Production of a bacterial extracellular L-glutaminase possessing high antioxidant capability

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Received: 18 September 2020
Revised: 9 October 2020
Accepted: 12 October 2020
Published: 15 February 2021

Egyptian Pharmaceutical Journal 2021, 20:59–71

Background and objectives
L-glutaminase has utmost practical importance in many fields, such as medicine, pharmacy, and some industries as an effective antioxidant, anticancer, flavor enhancer, and used as an analytical reagent in the determination of glutamate and glutamine. The objective of the present article was to formulate the production medium and to pinpoint the proper growth conditions for the most potent microorganism producing highly active glutaminase enzyme. The general properties of the crude enzyme preparation were determined to detect the proper conditions for enzyme activity. Under the specified conditions, the capabilities of the crude L-glutaminase preparation for antimicrobial and antioxidant activities were investigated.
Materials and methods
A total of 12 recommended microbial strains were screened for highly active L-glutaminase enzyme production. Factors influencing the production of L-glutaminase enzyme were optimized, and the important properties of the crude enzyme were pinpointed. Finally, biological activities of the crude enzyme were investigated as a preliminary index for the validity of the partially purified L-glutaminase form for medical applications.
Results and conclusion
Among all tested microorganisms, *Bacillus subtilis* NRRL 1315 was the most potent producer for L-glutaminase enzyme. The maximum glutaminase production was obtained after 48 h of incubation on a rotary shaker (150 rpm) with the medium containing 5 g/l glucose, 0.1 g/l sodium nitrate, and 10 g/l L-glutamine at 37°C and pH 7.5. The important properties of the crude L-glutaminase were duly pinpointed as follows: optimum enzyme protein concentration and substrate concentration were 2 mg/ml and 40 mM, respectively, and optimum reaction pH and temperature were 7.5 and 37°C, respectively. Under the specified conditions, the crude enzyme exhibited considerable 2, 2′-diphenyl-1-picrylhydrazyl radical scavenging activity.

Keywords:
2, 2′-diphenyl-1-picrylhydrazyl radical, antimicrobial, antioxidant, biological activities, L-glutaminase

Egypt Pharmaceut J 20:59–71

1687-4315

Introduction

L-glutaminase (EC.3.5.1.2) is an amidohydrolase that catalyzes the deamidation of L-glutamine, resulting in the production of L-glutamic acid and ammonia. L-glutaminases are ubiquitous in the biological world, and organisms ranging from bacteria to human cells produce this important enzyme [1,2].

L-glutaminase plays an important role in cellular metabolism of plant, animal, and microorganisms such as bacteria, fungi, and yeast [3]. It is widely used in food industry as a flavor enhancer [4,5] and in pharmaceutical sector as an effective therapeutic agent in the treatment of HIV [6,7] and acute lymphocytic leukemia [8]. In recent years, L-glutaminase in combination with or as an alternative to L-asparaginase could be used in enzyme therapy for cancer, particularly leukemia [9,10].

An enzyme that plays a dual role as an antioxidant and an anticancer agent may have a major influence on the proliferation of cancer cells. The enzyme causes selective death of glutamine-dependent tumor cells by blocking glutamine consumption and thus helps in the treatment of malignancies. According to this purpose, several studies were carried out to use L-glutaminase in cancer therapy [11–17]. Moreover, it was used as an analytical reagent in the determination of glutamate and glutamine [18,19].

Biological systems have highly reactive free radicals and oxygen species. Free radicals are able to oxidize nucleic

acids, proteins, and lipids; therefore, they can initiate degenerative diseases. On the contrary, antioxidants have the ability to scavenge and inhibit these radicals. Moreover, antioxidants have wide applications as dietary supplements and can prevent many diseases like cancer and coronary heart disease [20].

There are two known types of antioxidants: the natural and the synthetic antioxidants. Many studies reported the synthetic antioxidants as suspected to have carcinogenic probability. Therefore, natural antioxidants are the most preferred type of antioxidants. Antioxidants from natural sources play a paramount role in helping endogenous antioxidants to neutralize the oxidative stress. L-glutaminase is actually a vital ordinary antioxidant, and it is not related to lethal and cancer-causing effects like those of synthetic antioxidants [21,22].

Several studies were conducted to produce microbial L-glutaminase with antioxidant properties [16,17,23,24].

The production of L-glutaminase enzyme is influenced by a variety of nutritional factors. It is known that the factors involved in the process of production not only enhance the quantity but also the quality of enzyme, so it becomes more suitable for a specific application. Therefore, optimization of parameters can in turn influence enzyme synthesis and cell yield [25].

The aim of the present study was to screen some L-glutaminase-producing bacteria and fungi, to optimize the culture conditions for the maximum L-glutaminase production, to characterize the extracellular crude L-glutaminase, and then to study its antimicrobial and antioxidant capabilities.

Materials and methods

Materials

Microorganisms

A total of 12 recommended fungal and bacterial strains obtained from different sources were screened for their abilities to produce extracellular L-glutaminase. These strains were as follows:

Escherichia coli ATCC 8739 was obtained from Microbiological Resources Centre (Cairo Mircen), Faculty of Agriculture, Ain Shams University, Egypt, whereas *Bacillus cereus* NRC-20, *Aspergillus terreus* NRC-7, *Aspergillus oryzae* NRC-34, and *Penicillium janthinellum Biourge* are local strains obtained from the Culture Collection of the Department of the Natural and Microbial Products Chemistry, National Research Centre, Dokki, Egypt. In addition, *Bacillus subtilis* NRRL 1315, *Trichoderma reesei* NRRL 11460, and *Trichoderma viride* NRRL 6418 were obtained from the National Center for Agricultural Utilization Research, Peoria, Illinois, USA. Other four strains (*Pseudomonas aeruginosa* ASU-1, *Staphylococcus aureus*, *Aspergillus niger* ASU-a6, and *Penicillium cyclopium*) were local isolated strains provided by Microbiology Department, Faculty of Science, Ain Shams University.

Media

The following media (g/l) were applied, and it was autoclaved at 121°C for 20 min. All media prepared were made up to 1 l with distilled water and the pH was adjusted to the desirable value by 1N-NaOH or 1N-HCL.

Bacterial media

Culture maintenance medium

The tested bacterial strains were maintained on the nutrient agar slants and incubated at 37°C for 24 h. It had the following composition (g/l): peptone, five; beef extract, three; NaCl, eight; and agar, 12, with pH 7.3 ±0.2 [26].

Production medium

Mineral salt glutamine medium was applied for bacterial L-glutaminase production and had the following composition (g/l): L-glutamine, 10; D-glucose, five; NaCl, three; KH_2PO_4, one; $MgSO_4.7H_2O$, 0.5; $CaCl_2$, 0.1; $NaNO_3$, 0.1; trisodium citrate, 0.1, and pH was adjusted to 7.0 [27].

Fungal media

Culture maintenance medium

The fungal strains were maintained on potato-dextrose agar medium slants and incubated at 30°C for 7 days. It had the following composition (g/l): potato infusion, 200; D-glucose, 20; and agar, 15, with pH 5.6±0.2.

Production medium

Czapek Dox medium with some modification was used for fungal glutaminase production and was composed of the following (g/l): glucose, two; L-glutamine, 10; KH_2PO_4, 1.52; KCl, 0.52; $MgSO_4.7H_2O$, 0.52; $FeSO_4.7H_2O$, 0.01; and yeast extract, one, with pH adjusted to 7.0 [14].

Methods

Subculturing of fungal and bacterial strains and preparation of inoculum

The selected fungal strains were maintained on PDA slants and incubated at 28°C for 5–7 days, whereas the

selected bacterial strains were maintained on nutrient-agar medium and incubated at 37°C for 24 h. Spore suspension of each isolate was prepared by adding 5-ml sterile distilled water to 24-h-old bacterial slant or 7-day-old fungal slant, which were scratched and transferred to 250-ml Erlenmeyer flasks containing 50 ml of sterile enhancement growth mediums, and then the flasks were shaken in thermostatic rotary shakers (150 rpm) for 3 days at 28°C for fungi and 24 h at 37°C for bacteria.

Screening for enzyme production

Cultivation was carried out in 250-ml Erlenmeyer flasks, each containing 50 ml of the production medium. Flasks were autoclaved for 20 min. Each was inoculated with 5 ml inoculum (10^6–10^7 spore/ml). The culture flasks of the fungal strains were incubated at 28°C on a rotary shaker at 120 rpm for different periods (3, 5, and 7 days), whereas the bacterial cultures were shaken (120 rpm) at 37°C for 24, 48, and 72 h. The fungal fermented cultures were centrifuged by cooling centrifuge (4°C) at 2300*g* (the acceleration according to gravity) for 15 min, whereas the bacterial fermented cultures were centrifuged at 3980 g. for 15 min, and the presence of extracellular L-glutaminase in the clear supernatant was quantified by estimating the amount of ammonia liberated using the direct Nesslerization method described by Imada *et al.* [28].

Assay of L-glutaminase

An aliquot of 0.5-ml culture filtrate was mixed with 0.5 ml of 0.04-M L-glutamine solution in the presence of 0.5 ml distilled water and 0.5 ml phosphate buffer (0.1 M, pH 7.0). The mixture was incubated at 37°C for 30 min, and the reaction was stopped by the addition of 0.5 ml of 1.5 M-trichloro acetic acid. To 0.1 ml of the mixture, 3.7 ml of distilled water and 0.2 ml of Nessler's reagent were added. The absorbance was measured at 450 nm using a visible–ultraviolet (UV) spectrophotometer (V/UV spectrophotometer, Unico UV 2000). Then a standard curve was plotted using ammonium sulfate as the standard for estimation of ammonia liberated. One international unit of L-glutaminase was defined as the amount of enzyme that liberates 1 µm of ammonia under the optimum conditions. The enzyme yield was expressed as units/reaction.

Protein content assay

Protein was determined by spectrophotometer colorimetrically using Folin–Ciocalteu phenol reagent according to the method by Lowry *et al.* [29]. The standard curve of protein was constructed using the bovine serum albumin.

Effect of various parameters on L-glutaminase production by Bacillus subtilis NRRL 1315

Factors influencing the production of L-glutaminase enzyme were optimized by a single factor through varying the parameters one at a time. Experiments were carried out in Erlenmeyer flasks (250 ml) containing mineral salt glutamine broth. Factors include the effect of different carbon sources (glucose, fructose, maltose, lactose, galactose, sucrose, and starch), nitrogen sources (peptone, tryptone, yeast extract, urea, beef extract, ammonium chloride, ammonium nitrate, and ammonium sulfate), temperature (28, 37, 42, and 47°C), initial pH (4–10), inoculum size (2–20%), agitation rate (80, 120, 150, and 200 rpm), and different L-glutamine concentrations (0.35, 0.50, 0.75, 1, and 1.5%) on the enzyme productivity. Optimum conditions identified for one parameter were used for optimizing the other parameters.

Batch production of the crude L-glutaminase preparation by the bacterial isolate Bacillus subtilis NRRL 1315

The optimized medium was applied for batch enzyme production. Incubation lasted for 48 h in thermostatic rotary shaker (150 rpm at 37°C). At the end of any fermentation process, all cultures were collected, mixed, filtered, and centrifuged and then the clear filtrate was air dried. Many batches of the air-dried filtrates were collected, mixed, and homogenized and used as the crude L-glutaminase preparation used in all next studies.

Characterization of crude extracellular L-glutaminase

The characteristics specified included the effect of pH (3–10), reaction temperature (25–55°C), L-glutamine concentration (20–500 mM), and enzyme protein concentration (0.1–2.0 mg/ml enzyme) on L-glutaminase activity.

Biological activities of the crude L-glutaminase

Antimicrobial bioassay

The antimicrobial screening bioassay was determined by the agar well diffusion method described by Jorgensen and Turnidge [30].

Candida albicans ATCC 10231, *A. niger* ATCC 16404, and *Fusarium oxysporum* ATCC 62506 were used as the test fungal strains. The test fungal strains were slanted and maintained on the potato-dextrose agar medium. *B. subtilis* ATCC 6633, *S. aureus* ATCC 6538, *E. coli* ATCC 7839, and *P. aeruginosa* ATCC 9027 were used as the test bacterial strains. Bacteria were slanted on nutrient agar, and the measurement of the inhibition zones was done by placing 0.1 ml of the crude preparation separately in 10-mm diameter wells

cut in nutrient agar plates and then seeded by the tested bacteria, whereas potato-dextrose agar plates seeded by the tested fungi. After that, the bacterial plates were incubated at 37°C for 24 h, whereas the fungal plates were incubated at 30°C for 72 h, and the inhibition zones were measured in mm diameter.

Antioxidant activity of the crude L-glutaminase applying 2, 2′-diphenyl-1-picrylhydrazyl assay

Free radical scavenging activity of the crude L-glutaminase was determined by applying rapid, simple, and inexpensive method involving the use of the free radical, 2, 2-diphenyl-1-picrylhydrazyl (DPPH), according to the method of Peng *et al.* [31] and Sajitha *et al.* [23] with some modifications.

DPPH is a biological free radical, and its color is violet. The magnitude of antioxidation ability of sample can be expressed by their capacity to scavenge DPPH radical. Based on this property, antioxidants present in the sample will turn the free radical into yellow color, and the change of color from violet to yellow indicates a positive test. In brief, the crude enzyme stock ethanolic solution (10.0 mg/ml) was diluted with ethanol to final concentrations ranged from 0.25 to 9.0 mg enzyme/ml. Then, 0.5 ml of a 0.3 mM-DPPH ethanolic solution was added separately to 0.5 ml of each sample solution, and the reaction mixture was vortexed and incubated for 1 h at room temperature in dark place. Ascorbic acid was used as the standard. The degree of reduction of the reagent was measured as absorbance in UV spectrophotometer at 517 nm. The inhibitory percentage of DPPH was calculated according to the following equation:

$$(\%\text{inhibition}) = \left((\text{ABS}_{\text{control}} - \text{ABS}_{\text{sample}}) / \text{ABS}_{\text{control}}\right) \times 100$$

Where, $\text{ABS}_{\text{control}}$=absorbance of control and $\text{ABS}_{\text{sample}}$=absorbance of a tested sample at the end of the reaction. Each assay was carried out in triplicate, and the results were averaged.

Percentage of radical scavenging activity was plotted against the corresponding concentration of the crude enzyme to obtain IC_{50} value, where IC_{50} is the maximal concentration of the compound to cause 50% inhibition.

Statistical analysis

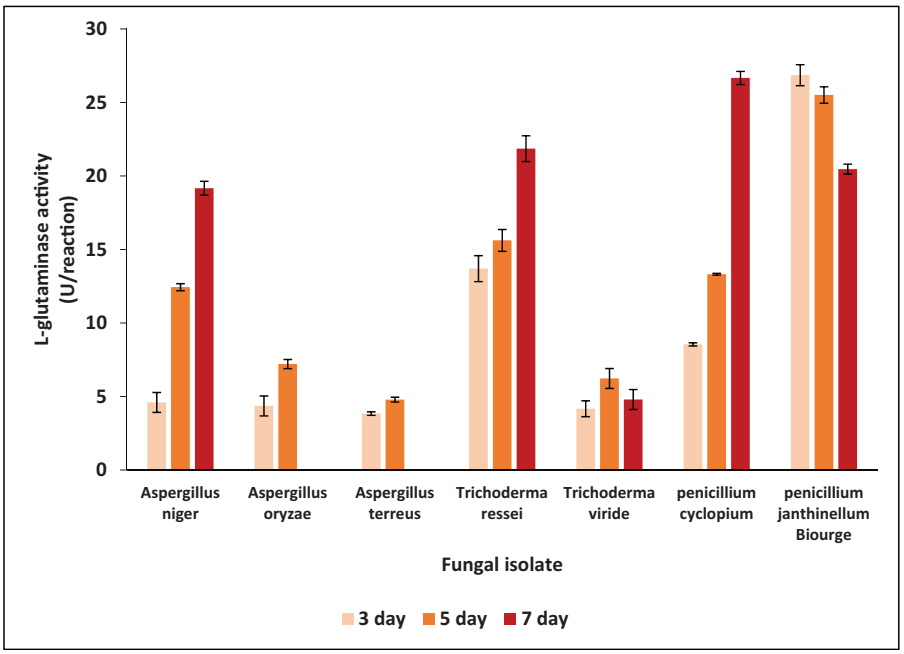
All experiments were repeated three times. The data shown in corresponding tables and figures were the mean values of the experiments. The data statistics were analyzed, and the standard error mean was determined.

Results and discussion

Screening of fungal and bacterial isolates for L-glutaminase production

The data in Figs 1 and 2 indicated that all the tested fungal and bacterial strains were able to produce the

Figure 1



Screening of some fungal strains for L-glutaminase production during different incubation periods applying shaken culture technique.

extracellular L-glutaminase, and the highest enzyme activity was exhibited by the bacterial species *B. subtilis* NRRL 1315 (27.13 U/reaction) after 48 h of incubation applying the shaken technique, and the protein content was 1.07 mg/ml, followed by the fungal strain *P. janthinellum Biourge* (26.70 U/reaction) after 3 days of incubation, and its protein content was 1.40 mg/ml. In most cases with the tested bacteria, the maximum L-glutaminase synthesis took place at 48 h of incubation, after which the enzyme synthesis decreased. In addition, most of the fungal species tested provided the highest productivity after 5 days of incubation. *A. niger* ASU-1, *T. reesei* NRRL 11460, and *P. cyclopium* afforded the highest enzyme activity after 7 days of incubation.

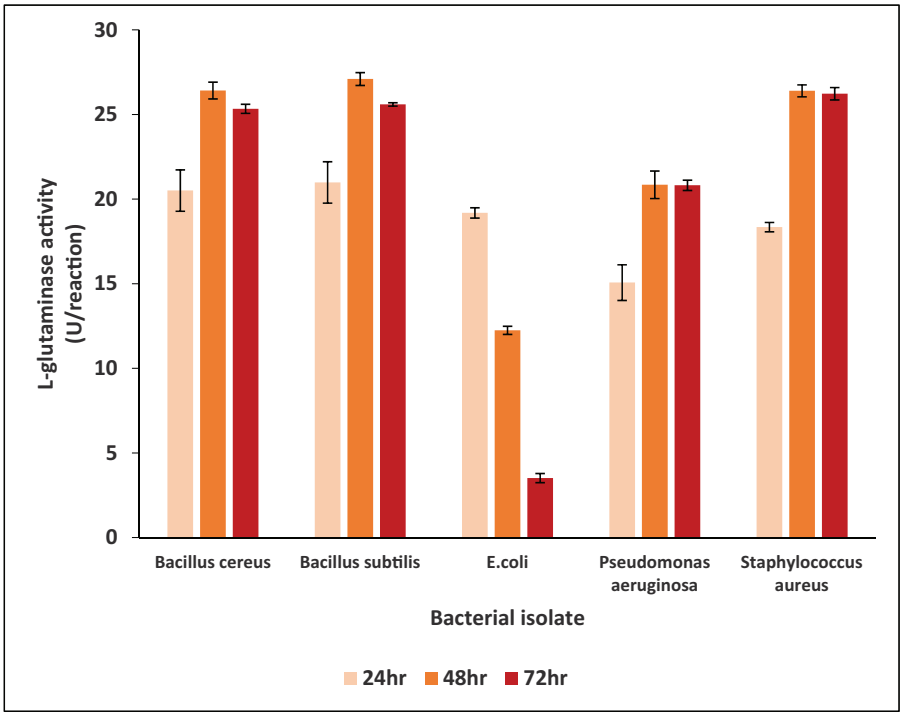
Optimization of the cultural conditions for maximum L-glutaminase production

The obtained results indicated that maximum L-glutaminase activity was seen after 48 h of incubation and then decreased after 72 h of incubation, and this may be attributed to the enzyme digestion by proteases when the enzyme substrate in the culture medium was consumed. In this respect, an optimum higher incubation period of 72 h was noted for L-glutaminase production by *P. aeruginosa* BGNAS-5 [32]; nevertheless, 18 h only was sufficient for L-glutaminase production by *Serratia marcescens* N1 [33]. Concerning streptomyces, the situation differs, where 5 days were reported for the enzyme production by *Streptomyces avermitilis* [34] and *Streptomyces* sp. [15].

Effect of carbon source on L-glutaminase production by *Bacillus subtilis* NRRL 1315

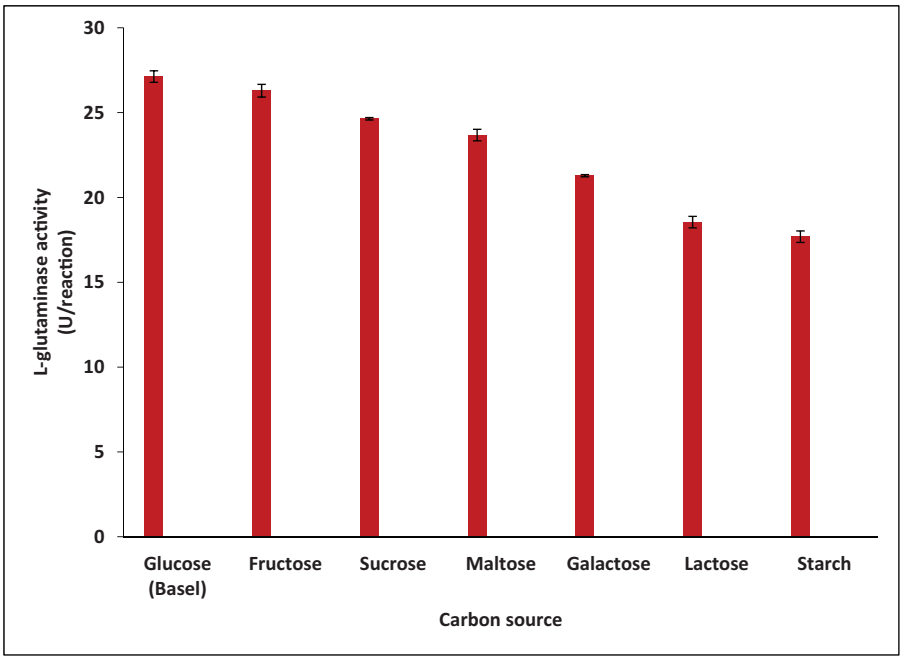
The mineral salts glutamine medium contains glucose as well as glutamine carbon source. It was seen to replace glucose by different sugars like fructose, galactose, lactose, maltose, sucrose, or starch. The data (Fig. 3) disclosed that glucose is still the favorable carbon source for enzyme production by *B. subtilis* NRRL 1315 and still affords the maximal levels for all the enzyme productivity, protein content, and growth level (27.13 U/reaction, 0.936 mg/ml, and 197 mg/culture, respectively) followed by fructose and sucrose, which led to little less in enzyme productivity (26.29 and 24.64 U/reaction, respectively). On the contrary, there was a slightly decrease in enzyme productivity with either galactose, lactose, or starch. In this regard, glucose was the proper carbon source for enzyme secretion by *Pseudomonas* sp. [35], *Streptomyces rimosus* [36], *S. avermitilis* [34], and *Streptomyces* sp. [37]. However, maltose was the proper carbon source for enzyme production by *P. aeruginosa* BGNAS-5 [32] and *B. cereus* LC13 [24]. In addition, lactose and sucrose enhanced the enzyme production by *A. oryzae* NRRL 32657 [38] and by *F. oxysporum* [39], respectively.

Figure 2



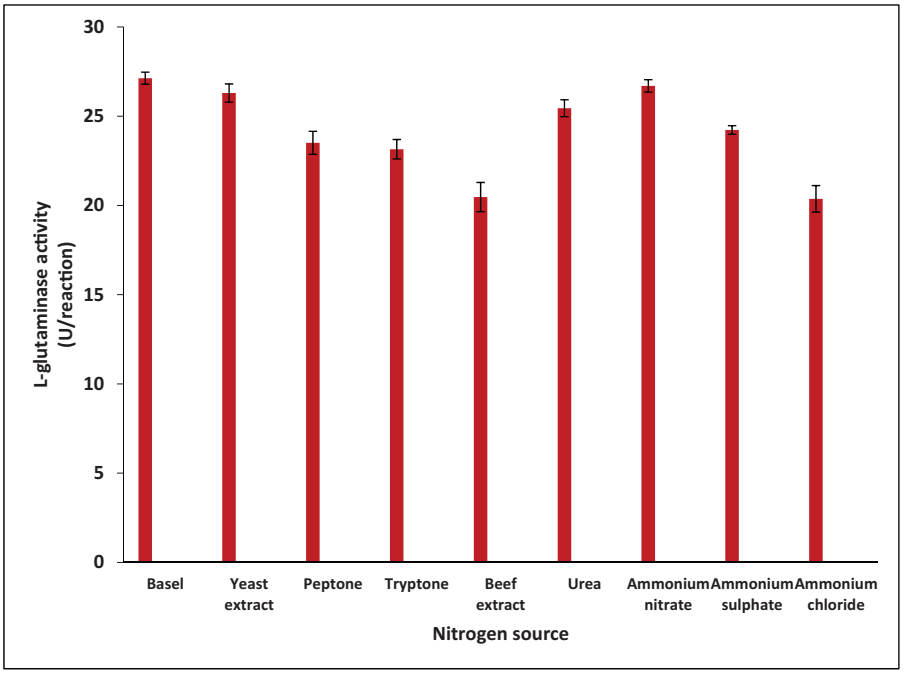
Screening of some bacterial strains for L-glutaminase production during different incubation periods applying shaken culture technique.

Figure 3



Effect of different carbon source on the production of L-glutaminase by *Bacillus subtilis* NRRL 1315.

Figure 4



Effect of different nitrogen sources on production of L-glutaminase by *Bacillus subtilis* NRRL 1315.

Effect of nitrogen source

The results in Fig. 4 clarified that among all N sources tested, L-glutamine and sodium nitrate in the basal production medium were the most suitable nitrogen sources for the enzyme production by *B. subtilis* NRRL 1315 and led to the maximum productivity and protein content, which amounted to 27.13 U/reaction and 0.936 mg/ml, respectively, followed by ammonium nitrate and yeast extract, which offered also high productivity, reaching 26.70 and 26.30 U/reaction, respectively. On the contrary, both of ammonium chloride and beef extract offered lowest L-glutaminase productivity. Compared with the aforementioned results, Abdallah *et al.* [34] reported that peptone and sodium nitrate were the most favorable organic and inorganic nitrogen sources,

respectively, for L-glutaminase production by *S. avermitilis*. Moreover, Desai *et al*. [37] disclosed that L-glutamine enhanced L-glutaminase productivity by *Streptomyces* sp. On the contrary, yeast extract was reported to enhance the enzyme secretion by all of *S. marcescens* [40], *A. oryzae* NRRL 32657 [38], and *Zygosaccharomyces rouxii* [2].

Effect of initial pH

The results in Fig. 5 clarified that *B. subtilis* NRRL 1315 afforded maximal L-glutaminase productivity (28.04 U/reaction) when pH of the culture medium was adjusted to 7.5, and even pH 7.0 and pH 8.0 led to similar records, whereas the enzyme productivity decreased at ether basic or acidic medium, and the lowest enzyme productivity was achieved at pH 10.0, at which the enzyme lost about 32.7% of its activity. The reports in this respect pointed out that the majority of the microorganisms produce L-glutaminases at neutral initial pH or slightly alkaline, whereas the enzyme synthesis decreased in both basic and acidic media. In this concern, *P. aeruginosa* BGNAS-5 [32], *A. oryzae* NRRL 32657 [38], *B. cereus* LC13 [24], *Streptomyces* sp. [37], and *Streptomyces* sp. D214 [15] brought out the highest glutaminase productivity at neutral pH, whereas all of *S. marcescens* N1 [33], *Streptomyces enissocaesilis* DMQ-24 [22], *Streptomyces variabilis* ASU319 [41], and *S. avermitilis* [34] afforded the highest L-glutaminase productivity at initial pH 8.0. The initial pH of the production medium strongly influences the growth and thus enzyme secretion by microorganisms [34].

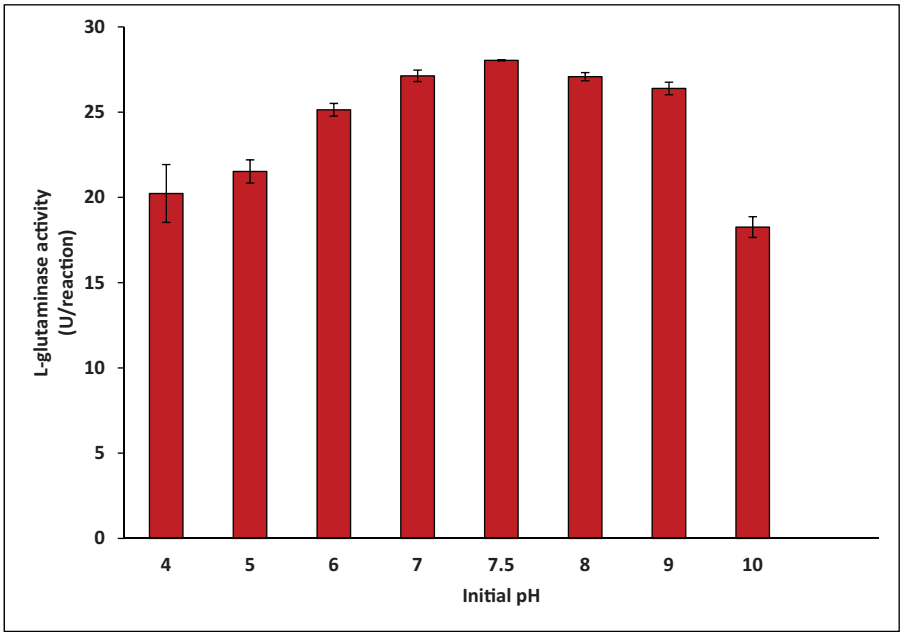
Effect of temperature

The results in Fig. 6 indicated that the optimum temperature for L-glutaminase production by *B. subtilis* NRRL 1315 was 37°C, which promised the highest enzyme productivity (28.04 U/reaction) accompanied with the highest protein content (0.956 mg/ml). At higher temperatures, the enzyme productivity and protein content decreased gradually. It was reported that the incubation temperature plays a significant role in cellular activities, including protein denaturation, enzyme release, and cell viability [42]. Temperature of 37°C was the optimum for L-glutaminase production by any of *P. aeruginosa* BGNAS-5 [32], *S. variabilis* ASU319 [41], and *B. cereus* LC13 [24]. However, lower optimum incubation temperatures of 28 and 30°C were reported for *Olivochromogenes* [27], *S. avermitilis* [34], *Vibrio azureus* [43], *S. marcescens* N1 [33], *A. oryzae* NRRL 32657 [38], and *Streptomyces rochei* [16], whereas 45°C was the optimum temperature for the enzyme production by *Streptomyces* sp. [15].

Effect of substrate concentration

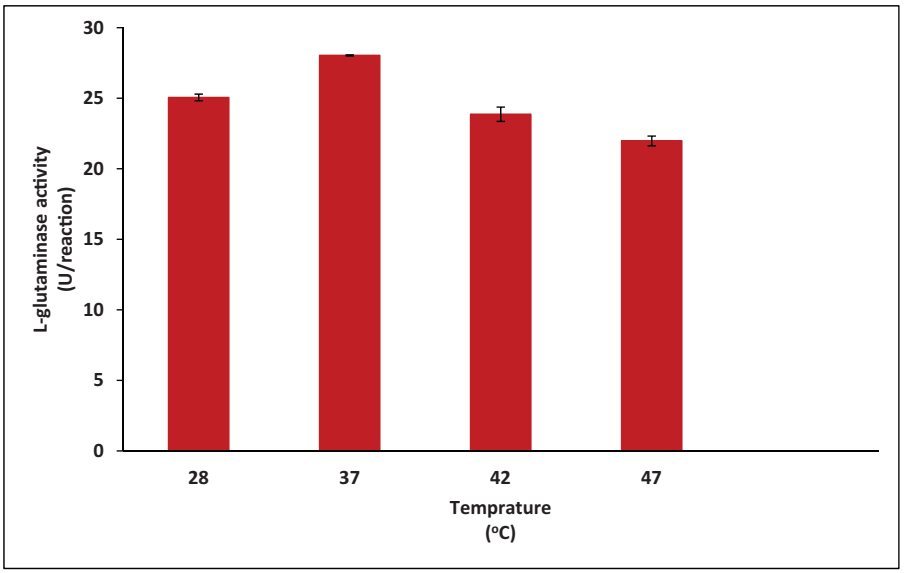
The results indicated that the enzyme productivity increased with L-glutamine concentration till 1.5% (w/v), which led to a considerable productivity, which amounted to 30.08 U/reaction, and above this concentration, the enzyme productivity decreased. In this concern, Kiruthika and Saraswathy [43] revealed that the maximum yield of *V. azureus* L-glutaminase production was achieved at 2% (w/v) L-glutamine concentration, but L-glutamine at concentration of

Figure 5



Effect of the initial pH on L-glutaminase production by *Bacillus subtilis* NRRL 1315.

Figure 6



Effect of the incubation temperature on the extracellular L-glutaminase production by *Bacillus subtilis* NRRL 1315.

1% (w/v) gave the maximum yield of enzyme produced by *S. enissocaesilis* DMQ-24 [22] and *P. aeruginosa* BGNAS-5 [32]. Nevertheless, More *et al.* [24] disclosed that L-glutaminase activity was produced maximally by *B. cereus* LC13 at a concentration of 0.3% (w/v), and 0.025% (w/v) L-glutamine was reported for the maximum enzyme production by *F. oxysporum* [39].

Effect of inoculum size on L-glutaminase production

The results indicated that the enzyme productivity and protein content increased gradually till 18% (v/v) inoculum size, which led to the highest activity and protein level (30.97 U/reaction and 0.990 mg/ml, respectively). However, inoculum concentration over the optimum resulted in slight reduction of the enzyme productivity. It is well known that the initial biomass controls the kinetics of growth and several biological metabolic functions, leading to the overall biomass and extracellular product production [44]. In this respect, the results obtained by Thadikamala and Reddy [45] for the enzyme produced from *B. subtilis* RSP-GLU indicated that the maximum enzyme productivity was at 2.0% (v/v) inoculum size, after which, the enzyme productivity decreased. Moreover, Kiruthika and Saraswathy [43] reported that the maximal production by *V. azureus* JK-79 was observed at 1% (v/v) initial inoculum size, and the results obtained by Abdallah *et al.* [34] revealed that the enzyme productivity increased as the inoculum size increased till it reached its maximum productivity at 5 ml (10% v/v). On the contrary, Kiruthika *et al.* [46] revealed that the maximal L-glutaminase production by marine *B. subtilis* JK-79 was obtained at 3% (v/v) inoculum size, and any further increase resulted in L-glutaminase production decrease, and this may be attributed to nutrient depreciation or accumulation of some toxic substance.

Effect of agitation speed on L-glutaminase production

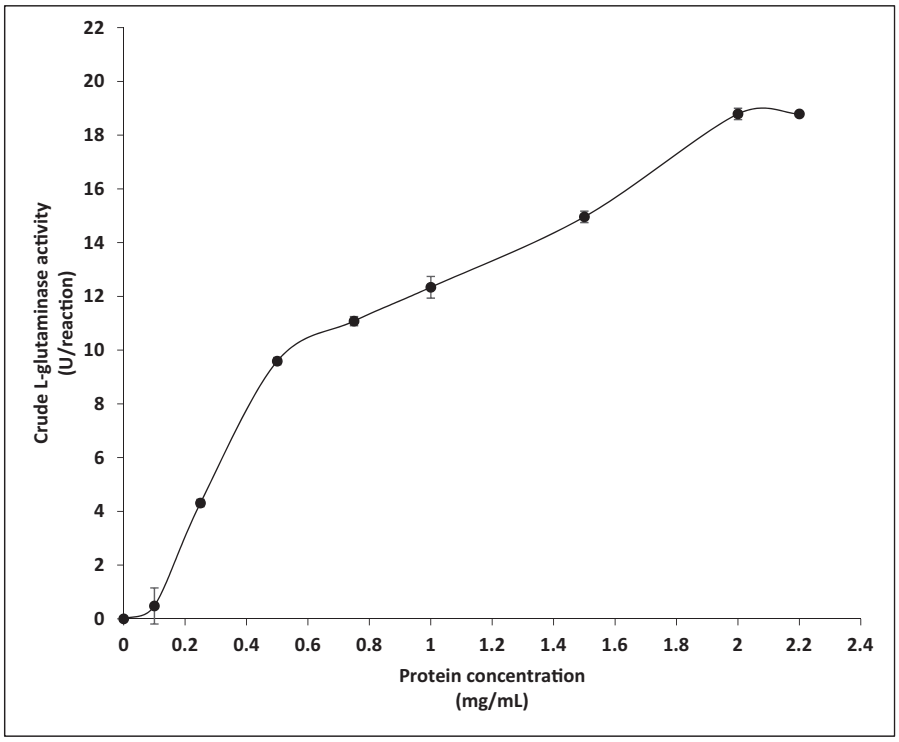
To evaluate the effect of agitation speed on L-glutaminase production by *B. subtilis*, experiments were carried out in different agitation speeds ranging from 80 to 200 rpm. The results indicated that all of the enzyme activity, protein content, and biomass increased with the shaking velocity till 150 rpm, where they reach their maximum value (31.60 U/reaction, 0.990 mg/ml, and 196 mg/culture, respectively), after which all values decreased. Understanding of mass transfer of substrates, products, nutrients, and gases among the system components is one of the important parameters to be considered for optimal production of any metabolite or product and exploitation of microbial capability under fermentation process [47], and this is achieved by agitating the culture components in controlled environmental setup. In this concern, L-glutaminase from *B. subtilis* RSP-GLU produced maximally at 100 rpm, and any change in agitation speed in either side of 100 rpm resulted in drop of enzyme activity as well as biomass; losses of the biomass and enzyme activity were high when the rpm was decreased [45]. However, L-glutaminase from *V. azureus* JK-79 produced maximally at 120 rpm [43].

General properties of the crude L-glutaminase

Effect of the crude L-glutaminase preparation protein concentration on the enzyme activity

Different protein concentrations (0.1–2.0 mg/ml enzyme) of the crude enzyme preparation were used

Figure 7



Effect of the crude L-glutaminase preparation protein concentration on the enzyme activity.

separately in the usual enzymatic reaction mixture. It seemed that a direct relationship existed between the enzyme activity and the protein concentration up to 2 mg/ml enzyme, which was sufficient to bring about the maximum enzyme activity (18.79 U/reaction) (Fig. 7). It is well known that the normal relationship of the enzyme protein concentration and the enzyme activity is linear, and the deviation from the linearity may be owing to the existence of some enzyme activators or inhibitors, which effectively act the elevated enzyme concentration, and the inhibition effect was clearly obvious in Fig. 7, which also indicates that the optimum protein concentration of the crude bacterial L-glutaminase preparation was 2 mg/ml enzyme.

Effect of L-glutamine concentration on the crude L-glutaminase activity

Concerning the effect of L-glutamine concentration on the enzyme activity, it was investigated within the concentration range of 20–500 mM. The results (Fig. 8) indicated that the substrate concentration enhanced the reaction rate till 40 mM (18.65 U/reaction), after which the activity slightly decreased. However, high L-glutamine concentration of 500 mM led to 23.17% loss of the activity. It seemed that 40 mM L-glutamine was enough to saturate all the available enzyme active sites of the applied enzyme molecules. The crude enzyme did not exhibit the normal hyperbolic relationship with the substrate, and this happened considering the enzyme impurity. In this regard, our results agree with the results of Singh and Banik [48] and Desai *et al.* [37] who found that concentration of 40 mM was sufficient to bring about the maximal L-glutaminase activity.

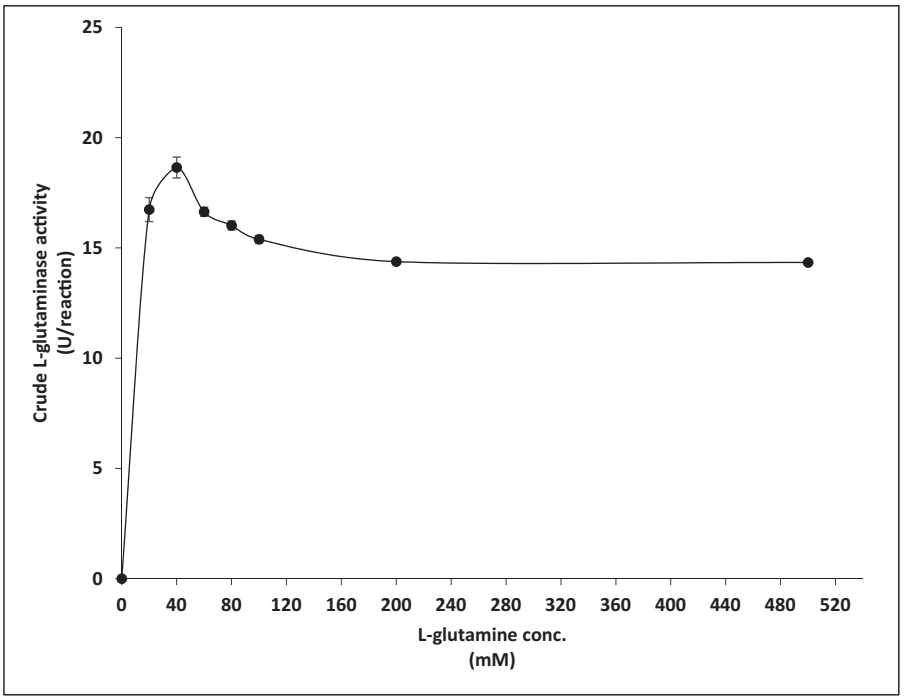
Effect of the reaction temperature on the crude enzyme activity

The effect of reaction temperature on the crude enzyme activity was studied within a range from 25 to 60°C. The optimum reaction temperature was found in the range of 30–40°C, as shown in Fig. 9, and the reaction temperature plot was semi-bell shape. At elevated reaction temperatures from 45 to 60°C, the enzyme still retained more than 58.44% of its optimal activity pointing out its good heat thermostability. Thus, 37°C was chosen as the optimum reaction temperature in all the following studies. In this concern, many of the microbial L-glutaminases have been found to be optimal and stable in this range. L-glutaminase of *Debaryomyces* sp. [49] and *Lactobacillus reuteri* KCTC3594 [50] exhibited their maximum activity at 37°C.

Effect of the reaction pH on the crude enzyme activity

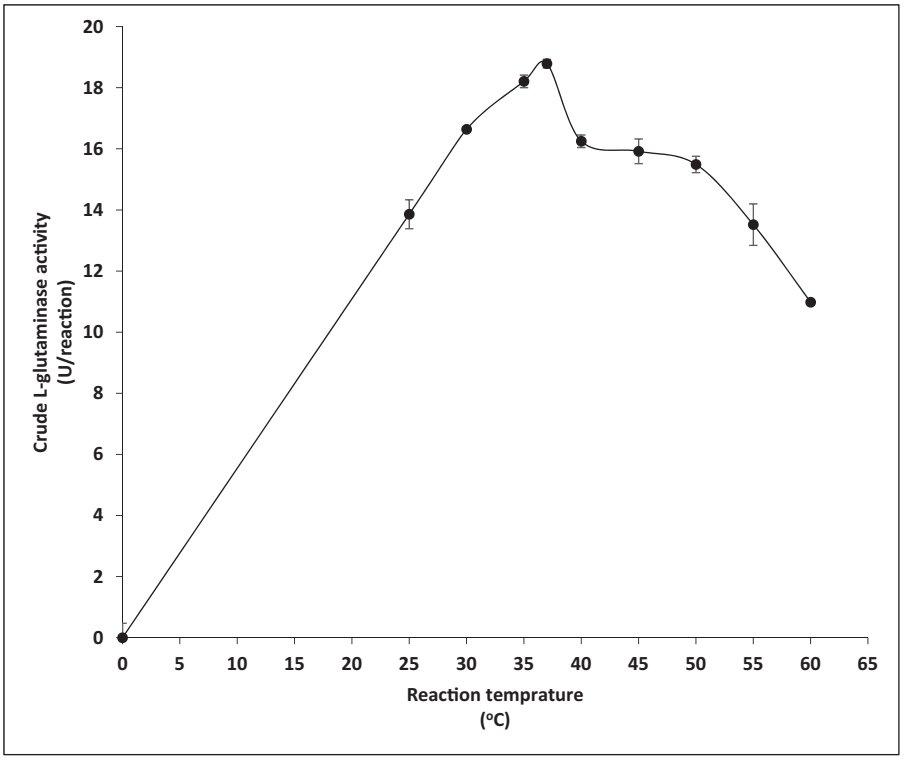
Applying the optimum conditions formerly outlined, the effect of the reaction pH on the crude enzyme activity was studied within a wide pH range involving

Figure 8



Effect of L-glutamine concentration on the crude enzyme activity.

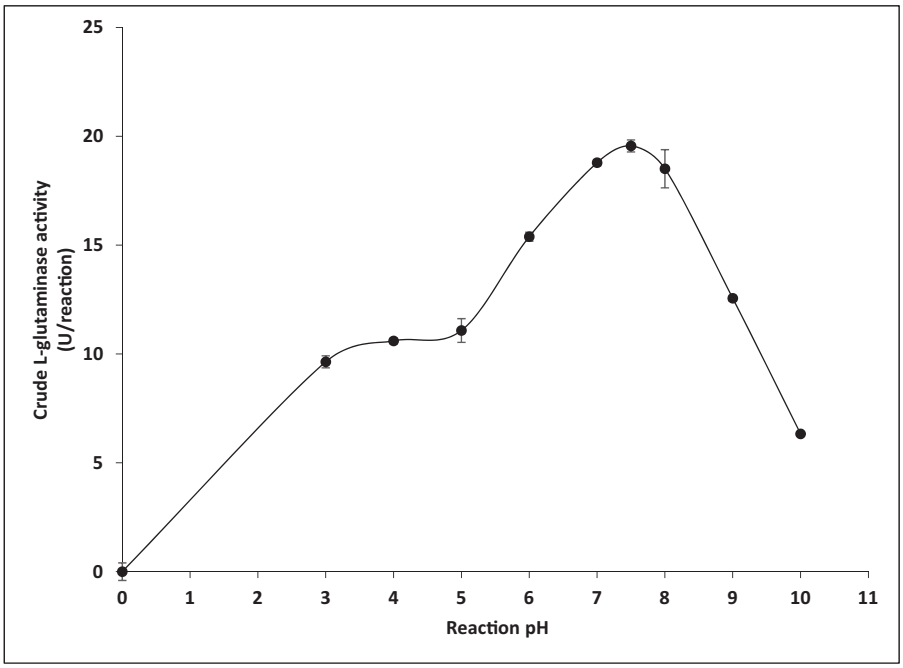
Figure 9



Effect of the reaction temperature on the crude enzyme activity.

each of the acidic, neutral, and alkaline values (3–10). The results in Fig. 10 indicated that the enzyme activity increased with reaction pH till the slight alkaline pH of 7.5, after which a gradual decrease took place. At extreme alkaline pH 10, more than 67% of enzyme activity loss was recorded. In this respect, it was reported that the optimum pH values for L-glutaminase from *Debaryomyces* sp. [51], *Aspergillus*

Figure 10



Effect of the reaction pH on the crude enzyme activity.

sp. [52], and *Vibrio* sp. [53] were in the range of 7.0–8.5.

Biological activities of the crude L-glutaminase

Antimicrobial activity of the crude L-glutaminase preparation

Concerning the expected antimicrobial activity of the crude enzyme preparation, unfortunately, the crude enzyme form exhibited no antifungal or antibacterial activities, and this was followed by other important activities, like antioxidant and anticancer.

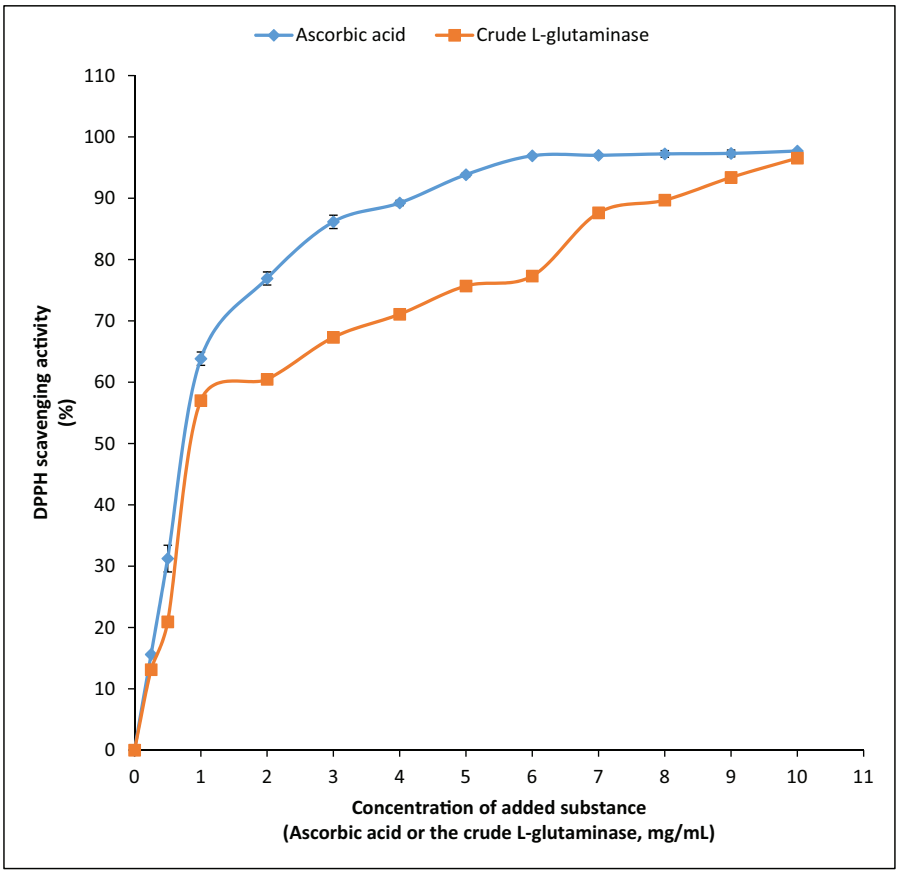
xxxxxx2, 2′-diphenyl-1-picrylhydrazyl radical scavenging activity of the crude L-glutaminase preparation

Antioxidant activity of the crude enzyme preparation was examined applying DPPH free radical scavenging activity, which has been widely accepted as a tool for estimating the free radical scavenging activities of antioxidants. The data in Fig. 11 disclosed that the DPPH radical scavenging activity of the crude L-glutaminase preparation ranged from 13.15 to 96.54% at concentrations from 0.25 to 10 mg/ml enzyme in ethanolic solution. However, DPPH radical scavenging activity of the standard ascorbic acid ranged from 15.60 to 97.70% at concentrations from 0.25 to 10 mg/ml.

It is worthy to decide here that the crude L-glutaminase form afforded scavenging activity very close to that of the standard ascorbic acid at concentration ranged from 9 to 10 mg/ml and from 0.25 to 1 mg/ml.

The IC_{50} value was calculated for the applied crude enzyme to be 0.8 mg/ml and IC_{50} of ascorbic acid was 0.65 mg/ml, and these values were calculated from the scavenging activity plots applying the equation previously mentioned in Materials and methods section. This result highlights the potential of L-glutaminase enzyme as potent antioxidant agent. L-glutaminase is actually a vital natural antioxidant, and it is not related to lethal and cancer-causing effects. In this respect, More *et al.* [24] reported that L-glutaminase of *B. cereus* LC13 was able to scavenge free radicals generated *in vitro* by DPPH, and IC_{50} values recorded were 17.06 and 57.07 mg/ml for L-ascorbic acid and L-glutaminase, respectively. Moreover, Awad *et al.* [16] reported that the enzyme purified from *S. rochei* was found to scavenge free radicals produced *in vitro* applying DPPH scavenging assay, and the IC_{50} value of L-glutaminase was 1.65 mg/ml, whereas the IC_{50} of L-ascorbic acid is 0.65 mg/ml. In addition, Kiruthika and Swathi [17] reported that the partially purified enzyme from *B. subtilis* JK-79 was found to scavenge free radicals produced *in vitro* applying DPPH scavenging assay, and IC?? value for DPPH radical was found to be 400 μg/ml.

Figure 11



DPPH scavenging activity of the crude L-glutaminase preparation and ascorbic acid. DPPH, 2, 2′-diphenyl-1-picrylhydrazyl.

Financial support and sponsorship
Nil.

Conflicts of interest
There are no conflicts of interest.

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