source for L-asparaginase production. In contrast, Deokar *et al.*<sup>37</sup> observed maximum L-asparaginase production by *Erwinia carotovora* at 1.13% maltose concentration.

Different nitrogen sources in culture media were investigated for enzyme production by *Erwinia carotovora*. L-asparagine was the most suitable nitrogen source for optimum L-asparaginase production by *Erwinia carotovora* with specific activity ( $4.7 \text{ U mg}^{-1}$ ). This result agreed with Nawaz *et al.*<sup>38</sup>, who reported that L-asparagine was the best nitrogen source for L-asparaginase enzyme production. Wakil *et al.*<sup>3</sup>, Verma *et al.*<sup>39</sup>, Narayana *et al.*<sup>29</sup> and Deokar *et al.*<sup>37</sup> showed that yeast extract was optimum for L-asparaginase synthesis by *Bacillus polymyxa* and *Erwinia carotovora*. However, Deshpande *et al.*<sup>32</sup> used peptone as the best nitrogen source.

By studying the effect of different concentration of the carbon source which represented in glucose, it was found that  $10 \text{ gm L}^{-1}$  (1%) was the best concentration and gave the maximum L-asparaginase production. These findings were comparable to that described by Amena *et al.*<sup>19</sup> and Narayana *et al.*<sup>29</sup>. However, Prakasham *et al.*<sup>26</sup> and Ghosh *et al.*<sup>30</sup> indicated that the best concentration of glucose for production high yields of L-asparaginase was  $20 \text{ gm L}^{-1}$ .

By studying the effect of different concentration of the nitrogen source which represented in L-asparagine, it was found that  $5 \text{ gm L}^{-1}$  (0.5%) was the best concentration and gave the maximum L-asparaginase production. These observations were equivalent to that had been found by Prakasham *et al.*<sup>26</sup>. But Amena *et al.*<sup>19</sup> had found  $2 \text{ gm L}^{-1}$  (0.2%) was the best concentration.

L-asparaginase enzyme produced by *Erwinia carotovora* was purified using ammonium sulphate precipitation (70%)<sup>40</sup>. Followed by gel filtration (fractionation) using Sephadex G100 (Patro and Gupta<sup>18</sup>). In the present study some biochemical

properties of L-asparaginase enzyme produced by *Erwinia carotovora* have been investigated. The optimum incubation period of high relative enzymes activities and the rate of substrate hydrolysis was investigated and found that L-asparaginase activity was obtained after incubation 10 min. These findings were comparable to that described by Moorthy *et al.*<sup>17</sup>. For temperature profile for L-asparaginase enzyme was found that this enzyme showed an optimum temperature at 37°C. These findings were comparable to that described by Abbas and Gupta<sup>24</sup>, Moorthy *et al.*<sup>17</sup> and Patro and Gupta<sup>18</sup>. For pH profile using broad pH range from pH 5-9 using different concentrations of borate buffer. L-asparaginase enzyme produced by *Erwinia carotovora* exhibited an optimum pH 8.0 and this result was similar to that deduced by Dhevagi and Poorani<sup>31</sup> but Moorthy *et al.*<sup>17</sup> had found pH 8 the best for L-asparaginase activity. The effect of activator MgCl<sub>2</sub> on L-asparaginase enzyme was determined by adding the activator in the reaction mixture. The enzyme was activated by MgCl<sub>2</sub> and these by 1.0 mL of activator. These similar to that described before by Moorthy *et al.*<sup>17</sup>. Our study recommended that soil can be a good source of L-asparaginase enzyme produced bacteria and evaluate the different conditions that can produce a maximum amount of L-asparaginase enzyme.

### CONCLUSION

This study clearly indicates that soil can provide a good source of L-asparaginase producing bacteria and *Erwinia carotovora* was the most active bacterial isolate for L-asparaginase production from the isolated bacterial species. Glucose was the best carbon source at conc. (10 gm L<sup>-1</sup>) and L-asparagine the best nitrogen source at conc. (5 gm L<sup>-1</sup>). A characterization of partially purified L-asparaginase from *Erwinia carotovora* has been done. It was investigated that this enzyme showed an optimum activity after 10 min at 37°C, pH 8 and highly affected by adding 1.0 mL MgCl<sub>2</sub> as activator.

### SIGNIFICANCE STATEMENT

This study discovered the new methods for isolation and production of L-asparaginase enzyme that can be beneficial for L-asparaginase enzyme production.

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