

## Optimization Production of L-asparaginase by Locally Isolated Filamentous Fungi from Egypt

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### ABSTRACT

The current study describes isolation and screening of microorganisms from soil for L-asparaginase activity, and for the selection a high potential strain, characterization and identification of the potential strain by combination approach and optimization of the process parameters for maximization of L-asparaginase production by the potential strain. Twenty-one fungal strains from Forty three isolates were isolated from different rhizosphere soils of Egypt showed positive for L-asparaginase production by producing pink coloration around the colony. Among various filamentous fungi tested by plate assay and broth studies the isolate BES1 and QW5 from the rhizosphere soil of Berket El-Sabaa (BES) and Quwaysna (QW) exhibited the highest zone of diameter (6.0 and 5.cm, respectively) and maximum activity (5.55 and 4.86 IU/ml, respectively) were considered as the potential strains and were used for further studies. Based on it's morphological and microscopy characteristics as well as 18S rRNA sequence analysis, the two isolates designated as BES1 and QW5 were identified as identified *Fusarium solani* strain FRC#s1162 and *Penicillium oxalicum* strain No. KUC1674, respectively. The observations made in this work hold great promise for maximum production of L-asparaginase enzyme after optimization of fermentation parameters such as fermentation time, temperature, pH, carbon source such as glucose, nitrogen source such as ammonium sulphate under fermentation. The optimum incubation time for maximum L-asparaginase production from *F. solani* and *P. oxalicum* were 48 h (6.422 IU) and 72 h (8.56 IU), temperature near to 45°C and 25°C, pH 7.0 (7.594 and 7.820 IU), glucose (6.81 IU at 0.5%) and sucrose (6.21 IU at 0.5%) ammonium sulphate (0.5%) and yeast extract (0.5%) appears to be the good inorganic and organic nitrogen source for *F. solani* and *P. oxalicum*, respectively.

**Key words:** L-asparaginase, *F. solani*, *P. oxalicum*, optimization of fermentation

### Introduction

L-asparaginase (L-asparagine amido hydrolase E.C.3.5.1.1) enzyme which converts L-asparagine to L-aspartic acid and ammonia has been used as a chemotherapeutic agent in the treatment of acute lymphoblastic leukemia for over 30 years (Sangita *et al.*, 2013). The clinical action of this enzyme is attributed to the reduction of L-asparagine, since tumor cells unable to synthesize this enzyme are selectively killed by L-asparagine deprivation (Amena *et al.*, 2010). Therefore, L-asparaginase has been commercially used to prevent acrylamide formation in fried food products (Mohan *et al.*, 2013). L-asparaginase from bacterial sources has been reported to be used especially as a therapeutic agent in the treatment of acute lymphoblastic leukemia in children (Pieters *et al.*, 2011). This enzyme depletes malignant cells by preventing the formation of essential growth factors for tumor development.

L-asparaginase is present and production from diverse types of microorganisms, plants and animals (Mohan *et al.*, 2013). Various microorganisms including, *Erwinia carotovora* (Maladkar *et al.*, 1993), *Escherichia coli* (Wei and Liu, 1998), *Aspergillus* sp. (Sarquis *et al.*, 2004) and also from marine derived fungal endophytes such as *Fusarium* sp., *Phomopsis* sp., *Trichoderma* sp. and *Sargasam wightii* (Thirunavukkarasu *et al.*, 2011) has been studied extensively for production of L-asparaginase. Most of the microbial L-asparaginase is intracellular in nature except few which are secreted outside the cell (Narayana *et al.*, 2008). Extracellular L-asparaginase is more advantageous than intracellular since they could be produced abundantly in culture under normal condition and could be purified economically (Deokar *et al.*, 2010; Joseph & Rajan 2011). On the other hand, L-asparaginase from bacterial origin can cause hypersensitivity in long term use leading to allergic reactions and anaphylaxis (Reynolds and Taylor, 1993). Therefore, the discovery of a new L- asparaginase immunologically different from that of bacteria has been greatly desired. It has been observed that eukaryotic microorganisms like yeast and filamentous fungi genera such as *Aspergillus*, *Penicillium* and *Fusarium* are commonly reported in scientific literature to produce L-asparaginase with less adverse effects (Fisher & Wray Jr 2002; Patro & Gupta 2012, Soniyambi *et al.*, 2011). Therefore, the process parameters play an important role in any bioprocesses.

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Yield and quality of the product depend mainly on these parameters. Optimization of the process parameters in order to achieve quality higher yield of the product is an important step for bioprocess development. In industrial production process, a small improvement as a result of process optimization may lead to big commercial success. Since there are very less reports of the production of L-asparaginase by the filamentous fungi, an attempt have been made to produce the L-asparaginase by employing fungal isolated from the rhizosphere soil of Egypt. The current study describes isolation and screening of microorganisms from soil for L-asparaginase activity, and for the selection a high potential strain, Characterization and identification of the potential strain by combination approach, Optimization of the process parameters for maximization of L-asparaginase production by the potential strain, Bench scale production and unstructured kinetic modeling of L-asparaginase production by the potential strain and Purification of L-asparaginase from production medium.

So, the aim of the present study is to evaluate the production of L-asparaginase from the locally isolated filamentous fungi for Egypt and optimization of the process parameters for maximization of L-asparaginase production by the potential strain.

## **Materials and Methods**

### *Sample Collection*

The soil samples were collected in sterilized polythene bags from the rhizosphere region of different plants at various locations around Shibin Al Kawm (SAK), Berket El-Sabaa (BES) and Quwaysna (QW), Al Monufia Governorate. Then the samples were transported to the laboratory for further filamentous fungi analysis (Dhevendaran and Annie, 1999). Potato Dextrose Agar (PDA) was used for recovering the fungal isolates from stock cultures. Then, maintaining them time to time as pure isolates by their sub-culturing and finally preservation of pure cultures in PDA slants (Agarwal and Hasija, 1986).

### *Isolation of filamentous fungi*

About 1 g of each of the above samples was taken into separate conical flasks each containing 100 ml of sterile water. The suspension was kept on rotary shaker for 30 min and kept aside to settle the suspending matter. One ml of the supernatant was serially diluted with sterile water and plated on modified rose bengal agar (MRB) plates by pour plate technique. The plates were incubated at  $28 \pm 2^\circ\text{C}$  for 96-120 hours. Individual colonies were regrown on MRB at  $28^\circ\text{C}$  for obtaining pure culture. The pure cultures were maintained at  $4-5^\circ\text{C}$  and were sub cultured once in a month.

### *Sub-culturing and preservation of pure culture*

Forty three fungi were aseptically sub cultured onto PDA plates and incubated at  $28 \pm 2^\circ\text{C}$  for 7-10 days till the profuse fungal growth was seen. The loop full of the metabolically active culture was aseptically inoculated on to PDA slants containing 10% glycerol for long term preservation.

### *Preliminary screening for L-asparaginase producers*

Primary screening of 43 fungal isolates was done for L-asparaginase production by using modified protocol as previously described by Patil *et al.*, 2012. For this assay, A modified Czapek Dox (CD) medium (glucose  $2 \text{ g L}^{-1}$ , L-asparagine  $10 \text{ g L}^{-1}$ ,  $\text{KH}_2\text{PO}_4$   $1.52 \text{ g L}^{-1}$  KCl  $0.52 \text{ g L}^{-1}$   $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$   $0.52 \text{ g L}^{-1}$ ,  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$   $0.01 \text{ g L}^{-1}$  and agar  $20 \text{ g L}^{-1}$ ) was used for plate assay (Thirunavukkarasu *et al.*, 2011). A 2.5% stock solution of phenol red was prepared in ethanol (pH 6.2) and 3 mL of this was added to 1000 mL of Czapek Dox medium. A mycelial disc (5 mm diameter) cut from the growing margin of the colony of an endophyte or phellophyte was placed in a petri dish containing 20 mL of this medium. After 72 h of incubation at  $26 \pm 1^\circ\text{C}$ , the appearance of a pink zone around the fungal colony in an otherwise yellow medium indicated L-asparaginase activity (Gulati *et al.*, 1997).

### *Secondary screening for L-asparaginase producers*

The cultures selected on the basis of preliminary testing were further screened on L-asparagine-agar plates containing 0.009% phenol red. L-asparagine acts as sole nitrogen source. All the positive fungal isolates were grown on L- asparagine-agar plates for 7 days at  $28^\circ\text{C}$ . The color change from yellow to pink was observed and diameter of zone was measured (Theantana *et al.*, 2007).

### *Shake flask culture filtrate activity*

The selected fungal isolates on the basis of secondary screening were subjected for culture filtrate production in Modified Czapek Dox (MCD) medium. 5 mm mycelial disc of 7 day old culture were inoculated in 25 ml pre-sterilized MCD broth in Erlenmeyer flask under aseptic conditions and were incubated in shaker incubator at 10.000 rpm,  $28^\circ\text{C}$  for 7 -10 days (Imada *et al.*, 1973). After the incubation is over, the fungal mycelium was separated from broth through filtration using Whatman filter paper No.1 followed by

centrifugation at 12,000 rpm for 15 min to get cell free culture filtrate. The cell free culture filtrate was then used for further qualitative testing by Agar well Diffusion assay.

#### *Determination of L-asparaginase activity (Ren et al., 2010)*

1.0ml of enzyme crude extract, 1.5ml of 50mM asparagine and 0.5ml potassium phosphate buffer (0.02 M and pH 7.8) were mixed well, the mixture was incubated in water bath at 37°C for 30 minutes, after the incubation, 0.5ml of 1.5M trichloroacetic acid was added to the mixture to stop the reaction, the mixture was centrifuged at 8000 rpm for 10 minutes and the supernatant was collected. After that, the supernatant was transported to clear test tubes to determining the concentration of ammonia which is liberated from the enzyme action by the method of direct Nesslerization. Then, the amount of ammonia liberated from protein sample of interest is calculated from Standard curve of ammonia in units/ ml.

#### *Identification of fungal isolates*

Identification of the selected fungal isolate was carried out by morphological and microscopic observations using the procedures described by Nelson *et al.*, (1983); Leslie and Summerell, (2006). Primers for the 18S rRNA were used to amplify a DNA sequence (primers NS1 [5'- GTAGTCATATGCT TGTCTC -3'] and NS2 [5'- TGCTGGCACCAGACTTG -3']) by Polymerase Chain Reaction (PCR) (White *et al.*, 1990). Primers were synthesized by GenScript Corporation Ltd. (NanJing, China). PCR amplifications were carried out in 50 uL reaction volumes with a PCR reactor (Eppendorf) with following program: initial denaturation at 94°C for 3 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 2 min followed by a final extension phase at 72°C for 10 min. The purified PCR product was sequenced in both the directions. The sequences of 18S rRNA genes were first analyzed using the BLAST searching program at the National Center for Biotechnology Information (NCBI) website: <http://www.ncbi.nlm.nih.gov/BLAST/>. Sequence alignments were performed using MEGA-5 software. The 18S rRNA sequence of the isolate was aligned with reference sequences and other close relatives from GenBank. A maximum likelihood phylogenetic tree was constructed using MEGA version 5.0. To estimate the confidence of the phylogenetic tree, bootstrapping was performed (Tamura *et al.*, 2007).

#### *Estimation of total protein*

Extracellular protein was estimated by Lowry method (Lowry *et al.*, 1951). The absorbance of the colour was measured at 660 nm in a pectrophotometer. Amount of protein in test samples was calculated by using the correlation equation obtained by BSA standard curve.

#### *Optimization of fermentation parameters*

L-asparaginase production was studied using Czapek-Dox medium for optimum incubation period (24, 48, 72, 96 and 120 h), temperature at (10, 25, 37, 45 and 55°C), pH (from 4.0 to 10.0), carbon sources (0.25 % to 1.25%) such as glucose, sucrose and fructose, inorganic nitrogen sources like ammonium chloride, ammonium sulphate and ammonium nitrate and organic nitrogen sources such as peptone, yeast extract and beef extract which were varied as 0.25 %, 0.5 %, 0.75 %, 1.0 % , 1.25 %, respectively. Enzyme assay was carried out as previously described and the optimum period achieved was taken for further experiments.

#### *Statistical analysis*

All the experiments were carried out in five replicates and mean values were presented. The data presented in graphs and tables corresponding to mean values + SEM and the statistical significant ( $P < 0.05$ ) was established by using GraphPad prism 5 software.

## **Results**

The results of fungal isolates from different soil samples revealed variations among different types of fungi. Forty three fungi isolated were obtained at following, nine isolates from Shibin Al Kawm1 (SAK1 to SAK9), seven isolates from Shibin Al Kawm2 (SAK10 to SAK16) (Fig1 and Table 1), eight isolates from Berket El-Sabaa (BES1 to BES8), six isolates from Berket El-Sabaa2 (BES8 to BES14), five isolates from Quwaysna (QW1 to QW5) and eight isolates from Quwaysna2 (QW6 to QW13). The major isolates belonging to *Acremonium*, *Aspergillus*, *Bipolaris*, *Curvularia* and *Fusarium*, were examined for L-asparaginase production.

#### *Preliminary screening for L-asparaginase production by plate assay method*

For agar plate assay, twenty-one fungal isolates from forty three isolated showed pink zone around the colonies on Modified Czapek Dox agar containing phenol red, indicating the increase in pH which originated from ammonia accumulation in the medium. The dye indicator is yellow at acidic condition and turns to pink at alkaline condition. Since this method is very simple and rapid for the detection of L-asparaginase activity, it has

been used for primary screening of L-asparaginase production from fungi isolates. This preliminary selection indicated that the isolates belonging to *Aspergillus*, *Alternaria*, *Penicillium*, *Fusarium*, *Acromonium*, *Bipolaris*, *Curvularia*, *Phaeotrichoconis* and *Botrytis* produced L-asparaginase (Table 1). *Aspergillus* (SAK9), *Fusarium* (BES1) *Bipolaris* (QW4) and *Penicillium* (QW5) isolated from Shibin Al Kawm, Berket El-Sabaa and Quwaysna show the highest zone diameter (6.0, 5.0, 4.0 and 5.0 cm, respectively).

*Secondary screening for L-asparaginase producers*

The twenty-two fungal isolates that showed pink zone around their colonies were tested for L-asparaginase production in liquid condition by Nesslerization method. All of them exhibited L-asparaginase activity between 0.13-5.55 IU/ml (Table 1). From our study, we found that enzyme activity of some isolates was not correlated to the size of pink zone diameter (Table 1). Therefore, the isolate BES1 and QW5 from the rhizosphere soil of Berket El-Sabaa (BES) and Quwaysna (QW) showed highest zone diameter (6.0 and 5.0 cm, respectively) and maximum activity (5.55 and 4.86 IU/ml, respectively), were considered as the potential strains and were used for further studies.

**Table 1:** Colony and pink zone diameters after 48 h and L-asparaginase activity of culture filtrate after 96 h incubation

	Fungi	Isolate	Colony diameter (cm)	Zone diameter (cm)	Enzyme activity(IU/ml)
1	SAK1	<i>Aspergillus</i> sp.	1.6±0.05	(-)	ND
2	SAK 2	<i>Aspergillus</i> sp.	1.2±0.00	(-)	ND
3	SAK 3	<i>Penicillium</i> sp.	1.6±0.10	(-)	ND
4	SAK 4	<i>Aspergillus Flavus</i>	1.4±0.10	(-)	ND
5	SAK 5	<i>Alternaria solani</i>	1.4±0.05	3.0±0.20	0.49±0.01
6	SAK 6	Unidentified	0.8±0.15	2.0±0.15	0.13±0.01
7	SAK 7	<i>Acromonium</i> sp.	1.2±0.00	(-)	ND
8	SAK 8	<i>Paecilomyces</i> sp.	2.0±0.00	3.5±0.10	1.03±0.00
9	SAK 9	<i>Aspergillus</i> sp.	2.1±0.10	6.0±0.10	3.50±0.00
10	SAK 10	<i>Fusarium</i> sp.	2.4±0.10	(-)	ND
11	SAK11	Unidentified	1.6±0.05	(-)	ND
12	SAK12	<i>Penicillium</i> sp.	1.8±0.00	(-)	ND
13	SAK13	<i>Aspergillus</i> sp.	1.5±0.10	3.5±0.05	0.98±0.01
14	SAK14	<i>Alternaria solani</i>	1.8±0.00	3.5±0.00	1.06±0.001
15	SAK15	<i>Fusarium</i> sp.	2.4±0.10	3.0±0.10	1.09±0.02
16	SAK16	<i>Acromonium</i> sp.	1.4±0.03	(-)	ND
17	BES1	<i>Fusarium</i> sp.	2.4±0.10	5.0±0.40	5.55±0.04
18	BES 2	<i>Phaeotrichoconis</i>	2.0±0.05	3.5±0.00	0.89±0.08
19	BES 3	<i>Fusarium</i> sp.	2.2±0.10	3.0±0.15	1.03±0.02
20	BES 4	<i>Bipolaris</i> sp.	0.7±0.03	(-)	ND
21	BES 5	Unidentified	1.0±0.00	4.8±0.00	1.21±0.001
22	BES 6	<i>Penicillium</i> sp.	2.0±0.05	3.0±0.25	1.05±0.01
23	BES 7	<i>Penicillium</i> sp.	2.0±0.00	2.5±0.25	0.94±0.00
24	BES 8	<i>Curvularia</i> sp.	1.8±0.03	2.0±0.00	0.83±0.02
25	BES 9	<i>Penicillium</i> sp.	2.5±0.10	2.0±0.20	0.83±0.00
26	BES 10	<i>Penicillium</i> sp.	2.2±0.10	(-)	ND
27	BES11	<i>Aspergillus</i> sp.	4.7±0.05	(-)	ND
28	BES12	Unidentified	2.1±0.10	(-)	ND
29	BES13	<i>Alternaria</i> sp.	6.8±0.25	(-)	ND
30	BES14	<i>Bipolaris</i> sp.	0.7±0.10	3.0±0.45	0.80±0.01
31	QW1	<i>Fusarium</i> sp.	0.7±0.00	2.0±0.00	1.00±0.00
32	QW2	<i>Phaeotrichoconis</i> sp.	1.8±0.00	(-)	ND
33	QW3	<i>Cochliobolus</i> sp.	0.7±0.03	(-)	ND
34	QW4	<i>Bipolaris</i> sp.	1.2±0.10	4.0±0.00	3.17±0.01

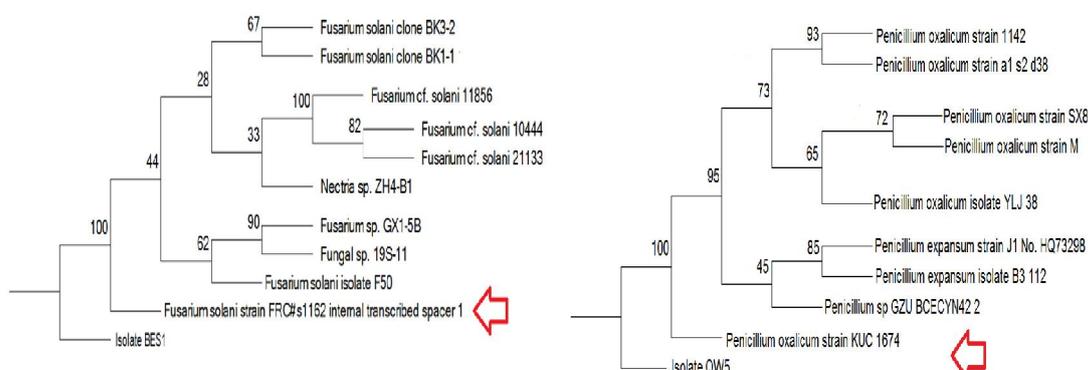
35	QW5	<i>Penicillium</i> sp.	2.0±0.00	5.0±0.00	4.86±0.12
36	QW6	<i>Curvularia</i> sp.	2.0±0.00	(-)	ND
37	QW7	<i>Aspergillus</i> sp.	1.6±0.12	1.92±0.04	0.15±0.01
38	QW8	<i>Bipolaris</i> sp.	1.3±0.41	1.61±0.07	0.54±0.00
39	QW9	Unidentified	1.8±0.22	1.61±0.10	0.33±0.02
40	QW10	<i>Alternaria</i> sp.	0.1±0.00	(-)	ND
41	QW11	<i>Botrytis</i> sp.	2.1±0.01	(-)	ND
42	QW12	<i>Acromonium</i> sp.	2.4±0.33	(-)	ND
43	QW13	Unidentified	1.2±0.22	(-)	ND

ND = Not determined; (-) = No zone produced

#### The identification of the fungi isolates

Based on its morphological the two isolates designated as BES1 and QW5 were identified as *Fusarium* sp. and *Penicillium* sp., respectively.

The sequence data showed that the isolates BES1 and QW5 have highest sequence similarity 99 and 98%, respectively with the genus *Fusarium solani* and *Penicillium oxalicum*. Hence, it is concluded that the isolated strains BES1 and QW5 are *Fusarium solani* strain FRC#s1162 and *Penicillium oxalicum* strain no. KUC1674 (Figure 1).



**Fig. 1:** Phylogenetic tree of fungal isolate BES1 and QW5 relationship among the selected strains based on sequencing analysis and the most closely related fungus species

### Optimization of cultural parameters for L-asparaginase production

#### Effect of incubation period on the production of L-asparaginase

The production of L-asparaginase by *F. solani* and *P. oxalicum* were studied along 7 days of incubation when cultivated on Czapek's Dox medium with 1% L-asparagine. Incubation period during the process fermentation is very much essential to study the optimum incubation time for maximum L-asparaginase production. *F. solani* showed maximum enzyme activity of 6.422 IU/ml at 48 h and further increase in the incubation period decreased the enzyme activity. On the other hand, Fig.2 reveals that the enzyme production by *P. oxalicum* was maximum at 72 h of incubation period (8.560 IU/ml).

#### Effect of temperature on the production of L-asparaginase

Highest activity of *F. solani* (Fig. 3) was observed at 45°C which showed 6.85 IU/ml at 48 h of fermentation period whereas lowest enzyme was produced at 10°C with maximum activity of 2.80 IU/ml respectively at 48 h. On the other hands, the production of L-asparaginase by *P. oxalicum* is strongly influenced by temperature. The production of L-asparaginase was maximum at temperature near to ambient temperature (25°C) with an activity of 7.148 IU/ml (Fig. 3). A lower enzyme activity was obtained with cultivation temperatures lower and higher than the optimum temperature.

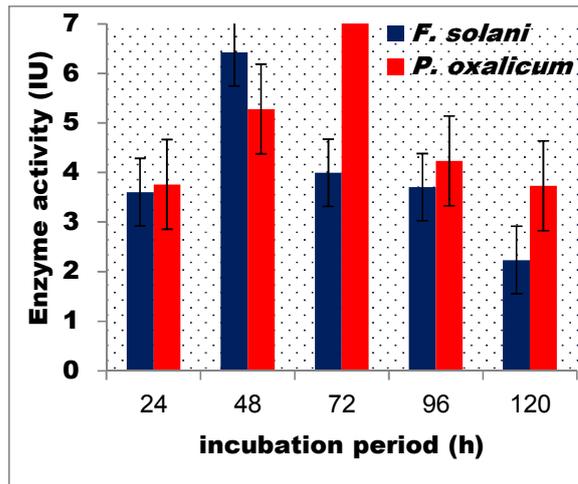


Fig. 2: Effect of incubation period on the production of L-asparaginase

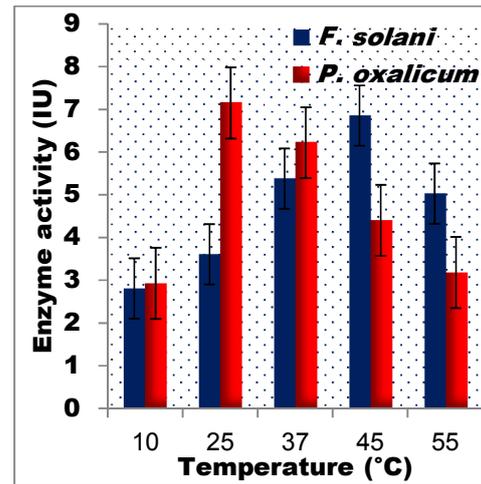


Fig. 3: Effect of temperature on the production of L-asparaginase

#### Effect of pH on the production of L-asparaginase

Production of L-asparaginase by *F. solani* and *P. oxalicum* at 48 h was found to be maximum at pH 7.0 with the activity of 7.59 and 7.820 IU/ml, respectively (Fig. 4). However, asparaginase activity decreased at low pH (3 and 6) and high pH range (above pH 7-8).

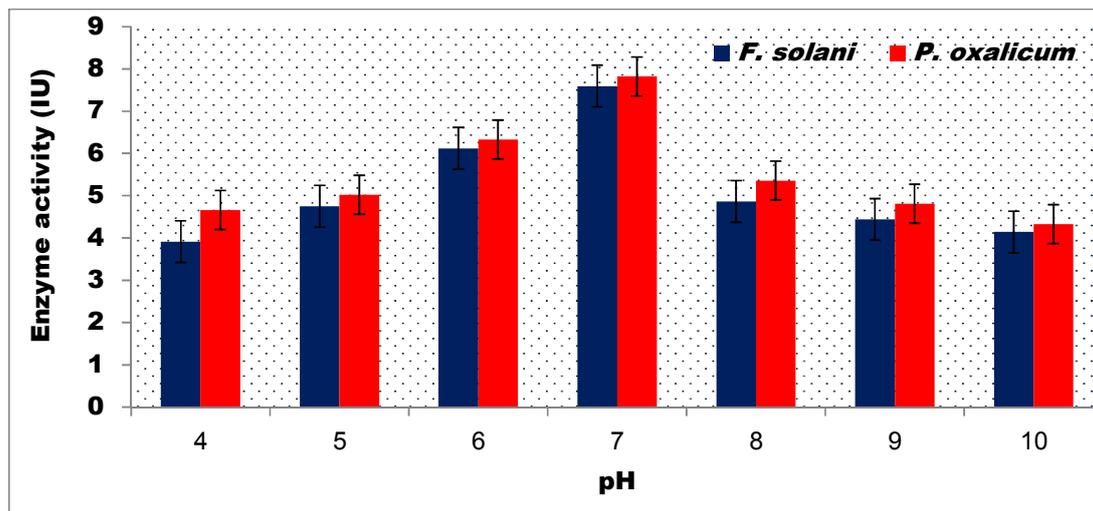


Fig. 4: Effect of pH on the production of L-asparaginase

#### Effect of carbon source on the production of L-asparaginase

The results indicated that at different concentrations of glucose there was significant increase in the enzyme activity with increase in the glucose concentration up to 0.5% and later decreased with increase in the fermentation period. The present results pertaining to the sucrose and fructose concentration reveals that there was significant increase in the enzyme activity up to 0.75 % at 48 h and on further incubation decreased the enzyme activity in case of *F. solani* (Fig 5A). On the other hand, data in Fig 5B indicates that *P. oxalicum* could produce maximum asparaginase when sucrose was used as the carbon source (6.21 IU/ml at concentration 0.5%), followed by glucose (5.826 IU/ml at concentration 0.75%) when compared to the control medium at 48 h of fermentation period. For instance glucose, sucrose and fructose were good in inducing the production of enzyme when compared to control (Fig. 5).

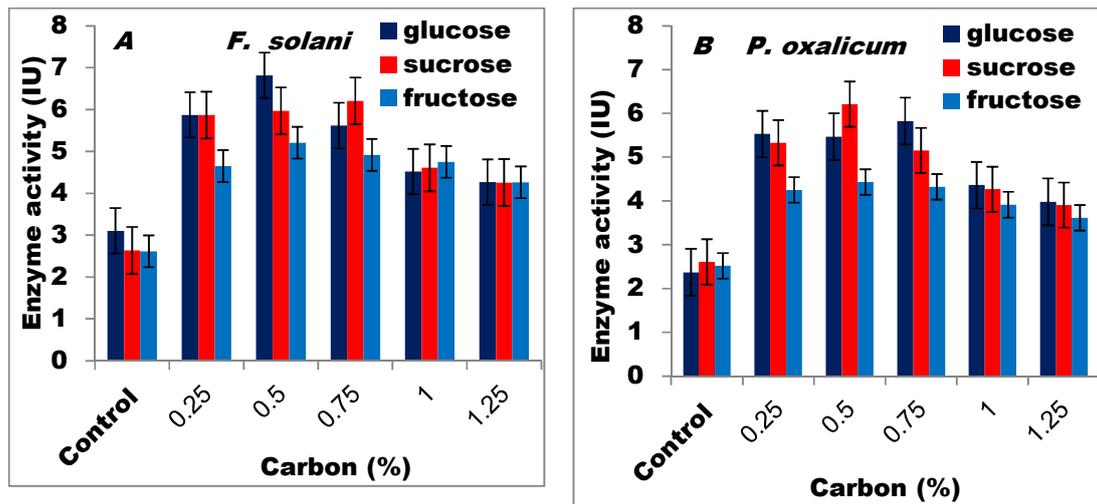


Fig. 5A&B: Effect of carbon sources and concentration on the production of L-asparaginase by *F. solani* and *P. oxalicum*

*Effect of nitrogen source on the production of L-asparaginase*

The nitrogen source is the limiting factor and plays key role in the L-asparaginase production (Fig 6). Most of the microorganisms utilize nitrogen source either inorganic or organic form or sometimes both. The results illustrated that the maximum enzyme activity production by *F. solani* was 7.36 IU at 0.5 %, ammonium sulphate at 48 h of fermentation period. On the other hand, the enzyme production by *P. oxalicum* was higher than *F. solani* in all levels of. The results obtained reveals that the enzyme production was high in ammonium sulphate at 0.50% (7.642 IU/ml) when compared to all levels of ammonium sulphate. The results revealed that the enzyme activity for production by *F. solani* was 4.79 IU at 0.5 % at 48 h of fermentation period. Therefore, the present results showed that there was significant increase in the enzyme activity production by *P. oxalicum* of 5.177 IU at 0.5 % ammonium chloride. The results pertaining to the above studies indicated that the enzyme activity production by *F. solani* and *P. oxalicum* were 6.27 and 7.334 IU/ml, respectively at 0.25 % ammonium chloride at 48 h of fermentation period. Thus, amongst all inorganic nitrogen sources provided for L-asparaginase production ammonium sulphate (0.5 %) appears to be the good nitrogen source for *F. solani* and *P. oxalicum*.

*Effect of organic nitrogen sources on the production of L-asparaginase*

Present investigation demonstrated that the maximum enzyme activity production by *F. solani* and *P. oxalicum* were 8.518 and 9.062 IU/ml, respectively with 0.5 % yeast extract at 48 h of fermentation period (Fig. 7A&B). The results of the enzyme activity for the effect of peptone and beef extract revealed that the enzyme activity was lowery compared to yeast extract. Thus, among all the organic nitrogen sources, yeast extract (0.5%) emerged as the best organic nitrogen source for the L-asparaginase production by *F. solani* and *P. oxalicum*. The results obtained also show significant increase in production of asparaginase by *F. solani* and *P. oxalicum* in organic than inorganic nitrogen (Fig. 7).

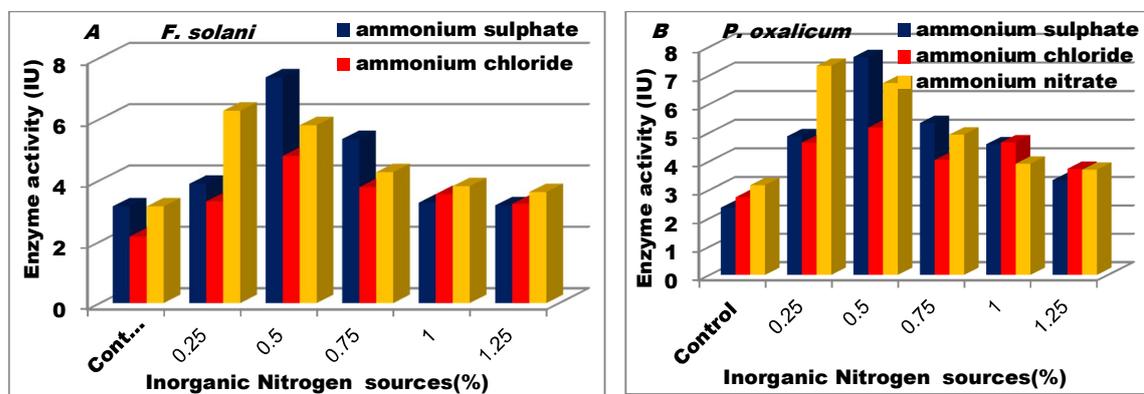


Fig. 6 A&B: Effect of different inorganic nitrogen sources on L-asparaginase production by *F. solani* and *P. oxalicum*

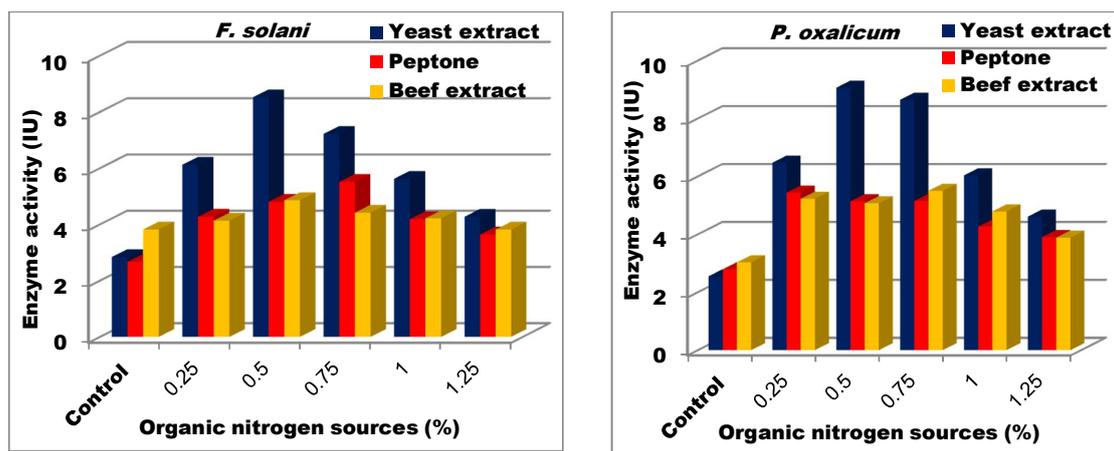


Fig. 7: Effect of different organic nitrogen sources on L-asparaginase production by *F. solani* and *P. oxalicum*

## Discussion

In the present study, L-asparaginase producing fungus was isolated from soil fields at various locations around Shibin Al Kawm (SAK), Berket El-Sabaa (BES) and Quwaysna (QW), Al Monufia Governorate. The modified Czapek-Dox agar contained L-asparagine as a sole nitrogen source and phenol red as an indicator. L-asparagine also served as an enzyme inducer. Out of forty three isolates collected from different rhizosphere soils, the twenty-one isolates showed positive for L-asparaginase production by producing pink coloration around the colony. The change in color (from yellow to pink) of the indicator resulted from the increase in pH due to ammonia release. The ammonia along with L-aspartic acid is formed by the deamidation reaction of the substrate L-asparagine by L-asparaginase (Gulati *et al.*, 1997). Gupta *et al.* (2007) have reported rhizosphere soil of mangrove plant isolate *Fusarium* FMG 13 was able to produce L-asparaginase. Among various filamentous fungi tested by plate assay and broth studies the isolate BES1 and QW5 from the rhizosphere soil of Berket El-Sabaa (BES) and Quwaysna (QW) showed highest zone diameter and maximum activity, were considered as the potential strains and were used for further studies (Table 1). It is proposed that strain exhibiting zone of diameter above 0.9 cm are referred as good L-asparaginase producers, those strains with zone diameter of 0.6-0.9 cm and those having below 0.6 cm zone diameter may be referred to as moderate and poor L-asparaginase producers respectively (Thakur *et al.*, 2014).

The positive L-asparaginase producing fungi were identified morphologically and microscopically as *Fusarium* sp. and *Penicillium* sp. The identity was further confirmed by phylogenetic analysis based on 18S rRNA gene sequencing by a National Research Centre-Cairo-Egypt (Agricultural Microbiology Department). Sequence data showed that the isolates BES1 and QW5 have highest sequence similarity (99% and 98%, respectively) with the genus *Fusarium solani* and *Penicillium oxalicum* (Fig 1). Hence it is concluded that the strain BES1 belongs to the genus *Fusarium solani* and QW5 belongs to the genus *Penicillium oxalicum*. Information, USA, accession number FRC#s1162 and KUC1674, respectively).

A *Fusarium* sp. and *Penicillium* sp. secreting extracellular L-asparaginase was also isolated from the marine sponge *Spirastrella* sp. (Mohapatra *et al.*, 1995). *Aspergillus*, *Penicillium*, *Fusarium*, *Helminthosporium*, *Scophulariopsis*, *Paecilomyces* and *Pestalotiopsis* screened from Bhitarkanika mangrove forest soil of Orissa coast (India) were found to be a good source of L-asparaginase (Gupta *et al.*, 2007). Many fungal species producing L-asparaginase were also isolated from soil. For example, *Emericella nidulans* from different soils of Tumkur university campus (Karnataka, India) (Jayaramu *et al.*, 2010), *Aspergillus flavus* (KUFS20) from garden soil of Coimbatore (Tamilnadu, India) (Rani *et al.*, 2012) and *Penicillium* sp. was screened from soil samples of Bangalore (Karnataka, India) (Mushtaq *et al.*, 2012). Hosamani and Kaliwal (2011) reported the screening of *Fusarium equiseti* from rhizosphere soil of various plants around Dharwad campus (Karnataka, India) and suggested that the presence of the fungus might be due to the presence of natural source of amino acid present in root exudates of the plants in the rhizosphere soil. Gupta *et al.*, (2007) have reported rhizosphere soil of mangrove plant isolate *Fusarium* FMG 13 was able to produce L-asparaginase. In the present study it is clear that filamentous fungi from rhizosphere have shown to produce extracellular L-asparaginase.

The various parameters influencing L-asparaginase secretion were optimized. The L-asparaginase-producing fungi must be provided with optimum growth conditions in order to improve and increase the enzyme production without increasing the cost. A balance between various medium components is maintained, reducing the amount of unused nutrients after fermentation completion. Incubation temperature, initial pH, nitrogen and

carbon source, and incubation temperature were optimized. The incubation temperature is a critical environmental factor for L-asparaginase production by microbes because it regulates microbial growth and consequently enzyme secretion. In the present study, *F. solani* production of L-asparaginase started at 24 hours and reached maximum at 48 hours and the decreased significantly with increase in the incubation time but *P. oxalicum* started at 24 hours and reached maximum at 72 hours. Similar results have been reported by Lapmak *et al.*, (2010) where the highest activity of 6.3 IU/ml for 72 hours using *Bipolaris* sp. BR438. The same incubation period was also noted for L-asparaginase of *Aspergillus* species (Siddalingeshwara and Lingappa 2011), Gurunathan B, Sahadevan 2011, Chandrasekhar 2012, Balasubramanian, *et al.*, 2012), *Bipolaris* sp. BR438 (Lapmak *et al.*, 2010) and *Penicillium* sp. (Rani *et al.*, 2011). The shorter incubation time makes the present submerged fermentation cost effective and reduces the chance of L-asparaginase decomposition by proteolytic enzymes. Contrastingly, a higher incubation time of 120 h was seen in the secretion of L-asparaginase by *Fusarium* sp. (Thirunavukkarasu *et al.*, 2011). In the present study, the prolonged incubation time led to a decrease in L-asparaginase secretion and this may be due to the exhaustion of some medium constituents or the production of inhibitory compounds (Fig 2). At longer incubation periods, the enzyme activity decreased which might be due to the depletion of nutrients, accumulation of toxic end products, and the change in pH of the medium, or loss of moisture. *F. solani* and *P. oxalicum* were able to grow and produce the enzyme on all the temperatures evaluated with maximum production at 45°C and 25 °C, respectively although statistically at par with 37 °C. However a noticeable decrease in enzyme yield was seen at 45°C in case of *P. oxalicum* (Fig. 3). The optimum temperature of 30 or 37 °C was reported in most of the L-asparaginase producing fungal species (Gurunathan and Sahadevan, 2012, Sarquis *et al.*, 2004, Lapmak *et al.*, 2010, Jayaramu *et al.*, 2010, Rani *et al.*, 2012). The low enzyme activity value recorded at 45 °C may be attributed to partial enzyme denaturation resulted from a change in metabolic activities. Any increase or decrease from optimum incubation temperature slows down the metabolic activities of microorganisms (Jayaramu, *et al.*, 2010). Any temperature beyond the optimum range is found to have an adverse effect on the metabolic activities of microorganisms and it is also reported that metabolic activities become slower at lower temperature (Carrizales and Jaffe, 1986). Mishra (2006) who have reported the highest activity of 40.9 U at 30°C whereas Maladkar *et al.*, (1993) have reported the optimum activity at 50°C. The thermostable asparaginase from *T. aquaticus* had an optimum temperature of 75°C have been reported by Curran *et al.*, (1985). The initial pH of the production medium is an important parameter affecting the enzyme production since it can indirectly act on the fungal growth by affecting the availability of medium nutrients. In order to find out there optimum pH for the L-asparaginase production, the initial pH of the fermentation medium was adjust to different levels and fermentation was carried out at 45°C and 25°C for *F. solani* and *P. oxalicum*, respectively. The maximum L-asparaginase production was noted at an initial pH of 7.0; thereafter a decline in enzyme production was seen (Fig. 4). Similarly, an initial pH of 7.0 led to maximum enzyme yield by *Fusarium equiseti* (Hosamani and Kaliwal, 2011) and *Aspergillus terreus* (Chandrasekhar, 2012). The initial pH reported for maximum L-asparaginase production is in the pH 6.0 - 9.0 range (Akilandeswari *et al.*, 2012, Balasubramanian *et al.* 2012). A decline in enzyme activity seen after optimum pH may due to partial denaturation of the enzyme resulted from dissociation of the ionizable groups of enzymes. The change in pH prevents the binding of a substrate to the enzyme owing to change of shape and properties of an enzyme and/or the substrate (Khalaf *et al.*, 2012). The fermentation efficiency was maximum at pH 7 by *Streptomyces plicatus* (Koshy *et al.*, 1997) while Narayana *et al.* (2008) have reported the maximum L-asparaginase production at pH 7.5 by *S. albidoflavus* and Siddalingeshwara and Lingappa (2010) have reported the maximum activity of 5.21 IU at pH 4.5. The influence of various carbon sources namely glucose, sucrose and fructose were studied for L-asparaginase production by *F. solani* and *P. oxalicum*. Optimal glucose concentration was observed at 0.5% with maximum activity of 6.81 IU and the least activity of 4.25 IU for 1.25% sucrose. Chanakya *et al.*, (2011) have reported the increase in activity of 6.02 IU to 6.92 IU at 0.3% glucose for L-asparaginase production by *Fusarium oxysporum*. The present results were in good agreement with Lapmak *et al.*, (2010) who has reported 0.4% glucose as the optimum concentration for the maximum enzyme activity. It has been reported that the microbial synthesis of L-asparaginase is under catabolic repression and requires less amount of carbon source (Baskar and Renganathann 2009). But the incorporation of glucose may be attributed to the positive influence of additional carbon sources on enhanced biosynthesis.

An extracellular L-asparaginase is produced during the post exponential and stationary growth phases by fungi under most culture conditions. It is therefore regulated by carbon and nitrogen sources. An important L-asparaginase production was recorded at 0.5% glucose concentration in case of *F. solani* but sucrose at 0.5% for *P. oxalicum* beyond which the enzyme activity gradually declined (Fig 5). Likewise 0.4% (w/v) was the best glucose concentration for the L-asparaginase production in *Aspergillus terreus* MTCC 1782 (Gurunathan and Sahadevan 2011) and *Bipolaris* sp. BR438 (Lapmak *et al.* 2010). A decline in L-asparaginase synthesis observed after optimum glucose concentration may be attributable to glucose catabolite repression. A stimulatory effect is thus seen at the low glucose concentrations whereas higher glucose concentrations are

inhibitory. The similar glucose effect was observed for the L-asparaginase of *Aspergillus terreus* MTCC 1782 (Gurunathan and Sahadevan 2011) and *Bipolaris* sp. BR438 (Lapmak et al. 2010).

Most of the microorganism utilize nitrogen sources either inorganic or organic form or sometimes both. Therefore in the present context, the L-asparaginase production was studied supplementing the inorganic nitrogen forms such as ammonium sulphate, ammonium chloride, ammonium nitrate and organic nitrogen forms such as yeast extract, peptone and beef extract respectively. 0.5% ammonium sulphate and 0.5% yeast extract gave the optimum activity (Fig 6&7). Hence in the present study ammonium sulphate and yeast extract can be used as best nitrogen source. Narayana *et al.* (2008) who have reported 2% of yeast extract as the best nitrogen source for L-asparaginase production by *S. albidoflavus*. Amena *et al.* (2010) have reported 0.25% ammonium sulphate can be used as best inorganic nitrogen source for L-asparaginase production by *S. gulbargensis*. Baskar and Rangathan (2009) have reported ammonium chloride was found to be the best nitrogen source for L-asparaginase production using *Aspergillus terreus* MTCC 1782. Hence, from the present findings it was clear that ammonium sulphate and yeast extract were the best inorganic and organic nitrogen sources respectively that can be used for L-asparaginase production by *F. solani* and *P. oxalicum*.

## Conclusion

The enzyme production was achieved by using two different fungal species *F. solani* and *P. oxalicum*. With respect to organisms, and the production of L-asparaginase by submerged fermentation, it was found that *P. oxalicum* showed higher specific activity when compared to *F. solani*.

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