

Optimization of L-asparaginase Production from Some Filamentous Fungi with Potential Pharmaceutical Properties

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ONE HUNDRED and forty three isolates related to 16 fungal species were recovered from garden soil and indoor air of the medical chambers of Tumor Institute, Kasr El Ainy, Egypt. Quantitative L-asparaginase assay for isolated species was determined by Nesslerization. The fungal isolates exhibited different L-asparaginase activities. *Aspergillus sydowii* and *Fusarium oxysporum* showed the highest L-asparaginase activity where the activity reached 3.98 and 3.91 U/ml in the assay mixture after 48 h incubation period, respectively. The identification of *A. sydowii* and *F. oxysporum* was confirmed molecularly using Internal Transcribed Spacer Regions sequences. The optimization of the cultural conditions for the production of L-asparaginase was performed by using Taguchi design. Eight component factors of a modified medium were evaluated in influencing L-asparaginase yield. The highest L-asparagenolytic activity was achieved in culture media containing 1% L-asparagine, 0.2% glucose, 0.1% KH₂PO₄, 0.025% KCl, 0.052% MgSO₄.7H₂O, pH 5.0 and incubation at 40°C in a rotatory incubator (100 rpm) for 5 days, where enzyme activities of 146 and 143 U/ml were measured for *A. sydowii* and *F. oxysporum*, respectively. Anti-leukemic activity of the crude enzyme of either *A. sydowii* or *F. oxysporum* against murine RAW264.7 leukemia cells line using MTT viability assay with IC₅₀ of 50.0 and 62.5 U/ml, respectively for the two tested fungi. The anti-cancer activity was determined against cell lines of human colon carcinoma, liver carcinoma and breast carcinoma. About 70-80% cell death of carcinoma cell lines was obtained by using the crude L-asparaginase of either *A. sydowii* or *F. oxysporum*.

Keywords: Taguchi design, L-asparaginase, Filamentous fungi, Optimization, Anti-leukemic and anti-cancer cytotoxicity.

Introduction

L-asparaginase (L-asparagine amidohydrolase; E.C. 3.5.1.1) is a tetrameric protein, belongs to an amide group which catalyzes the hydrolysis of L-asparagine into L-aspartate and ammonia. The hydrolysis proceeds in two steps *via* a beta-acyl-enzyme intermediate in a mechanism described by Hill (1967). The catalytic reaction is essentially irreversible under physiological conditions (Nakahama et al., 1973 and Lubkowski et al., 1996).

The enzyme is considered to participate a major role in the asparagine metabolism of the cell (Imada et al., 1973 and Zuo et al., 2014). L-Asparaginase constitutes the largest group of therapeutic enzymes as they account for about 40% of the total worldwide sale of anti-leukemic and anti-lymphoma agents (Clavell et al., 1986

and Shrivastava et al., 2016).

L-asparagine is an amino acid required both by normal cells as well as cancer cells for the production of protein. Normal cells synthesize this amino acid by the catalytic activity of asparagine synthetase from aspartic acid and glutamine. However, neoplastic cells can not produce L-asparagine due to the low levels of L-asparagine synthetase and they depend on cellular pools of L-asparagine for their growth (Keating et al., 1993 and Shrivastava et al., 2016). For this reason, L-asparaginase is an anti-neoplastic agent, used in the lymphoblastic leukemia chemotherapy and the commonest therapeutic practice is to inject intravenously free enzyme in order to decrease the blood concentration of L-asparagine affecting selectively the neoplastic cells (Hosamani

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& Kaliwal, 2011; Dange & Peshwe, 2015; Cachumba et al., 2016; Sanjotha & Manawadi, 2017 and Vidya et al., 2017).

L-asparaginase can be obtained from animals, plants and microorganisms. Microbial L-asparaginase was isolated from *Escherichia coli* or *Erwinia carotovora* with anti-leukemic activity. L-asparaginase hydrolyzes L-asparagine into L-aspartic acid and ammonia in leukemic cells, resulting in the depletion of asparagine, inhibition of protein synthesis, cell cycle arrest in the "G1" phase, and apoptosis in susceptible leukemic cell population (Krasotkina et al., 2004; Narta et al., 2007 and El-Naggar et al., 2016). Verma et al. (2007) classified bacterial-type L-asparaginase into subtypes I and II, which are defined by their intra-or extracellular localization. Type-I (cytosolic) has a lower affinity for L-asparagine, whereas type-II (periplasmic) has a high substrate affinity. However, L-asparaginase from bacterial origin can cause hypersensitivity in the longterm use, leading to allergic reactions and anaphylaxis. The search for other asparaginase sources, like eukaryotic microorganisms, may lead to an enzyme with less adverse effects. It has been observed that eukaryotic microorganisms like yeast, and filamentous fungi have a potential asparaginase production (Dhale & Mohan-Kuman, 2014; Indira et al., 2015; ELshafei & El-Ghonemy, 2015; Isaac & Abu-Tahon, 2016 and Bedaiwy et al., 2016). Therefore, there is a continuing need to screen new microorganisms in order to obtain strains capable of producing new and high yield of L-asparaginase.

The objective of the present work was to search for L-asparaginase from eukaryotic fungal sources for production of enzyme with less immunogenic and non-toxic properties.

Materials and Methods

Isolation and identification of fungi

Fungi were isolated from soil samples collected from garden and from air of the medical chambers of Tumor Institute, Kasr El Ainy, Cairo Governorate, Egypt. Dilution plate method (Johnson et al., 1959) was employed for isolation. The growing fungi were counted and maintained on potato dextrose agar (PDA) slants for further studies.

The developed fungal colonies were identified up to species level by macro-and microscopic examination through the help of the

following references: Raper & Fennell (1965) for the genus *Aspergillus*, Booth (1971, 1977) for the genus *Fusarium* and Moubasher (1993) for soil fungi in Qatar and other Arab countries. The identification of fungi was confirmed by Assuit University Mycological Centre (AUMC), Egypt.

Molecular identification

The two most active fungi producing L-asparaginase were identified on the basis of 18S r DNA amplification and sequencing as described by White et al. (1990). Sequencing was carried out and the results were compared to sequences held in GenBank databases using the BLAST program (Altschul et al., 1997). Closely related or phylogenetically relevant sequences were obtained from the GenBank databases and sequencing were aligned using ClustalW programs (Tamura et al., 2011). The evolutionary tree was inferred by using Neighbor Joining method using MEGA 5.05 software. The 18S rDNA gene sequence of strain (975 nucleotides) has been deposited in GenBank with nucleotide sequence (Wilson & Walker, 1995; Oliver & Schweizer, 1999 and Edward, 2001).

Qualitative and quantitative screening of L-asparaginase

All isolated fungi were assayed qualitatively for their L-asparaginase activity. A 2.5% stock of phenol red indicator was prepared in ethanol (96%) and the pH was adjusted to 7.0 using 1.0 N NaOH. Different volumes of the stock solution of the phenol red dye, ranging from 0.05 to 0.2%, were added to 100 ml of modified Czapek's Dox Agar medium (i) without dye and (ii) without L-asparagine (instead of NaNO_3) as nitrogen source according to the method described by Gulati et al. (1997). The modified Czapek's Dox Agar medium (g/L) consisting of glucose, 2.0; L-asparagine, 10.0; KH_2PO_4 , 1.52; KCl, 0.52; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.52 and Agar, 20. The medium was sterilized by autoclaving, then the desired phenol red concentrations containing sterilized medium was poured in each plate and left to solidify. The plates were then inoculated with a mycelium disc, cut from the margin of actively growing colonies of 96 hours old culture of the test fungi by a sterile cork borer and incubated at $28 \pm 1^\circ\text{C}$ for 48 h. The L-asparaginase activity was measured by the diameter of pink zone resulted from ammonia liberation which turns the phenol red to pink color.

Quantitative assay of L-asparaginase

activity in submerged cultures was carried out using modified Czapek's Dox liquid medium. Erlenmeyer conical flasks 250 ml containing 50 ml of the appropriate medium were inoculated with each of the test organisms. Cultures were incubated under shaking at 100 rpm at $28\pm 1^\circ\text{C}$ for 48 h in a sterile controlled environment shaking incubator (Model G25R; New Brunswick Scientific Co. Inc., Edison, NJ). Non-inoculated media served as control. The fungal cultures were harvested by filtration through Whatman No. 1 filter paper in Büchner funnels. The resulting mycelial mat of each fungal culture was rinsed with hot water, oven dried to constant weight at 80°C , to determine dry mass values. The culture filtrates were clarified through four layers of cheesecloth and centrifuged at $15000 \times g$ for 20 min at -5°C using a Beckman J-21 B cooling centrifuge. The supernatant (crude enzyme source) was used immediately in the assay. L-asparaginase activities were estimated in culture filtrates by Nesslerization (Imada et al., 1973). A reaction mixture containing 0.9 ml of Tris-HCl buffer (0.05 M and pH 8.6), 0.1 ml of crude enzyme source of fungal species and 0.5 ml of 0.01 M L-asparagine as substrate (Sigma, St. Louis, MO., USA) were added and the volume was completed to 2.0 ml with distilled water. The reaction was incubated at 30°C for 30 min. After the incubation period, the reaction mixture was stopped by adding 0.5 ml of 1.5 M trichloroacetic acid (TCA). From the reaction mixture aliquots of 0.2 ml were added to distilled water (3.4 ml) and to that Neesler's reagent (0.4 ml) and incubated for 20 min at room temperature ($28\pm 1^\circ\text{C}$). The absorbance was recorded at a wavelength of 450 nm with a UV-spectrophotometer. Blank tubes were prepared by adding the enzyme source after the addition of TCA. One international unit of L-asparaginase (IU) is the amount of enzyme required to release $1\mu\text{mol}$ of ammonia per min per ml under the assay conditions, calculated by the following equation:

$$\text{Units/ml enzyme} = \frac{(\mu\text{M of NH}_3 \text{ Liberated})(2.5)}{(0.1)(30)(1)}$$

where: 2.5 = Initial volume of reaction mixture (ml); 0.1 = Volume of reaction mixture in final reaction (ml); 30 = Incubation time (min); 1 = Volume of enzyme used (ml).

Optimization of culture conditions for L-asparaginase production by two selected fungal species

Based on their high ability to produce L-asparaginase, *A. sydowii* and *F. oxysporum* were selected for further studies to optimize the cultural conditions for production and activity of L-asparaginase, according to Taguchi design (Prasad et al., 2005). The experimental design and methodology involve the establishment of large number of experimental situation described as orthogonal arrays to reduce experimental errors and to enhance the efficiency and reproducibility of the laboratory experiments. In the present investigation, the three levels of variation factors were considered and the size of experimentation was represented by symbolic arrays indicating 18 experimental trails. All the factors have been assigned with three levels except L-asparagine with two levels (2^1) with a layout of L18 ($2^1 \times 3^7$). The total degree of freedom is equal to the number of trials minus one i.e., 17.

Submerged fermentation experiments were carried out in 250 ml Erlenmeyer conical flasks, each, containing 100 ml of production medium composed of different levels of the following (g/L) L-asparagine (5.0, 10), glucose (1.0, 2.0, 3.0), KH_2PO_4 (1.0, 1.52, 2.0), KCl (0.25, 0.52, 0.75), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.25, 0.52, 0.75). Temperature was adjusted at 20, 30 and 40°C , agitation speed was adjusted at 50, 100 and 200 rpm and pH was adjusted at 5.0, 7.0 and 9.0. Erlenmeyer conical flasks containing different media were sterilized as a previously described. Fungal disc (5 mm), cut from the growing margin of 96 hours-old culture, was inoculated for each flask. The inoculated flasks were incubated at 20, 30 and 40°C for 5 days in orbital shaking incubator. Mycelial mats were collected by filtration and the filtrate was used for L-asparaginase assay (Prasad et al., 2005).

Eighteen media used in this experimentation were prepared according to Taguchi method of orthogonal array experimental design, where 18 probabilities were resulted from the interaction of eight factors (L-asparagine, glucose, KH_2PO_4 , KCl, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, temperature, agitation speed and pH) in addition to the best medium no. "19" as shown in Table 1.

TABLE 1. Optimization factorial design of modified Czapek's Dox media for maximization of L-asparaginase production by *Aspergillus sydowii* and *Fusarium oxysporum*

Modified Czapek's Dox media number	Components (g/L)					Temp. (°C)	Agitation speed (rpm)	pH
	L-asparagine	Glucose	KH ₂ PO ₄	KCl	MgSO ₄ ·7H ₂ O			
1	5.0	1.0	1.0	0.25	0.25	20	50	5.0
2	5.0	1.0	1.52	0.52	0.52	30	100	7.0
3	5.0	1.0	2.0	0.75	0.75	40	200	9.0
4	5.0	2.0	1.52	0.25	0.25	40	200	7.0
5	5.0	2.0	2.0	0.52	0.52	20	50	9.0
6	5.0	2.0	1.0	0.75	0.75	30	100	5.0
7	5.0	3.0	1.0	0.25	0.52	30	200	9.0
8	5.0	3.0	1.52	0.52	0.75	40	50	5.0
9	5.0	3.0	2.0	0.75	0.25	20	100	7.0
10	10.0	1.0	2.0	0.75	0.75	30	50	7.0
11	10.0	1.0	1.0	0.25	0.25	40	100	9.0
12	10.0	1.0	1.52	0.52	0.52	20	200	5.0
13	10.0	2.0	2.0	0.25	0.52	40	100	5.0
14	10.0	2.0	1.0	0.52	0.75	20	200	7.0
15	10.0	2.0	1.52	0.75	0.25	30	50	9.0
16	10.0	3.0	1.52	0.25	0.75	20	100	9.0
17	10.0	3.0	2.0	0.52	0.25	30	200	5.0
18	10.0	3.0	1.0	0.75	0.52	40	50	7.0
19	10.0	2.0	1.0	0.25	0.52	40	100	5.0

Dry mass yields (g/100 ml) and L-asparaginase activities (U/ml) of *A. sydowii* and *F. oxysporum* were determined as previously described.

In vitro assay of the anti-leukemic activity of the crude L-asparaginase

Cell culture

Raw murine macrophage (RAW 264.7) was purchased from the American Type Culture Collections. Cells were routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2.0 mM L-glutamine, containing 100U/ml penicillin G sodium, 100 µg/ml streptomycin sulphate, and 250 µg/ml amphotericin B. Cells were maintained in humidified air containing 5.0% CO₂ at 37°C. RAW 264.7 cells were collected by scraping. All experiments were repeated four times and the data was represented as mean ± S.D. All cell culture materials were obtained from Cambrex, Bioscience (Copenhagen, Denmark).

Cell viability and cytotoxicity assay

The effect of tested samples on the viability of RAW264.7 Leukemia cells was measured according to the method described by Hansen et al. (1989) using the MTT cells assay (3-(4,5-dimethylthiazol-2-yl)-[2,5-diphenyltetrazolium bromide]). MTT assay is based on the ability of active mitochondrial dehydrogenase enzyme of living cells to cleave the tetrazolium rings of the yellow MTT and form a dark blue insoluble formazan crystals which can not

penetrate through cell membranes, resulting in their accumulation within healthy cells. Lysis of the cells results in the liberation of crystals, which are then solubilized. The number of viable cells is directly proportional to the level of soluble formazan dark blue color. The extent of the reduction of MTT was quantified by measuring the absorbance at 570 nm. MTT solution was prepared as follows: 5.0 mg/ml of MTT in 0.9% NaCl; acidified isopropanol: 0.04N HCl in absolute isopropanol.

Cytotoxicity assay was studied as follows: cells 0.5 µL of 10⁵ cells/well in serum-free media were plated in a flat bottom 96-well microplate, and treated with 20 µL of different concentrations of each tested crude L-asparaginase produced by *A. sydowii* or *F. oxysporum* for 24 h at 37°C, in humidified air containing 5.0% CO₂. After incubation, media were removed and 40 µL MTT solution/well were added and incubated for an additional 4 h. MTT crystals were solubilized by adding 180 µL of acidified isopropanol/well and plate was shaken at room temperature, followed by photometric determination at the absorbance of 570 nm using 96 wells microplate ELISA reader. Triplicates were performed for each crude enzyme concentration and the average was calculated. Data were expressed as the percentage of relative

viability compared with the untreated control cells, as relative fold of increase in the cell viability (Hansen et al., 1989).

The fold increase in the relative viability (R.V.) was calculated using the following equation:

$$\text{Relative Viability} = \frac{\text{R.V.} - \text{Absorbance of treated cells}}{\text{Absorbance of control untreated cells}} \times 100$$

Cytotoxicity assay against tumor cell lines

The cellular toxicity of the crude L-asparaginase produced by either *A. sydowii* or *F. oxysporum* were assessed *in vitro* by Skehan et al. (1990). The assay was done on cell monolayers of human colon carcinoma cell line (HCT116), liver carcinoma cell line (HEPG2), and breast carcinoma cell line (MCF-7) in the tissue culture plates. The cells were plated in 96-multiwell plate (10^5 cells/well) for 24 h before treatment with the crude L-asparaginase. The crude enzyme of each tested fungus was added to the cell monolayers triplicate to reach a final concentration of 50 $\mu\text{g/ml}$. The monolayer cells were incubated with the fractions for 48 h at 37°C and in humidified atmosphere of 5.0% CO_2 . After 48 h, cells were fixed by adding trichloroacetic acid (TCA) in each well for 1 h and washed five times with tap water to remove TCA. Thereafter, the cells were stained for 30 min with 0.4% (w/v), SulfoRhodamine-B (SRB) stain dissolved in 1.0% acetic acid. Unbound dye was removed by four washes with 1.0% acetic acid, and protein-bound dye was extracted with 10 mM buffered Tris-base [tris-(hydroxymethyl)aminomethane] for determination of optical density in a computer-interfaced. The color intensity was measured in ELISA reader at 564nm. Cells free blank was used for background correction of the absorbance. The viability was calculated as percentage relative to the solvent control absorbance value after subtracting the contribution from cell-free blank.

The results are represented as viability % or cytotoxicity % (obtained by subtraction of the % viability value from 100).

Results

Air and soil-borne mycobiota isolated in the present study

The data in Table 2 revealed that a total of 16 fungal species belonging to 8 genera were isolated from the two studied localities with total count of 143 isolates. Sixty seven isolates were recovered

from the air constituting related to 14 species while seventy six isolates were recovered from the soil related to 16 species. *Aspergillus sydowii* was the most dominant species in air and soil with 26.87% and 21.06% relative density, respectively. *A. terreus* shared *A. sydowii* with 16 isolates from air and soil with relative density of 11.19%. *A. ochraceus* was represented by 15 isolates constituting 10.49% of which nine isolates were recorded from air and six from soil. According to the relative density, *F. poae*, *F. incarnatum*, *F. oxysporum* and *A. flavus* ranked fourth constituting 7.69, 6.99, 6.99 and 6.29%, respectively, with the major count isolated from the soil. Each of the remaining fungal species constituted less than 5.0% relative density including *A. japonicus*, *A. parasiticus*, *A. ustus*, *Epicoccum nigrum*, *Paecilomyces variotii*, *Saccharomycopsis fibuligera* and *Ulocladium consortiale*. It is noticed that *Curvularia lunata* and *Trichoderma longibrachiatum* were recovered from soil locality but were completely missed from air environment.

L-asparaginase assay

All isolated fungal species were tested for their ability to produce extracellular L-asparaginase using diffusion plate method.

Results in Table 3 indicated that *F. oxysporum*, *F. incarnatum*, *A. terreus* and *A. sydowii* were strong asparaginase producers. However, *Epicoccum nigrum*, *F. poae*, *A. ustus*, *A. japonicus* and *A. ochraceus* were of moderate asparaginolytic activities. Weak L-asparaginase production was observed in the case of *Saccharomycopsis fibuligera* and *Ulocladium consortiale*. L-asparaginase induction and activity were significantly affected by phenol red concentration. Regardless of *Paecilomyces variotii*, *Saccharomycopsis fibuligera* and *Ulocladium consortiale* which showed their maximum activity of L-asparaginase at 0.05% phenol red, all tested fungi exhibited better L-asparaginase production at 0.07%. The maximum production of L-asparaginase was achieved by *F. oxysporum*, *A. sydowii* and *F. incarnatum* at 0.07% measuring 4.9, 4.6 and 4.5 cm, respectively. At higher levels of phenol red concentrations (0.09 and 0.2%) L-asparaginase production was markedly retarded. Considering linear growth, it was observed that addition of phenol red was inhibitory for all fungal species, regardless of all concentrations used, as compared to control which lack the phenol red. Appearing of pink zone around the colonies indicates an increase in pH, which originate from ammonia accumulation in the medium.

TABLE 2. Fungi recovered from air and soil of tumor institute, KasrEl Ainy, Egypt .

Fungal species	Air fungi		Soil fungi		Total	
	Total count	Relative density (%)	Total count	Relative density (%)	Total count	Relative density (%)
<i>Aspergillus flavus</i> var. <i>columnaris</i> Link	3	4.48	6	7.89	9	6.29
<i>Aspergillus japonicus</i> Link	2	2.99	4	5.26	6	4.20
<i>Aspergillus ochraceus</i> Wilhelm	9	13.43	6	7.89	15	10.49
<i>Aspergillus parasiticus</i> link	1	1.49	3	3.95	4	2.80
<i>Aspergillus sydowii</i> (Bainier&Sartory) Thom & Church	18	26.87	16	21.06	34	23.78
<i>Aspergillus terreus</i> Thom	13	19.40	3	3.95	16	11.19
<i>Aspergillus ustus</i> (Bainier) Thom & Church	3	4.48	2	2.63	5	3.49
<i>Curvularia lunata</i> (Walker) Boedijn	0	0.0	4	5.26	4	2.80
<i>Epicoccum nigrum</i> Link	3	4.48	1	1.32	4	2.80
<i>Fusarium incarnatum</i> (Rob.) Sacc.	3	4.48	7	9.21	10	6.99
<i>Fusarium oxysporum</i> Schl. F. I. Wollenw.	4	5.97	6	7.89	10	6.99
<i>Fusarium poae</i> (Peck) Wollenw.f. pallens Wollenw.	3	4.48	8	10.53	11	7.69
<i>Paecilomyces variotii</i> (Bainier)	2	2.99	1	1.32	3	2.10
<i>Saccharomycopsis fibuligera</i> (Lindner) Klocker	1	1.49	1	1.32	2	1.39
<i>Trichoderma longibrachiatum</i> Pers	0	0.0	5	6.58	5	3.50
<i>Ulocladium consortiale</i> (Thumen) Simmons	2	2.99	3	3.95	5	3.50
Total isolates	67	100	76	100	143	100
Total number of species	14	-	16	-	16	-

The fungal species under test were quantitatively assayed for L-asparaginase activity in the culture filtrate (Table 4). *A. sydowii* and *F. oxysporum* were the most potent species in L-asparaginase production where the enzyme activity reached 3.98 and 3.91 U/ml, respectively. *A. terreus*, *F. incarnatum* and *F. poe* showed considerable activity recording 2.91, 2.41 and 2.21 U/ml, respectively. *Saccharomycopsis fibuligera*, *Ulocladium consortiale* and *Trichoderma longibrachiatum* recorded the least enzyme activity (less than 1.0 U/mL for each).

Molecular identification of the most potent fungal isolates

The identification of the most potent fungal isolates producing L-asparaginase (*Aspergillus sydowii* and *Fusarium oxysporum*) was further confirmed by performing 18S rDNA sequencing. The obtained sequences of both fungal strains

were deposited in the GenBank given accession Numbers of MG846707 for *Aspergillus sydowii* strain D11 and MG846495 for *Fusarium oxysporum* strain D3.

The phylogenetic tree (Fig. 1) showed high similarities of these fungi with closely related strains accessed from the GenBank.

Optimization of L-asparaginase production

High L-asparagenolytic activity was achieved in modified medium "19" composed of: (g/L) L-asparagine, 10; glucose, 2.0; KH_2PO_4 , 1.0; KCl, 0.25; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.52; after 5 day-incubation at 40°C and agitation speed of 100 rpm with the adjustment of pH at 5.0 as shown in Table 5. In this medium, enzyme activity of 146 and 143 U/ml was detected in the assay mixture, and the dry mass yield measured 1.26 and 1.19 g/100 ml for *A. sydowii* and *F. oxysporum*, respectively.

TABLE 3. Qualitative L-asparaginolytic enzyme assays of isolated fungal species under different concentrations of phenol red using diffusion plate method.

Fungal species	Phenol red concentrations (%)										L.S.D. 5 %	
	Control (0.0)		(0.05)		(0.07)		(0.09)		(0.2)			
	Lysing area (cm)	Growth diameter (cm)	Lysing area (cm)	Growth Diameter (cm)	Lysing area (cm)	Growth diameter (cm)	Lysing area (cm)	Growth diameter (cm)	Lysing area (cm)	Growth diameter (cm)		
<i>Aspergillus flavus</i> var. <i>columnaris</i>	0.0	1.6	1.4	1.4	1.8	1.7	1.5	1.6	1.1	1.0	0.11	0.14
<i>A. japonicus</i>	0.0	1.4	1.6	1.5	2.1	1.8	1.8	1.8	1.3	1.1	0.13	0.16
<i>A. ochraceus</i>	0.0	1.5	1.4	1.5	2.0	1.8	1.7	1.6	1.2	1.1	0.12	0.15
<i>A. parasiticus</i>	0.0	1.6	1.3	1.4	1.6	1.7	1.3	1.7	0.9	0.7	0.17	0.19
<i>A. sydowii</i>	0.0	1.8	1.5	1.6	4.6	1.9	1.6	1.5	1.0	0.8	0.19	0.24
<i>A. terreus</i>	0.0	1.5	1.6	1.4	3.6	1.6	1.4	1.4	0.9	0.7	0.14	0.17
<i>A. ustus</i>	0.0	1.4	1.5	1.2	2.7	1.5	1.3	1.4	0.8	0.7	0.15	0.18
<i>Curvularia lunata</i>	0.0	3.2	1.4	1.9	1.4	2.4	1.1	2.9	0.7	2.4	0.13	0.16
<i>Epicoccum nigrum</i>	0.0	3.4	2.2	2.6	2.9	3.7	2.5	4.2	2.1	3.6	0.11	0.14
<i>Fusarium incarnatum</i>	0.0	2.9	2.9	2.9	4.5	3.6	3.3	4.1	2.9	3.2	0.12	0.17
<i>F. oxysporum</i>	0.0	2.7	2.7	2.5	4.9	2.9	3.1	3.6	2.6	3.1	0.14	0.16
<i>F. poae</i>	0.0	2.6	2.6	2.4	2.8	2.9	2.2	3.4	1.7	2.8	0.13	0.15
<i>Paecilomyces variotii</i>	0.0	2.1	1.5	1.9	1.3	2.2	1.1	2.6	0.7	1.9	0.16	0.19
<i>Saccharomycopsis fibuligera</i>	0.0	1.8	1.3	1.6	0.3	2.1	0.1	2.4	0.0	1.9	0.11	0.13
<i>Trichoderma longibrachiatum</i>	0.0	1.9	1.4	1.7	1.4	2.1	1.0	2.4	0.6	1.7	0.12	0.16
<i>Ulocladium consortiale</i>	0.0	1.7	1.7	1.5	0.7	2.0	0.3	2.4	0.0	1.7	0.10	0.11
LSD												
5%	-	0.31	0.33	0.22	0.23	0.32	0.42	0.33	0.32	0.51		
1%	-	0.52	0.76	0.57	0.53	0.72	0.97	0.62	0.74	0.96		

TABLE 4. Quantitative L-asparaginolytic enzyme assay of the investigated isolated fungal species in culture liquid media after 48 h incubation period.

Fungal species	L-asparaginase activity (U/ml)	Dry mass yield (g/100 ml)
<i>Aspergillus flavus</i> var. <i>columnaris</i>	1.69	0.631
<i>A. japonicus</i>	1.71	0.592
<i>A. ochraceus</i>	1.92	0.693
<i>A. parasiticus</i>	1.42	0.614
<i>A. sydowii</i>	3.98	0.966
<i>A. terreus</i>	2.91	0.914
<i>A. ustus</i>	1.40	0.841
<i>Curvularia lunata</i>	1.31	0.632
<i>Epicoccum nigrum</i>	1.72	0.924
<i>Fusarium ncarinatum</i>	2.41	0.822
<i>F. oxysporum</i>	3.91	0.941
<i>F. poae</i>	2.21	0.871
<i>Paecilomyces variotii</i>	0.82	0.552
<i>Saccharomycopsis fibuligera</i>	0.51	0.286
<i>Trichoderma longibrachiatum</i>	0.68	0.456
<i>Ulocladium consortiale</i>	0.62	0.393
LSD		
5%	0.72	0.31
1%	1.21	0.52

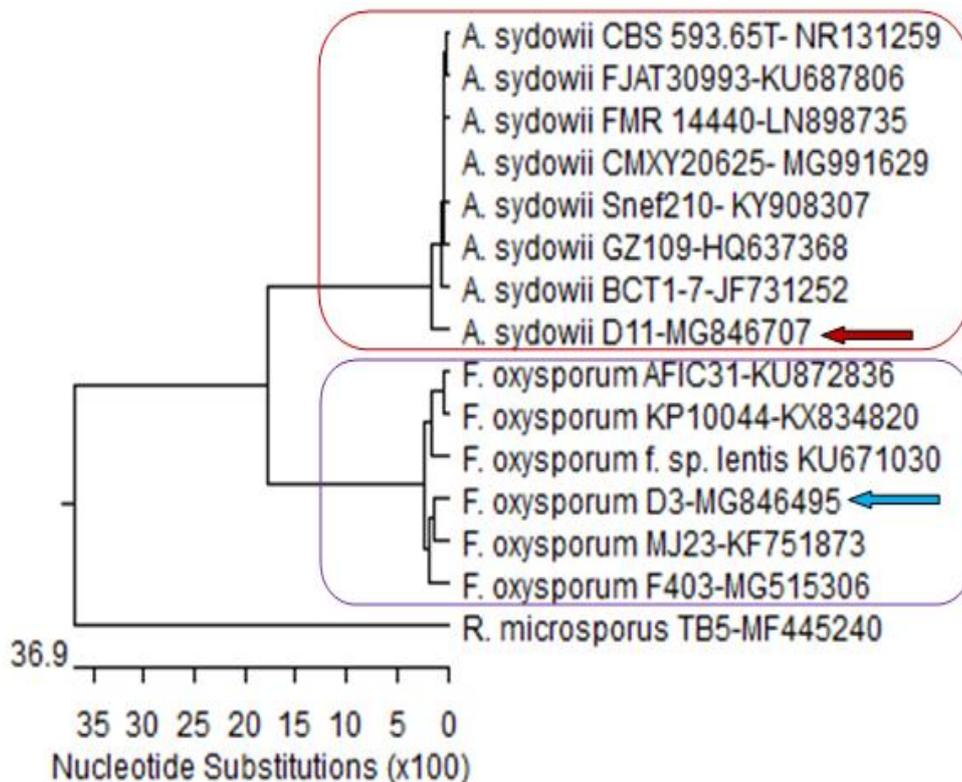
**Fig.1. Phylogenetic tree showing genetic relationship between *Aspergillus sydowii* (MG 846707) and *Fusarium oxysporum* (MG846495) isolated in the present study (see arrows) and other closely related strains accessed from the GenBank. *R. microsporus* is included in the tree as an outgroup strain. (A= *Aspergillus*, F= *Fusarium*, R=*Rhizopus*).**

TABLE 5. Optimization factorial design for maximization of production and activity of L-asparaginase (U/ml) in culture filtrate of *Aspergillus sydowii* and *Fusarium oxysporum* after 5 days incubation period.

No of experiments	<i>A. sydowii</i>		<i>F. oxysporum</i>	
	L-asparaginase activity	Dry mass yield (g/100 ml)	L-asparaginase activity	Dry mass yield (g/100 ml)
1	69	0.861	67	0.840
2	46	0.820	44	0.801
3	37	0.621	33	0.603
4	76	0.892	73	0.874
5	83	0.961	80	0.947
6	123	1.07	121	1.03
7	48	0.811	46	0.800
8	45	0.807	43	0.796
9	39	0.632	36	0.620
10	46	0.826	43	0.802
11	88	0.973	85	0.952
12	47	0.829	44	0.801
13	127	1.12	124	1.02
14	85	0.952	81	0.933
15	53	0.831	50	0.811
16	67	0.842	63	0.820
17	63	0.836	60	0.817
18	56	0.847	52	0.828
19	146	1.26	143	1.19
LSD 5%	0.9	0.12	0.8	0.31
1%	1.82	0.45	1.94	0.83

Among the 18 different types of media in the experimental factorial design, medium "13" composed of (g/L) L-asparagine, 10; glucose, 2.0; KH_2PO_4 , 2.0; KCl, 0.25; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.52; in addition to medium "6" composed of (g/L) L-asparagine, 5.0; glucose, 2.0; KH_2PO_4 , 1.0; KCl, 0.75; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.75; temp., 30°C; agitation speed, 100 rpm and pH 5.0 were also suitable for L-asparaginase production. The recorded results showed L-asparaginolytic activity of 127 and 123 for *A. sydowii* and 124 and 121 U/ml for *F. oxysporum* in case of media no "6 and 13", respectively. Media "4, 5, 11 and 14" induced moderate L-asparaginolytic activity in the culture filtrates of the tested fungi. The remaining medium types resulted in weak activity of L-asparaginase with a minimum value recorded by at medium "3" with 37 and 33 U/ml enzyme activities for *A. sydowii* and *F. oxysporum*, respectively.

The dry mycelial mass of *A. sydowii* and *F. oxysporum* ranged from 0.621 to 1.26 and from 0.603 to 1.19 g/100 ml respectively with the highest

amounts being observed in cultures grown in the modified medium No. 19 as shown in Table 5.

Therapeutic efficacy

From the previous data (Table 6), the optimum modified medium (19) was selected for anti-leukemic activity and anti-cancer assay of L-asparaginase produced by *A. sydowii* and *F. oxysporum*.

Anti-leukemic activity of the crude L-asparaginase was tested against murine RAW 264.7 leukemia cells line using MTT viability assay. The results in Fig. 2 indicated that both of the crude L-asparaginase from *A. sydowii* and *F. oxysporum* exhibited anti-leukemic activities depending on IC_{50} of 50.0 and 62.5U/ml, respectively. The lower dose of the crude enzyme (12.5U/ml) reduced the cell viability by about 20% with relative viability of 80.0% of the control, while the high dose (100U/ml) reduced cell viability by 75-85% with relative viability about 15-25% of the control for both fungi.

In vitro anti-cancer test for the crude L-asparaginase (50 µg/ml) produced by *A. sydowii* and *F. oxysporum*, of either crude L-asparaginase was carried out on human colon carcinoma cell line (HCT116), liver carcinoma cell line (HEPG2) and breast carcinoma cell line (MCF-7). The

results revealed that the cell death of human colon carcinoma cell line reached 70 and 73% in case of crude enzyme from *A. sydowii* and *F. oxysporum*, respectively. Higher anti-cancer activity (up to 80%) was observed in case of liver and breast carcinoma cell lines (Fig. 3).

TABLE 6. Comparison between media composition exerting high L-asparaginase activity from *A. sydowii* and *F. oxysporum* after 5 days incubation.

Modified Czapek's Dox media number	L-asparagine (g/L)	Glucose (g/L)	KH ₂ PO ₄ (g/L)	KCl (g/L)	MgSO ₄ .7H ₂ O (g/L)	Temp. (°C)	Agitation speed (rpm)	pH	L-asparaginase activity (U/ml)	
									<i>A. sydowii</i>	<i>F. oxysporum</i>
									19	10
6	5.0	2.0	1.0	0.75	0.75	30	100	5	123	121
13	10	2.0	2.0	0.25	0.52	40	100	5	127	124
3	5.0	1.0	2.0	0.75	0.75	40	200	9	37	33

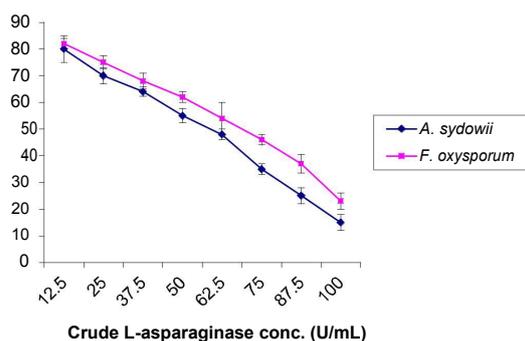


Fig. 2. Cell viability of RAW 246.7 Leukemia cells after the treatment with different concentrations of the crude L-asparaginase produced from *A. sydowii* and *F. oxysporum* for 48 h as measured by MTT assay.

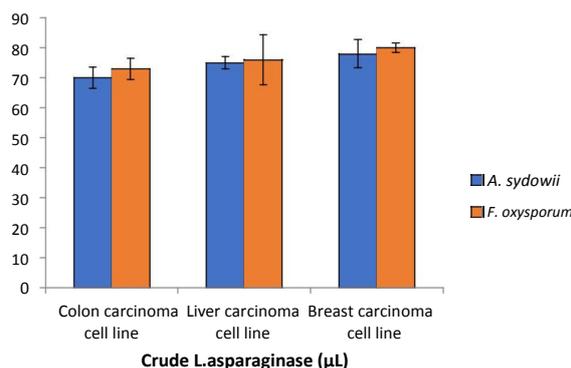


Fig. 3. Effect of the crude L-asparaginase produced by either *A. sydowii* or *F. oxysporum* on different carcinoma cell lines in vitro.

Discussion

Recently a considerable attention has been paid to the isolation of bioactive compounds from eukaryotic microorganisms. Among the bioactive compounds produced by *A. sydowii* and *F. oxysporum* L-asparaginase were studied. Sixteen fungal species were isolated from air and soil environments of Tumor Institute, Kasr El Ainy, Cairo University, Egypt. Several of the isolated fungi were confirmed earlier by many investigators to be L-asparaginase producers. In the present study, *A. sydowii* and *F. oxysporum* were found to be the most efficient species in L-asparaginase production. In this respect, several species of *Aspergillus* including

A. tamarii, *A. niger*, *A. nidulans* and *A. terreus* were recorded as L-asparaginase producers (Bedaiwy et al., 2016; Dange & Peshwe, 2015; Archana & Raja, 2014 and Farag et al., 2015). Similar investigations have been done with *Fusarium* spp. including *F. anguoides*, *F. oxysporum*, *F. roseum*, *F. solani* and *F. equiseti* (Nakahama et al., 1973; Niharika & Supriya, 2014; Hosamani & Kaliwal, 2011; Radhika & Girisham, 2012 and Ruma et al., 2017).

The screening system was comparable with those previously reported for production of L-asparaginase by filamentous fungi (El-Sayed, 2008). Also, the differences in the production level

of L-asparaginase by the different investigated species may be attributed to either the fermentation conditions or to the tested fungi strain (Bedaiwy et al., 2016). Several fungi isolated from different environments have efficiency and potentiality to produce L-asparaginase including *Penicillium* isolated from mangrove ecosystem of Bhitarkanika (Gupta et al., 2009), *Penicillium citrinum*, *Aspergillus ochraceus*, *Fusarium oxysporum*, *Alternaria alternata*, *Rhizopus* spp. and *Trichoderma viride* isolated from the soil (Niharika & Supriya, 2014 and Lincoln et al., 2015), *Aspergillus*, *Penicillium* and *Fusarium* isolated from the marine environment of the Red Sea Coast of Egypt (Farag et al., 2015), *Aspergillus terreus* as endophytic fungus from the salt marsh plant (*Sueada maritima*) occurring along the velar estuary of southeast coast of India (Indira et al., 2015), *Colletotrichum*, *Fusarium*, *Phoma* and *Penicillium* isolated from plants (Chow & Ting, 2015).

The optimization of cultural and environmental condition is an important requirement for the production of L-asparaginase. In this research Taguchi design was employed to try 18 different media for L-asparaginase production in addition to the medium no "19". Many statistical experimental design methods have been employed for optimization bioprocess (Ferreira et al., 2004 and Prasad et al., 2005). These methods have been successfully applied to optimize different process parameters (Vanot et al., 2002 and Dutra et al., 2006). Applications of such designs minimize the error in determining the effect of parameter than the classic time-by-time optimization experiments (Zheng et al., 2008). The significant factors differ, depending on the tested microorganisms. Glucose, pH, aeration and agitation speed were determining factors for L-asparaginase production from *Fusarium equiseti* (Hosamani & Kaliwal, 2011) and *Aspergillus* sp. (Ahmed et al., 2015). Statistical methodologies are generally preferred by several investigators in determining the effect of interacting factors (Sharma & Satyanarayana, 2006). Hence statistical optimization design for maximization of L-asparaginase production and activity of *A. sydowii* and *F. oxysporum* was carried out using Taguchi design. Our results revealed that *A. sydowii* and *F. oxysporum* exhibited the highest L-asparaginase activities in the culture filtrate after 5 days incubation. The data also showed that the dried biomasses of both fungal species are matched with L-asparaginase activities. Also the modified medium "19" was the optimum for production

of L-asparaginase followed by medium "13" and medium "6". The poorest L-asparaginolytic activity of *A. sydowii* or *F. oxysporum* was recorded in case of medium "3". Addition of relatively higher amount of L-asparagine was found to be suitable for maximum L-asparaginase activity reaching 146 and 143 U/ml after 5 days of incubation in case of *A. sydowii* and *F. oxysporum*, respectively. Our observation confirmed the data published earlier by Basker & Renganathan (2009) working with production of L-asparaginase from *A. terreus* MTCC 1782. Their study revealed that 1.0% sodium nitrate and 1.2% L-asparagine induced considerable L-asparaginase activity of 30.53 U/ml on the fourth day. However, glucose was the preferred carbon source for production of L-asparaginase by Bedaiwy et al. (2016).

From the previous data the optimum modified Czapek's Dox medium "19" was selected for anti-leukemic and anti-cancer assays of L-asparaginase produced by *A. sydowii* and *F. oxysporum*. The crude L-asparaginase from both fungi exhibited anti-leukemic concentrations activities dependent with the concentration required for a 50% inhibition of viability (IC_{50}) of 50.0 and 62.5 U/ml, respectively. It is well known that, the L-asparaginase is very effective anti-leukemic agent and for most patients and probably administered once every two weeks (Graham, 2003 and Oza et al., 2010). The relative selectivity with regard to metabolism of malignant tumor cell forces to look for novel L-asparaginase with little L-glutaminase producing system compared to existing enzymes (Parkasham et al., 2010). L-asparaginase has revolutionized the anti-leukemic therapy of acute lymphoblastic leukemia (ALL). It is early sustained that L-asparagine depletion extends at least up to the first 30 weeks of therapy (Patil et al., 2011). Performing *in vitro* anti-cancer test, our results revealed that the cell death percent of carcinoma cell lines reached between 70 to 80% after treatment with the crude L-asparaginase of either *A. sydowii* or *F. oxysporum*, respectively. In this respect, tumor cells especially, lymphatic cells, require huge amounts of L-asparagine to keep up their malignant growth. L-asparaginase, as a drug, exploits this unusually high requirement of L-asparagine because the malignant cells lack L-asparagine. Hence, L-asparagine in the tumor cells forms an essential factor for protein synthesis. Healthy cells, however, not affected, as they are capable of synthesizing L-asparagine with the help of L-asparagine synthetase which is present in sufficient amounts (Narta et al., 2007).

L-asparaginase arrest cell cycle at "G1" phase in the leukemia murine L5178 cell line (Ueno et al., 1977) and in the MOLT-4 human T-lymphoblastic line (Shimizu et al., 1992). Ammonia produced from L-asparagine hydrolysis diffuse into the cytosol and change the pH which activate signal transduction pathway associated apoptosis (Ueno et al. 1977 and Shimizu et al., 1992). Moreover, L-asparaginase have anti-carcinogenic effects in animal and cell culture by decreasing the expression of nuclear factor-Kapp- B, suppressing intercellular tumor necrosis factor and potentiating apoptotic signaling in cancerous cells (Story et al., 1993 and Ichikawa et al., 2006). L-asparagine deficiency rapidly impairs the protein synthesis and leads to inhibition of DNA and RNA synthesis and hence an impairment of cellular function resulting in cell death (Narta et al., 2007).

In conclusion, the present study showed that L-asparaginase could be produced from local strains of filamentous fungi. Maximization of enzyme production can be achieved under suitable nutritional and environmental fermentation conditions. More trials are needed to concentrate, purify, lyophilize and preserve this important product to find its way for application in the field of medicine.

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الإنتاجية المثلى لانزيم ال- اسباراجينيز من بعض الفطريات الخيطية ولها خواص علاجية قوية

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في هذه الدراسة تم عزل مائة و ثلاثة و اربعون عزلة من الفطريات من مواقع مختلفة تتمثل في (14 عزلة فطرية من الهواء و 16 عزلة فطرية من التربة) من وحدة الأورام التابعة للقصر العيني محافظة القاهرة بمصر. وتم تعريف العزلات الفطرية مستخدمة الطرائق القياسية في التعريف. تم اختبار و مسح ستة عشر من العزلات الفطرية لمعرفة مدى افرازها لإنزيم ال-اسباراجينيز معمليا على بيئة وسط غذائي اجارى من شابك دو كس تحتوي على ال- اسباراجين كمصدر للنيتروجين في وجود كاشف فينول الأحمر .

اشارت النتائج أن جميع الفطريات المعزولة مفرزه لإنزيم ال-اسباراجينيز بكميات متفاوتة معتمدا على قطر اللون القرمزي المصاحب لإفراز الإنزيم حيث يتم تغير لون الوسط الغذائي من اللون الصفير إلى اللون القرمزي و سجلت النتائج أن أعلى إنتاجية للفطريات المعزولة هما فطره *اسبرجيللس سيدوفى* و فطره *فيوزاريوم اوكسيسبورم*.

تم تعين الإفراز الكمي للإنزيم مستخدما محللول نسلر وقياس كمية الأمونيا المتصاعد حيث تم تسجيل أعلى كمية لافراز الأنزيم بواسطة فطره *اسبرجيللس سيدوفى* و فطره *فيوزاريوم اوكسيسبورم* حيث قدرت كمية افراز الأنزيم على وسط غذائي سائل من شابك دو كس وفترة تحضين 48 ساعة هي 3.98 ، 3.91 وحدة/ملى للفطرتين على الترتيب. اشارت النتائج وجود علاقة طردية بين كمية الأنزيم المفرز والوزن الجاف لجميع الفطريات المعزولة المختبرة.

تم تأكيد تعريف الفطرتين *اسبرجيللس سيدوفى* و فطره *فيوزاريوم اوكسيسبورم* على المستوى الجزيئى اعتمادا على هوية منطقة 18 اس الموجودة في الحامض النووى دنا.

تم عمل محاولات لإنتاج البيئة الغذائية المثلى مستخدما تصميم تاجوشى على شابك دو كس مع تغير المعاملات الموجودة في البيئة الغذائية و تم عمل عدد 18 بيئة غذائية بجانب البيئة الغذائية رقم 19 حيث سجلت أعلى إنتاجية للإنزيم لكلا الفطرتين بعد خمسة أيام فترة تحضين الفطريات مقارنة بسبعة أيام فترة تحضين و قدرت أعلى إنتاجية للأنزيم الخام هي 146 و 143 وحدة/ملى في الرشيج على الترتيب.

تم اختبار مضادات إنزيم ال-اسباراجينيز المفرز بأعلى إنتاجية بواسطة الفطرتين مستخدما قياس النشاط الحيوى بطريقة (ام تى تى) عند تركيز 50 ميكرو جرام/ملى ضد خلايا لوكيميا حيث قدرت القيمة الحيوية لنشاط عند 50 و 62,5 وحدة انزيم/ملى للفطرتين على الترتيب.

تم اختبار وتعين مضادات الأورام لإنزيم اسباراجينيز الخام المفرز لكلا الفطرتين عند تركيز 50 ميكروجرام/ملى ضد الخلايا البشرية للقولون السرطانية HCT116 وخلايا الكبد السرطانية HEPG2 وكذلك خلايا الثدي السرطانية MCF-7 معمليا حيث سجلت النتائج أن نسبة موت الخلايا السرطانية المختلفة تتراوح بين 70 إلى 80% باستخدام الأنزيم المفرز الخام لكلا الفطرتين.