



# Screening and Optimization of Process Parameters for the Production of L-asparaginase by Indigenous Fungal-Type Strains

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## Abstract

Microbial L-asparaginases have received significant attention due to their potential for applications in cancer treatment and for preparation of acrylamide-free baked and fried food. Eleven fungal-type strains were obtained from Iranian Biological Resource and screened for production of L-asparaginase using dye-based plate assay on modified Czapek Dox medium. The efficiency of three different dye indicators including phenol red, bromothymol blue and cresol red was evaluated. Production mode was also evaluated by the agar well diffusion method. A comparative study was carried out to evaluate L-asparaginase production in submerged condition by enzymatic assay using Nessler's reagent. Optimization of the enzyme production by the selected superior strain was performed using one factor at a time method for temperature, inoculum and different carbon and nitrogen sources. Five-type strains showed positive L-asparaginase activity in preliminary screening. Phenol red and bromothymol blue showed better differentiable zone of hydrolysis and consistent results in comparison with cresol red. The results also indicated that L-asparaginase production mode is extracellular. L-asparaginase production by *Fereydownia khargensis* IBRC-M 30116<sup>T</sup> which showed the highest zone of color change was then optimized. The optimum enzyme activity of 61.3 U. mL<sup>-1</sup> was obtained with 9% inoculum, at a temperature of 30 °C and at the presence of ammonium chloride as nitrogen source and with glycerol as the carbon sources. The study revealed that, apart from bacteria, yeast and yeast-like microorganisms could be considered as novel and appropriate sources for production of the high yield of L-asparaginase.

**Keywords** Dye-based indicators · Extracellular enzyme · L-Asparaginase · Submerged fermentation · Therapeutic agent · Yeast and yeast-like microorganisms

## 1 Introduction

L-asparaginase is a hydrolase that primarily catalyzes the conversion of L-asparagine to L-aspartic and ammonia (Qeshmi et al. 2018). L-asparaginase has received

considerable attention as a primary component in the treatment of a variety of lymphoproliferative disorders and lymphomas particularly acute lymphoblastic leukemia (ALL) (Doriya and Kumar 2016; Qeshmi et al. 2018). Normal cells can synthesize L-asparagine with the help of asparagine synthetase, whereas certain sensitive malignant cells cannot synthesize it by itself and require an external source of L-asparagine for growth (Doriya and Kumar 2016). As a result, the enzyme activity can inhibit the growth of tumor cells by consuming the L-asparagine and restrict the protein synthesis leading to apoptosis and G1 cell-cycle arrest (Vimal and Kumar 2017a; Ashok et al. 2019). L-asparaginase contributes to 40% of the total enzyme demands worldwide and one-third of the global requirements as anti-leukemic and anti-lymphoma agents (Qeshmi et al. 2018; Chand et al. 2020). The market size of the enzyme in the healthcare sector expected to reach from \$5.0 billion in 2016 to \$6.3 billion by 2021 with 4.7%

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annual growth rate (Chand et al. 2020). In addition to medicine and pharmaceuticals, the enzyme received remarkable attention in the food industry due to the ability to reduce the formation of acrylamide in the fried and baked food and hence, to decrease the risk of carcinogenicity (Vala and Dave 2016). L-asparaginase is also used for the development of biosensor technology to analyze asparagine levels and/or to determine the ammonium ions levels either in leukemia or the food industry (Batool et al. 2016; Chand et al. 2020).

Asparaginase activity is widely observed in plants, animal tissues and microorganisms including bacteria, archaea, algae and fungi (Doriya and Kumar 2016; Vimal and Kumar 2017a; Brumano et al. 2019). The microbial enzymes production is preferred over plant and animal sources because their production process is more economically feasible and process modification, optimization, extraction and purification is easier in microbial strains (Trivedi and Padalia 2016; Vimal and Kumar 2017a, Brumano et al. 2019). The production of this enzyme is mainly carried out by submerged fermentation (Vimal and Kumar 2017a, b; Cachumba et al. 2016). Currently, all available commercial L-asparaginase drugs in the market derived from bacterial sources such as *Escherichia coli* and *Erwinia chrysanthemi* (Vala and Dave 2016; Ashok et al. 2019). However, clinical usage of bacterial L-asparaginase has led to unpleasant side effects such as normal cell toxicity, patient hypersensitivity, development of allergic reactions and immunogenic complications. Besides, the rapid clearance of the enzyme from the bloodstream which requires multiple administrations makes the treatment very costly (Vala and Dave 2016, El-Naggar et al. 2018). Considering the higher similarity of fungi to humans which could decrease the chance of immunological reactions, L-asparaginase production by fungi seems more promising. Furthermore, the possibility of extracellular production of the enzyme in fungal strains could facilitate the purification process and eventually reduces the final production cost (Vala and Dave 2016). Understanding these limitations, this study aims to investigate the alternative unexplored microbial sources for the extracellular production of L-asparaginase that can offer better production yields and avoid undesired side effects to meet the market requirements.

## 2 Materials and Methods

### 2.1 Microorganisms

Eleven yeast and yeast-like-type strains used in the present study were isolated by Iranian researchers from various environmental sources and the cultures were obtained from

Microorganisms Bank, Iranian Biological Resource Center (IBRC), ACECR, Tehran, Iran (Table 1).

### 2.2 Preliminary Screening of L-Asparaginase Production Using Semi-quantitative Assay

Screening of L-asparaginase production was carried out by rapid plate assay. Modified Czapek Dox (MCD) agar medium (glucose 2 g, L-asparagine 10 g,  $\text{KH}_2\text{PO}_4$  1.52 g, KCl 0.52 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.52 g, agar 20 g, distilled water 1000 mL) was used (Thirunavukkarasu et al. 2011). The medium was separately supplemented with 0.009% phenol red (PR), 0.009% cresol red (CR) and 0.008% bromothymol blue (BTB) as pH indicators, respectively (Mihooliya et al. 2017). The initial pH of each medium was adjusted at 6.2. Uninoculated plates were set as control (Mahajan et al. 2013). In order to inoculate the plates for the semi-quantitative assay, the fungi were cultured in 5 mL YPG broth for 48 h, the cultures were centrifuged at  $10\,000 \times g$  for 10 min, and the cells were resuspended in 500- $\mu\text{L}$  sterile distilled water that contained agar and tween 80 to avoid spreading of the inoculum (Nasr et al. 2018a, b, c). All plates were point-inoculated in the center with 5  $\mu\text{L}$  of the cell suspension and incubated at 25 °C for 5 d. The diameter of zone of color change was measured as zone of hydrolysis (Vimal and Kumar 2017b; Ashok et al. 2019).

### 2.3 Plate assay for L-glutaminase Activity

L-Glutaminase activity of the L-asparaginase producing fungal strains was detected by supplementing MCD agar medium with L-glutamine as sole nitrogen source. The test strains were inoculated and observed for color change in presence of PR and BTB dyes as an indicator after 5 d incubation (Doriya and Kumar 2016).

### 2.4 Inoculum Preparation

In order to inoculate the production medium, the fungi were cultured in tubes containing 3 mL MCD broth and incubated on a rotary shaking incubator at 150 rpm and 25 °C for 72 h. Then, the culture broth was centrifuged at  $10\,000 \times g$  for 15 min and the supernatant were discarded. The harvested pellet was resuspended in MCD broth and used to reach the culture absorbance of 0.1 at 600 nm ( $A_{600\text{ nm}}$ ) in the production flask.

### 2.5 L-asparaginase Production by Submerged Fermentation

Five fungal strains which showed positive results during primary screening were further tested for production of L-asparaginase. The 100-mL flasks containing 20 mL broth

**Table 1** Accession number and source of isolation of the yeast and yeast-like strains used in this study

Accession No. IBRC-M	Scientific name	Type	Isolation source	References
30078	<i>Basidioascus persicus</i>	Y	Soil	Nasr et al. (2014a)
30116	<i>Fereydownia khargensis</i>	YL	Plant material	Nasr et al. (2014b)
30204	<i>Starmerella orientalis</i>	Y	Flower	Alimadadi et al. (2016)
30103	<i>Wickerhamomyces orientalis</i>	Y	Soil	Nasr et al. (2016)
30238	<i>Blastobotrys persicus</i>	Y	Soil	Nouri et al. (2017)
30284	<i>Jaminaea pallidilutea</i>	Y	Healthy plant of <i>Avicennia marina</i>	Nasr et al. (2017)
30265	<i>Aureobasidium mangrovei</i>	YL		Nasr et al. (2018a)
30187	<i>Coniochaeta iranica</i>	YL	Healthy plant of <i>Euphorbia polycaulis</i>	Nasr et al. (2018b)
30188	<i>Coniochaeta euphorbiae</i>	YL		
30207	<i>Collophorina euphorbiae</i>	YL		
30158	<i>Graphioliella fimbriata</i>	YL	Plant material	Nasr et al. (2018c)

Y Yeast, YL Yeast-Like

medium were incubated on a rotary shaker incubator at 150 rpm and 25 °C. After 4-day incubation, the broth was centrifuged with cooling centrifuge at 10,000×g for 15 min at 4 °C and the clear supernatant served as crude enzyme.

## 2.6 Evaluation of Enzyme Production Mode

The modified agar well diffusion method was employed to measure the L-asparaginase activity of the crude enzyme. Modified Czapek Dox (MCD) agar medium supplemented with 0.009% PR or 0.008% BTB was punched for 8-mm-diameter wells. Wells were filled with 100 µL of the filtrated crude enzyme (0.2 µm Cellulose Acetate Membrane Filters; Sartorius). and sterile distilled water was used as negative control. The crude enzyme was incubated at 25 °C for 72 h. A clear zone formation of color change around the well indicated as the extracellular L-asparaginase activity.

## 2.7 Quantitative Assay for L-asparaginase Production

L-Asparaginase activity assay was quantified in the five isolates that produced the five highest zone of color change according to the method of Wriston and Yellin (1973), Imada et al. (1973) using nesslerization to measure the amount of liberated ammonia. The reaction mixture containing 0.5 mL of 0.4 M asparagine, 0.5 mL of 0.05 M Tris-HCl buffer (pH 8.6) and 0.5 mL of a proper dilution from the crude enzyme was incubated at 37 °C for 30 min. The reaction was terminated by the addition of 0.5 mL of 1.5 M trichloroacetic acid (TCA). The negative control was run by adding the inactive crude enzyme after

incubation. To remove the precipitated protein, the reaction contents were centrifuged at 10,000×g for 5 min and the filtrate was collected. For quantification of liberated ammonia, 0.1 mL filtrate was diluted with 3.75 mL distilled water and 0.2 mL Nessler's reagent was added to the mixture. The color reaction was allowed to proceed for 10 min before measuring the absorbance at 450 nm using UV-1800 spectrophotometer (Shimadzu, Schweiz GmbH). The amount of liberated ammonia by the test sample was calculated by comparing the absorbance with a standard curve prepared from solutions of ammonium sulfate (0–0.9 mM) as the ammonia source. One unit (U) of L-asparaginase was defined as the amount of enzyme which generates 1 µmole of ammonia in 1 min at 37 °C and pH 8.6.

## 2.8 Optimization of Fermentation Parameters

The optimization of L-asparaginase production process for *F. khargensis* IBRC-M 30116<sup>T</sup> was carried out based on one factor at a time method by varying only a single factor and keeping the remaining factors constant. Fifty milliliters MCD broth in a 250-mL flask was used as the initial production medium. The selected parameters including inoculum concentration (1, 3, 6, 9% v/v), incubation temperature (at 15, 20, 25, 30, 35 and 37 °C), different carbon sources (by adding 1% w/v of each one of the glucose, sucrose, citric acid and glycerol into the production medium) different inorganic nitrogen sources (by adding 1% w/v of each one of the ammonium chloride and sodium nitrate) and organic nitrogen sources (by adding 1% w/v of each one of yeast extract, proline and asparagine) were evaluated, respectively. Samples were withdrawn after

4 days and the cell density ( $A_{600\text{ nm}}$ ) and enzyme activity was determined.

## 2.9 Statistical Analysis

All the above-mentioned experiments were conducted in triplicate and the experimental results presented as means of  $\pm$  S.E.M (standard error mean) of three independent trials. The data were analyzed by one-way analysis of variance and Tukey test with 95% confidence level using Minitab 17 software. *P* values less than 0.05 were considered as significant.

## 3 Results

A total of 11 yeast and yeast-like-type strains were investigated for L-asparaginase production (Table 1). The screening was carried out by plate assay method using MCD agar medium which separately supplemented with phenol red, cresol red and bromothymol blue as pH indicators. Five-type strains out of eleven showed positive results for the production of L-asparaginase enzyme (Table 2). The medium containing BTB and PR as pH indicators showed better contrast in comparison to CR (Fig. 1). Growth of *F. khargensis* and *A. mangrovei* resulted in the largest zone of color change on the dye containing media. The slight amount of L-glutaminase production was observed in both *A. mangrovei* IBRC-M 30265<sup>T</sup> and *F. khargensis* IBRC-M 30116<sup>T</sup>.

The extracellular production mode was detected based on agar well diffusion method in all the positive enzyme producer strains except *S. orientalis* IBRC-M 30204<sup>T</sup>. Since low activity was observed for crude enzyme obtained from *S.orientalis* in quantitative asparaginase assay, this negative result may be due to low enzyme activity or low enzyme production. The diameter of the zone of hydrolysis by extracellular enzyme was ranging from 20 to 30 mm. The highest extracellular L-asparaginase activity was

obtained from *F. khargensis* IBRC-M 30116<sup>T</sup>, and the enzyme production of this type strain was  $13.6 \pm 5.7$  U/mL.

The highest level of L-asparaginase production of *F. khargensis* IBRC-M 30116<sup>T</sup> was observed with 9% inoculum (Fig. 2a). The reduction in inoculum concentration led to decrease in enzyme production level in this strain. A steady increase in L-asparaginase production was observed with increasing in temperature from 20 °C and reached the maximum at 30 °C. Further increase in temperature resulted in decline in production of the enzyme (Fig. 2b). The influence of different carbon sources on the production of L-asparaginase was recorded for *F. khargensis* IBRC-M 30116<sup>T</sup>. The optimum growth and L-asparaginase production was observed while using glycerol as a carbon source, whereas the lower amount of enzyme production was observed with sucrose followed by glucose and citric acid (Fig. 2c). Among the nitrogen sources tested, ammonium chloride was found to significantly enhance L-asparaginase production when the strain was grown at 30 °C. Among the organics nitrogen sources, asparagine was the proper choice that increases the enzyme production to  $51.9 \pm 4.9$  U/mL (Fig. 2d).

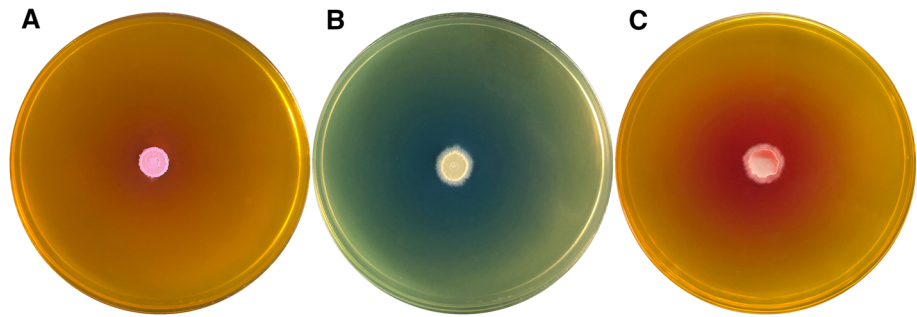
## 4 Discussion

Different type of microorganisms such as archaea, bacteria, actinomycetes, yeast and fungi have been found to produce L-asparaginase in significant quantities. L-asparaginase production using microbial systems has attracted significant attention due to its cost-effective and ecofriendly nature and ease of production in industrial scale using submerge or solid-state fermentation (Krishnapura et al. 2016). Apart from bacteria, fungi could be considered as a proper potential source for L-asparaginase production owing to two main reasons: first, less chance of immunological reactions expected for fungal strains as they are more closely related to human beings and second,

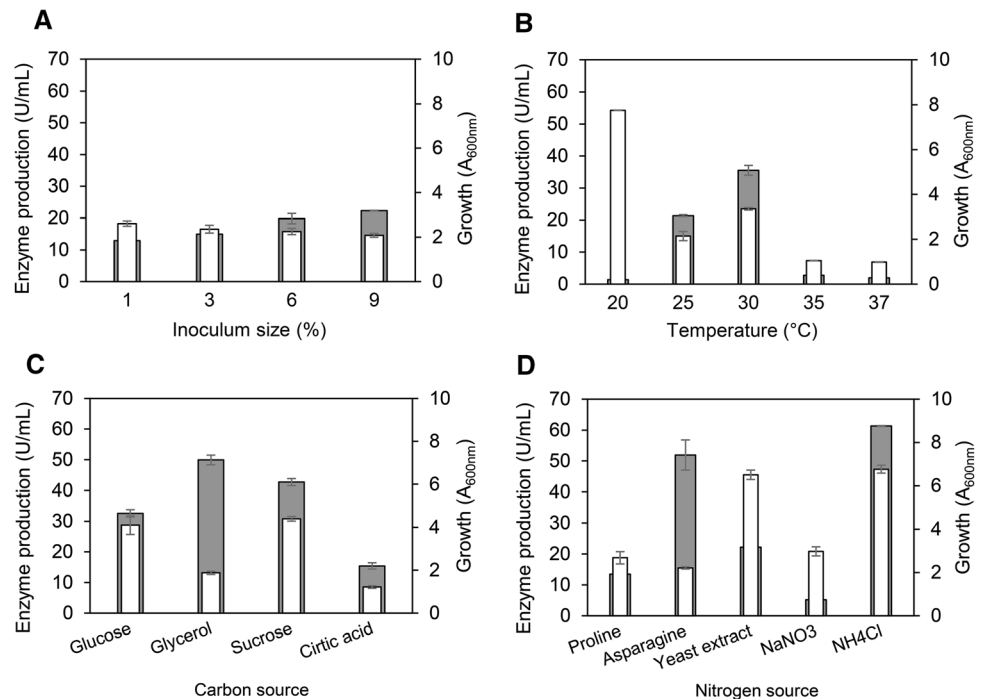
**Table 2** Fungal-type strains and their respective L-Asparaginase production potential (+, 1–10 mm; ++, 11–20 mm; +++, > 20 mm; –, No Color Change)

Type strain	L-Asparaginase production			L-Asparaginase activity (U mL <sup>-1</sup> )	L-Glutaminase production	
	BTB	PR	CR		BTB	PR
<i>F. khargensis</i>	+++	++	++	$13.6 \pm 5.7$	+	–
<i>A. mangrovei</i>	+++	+++	++	$9.3 \pm 2.6$	+	–
<i>C. iranica</i>	++	++	++	$7.2 \pm 1.6$	–	–
<i>G. fimbriata</i>	++	++	–	$3.4 \pm 2.2$	–	–
<i>S. orientalis</i>	+	–	–	$1.0 \pm 0.1$	–	–

**Fig. 1** Comparative effectiveness of different dyes in screening of L-asparaginase production in *F. khargensis* IBRC-M 30116<sup>T</sup> after 3-day incubation at 25 °C. **a** CR, **b** BTB, **c** PR



**Fig. 2** Effect of inoculum concentration **a** temperature, **b** different carbon sources **(c)** and different nitrogen sources **(d)** on growth and L-asparaginase production *F. khargensis* IBRC-M 30116<sup>T</sup> using one factor at the time method. Gray columns: enzyme production. White columns: cell growth



extracellular production of L-asparaginase will lead to easier purification process and reducing production cost (Vala and Dave 2016). Overall 45% of tested yeast and yeast-like-type strains were found positive for L-asparaginase production. In this study, the species *F. khargensis*, *A. mangrovei*, *C. iranica*, *G. fimbriata*, *S. orientalis* are reported for the first time as L-asparaginase producing fungi.

The plate assay method was chosen as a semi-quantitative, simple and rapid screening procedure for L-asparaginase production. The method is suitable for preliminary large-scale screening due to the fact that it is easy and L-asparaginase production can be visualized directly from the plates without performing time-consuming assays (Rani et al. 2012). Previously, multiple studies were carried out with the objective to improve the plate assay method using different pH indicator dyes for the screening of asparaginase producer strains; however, the

results are not always compatible (Mahajan et al. 2013; Mihooliya et al. 2017; Vaishali and Bhupendra 2017).

In the present study, PR, BTB and CR were used as pH indicator dyes for the screening of L-asparaginase producing microorganisms. PR shows a yellow color at pH range of 6.8–8.2 and turns red at the pH level above 8 (Merck). CR is a maroon to dark red crystals and soluble in water. It shows a yellow color at pH 7.2 and turns to a dark red or maroon when pH increases to 8.2 (Mihooliya et al. 2017). BTB gives a transient green color at the pH range of 6.0–7.6 and it turns to blue at the pH level above 7.6 (Mahajan et al. 2013), thus, is more sensitive to alkalinizing agents. The hydrolytic action of L-asparaginase on L-asparagine causes an increase in pH from acidic to basic (in the range of 7.0–9.0; data not shown) due to the release of ammonia. This formation of ammonia is responsible for the development of a zone of hydrolysis in plates supplemented with different pH indicator dyes. In this study, plates containing Modified Czapek Dox agar medium were

supplemented with 0.008% bromothymol blue were observed to produce sharp and clear hydrolysis zone which might be attribute to the lower turning point of BTB in alkaline zone. However, the plates with phenol red were observed to produce a zone of hydrolysis with the lower intensity. In contrast, cresol red has the lowest efficiency among the tested pH indicator dyes. These results seem to contradict Mihooliya et al. 2017 that claimed CR is better than PR and BTB relating to the sensitivity and consistency of the results. In addition to L-asparaginase production, a quantitative assessment for the production of glutaminase was also done. Glutaminase activity was present in two-type strains which show better results in the preliminary screening. However, the glutaminase activity was not significant in comparison with L-asparaginase activity. The concomitant presence of the activity of glutaminase is not ideal for clinical application due to the toxic side effects that are generally attributed to the glutaminase activity (Ashok et al. 2019).

Many factors such as type and concentration of carbon and nitrogen sources, pH, aeration, temperature, fermentation time, and, mainly, the microbial sources, have great influence on the efficiency of industrial production process aiming to reach higher production yield and economic viability (Trivedi and Padalia 2016). Therefore, fermentation process and fermentation media have been continuously updated to obtain the highest production level of the selected enzyme (Talluri et al. 2019). There are few reports on media and optimization process for production of L-asparaginase by yeast strains. Four significant parameters (inoculum concentration, temperature, carbon source; nitrogen source) were evaluated using classical One Factor at a Time (OFAT) optimization method to increase the level of L-asparaginase production in the *F. khargensis* IBRC-M 30116<sup>T</sup>. In this method, optimization process was done by varying a single factor only and keeping the remaining factors constant enzyme (Talluri et al. 2019). In the first step, the optimal level of inoculum was studied by varying the concentration 1–9%. Enzyme production increased with increasing inoculum size and it seems lower level of an inoculum may not be sufficient for initiating the growth of the producer strain, whereas, much higher level of inoculum may cause competitive inhibition (Nandal et al. 2013). Positive effect of increasing inoculum size on L-asparaginase production by the yeast *Leucosporidium scottii* has been reported by Moguel (2018). One of the most significant factors for L-asparaginase production is the incubation temperature which affects the microbial growth, enzyme secretion, and rate of the chemical reaction, which finally affects the rate of enzymatic activity (El-Hadi et al. 2019). To determine the optimum temperature for L-asparaginase production, the type strain was cultured in a modified Czapek Dox broth medium supplied with L-

asparagine at different temperatures ranging from 20 to 37 °C for an incubation period of 4 days. A steady escalation in the enzyme activity with increasing in temperature from 20 °C observed, and the maximum activity obtained ( $35.5 \pm 1.5$  U/mL) at 30 °C (Fig. 2b). Further increase in the temperature resulted in significant reduction in enzyme activity. This may be due to the heat accumulation in the medium and subsequent denaturation of the enzyme. Selection of the appropriate substrate based on nutritional value, availability and cost plays an important role in enzyme production in industrial scale (Ghosh et al. 2013). In the next step, different carbon sources (glucose, sucrose, citric acid and glycerol) and then, different nitrogen sources (yeast extract, proline and asparagine, ammonium chloride, sodium nitrate) at a final concentration of 1% w/v were used. The highest level of enzyme activity obtained was  $49.9 \pm 1.6$  U/mL when glycerol was used as a carbon source. On the other hand, citric acid recorded the lowest value in L-asparaginase production ( $15.4 \pm 1.0$  U/mL). Sucrose, glycerol and citric acid have been reported as positive factors on L-asparaginase production by *L. scottii* (Moguel 2018). Improving the production of L-asparaginase is highly affected by the nitrogen sources either the organic form or inorganic form. In this study, ammonium chloride in the medium provides the highest level of L-asparaginase production ( $61.3 \pm 0.2$  U/mL). However, the researchers don't share the same opinion regarding the proper choice of suitable nitrogen sources. Some researchers such as Venil and Lakshmanaperumalsamy (2008), El-Naggar et al. (2018) reported that the addition of organic nitrogen sources in production medium will induce maximum L-asparaginase production and inorganic nitrogen supplements reduce L-asparaginase production significantly while others such as El-Hadi et al. (2017) contradict this notion. Finally, the results of this study indicated that the yeast-like *F. khargensis* IBRC-M 30116<sup>T</sup> possessed an activity level of 13.6 U/mL before obtaining optimal conditions, and after reaching optimal conditions this increased to 61.3 U/mL, which represents a 4.5-fold increase.

## 5 Conclusion

L-asparaginase is one of the most potential industrial enzymes that usually produce by bacterial sources. However, recognition of several adverse side effects linked with bacterial asparaginase requests a universal search for L-asparaginase from new sources. Asparaginase production has been reported from the various yeasts and yeast-like genera including *Saccharomyces* sp., *Candida* sp., *Pichia* sp., *Rhodotorula* sp., *Hansenula* sp., and *Spobolomyces* sp. (Batoool et al. 2016). However, to the best of our knowledge, this is the first report which describes an unexploited

source for production of L-asparaginase enzyme by type species of *Fereydounia* genus. Nevertheless, further studies are required to characterize this microbial enzyme and to fully optimize the production process using statistical design of experiment approaches and also to use latest recombinant technologies which could improve the efficiency of large-scale production of the enzyme for commercial purposes in the future.

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**Availability of Data and Material** The data that support the finding of this study are openly available in NCBI.

## Compliance with Ethical Standards

**Conflict of interests** The authors declare that they have no conflict of interest.

**Consent to Participate** The authors declare that they voluntarily agree to participate in this research study.

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