Biosynthesis and characterization of a novel *penicillium janthinellum Biourge* L-asparaginase as a diverse biological activities agent

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Background and objectives
L-asparaginase (L-ASP) is a therapeutic enzyme used in the treatment of certain human cancers, especially acute lymphoblastic leukemia, as a chemotherapeutic agent. Other than as an anticancer agent, it has many applications, including in the treatment of autoimmune disorders, infectious diseases, and antibacterial activity. Microorganisms such as bacteria, fungi, and actinomycetes are very effective producers and a better source of L-ASP because they can be easily cultivated, and it is also easy to extract and purify L-ASP. The aim of this study is to formulate the production medium and to pinpoint the proper growth conditions for the chosen microorganism producing highly active L-ASP enzyme. The general properties of the crude L-ASP enzyme preparation were also determined to define the proper conditions for enzyme action. Under the specified conditions, the opportunity of the crude L-ASP enzyme for antimicrobial and antioxidant activities was determined.
Materials and methods
Eight recommended microbial isolates were screened for biologically active L-ASP enzyme productivity. Optimization of the cultural conditions for extracellular L-ASP production and also the important properties of the crude L-ASP were duly pinpointed. Finally, biological activities of the crude enzyme were explored.
Results and conclusion
Among all the screened organisms, the fungal strain *Penicillium janthinellum Biourge* was the most potent producer of an influential L-ASP enzyme. The maximum L-ASP activity of 17.85±0.579 U/reaction was obtained from medium containing glucose 0.2% (w/v) and L-asparagine 1% (w/v) at 30°C and pH 6.2. The important properties of the crude *P. janthinellum Biourge* L-ASP were duly pinpointed as follows: optimum enzyme and substrate concentrations were 1 mg/ml and 1% (w/v), respectively, and optimum reaction pH and temperature were 10.7 and 45°C, respectively. Under the specified conditions, at varying concentrations, the enzyme preparation exhibited considerable 2, 2-diphenyl-1-picrylhydrazyl radical scavenging activity accompanied with nonantimicrobial activity, and this pointed out the necessity of partial purification of the crude fungal enzyme for further studies.

Keywords:
anticancer activity, antimicrobial, antioxidant activity, biological activities, L-asparaginase, *Penicillium janthinellum Biourge*

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Introduction

L-asparaginase (L-ASP, L-asparagine amino hydrolase, EC 3.5.1.1) is a hydrolytic enzyme that catalyzes the conversion of L-asparagine to L-aspartic acid and release of ammonia [1]. L-ASP enzymes are widely distributed in animal, plant tissues, and algae. It can be obtained also from microorganisms, such as bacteria, fungi, and actinomycetes, which are recognized as very effective producers and are a better source of L-ASP, because they can be easily cultivated, and it is also easy to extract and purify L-ASP from the mare, enabling large-scale production [2]. L-ASP is the first therapeutic enzyme with antineoplastic properties and has been studied broadly by researchers and scientists far and wide. L-ASP was first observed by Lang [3]. In 1922, Clementi [4] made the pioneering observation that proved to be significant for the production of L-ASP as a potential antineoplastic agent, revealing that guinea pig serum is a rich source of L-ASP. In addition, Kidd [5] showed the ability of guinea pig serum to inhibit the growth of transplantable lymphoid tumors in mice and rats, as well as some spontaneous and

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radiation-induced leukemias in mice. These enzymes acquired their extreme importance owing to their different applications in medical and in healthy food industry fields [6,7]. L-ASPs are highly influential affinity enzymes in certain types of cancer therapy [8]. Moreover, L-ASP has recently found the way to healthy food industries as the most beautiful and novel agent preventing the formation of powerful carcinogenic acrylamide, which is extensively developed in many fried, roasted, and baked food, particularly those in common hurried meals [9,10]. In addition, L-ASP has high antioxidant capacity [11,12].

Accordingly, the increasing demands for these enzymes justified the broad and comprehensive worldwide research studies to produce effective, applicable, and low-priced L-ASPs.

Materials and methods

Materials

Microorganisms

Eight recommended bacterial and fungal isolates were screened for L-ASP production in the present study. The bacterial strains *Bacillus subtilis*, *Escherichia coli*, and *Pseudomonas aeruginosa* were obtained from Microbiology Department, Faculty of Science, Ain Shams University, Egypt, whereas the fungal strain *Penicillium janthinellum Biourge* was isolated from agricultural soil in Zagazig City, Al Sharqiyah Governorate, Egypt, and completely identified by Prof. Dr Gamal Eldin Helal of the Microbiology Department, Faculty of Science, Zagazig University, Egypt, whereas the other four fungi *Aspergillus niger*, *Aspergillus terreus*, *Aspergillus oryzae*, and *Penicillium sp.* were provided by the Culture Collection Center of the National Research Center, Egypt.

Media

The following media were used in the present study and had the following composition (g/l):

Bacterial media

Bacterial maintenance medium (nutrient agar), medium 1

This medium was used for stock culture and maintenance of bacterial strains and was composed of the following: peptone, 5.0; beef extract, 3.0; NaCl, 8.0; agar, 12.0 and pH 7.2±0.2.

Bacterial growth enhancement medium (tryptone–yeast extract), medium 2

This medium was used for bacterial growth enhancement and had the following composition: tryptone, 10.0; yeast extract, 5.0; NaCl, 10.0; and pH 7 [13].

L-asparaginase production medium, medium 3 [14]

This was applied for bacterial L-ASP production and had the following composition: $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; KCl, 0.5; K_2HPO_4, 1.0; yeast extract, 0.5; L-asparagine, 1.5; and pH 7.0±0.2.

Fungal media

Fungal cultural maintenance medium (potato-dextrose-agar), medium 4

This medium was applied for stock cultures and culture maintenance of fungal isolates and composed of the following (g/l): potato infusion, 200; D-glucose, 20.0; agar, 15.0; and pH 5.6±0.2.

Fungal cultural enhancement medium, medium 5

This medium was used for the preparation of active fungal inoculum and composed of the following: glucose, 16.0; peptone, 5.0; yeast extract, 1.0; $MgSO_4.7H_2O$, 0.5; KH_2PO_4, 1.0; and pH7.0±0.2 [13].

L-asparaginase production medium (modified Czapek–Dox medium), medium 6 [15]

The following medium was used in the present investigation for L-ASP production from fungi and composed of the following: glucose, 2.0; L-asparagine, 10.0; KH_2PO_4, 1.52; KCl, 0.52; $MgSO_4.7H_2O$, 0.52; and traces of Cu $(NO_3)_2.3H_2O$, $ZnSO_4.7H_2O$, and $FeSO_47.H_2O$, at pH 6.2.

Chemicals

L-asparagine and ammonium sulfate were purchased from Sigma Aldrich-Chemi GmbH & Co KG, Steinheim, Germany, and all the other chemicals were of analytical grade.

Buffers

The following buffer solutions were applied for different reaction pHs: 0.05 M-acetate, pH 4–5; 0.05M-phosphate, pH 6–7; 0.05M-Tris-HCl, pH 8.6; and 0.05M-carbonate-bicarbonate, pH 9.9–10.7.

Methods

Maintenance of the tested microorganisms and stock cultures

The tested bacteria were maintained on the nutrient agar slants and incubated at 37°C for 24 h, whereas the fungi were maintained on slants of potato-dextrose-agar medium and incubated at 30°C for 7 days.

Production of extracellular L-asparaginase

Each 250-ml Erlenmeyer flask contained 50 ml of the recommended culture media shaken at 200 rpm. The incubation period lasted for 24, 48, and 72 h at 37°C for bacteria and for 3, 5, and 7 days at 30°C for fungi.

Crude enzyme preparation

This was done either by filtration through Whatman filter paper No. 1 or centrifugation at 2300 g for 20 min. L-ASP activity, protein content, and final pH were determined in the clear supernatant. The dry weight of cells or mycelium was also determined.

Estimation of protein

The protein content was determined colorimetrically by Folin-Ciocalteu phenol reagent (Merck Company, Schuchardt, Germany) by the method of Lowry *et al.* [16] using bovine serum albumin as the standard.

Assay of L-asparaginase activity

L-ASP activity was determined by hydrolysis of L-asparagine to aspartic acid and ammonia, which was measured by Nesslerization according to modified method of Imada *et al.* [17]. The reaction was started by adding 0.5-ml enzyme supernatant to 0.5 ml 0.04 M-L-asparagine solution, 0.5 ml distilled water, and 0.5 ml 0.05 M-Tris-HCL buffer (pH 8.6) followed by incubation at 37°C for 30 min. The reaction was stopped by the addition of 0.5 ml of 1.5 M-trichloroacetic acid. The ammonia released in the supernatant was determined colorimetrically by adding 0.2 ml of Nessler's reagent into tube containing 0.1 ml supernatant and 3.7 ml of distilled water and incubated at room temperature for 10 min. The absorbance was recorded at 450 nm in ultraviolet–visible spectrophotometer. Standard curve was prepared applying varied dilutions of ammonium sulfate solution. One unit of L-ASP activity is defined as the amount of the enzyme that releases 1 μmol of ammonia per reaction under the assay conditions [18].

Optimization of fermentation parameters for L-asparaginase production by Penicillium janthinellum Biourge

Production of L-ASP enzyme is affected by various factors and fermentation conditions such as fermentation period, initial pH, inoculum size and age, agitation speed, and C and N sources. One factor at a time was optimized and then incorporated in the next experiments.

Effect of fermentation periods

This was assessed by incubation of the cultures for different periods of 2, 3, 4, 5, 6, and 7 day at 200 rpm and 30°C.

Effect of the initial pH

Effect of the initial pH value of the fermentation medium was studied through adjusting the initial pH value to 4, 5, 6.2, 7, or 8 using either 1 N-NaOH or 1 N-HCl with a digital pH meter, and after that, the medium was investigated for the production of the enzyme and compared with control (pH 6.2).

Effect of inoculum size and inoculum age

To study the fungal inoculum size effect on the L-ASP productivity, different inoculum sizes were separately employed, that is, 5, 10, 15, and 20% (v/v) and compared with control (10%, v/v). On the other side, the effect of inoculum age was also investigated by testing three inoculum ages (24, 48, and 72 h) compared with control of 72-h age.

Effect of agitation speed

Each 250-ml fermentation flask containing 50 ml of the production medium was subjected to various shaking speeds (100, 150, and 200 rpm) and compared with the static culture to find out the effects of shaking speed on L-ASP production.

Effect of nitrogen source

Effect of nitrogen source was tested in the production medium using the following nitrogen sources: inorganic (ammonium sulfate and ammonium chloride) and organic N sources (L-glycine and urea) in the medium by replacement of L-asparagine in the basal medium by any of the previously mentioned sources and compared with control (L-asparagine).

Effect of carbon source

To determine the effect of carbon source on L-ASP productivity, various 0.2% (w/v) carbon sources (glucose, sucrose, fructose, sodium acetate, soluble starch, and starch) were separately employed in the culture medium and compared with the control (0.2%, w/v glucose).

General properties of the crude L-ASP

Effects of enzyme protein and substrate (L-asparagine) concentrations and reaction pH and temperature were studied.

Biological activities of the crude L-asparaginase

Free radical scavenging activity

Free radical scavenging activity of the crude L-ASP enzyme was determined by modified, simple, rapid, and inexpensive method of Peng *et al.* [19] with using 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical. DPPH is a free radical of violet color. The antioxidants present in the sample scavenge the free radicals and convert them into yellow color.

The change of color from violet to yellow is proportional to the radical scavenging activity. In brief, the crude stock enzyme ethanol solution (10.0 mg/ml) was diluted with ethanol to final concentrations ranged from 0.25 to 9.0 mg enzyme (wt/ml). Overall, 0.5 ml of 0.3 mM-DPPH ethanolic solution was separately added to 0.5 ml of each sample solution. The reaction mixture was vortexed and incubate for 1 h in a dark room temperature. The absorbance of the solution was measured at 518 nm, using ascorbic acid as the standard. The percentage of DPPH inhibition was calculated according to the following equation:

$$I\ (\%) = \{(\text{Abs control} - \text{Abs test}) / \text{Abs control}\} \times 100.$$

Where I%=DPPH inhibition %, Abs control=absorbance of the control (t=0 h), and Abs test=absorbance of tested sample at the end of the reaction (t=1 h).

Assay was carried out in triplicate, and the result was averaged. The percentage of radical scavenging activity was plotted against the corresponding crude L-ASP concentration to obtain the value of IC_{50}. IC_{50} is the maximal concentration of the compound causing 50% inhibition.

Antimicrobial activity

The antimicrobial activity of the crude L-ASP was determined by well diffusion method [20] with slight modification, where the crude L-ASP was tested against different strains other than used in the screening studies including gram-positive and gram-negative bacteria (*B. subtilis*, *Staphylococcus aureus*, *E. coli*, and *P. aeruginosa*) and some pathogenic fungi (*A. niger*, *Candida albicans*, *Fusarium oxysporum*, and *Rhizoctonia solani*). Measurement of the inhibition zone was carried out by adding 100 μl of the crude enzyme preparation separately of each one in 10-mm diameter wells, cut out in nutrient agar plates, which were seeded separately with test bacteria, and in potato-dextrose-agar plates, which were seeded separately with test fungi. All plated were incubated at 37°C for 24 h for bacteria and at 30°C for 72 h for fungi. The inhibition zone was measured in mm diameter.

Statistical analysis

All the experiments were carried out in triplicates, and statistical analysis was performed. The values of the data shown in the corresponding tables and figures were expressed as mean±SD, the data statistics were analyzed, and the SEM was determined.

Results and discussion

Screening for L-asparaginase production by some recommended microorganisms

The screening of L-ASP production by eight recommend microorganisms was done in shaken cultures lasting for different periods (1–3 and 3, 5, and 7 days) for bacteria and fungi, respectively (Tables 1 and 2). The data showed that with most cultures of different ages, the protein level was in the usual range and had no consistent relationship with both the microbial growth and L-ASP activity. For all cultures, final pH slightly varied within the alkaline range. Among the tested microorganisms, 3-day shaken *P. janthinellum Biourge* culture afforded the highest L-ASP productivity (11.43±0.099 U/reaction) followed by *A. niger* (10.10±0.028 U/reaction), and finally, 2-day aged bacterial species *E. coli* (7.33±0.09 U/reaction). In this respect, many authors reported *Penicillium sp.* and *Aspergillus sp.* as potent L-ASP producers [21–23]. Moreover, Mohamed *et al.* [24] reported that among all screened bacterial isolates, *E. coli* was the most active in the production of L-ASP enzyme.

Table 1 Screening of some bacterial isolates during different incubation periods for the production of an extracellular L-asparaginase enzyme

Bacterial isolate	Incubation period (h)	L-ASP activity (U/reaction)	Final pH of CF	Protein content of CF (mg/ml)	Dry weight of cells (mg/culture)
B. subtilis	24	2.86±1.013	8.1	0.345	260
	48	2.39±0.678	8.3	0.374	100
	72	2.30±0.954	8.6	0.463	40
Escherichia coli	24	0.93±0.0989	7.3	0.518	180
	48	7.33±0.0919	8.1	0.297	220
	72	3.43±0.183	8.2	0.340	70
Pseudomonas aeruginosa	24	2.55±0.721	8.8	0.384	330
	48	2.34±0.205	8.6	0.478	60
	72	1.83±1.018	8.9	0.522	30

CF, culture filtrate; L-ASP, L-asparaginase.

Table 2 Screening of some fungal isolates during different incubation periods for the production of an extracellular L-asparaginase enzyme

Fungal isolate	Incubation period (day)	L-ASP activity (U/reaction)	Final pH of CF	Protein content of CF (mg/ml)	Dry weight of mycelium (mg/culture)
Penicillium sp.	3	0.70±0.063	8.3	0.357	700
	5	0.93±0.007	8.4	0.441	650
	7	1.26±0.084	8.7	0.576	400
Penicillium janthinellum Biourge	3	11.43±0.099	8.8	0.606	690
	5	9.50±0.141	8.6	0.570	600
	7	5.10±0.282	8.2	0.520	570
Aspergillus terreus	3	2.90±0.007	9.1	0.639	1820
	5	1.87±0.007	8.6	0.607	1100
	7	1.56±0.155	8.5	0.591	960
Aspergillus niger	3	10.10±0.028	9.0	0.743	2500
	5	5.84±0.791	8.8	0.775	2000
	7	1.71±0.516	8.0	0.692	1800
Aspergillus oryzae	3	NA	8.0	0.408	1220
	5	6.40±0.212	8.7	0.432	2190
	7	7.50±0.028	8.9	0.513	1490

CF, culture filtrate; L-ASP, L-asparaginase; NA, no activity.

Conclusively, among all investigated microbes, the fungal strain *P. janthinellum Biourge* was the most promising and afforded the highest extracellular L-ASP enzyme productivity (11.43±0.099 U/reaction) and was chosen for the succeeding studies.

Effect of the fermentation period on L-asparaginase production by *Penicillium janthinellum Biourge*

P. janthinellum Biourge shaken culture incubation was lasted for different periods, that is, 2, 3, 4, 5, 6, and 7 days for the maximum production of L-ASP enzyme at 200 rpm and 30°C. The result (Fig. 1) showed that a good L-ASP activity of 8.83±0.381 U/reaction was offered by *P. janthinellum Biourge* after 2 days of incubation, and this value increased to reach its peak activity of 11.65±0.657 U/reaction after 3 days of incubation and gradually decreased after the extended periods, where 54.5% L-ASP productivity was lost after the seventh day, and this may be attributed to the enzyme digestion by proteases when the enzyme substrate in culture medium was consumed [25] or may be owing to depletion of nutrients and accumulation of toxic end products. Similar results have been reported by El-Refai *et al.* [26] where the highest activity of *Penicillium cyclopium* L-ASP was 68 U/ml after incubation for 72 h. In addition, Lincoln and More [27] found that the maximum L-ASP productivity from *Trichoderma viride sp*. under submerged fermentation conditions occurred in the third day. However, fifth day was the optimum fermentation period for L-ASP production by *A. terreus* [28]. Moreover, L-ASP produced by *Streptomyces sp.* reached its maximal activity after 10 days of fermentation [2].

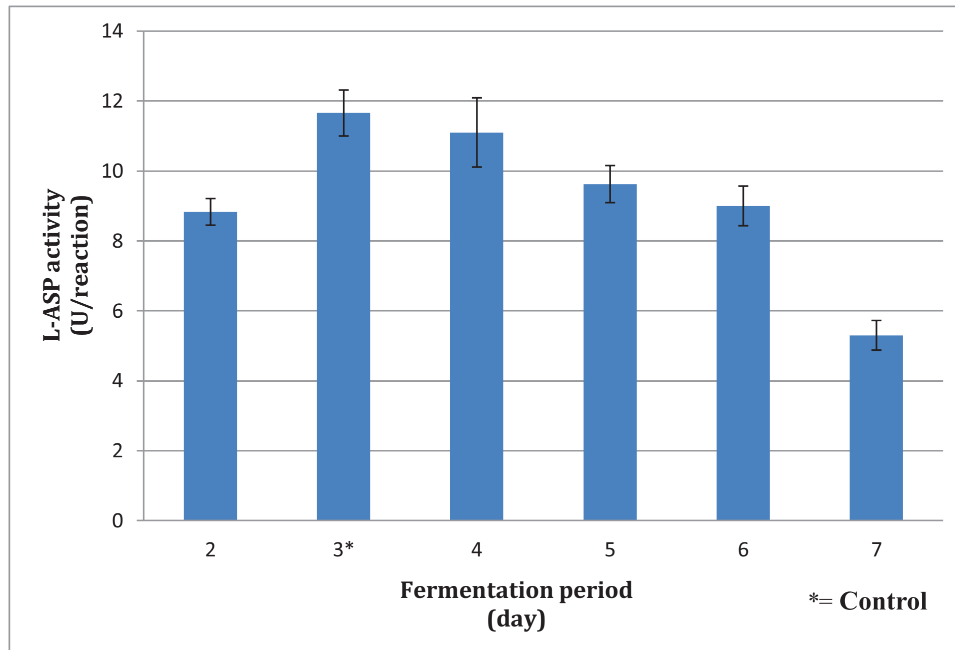
Effect of the initial pH on *Penicillium janthinellum Biourge* L-asparaginase productivity

The effect of the initial pH of the culture medium was studied within a wide pH range 5–8 using 1 N-NaOH and 1 N-HCl. Data (Fig. 2) exhibited two initial pH optima, 6.2 and 8.0, which afforded 11.65±0.657 and 10±1.202 U/reaction, respectively. However, the acidic pH (5.0) had the most adverse effect on the enzyme production, and more than 56.05% loss in enzyme productivity was recorded. The pronounced productivity was at the slight acidic pH 6.2; therefore, the initial pH 6.2 of the culture medium was chosen in all the succeeding experiments. It is worthy to mention that, in all cases, the final pH was independent to the initial pH and lied in the alkaline range 7.3–9.4. This was in accordance to great extent with that reported by Mohsin *et al*. [29], who reported that initial pH 6.0 was the optimum for *Penicillium sp.* L-ASP production. Moreover, the initial pH 6.5 was selected for effective L-ASP productivity from *T. viride sp*. [12]. In addition, Abd EL Ghany [30] observed that the production of *Aspergillus tamarii* NRRL 26258 L-ASP exhibited two initial pH optima: the first was highly acidic (2.7) and the second was alkaline (8.0).

Effects of inoculum size, age, and agitation rate on *Penicillium janthinellum Biourge* L-asparaginase production

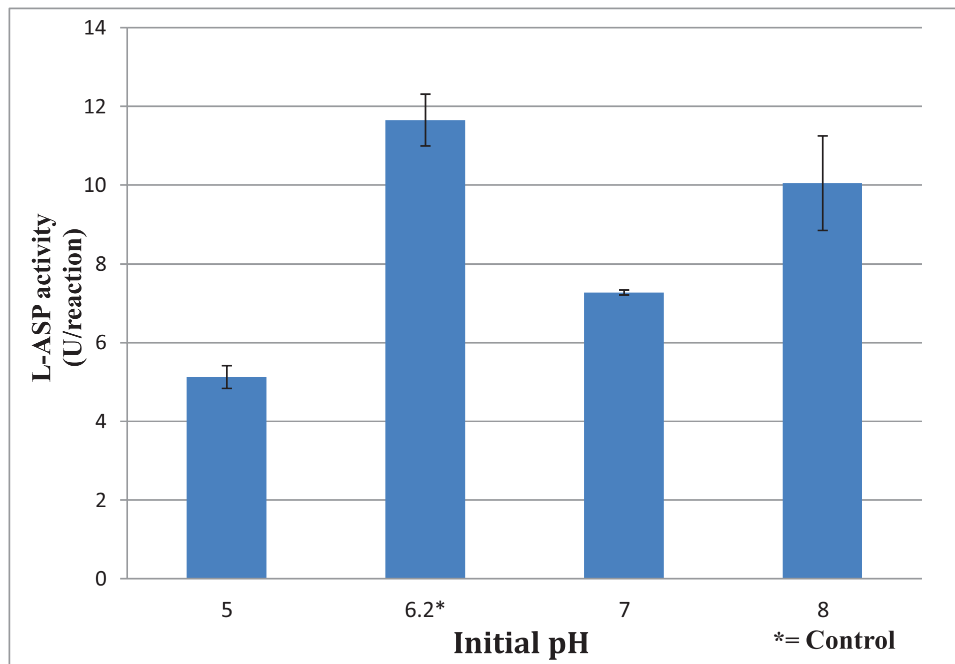
The effect of inoculum size (5–20%, v/v) and age (24–72 h) on *P. janthinellum Biourge* L-ASP production was studied. The data (Fig. 3) declared that 10% v/v (control) was the most proper and led to the highest L-ASP production (11.65±0.657 U/reaction). Moreover, the inoculum age of 72 h was

Figure 1



Effect of the fermentation period on *P. janthinellum Biourge* L-ASP productivity.

Figure 2

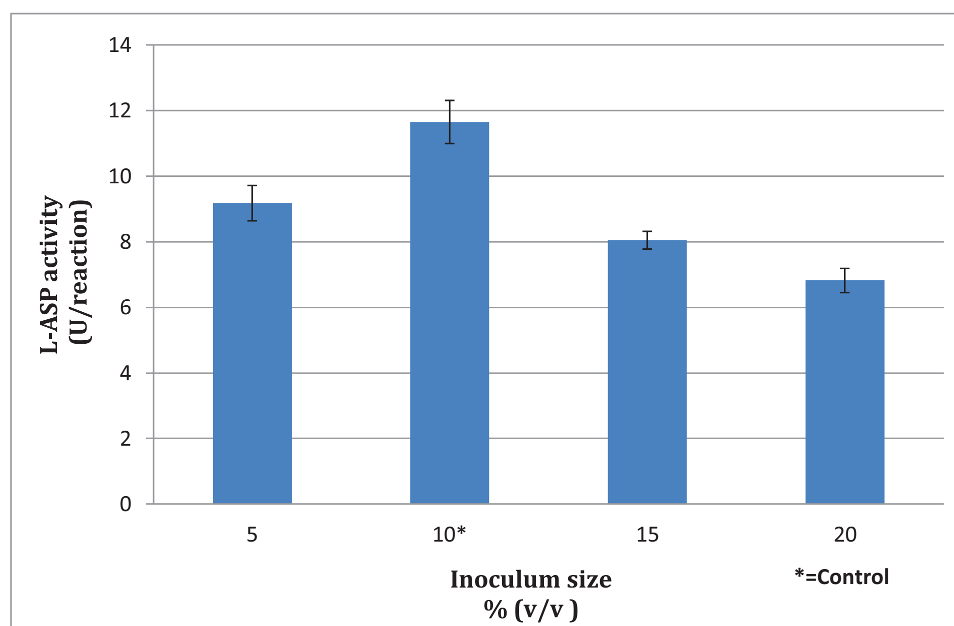


Effect of the Initial pH on *P. janthinellum Biourge* L-ASP productivity.

appropriate for the maximal *P. janthinellum Biourge* L-ASP production (11.65±0.657 U/reaction), and the younger inoculum than 72 h produced lower enzyme yields (10.91±0.120 and 10.21±0.926 U/reaction, respectively). In this connection, Mohsin *et al* [29] reported that the culture medium with 1% (v/v) 168-h-old inoculum was the optimal for *Penicillium sp.* L-ASP productivity. In addition, Pradhan *et al.* [31] reported that the maximum L-ASP production by *P. aeruginosa* strain F1 was obtained with inoculum size of 6% (v/v) and inoculum age of 16 h. Moreover, Kenari *et al.* [32] showed that the inoculum size of 10% (v/v) of 18-h age was the most suitable for maximum L-ASP activity from *E. coli* ATCC 11303. However, Sharma

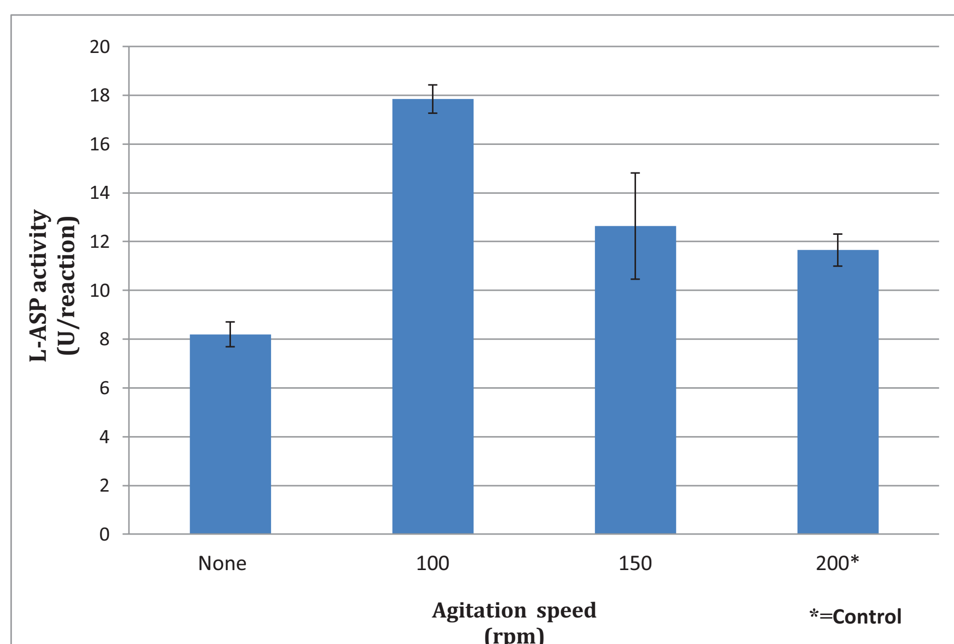
and Husain [33] applied 2% (v/v) for L-ASP production by *Enterobacter cloacae* with the inoculum age of 15 h. Concerning the effect of culture agitation rate, the rates from 100 to 200 rpm were applied in a bench-top thermostatic shaker and compared with a stationary culture. The shaking speed of 100 rpm resulted in the maximal L-ASP productivity and growth yield (17.85±0.579 U/reaction and 1170 mg/culture, respectively), above which the productivity considerably decreased (Fig. 4), and this was similar to those obtained by Ali *et al.* [34], who reported that the maximum L-ASP production by *Aspergillus sydowii* and *F. oxysporum* was achieved at 100 rpm shaking speed. Moreover, Mostafa *et al.* [35] showed that the greatest L-ASP production by marine *Bacillus velezensis* occurred at 100 rpm and the increase in the agitation rate above this value reduced L-ASP productivity. However, Mihooliya *et al.* [36] reported that the maximum production of *Pseudomonas resinovorans* L-ASP (38.88 IU/ml) was at 400 rpm.

Figure 3



Effect of the inoculum size on *P. janthinellum Biourge* L-ASP productivity.

Figure 4



Effect of the agitation speed on *P. janthinellum Biourge* L-ASP productivity.

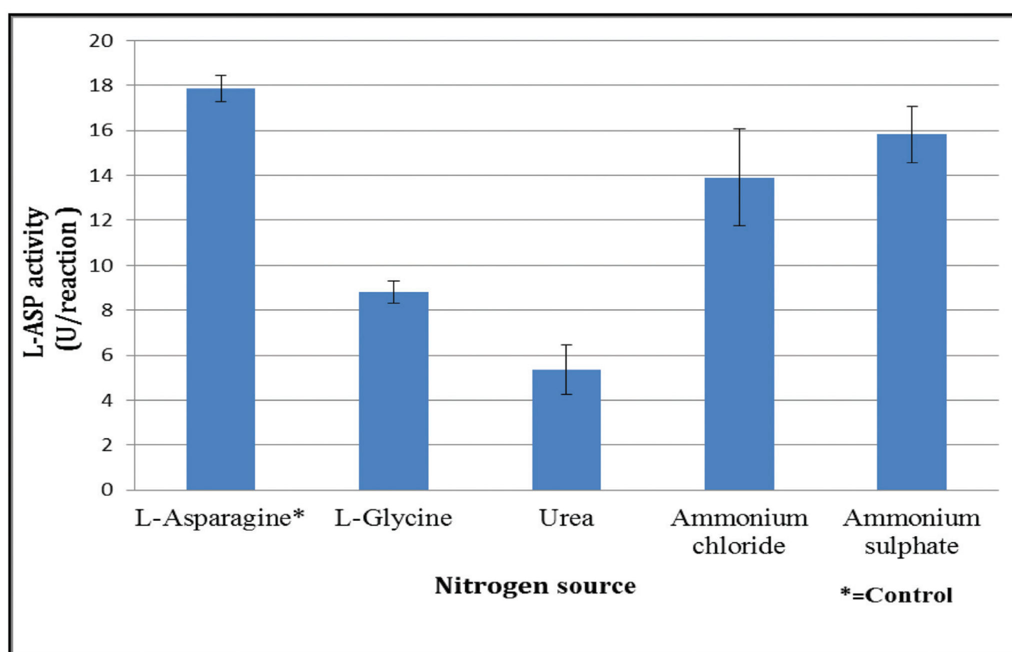
Effect of nitrogen source on L-asparaginase production

Different N sources (organic and inorganic) were separately employed in the production medium on equal N basis, that is, inorganic (ammonium chloride – ammonium sulfate) and organic (urea – L-glycine) by replacement of L-asparagine in the basal medium with any of the preceding N sources. The data (Fig. 5) disclosed that L-asparagine (control) led to the highest L-ASP activity (17.85±0.579 U/reaction) and also both the highest protein content and growth yield followed by inorganic sources ammonium sulfate and ammonium chloride, which also resulted in a considerable productivity (15.83 ±1.251 and 13.91±2.142 U/reaction, respectively), whereas L-glycine and urea led to the lowest enzyme productivity (8.8±0.495 and 5.37±1.103 U/reaction, respectively). These results are in agreement with those reported for the production of L-ASP from *A. tamarii*, where L-asparagine proved to be the favored nitrogen source [37]. However, El-Hefnawy *et al.* [38] reported that ammonium sulfate and yeast extract were the favorable nitrogen sources that can be used for L-ASP production by *Fusarium solani and Penicillium oxalicum.* Concerning, the effect of urea as N source, the present results accorded with those reported by Farag *et al.* [39] who found that *A. terreus* L-ASP productivity was declined when urea was applied as nitrogen source.

Effect of carbon source on L-asparaginase production

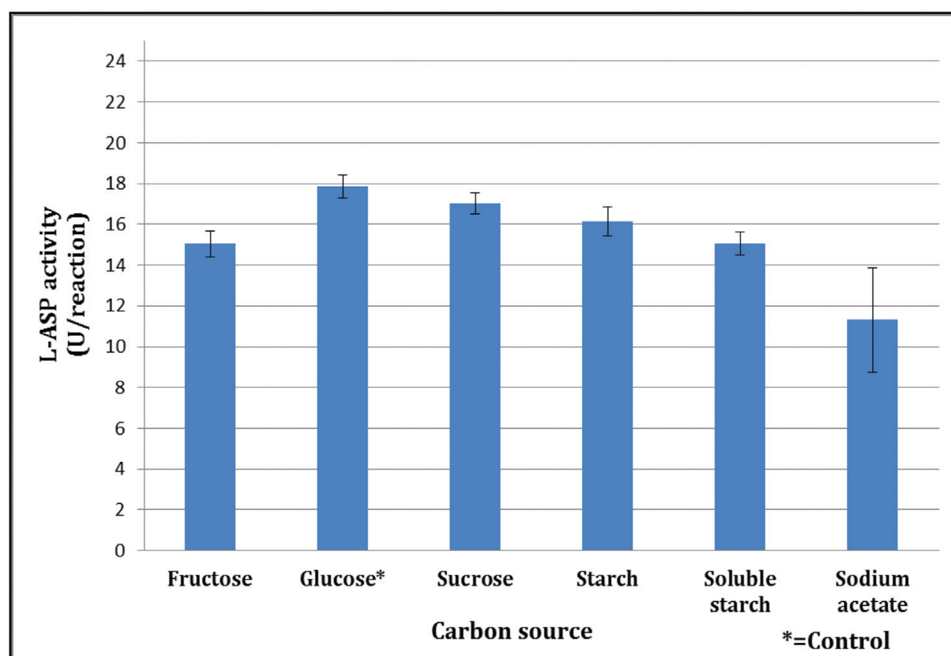
The effect of different carbon sources on *P. janthinellum Biourge* L-ASP productivity was investigated by substitution of the main carbon source (glucose) in the production medium with different carbon sources on equal C basis at 0.2% (w/v) concentration; these included fructose, sucrose, starch, soluble starch, and sodium acetate. The data (Fig. 6) revealed that all the tested carbon sources were appropriate for *P. janthinellum Biourge* L-ASP productivity, specifically glucose (control), which resulted in the maximal level of L-ASP production (17.85±0.579 U/reaction) followed by sucrose (17.03 ±0.523 U/reaction), starch (16.14±0.728 U/reaction), soluble starch (15.08±0.565 U/reaction), and fructose (15.04±0.622 U/reaction), whereas sodium acetate gave the lowest L-ASP productivity (11.31 ±2.552 U/reaction). In addition, both of the protein content and growth level were recorded in the presence of sucrose to be 0.571 mg/ml and 730 mg/culture, respectively. Consequently, glucose (control) was chosen as the suitable carbon source. These results more or less coincided with those reported by many authors The maximum L-ASP production by *F. solani* and *P. oxalicum* was achieved in the production medium containing 5% (w/v) of glucose (6.81 IU) and sucrose (6.21 IU) [38]. Moreover, glucose was found to be the optimum carbon source for maximum L-ASP production by *A. terreus* MTCC [40]. In addition, sucrose was the

Figure 5



Effect of N source on the *P. janthinellum Biourge* L-ASP productivity.

Figure 6



Effect of C source on the *P. janthinellum Biourge* L-ASP productivity.

recommended carbon source followed by glucose for *A. tamarii* L-ASP productivity [37]. On the contrary, Akilandeswari *et al*. [41] reported that starch was the optimum carbon source for L-ASP production by *A. niger*. In addition, Farag *et al*. [39] reported that dextrose brought the highest *A. terreus* L-ASP productivity (8.26 U/mg protein) compared with other carbon sources.

General properties of the crude enzyme preparation

The general properties of the crude L-ASP include effects of the enzyme protein concentration, the substrate concentration, and the reaction pH and temperature. The results in Table 3 and Fig. 7 showed that a parallel relationship existed between the enzyme protein concentration and the apparent L-ASP activity, thus gradual activity increase with the enzyme protein was recorded till 1000 μg/reaction, which afforded the maximum enzyme activity (27.21±1.103 U/reaction), and above this optimum concentration, the enzyme activity began to decline. This pointed out that 1.0 mg enzyme protein/reaction was enough to consume most of the substrate applied in the reaction mixture. The deviation of the plot from the normal relationship (straight line) may be owing to one or more of the following: presence of some inhibitors

or activators in the enzyme preparation and exhausting of substrate or presence of heavy metals such as Zn^{+2}, Ca^{+2}, Hg^{+2}, Pb^{+2} and other in the reaction mixture [42].

Table 3 General properties of the crude L-asparaginase preparation

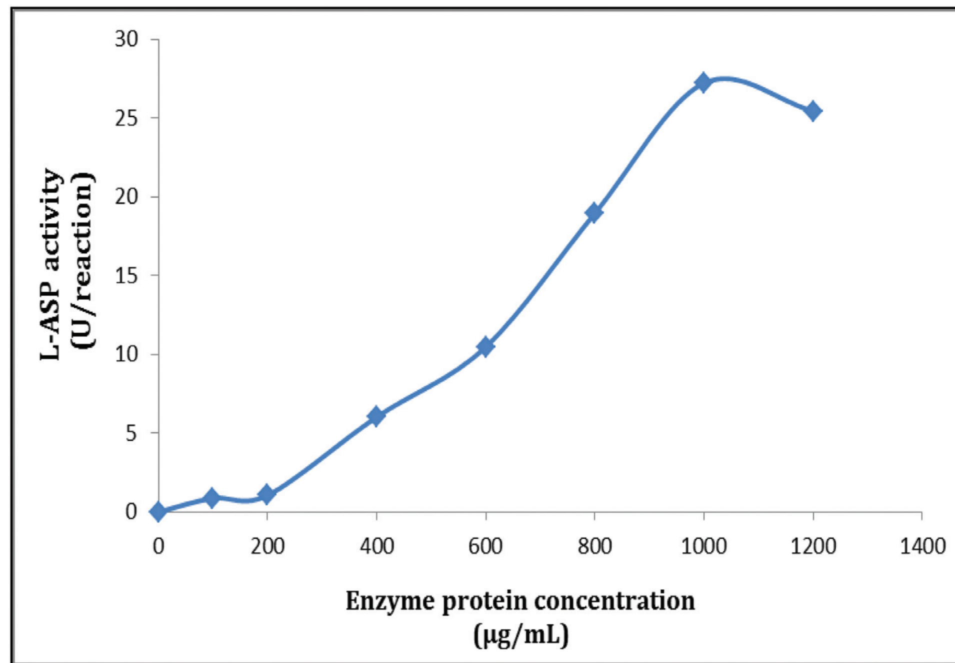
Property	L-ASP activity (U/reaction)
Enzyme protein concentration(μg/ml)	
100	0.88±0
200	1.06±0
400	6.03±2.050
600	10.47±1.346
800	18.95±0.367
1000	27.21±1.103
1200	25.44±0.629
Substrate concentration (%,w/v)	
0.2	25.28±1.400
0.4	26.90±0.325
0.6	27.21±1.103
0.8	27.52±0.162
1.0	29.36±0.551
1.5	28.87±0.0353
2.0	28.38±0.275
Reaction temperature (°C)	
30	24.94±0.586
35	26.12±0.374
37	29.36±0.551
40	29.78±0.579
45	33.13±0.573
50	30.77±0.834
Reaction pH	
4.0	31.86±1.116
5.0	34.37±0.573
6.0	33.15±1.265
7.0	32.53±0.530
8.6	33.13±0.573
9.9	36.29±0
10.7	38.44±0.219

L-ASP, L-asparaginase.

The effect of substrate (L-asparagine) was illustrated in Table 3 and Fig. 8, where the substrate concentration affected the reaction rate according to the mass action law, to reach the maximal 29.36±0.551 U/reaction at 1% w/v L-asparagine, indicating saturation of all enzyme active sites with L-asparagine molecules. It is worthy to mention that the L-ASP activity plot versus the substrate concentration came close to the normal hyperbolic phase.

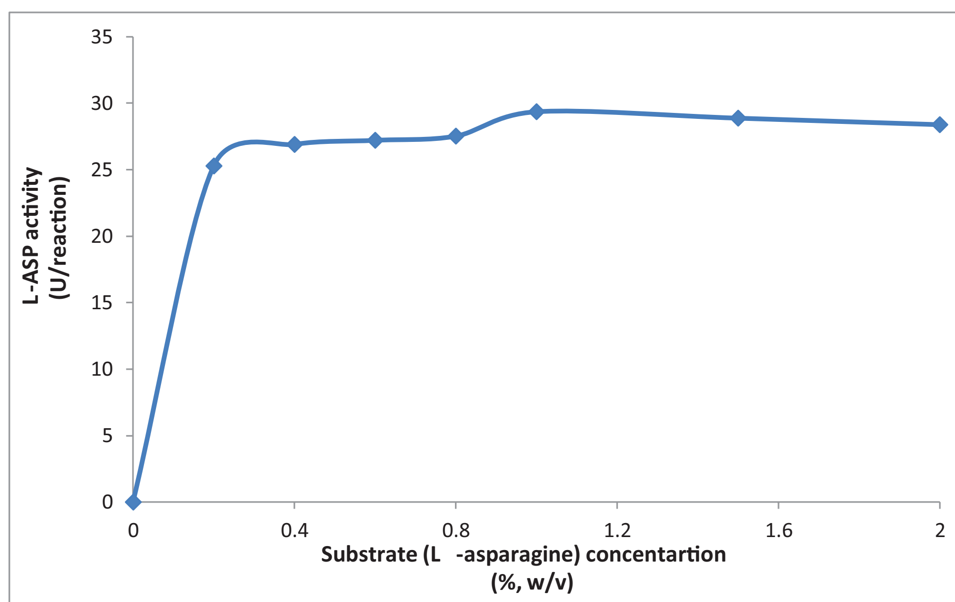
Concerning the effect of reaction temperature on the L-ASP activity, identical reaction mixtures were incubated at the following temperatures: 30, 35, 37, 40, 45, and 50°C for 30 min at pH 8.6, applying all the optimized previous conditions. The data recorded (Table 3) clarified that the activity increased with temperature till 45°C, at which the maximum value (33.13±0.573 U/reaction) of the crude L-ASP activity was reached. The elevation of temperature from 37°C

Figure 7



Effect of the enzyme protein concentration on the crude *P. janthinellum Biourge* L-ASP activity.

Figure 8



Effect of the substrate (L-asparagine) concentration on the crude *P. janthinellum Biourge* L-ASP activity.

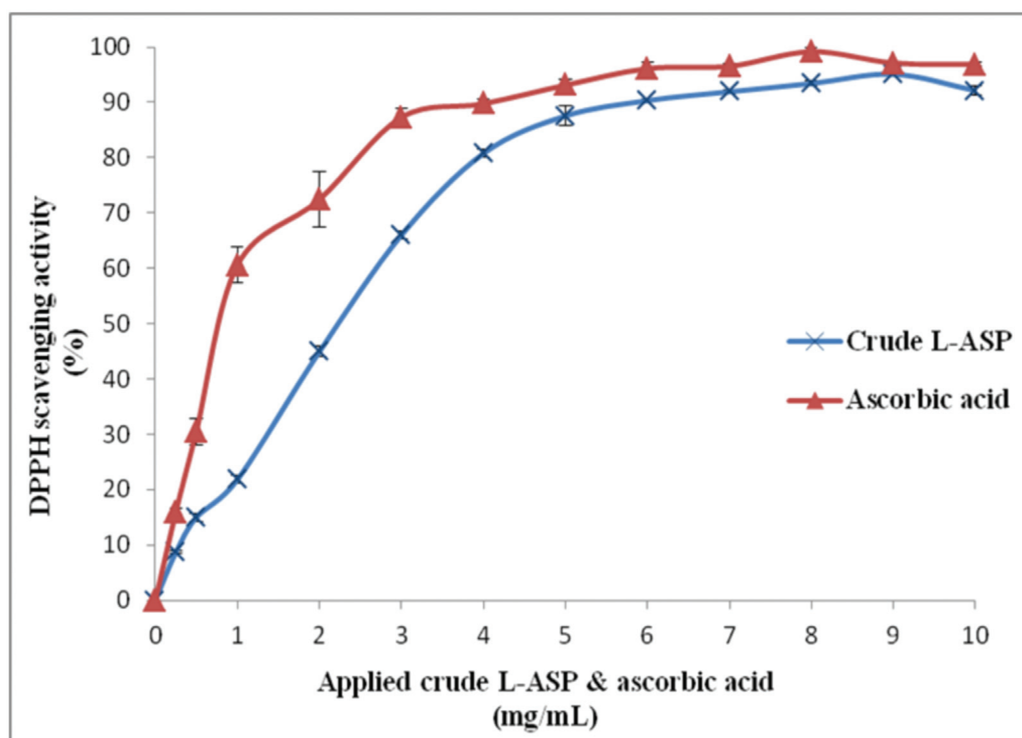
(control) to 45°C led to more than 12.84% activation for the crude L-ASP, and at the higher temperature of 50°C, the crude L-ASP retained about 92.87% of its activity at optimum temperature of 45°C. In this respect, the optimum temperature of 37°C was reported for *Penicillium* sp. L-ASP [23], and also other *A. terreus* L-ASPs were actively optimum at 37°C and 40°C [43,44]. On the contrary, many authors reported that the reaction temperatures from 37 to 60°C were the optimum temperatures for maximal L-ASP activity [45–47]. Generally, the high activity of the enzyme at 50°C pointed out its excellent thermostability.

The data recorded in Table 3 display the effect of reaction pH on the *P. janthinellum Biourge* L-ASP activity at a wide pH values, ranging from 4 to 10.7, applying 0.05 M-acetate pH (4–5), 0.05 M-phosphate pH (6–7), 0.05 M-Tris-HCl (8.6), and 0.05 M-carbonate-bicarbonate (9.9–10.7). The data displayed that the crude *P. janthinellum Biourge* L-ASP showed two peaks for activity at two different pH values of 5 and 10.7, with activities of 34.37±0.573 and 38.44±0.219 U/reaction, respectively, indicating the existence of two L-ASP enzyme forms in the crude enzyme preparation produced by the fungal strain, *P. janthinellum Biourge*, where one is acidic L-ASP the other is alkaline one. The two enzyme forms exhibited their maximal velocity at pH 5 and 10.7, respectively. In this respect, many authors reported on the production of the acidic and alkaline microbial L-ASP in the same microbial culture. Ahmed *et al*. [48] reported that the marine endophytic *Aspergillus* sp. ALAA-2000 strain produced two types of L-ASPs and showed two-peak activities curve at two different pH values of 6 and 10. Moreover, the crude and partially purified *A. tamarii* NRRL 26258 L-ASP exhibited two optimum reaction pH values: one acidic at 2.7 and other in alkaline form 8.0 [30].

Antioxidant activity of the crude enzyme preparation

The DPPH assay is the most commonly procedure for determination of the antioxidant activity as it is a simple, sensitive, and fast approach. The data in Fig. 5 show that the DPPH radical scavenging activity of the crude L-ASP ranged from 8.7 to 92.14 at varying concentrations from 0.25 to 10 mg dry enzyme preparation (wt/ml ethanol solution), whereas ascorbic acid (the standard) displayed over 90% activity at the concentration 5 mg/ml. The DPPH radical scavenging activity to a great extent dependently increased with the enzyme dose. According to Fig. 9, the IC_{50} value was calculated for the applied crude L-ASP to be at 2.2 mg/ml, which contains 198 μg enzyme protein and for the standard

Figure 9



DPPH scavenging activity of the crude *Penicillium janthinellum Biourge* L-ASP enzyme and ascorbic acid. DPPH, 2, 2-diphenyl-1-picrylhydrazyl.

was 0.86 mg/ml. In this connection, L-ASP produced from *Penicillium sp*. showed antioxidant property, with 64.96% against DPPH radical [21]. In addition, recombinant *P. resinovorans* L-ASP showed antioxidant activity of 62% against DPPH radical [36]. Furthermore, *Bacillus halotolerans* L-ASP showed a good antioxidant activity against DPPH, with IC_{50} of 64.07 mg/ml [49]. It could be decided here that the crude *P. janthinellum Biourge* L-ASP had an excellent scavenging activity, which was very close to that of the standard ascorbic acid. Concerning the antimicrobial activity of the crude enzyme form, many trials were performed with varied enzyme concentrations, but unfortunately, the crude L-ASP exhibited no antifungal or antibacterial activities by any of the crude enzyme preparation concentrations; therefore, this should be followed up by the partially purified enzyme form side by side with its expected anticancer activity in extended studies.

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Conflicts of interest

There are no conflicts of interest.

References

1 Mohideen AK. Molecular docking study of L-asparaginase I from *Vibrio campbellii* in the treatment of acute lymphoblastic leukemia (ALL). Euro Biotech J 2020; 4:8–16.

2 El-Hadi AA, Ahmed HM, Hamzawy RA. Optimization and characterization of l-asparaginase production by a novel isolated streptomyces spp. strain. Egypt Pharma J 2019; 18:111–122.

3 Lang S, Uber. Deamidation in the animal body. Beitra Chemi Physio Patholo 1904; 5:321–345.

4 Clementi A. The enzymatic deemidation of L-asparaginase in different animal species and the physiological significance of its presence in the organisms. Arch Inter Physio 1922; 19:369–398.

5 Kidd JG. Regression of transplanted lymphomas induced in vivo by means of normal guinea pig serum I. Course of transplanted cancers of various kinds in mice and rats given guinea pig serum, horse serum, or rabbit serum. J Exp Med 1953; 98:565–582.

6 Ahmad N, Pandit NP, Maheshwari SK. L-asparaginase gene-a therapeutic approach towards drugs for cancer cell. Int J Biosci 2012; 2:1–11.

7 Cachumba JM, Antunes FA, Peres GF, Brumano LP, Dos Santos JC, Da Silva SS. Current applications and different approaches for microbial L-asparaginase production. Brazill Microbiol J 2016; 47:77–85.

8 Verma N, Kumar K, Kaur G, Anand S. L-asparaginase: a promising chemotherapeutic agent. Crit Rev Biotech 2007; 27:45–62.

9 Krishnapura PR, Belur PD, Subramanya S. A critical review on properties and applications of microbial l-asparaginases. Crit Rev Microbiol 2016; 42:720–737.

10 Jiao L, Chi H, Lu Z, Zhang C, Chia SR, Show PL, *et al.* Characterization of a novel type I L-asparaginase from *Acinetobacter* soli and its ability to inhibit acrylamide formation in potato chips. J Biosci Bioeng 2020; 129:672–678.

11 Moharam ME, Gamal-Eldeen AM, El-sayed ST. Production, immobilization and anti-tumor activity of L-asparaginase of *Bacillus* sp.R36. J Am Sci 2010; 6:157–165.

12 Lincoln L, Niyonzima FN, More SS. Purification and properties of a fungal L-asparaginase from *Trichoderma viride* pers: SF GREY. J Microbiol Biotechnol Food Sci 2015; 4:310–316.

13 Gherna RL, Pienta P, Jong SC, Hsu HT, Daggett PM. The American type culture collection: catalogue of strains I. 13th ed. Rockville, MD: American Type Culture Collection; 1978; p. 445.

14 Geckil H, Gencer S, Uckun M. Vitreoscilla hemoglobin expressing *Enterobacter aerogenes* and *Pseudomonas aeruginosa* respond differently to carbon catabolite and oxygen repression for production of L-asparaginase, an enzyme used in cancer therapy. Enzyme Microb Technol 2004; 35:182–189.

15 Saxena RK, Sinha U. L-asparaginase and glutaminase activities in the culture filtrates of *Aspergillus nidulans*. Curr Sci 1981; 50:218–219.

16 Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Measurement of protein with the Folin phenol reagent. J Biol Chem 1951; 193:265–275.

17 Imada A, Igarasi S, Nakahama K, Isono M. Asparaginase and glutaminase activities of micro-organisms. J Gen Microbiol 1973; 76:85–99.

18 Jetti J, Jetti A, Perla R. Production of L-asparaginase by using *Pectobacterium carotovorum*. J Prob Health 2017; 5:1–6.

19 Peng CL, Chen SW, Lin ZF, Lin GZ. Detection of antioxidative capacity in plants by scavenging organic free radical DPPH. Prog Biochem Biophys 2000; 27:658–661.

20 Jorgensen JH, Turnidge JD. Susceptibility test methods: dilution and disk diffusion methods. In Manual of Clinical Microbiology. 11th ed. Washington, USA: American Society of Microbiology; 2015; 15. pp. 1253–1273.

21 Soniyambi AR, Lalitha S, Praveesh BV, Priyadarshini V. Isolation, production and anti-tumor activity of L-asparaginase of *Penicillium* sp. Int J Microbiol Res 2011; 2:38–42.

22 Siddalingeshwara KG, Karthic J, Sanil DP, Naveen M, Prathiba KS. Rapid screening and confirmation of l-asparaginase from *Penicillium spp*. Int J Res Pharmacol Pharmacother 2012; 1:147–150.

23 Patro KR, Gupta N. Extraction, purification and characterization of L-asparaginase from *Penicillium* sp. by submerged fermentation. Int J Biotechnol Mol Biol Res 2012; 3:30–34.

24 Mohamed ZK, Elnagdy SM, Seufi AE, Gamal M. Production and optimization of L-asparaginase in *Escherichia coli*. Egypt J Bot 2016; 56:203–224.

25 Ferdinand W. The enzyme molecule. London, New York: Wiley J and Sons Company; 1976; 224–227

26 El-Refai HA, El-Shafei MS, Mostafa H, El-Refai HAM, El-Beih FM, Awad GE, *et al.* Statistical optimization of anti-leukemic enzyme L-asparaginase production by *Penicillium cyclopium*. Curr Trends Biotechnol Pharma 2014; 8:130–142.

27 Lincoln L, More SS. Isolation and production of clinical and food grade L-asparaginase enzyme from fungi. J Pharmacognosy Phytochem 2014; 3:177–183.

28 Kalyanasundaram I, Nagamuthu J, Srinivasan B, Pachayappan A, Muthukumarasamy S. Production, purification and characterisation of extracellular L-asparaginase from salt marsh fungal endophytes. World J Pharma Pharma Sci 2015; 4:663–677.

29 Mohsin SM, Sunil PLNSN, Siddalingeshwara KG, Karthi J, Jayaramu M, Mani N, *et al.* Optimization of fermentation conditions for the biosynthesis of L-asparaginase by *Pencillium* sp. J Acad Indus Res 2012; 1:180–82.

30 Abd El Ghany MI. Studies on microbial asparaginases as prevalent anticancer agents [MSc Thesis]. Cairo: Faculty of Pharmacy, Cairo University; 2009.

31 Pradhan B, Dash S, Sahoo S. Optimization of some physical and nutritional parameters for the production of L-asparaginase by isolated thermophilic *Pseudomonas aeruginosa* strain F1. Biosci Biotech Res Asia 2013; 10:389–395.

32 Kenari SL, Alemzadeh I, Maghsodi V. Production of l-asparaginase from *Escherichia* coli ATCC 11303: Optimization by response surface methodology. Food Bioprod Process 2011; 89:315–321.

33 Sharma A, Husain I. Optimization of medium components for extracellular glutaminase free asparaginase from *Enterobacter cloacae*. Int J Curr Microbiol App Sci 2015; 4:296–309.

34 Ali D, Ouf S, Eweis M, Solieman D. Optimization of L-asparaginase production from some filamentous fungi with potential pharmaceutical properties. Egypt J Bot 2018; 58:355–369.

35 Mostafa Y, Alrumman S, Alamri S, Hashem M, Al-izran K, Alfaifi M, *et al.* Enhanced production of glutaminase-free L-asparaginase by marine *Bacillus velezensis* and cytotoxic activity against breast cancer cell lines. Electr J Biotech 2019; 42:6–15.

36 Mihooliya KN, Nandal J, Kumari A, Nanda S, Verma H, Sahoo DK. Studies on efficient production of a novel l-asparaginase by a newly isolated *Pseudomonas resinovorans* IGS-131 and its heterologous expression in *Escherichia coli*. Biotechnology 2020; 10:1–11.

37 Bedaiwy MY, Awadalla OA, Abou-Zeid AM, Hamada HT. Optimal conditions for production of L-asparaginase from *Aspergillus tamarii*. Egypt J Exp Biol (Botany) 2016; 12:229–237.

38 El-Hefnawy MAA, Attia M, El-Hofy ME, Ali SM. Optimization Production of L asparaginase by locally isolated filamentous fungi from Egypt. Curr Sci Int 2015; 4:330–341.

39 Farag AM, Hassan SW, Beltagy EA, El-Shenawy MA. Optimization of production of anti-tumor l-asparaginase by free and immobilized marine *Aspergillus terreus*. Egypt J Aqua Res 2015; 41:295–302.

40 Gurunathan B, Sahadevan R. Production of l-asparaginase from natural substrates by *Aspergillus terreus* MTCC 1782: Optimization of carbon source and operating conditions. Int J Chem React Eng 2011; 9:1.

41 Akilandeswari K, Kavitha K, Vijayalakshmi M. Production of bioactive enzyme L-asparaginase from fungal isolates of water sample through submerged fermentation. Int J Pharma Pharma Sci 2012; 4:363–366.

42 Plummer DT. An introduction to practical Biochemistry. 2nd (ed). London: Tata McGraw-Hill book Publishing Company (UK) Limited; 1978. 6:227–234.

43 Siddalingeshwara KG, Lingappa K. Production and characterization of L-asparaginase-a tumor inhibitor. Int J Pharm Tech Res 2011; 3:314–319.

44 Hassan SW, Farag AM, Beltagy EA. Purification, characterization and anticancer activity of L-asparaginase produced by marine *Aspergillus terreus*. J Pure Appl Microbiol 2018; 12:1845–1854.

45 El-Naggar NEA, Deraz SF, El-Ewasy SM, Suddek GM. Purification, characterization and immunogenicity assessment of glutaminase free L-asparaginase from *Streptomyces brollosae* NEAE-115. BMC Pharmacol Toxicol 2018; 19:1–15.

46 Phetsri K, Furukawa M, Yamashiro R, Kawamura Y, Hayashi J, Tobe R, *et al.* Comparative biochemical characterization of L-asparaginases from four species of lactic acid bacteria. J Biotech Biomed 2019; 2:112–124.

47 Maqsood B, Basit A, Khurshid M, Bashir Q. Characterization of a thermostable, allosteric L-asparaginase from Anoxybacillus flavithermus. Int J Biol Macromol 2020;152:584–592.

48 Ahmed MMA, Dahab NAF, Taha MT, Hassan SMF. Production, purification and characterization of L-Asparaginase from marine endophytic *Aspergillus* sp. ALAA-2000 under submerged and solid state fermentation. J Microb Biochem Technol 2015; 7:165–172.

49 El-Fakharany E, Orabi H, Abdelkhalek E, Sidkey N. Purification and biotechnological applications of L-asparaginase from newly isolated Bacillus halotolerans OHEM18 as antitumor and antioxidant agent. J Biomol Struct Dyn 2020; 23:1–13.