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Anticancer Efficacy of Purified Extracellular L-asparaginase from *Aspergillus niger* and Yield Enhancement by Agro-industrial Wastes

Asmaa S. Yassein[#], Amany A. El-Shahir
Botany and Microbiology Department, Faculty of Science, South Valley University, 83523 Qena, Egypt.

L-ASPARAGINASE enzyme is important medically as an anticancer agent and in the food industry. The enzyme acts via degradation of l-asparagine and mitigation of acrylamide. This work screened 31 fungal isolates recovered from rhizosphere soil for l-asparaginase production using the plate dilution medthod. Twenty-four isolates (77.4%) were l-asparaginase producers. *Aspergillus niger* and *A. quadrilineatus* were the highest producers with enzyme activities were 9.808±0.18930 and 7.348±0.12328U/mL, respectively. Optimum conditions for enzyme production were 30°C for 72h, with pH 6 at 160rpm, and 0.1% of KH_2PO_4 in presence of 2% glucose and 1.5% sucrose as carbon source and 1% L-asparagine by *A. niger* and *A. quadrilineatus*, respectively. Ammonium sulfate precipitation, Sphedax G-200, and SDS-PAGE were performed for L- asparaginase purification and molecular weight determination. Enzyme from *A. niger* displayed a MW of 50.36kDa and a specific activity of 50.4U/mg. The MW of *A. quadrilineatus* enzyme was 27.8kDa with a specific activity 37.4U/mg. Purified l-asparaginase significantly inhibited the proliferation of HCT-116, HePG-2, and MCF-7cells with IC50 concentrations of 28.9, 36.1, and 82.6µg/mL, respectively. The enzyme did not exhibit antibacterial activity. Enhancement of l-asparaginase production using agro-industrial wastes produces a maximum of 23.548 ± 0.00000U/mL when *A. niger* is cultivated on a mixture of onion and pomegranate peel powders (50%: 50% w/w) and cultivation of *A. quadrilineatus* on pomegranate peel alone.

Keywords: Agro-industrial wastes, Antibacterial, IC50, L-asparaginase, Optimum conditions, SDS-PAGE.

Introduction

L-asparaginase is a hydrolytic enzyme that catalyzes l-asparagine to aspartic acid and ammonia (Verma et al., 2007). The high global demand for l- asparaginase enzyme was 380 million USD in 2017 and may reach 420 million USD by 2025 (Alam et al., 2019). L-asparaginase is widely distributed in microbes, plants, and mammals but not is not found in humans (Cachumba et al., 2016). Microbes are the better source compared with animals or plants; microbes grow easily on rather simple and low-cost substrates (Lopes et al., 2015). Filamentous fungi, such as *Aspergillus*, *Penicillum*, and *Fusarium* are the prevalent sources of l-asparaginase that causes fewer adverse effects in comparison with enzymes obtained from bacterial sources (Jenila & Gnanadoss, 2018).

L-asparagine is typically formed within the normal cell by asparagine synthetase; however, the tumor cells growth and proliferation is essentially dependent on exogenous l-asparagine. L-asparaginase kills tumor cells by consuming l-asparagine (Brumano et al., 2019). L- asparaginase was used for the treatment of many tumors in combination with other chemotherapeutic agents such as acute lymphoblastic leukemia, non-Hodgkin's lymphoma, and breast cancer (Krishnapura et al., 2016; Kaminsky, 2017; Benchamin et al., 2019).

[#]Corresponding author email: asmaa_fungi@yahoo.com; asmaa.mohamed11@sci.svu.edu.eg Phone: +201012456864.
Fax: +2096 5211 279 .ORCID iD: 0000-0002-3809-5960
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Another application of l-asparaginase in the food industry is to reduce acrylamide formation (Xu et al., 2016; Ray et al., 2019).

Specific environmental conditions and medium constituents are essential for optimizing growth and l-asparaginase production by microorganisms (Hymavathi et al., 2009; Venil et al., 2009).

Wastes such as corncob, orange peel, wheat straw, rice straw, and soybean meal have been used to enhance l-asparaginase production, (Couto & Sanromán, 2006; Sanghvi et al., 2016; Shakambari et al., 2017; Ravindran et al., 2018).

The present study estimates the l-asparaginase production by filamentous fungi isolated from rhizosphere soil in Qena, Egypt. The work included molecular identification of the highest producers and determination of optimum conditions for l-asparaginase production. Further, extracellular l-asparaginase from the highest producer isolates was purified and evaluated for antitumor activity *in vitro* and antibacterial properties against pathogenic bacteria. Enhancing l-asparaginase yield using inexpensive agro-industrial wastes was also evaluated. We will purify L- asparaginase in our future studies by cultivating isolates on agro-industrial wastes and characterizing enzyme physicochemical properties for reduce acrylamide mitigation and suppression of biofilm formation by pathogenic bacteria.

Materials and Methods

Tested fungal isolates

Thirty-one fungal isolates were recovered from rhizosphere soil samples around various plants at Qena Governorate, Egypt using the dilution plate method on malt extract agar medium containing gL^{-1} (malt extract, 30; peptone, 6 and agar, 15). Isolates were identified macro-and microscopically (Johnson & Curl, 1972). These strains were maintained on potato dextrose agar slants (with 200, 20, $15gL^{-1}$ of potato, dextrose and agar, respectively).

Screening of fungal isolates

The method described by Gulati et al. (1997) was used to screen for l-asparaginase on Modified Czapek Dox (MCD) medium containing phenol red as an indicator.

L-asparaginase assay

The Nesslerization method described by Imada et al. (1973) was applied for estimation of l-asparaginase activity in fungal filtrates.

Molecular identification

PCR products were purified using a QIA Quick PCR Product extraction kit. (Qiagen, Valencia). The sequence reaction was obtained by Bigdye Terminator V3.1 cycle sequencing kit(Perkin-Elmer) and then it was purified using Centrisep spin column. Applied Biosystems3130 genetic analyzer (HITACHI, Japan), a BLAST® analysis (Basic Local Alignment Search Tool) was used for getting DNA sequences (Altschul et al., 1990) to establish sequence identity with GenBank sequences.

Accession numbers

Sequences were deposited in GenBank under accession numbers MW695524 for *A. niger* and MW695525 for *A. quadrilineatus*.

Optimization of l-asparaginase production

Effect of incubation period

The selected isolates were incubated for 12, 24, 36, 48, 60, 72, 84, and 96h and enzyme activity was estimated after each incubation period.

Effect of carbon sources

The fermentation medium was supplemented (glucose, sucrose, fructose, maltose, and lactose) to assess the impact of carbon source on l-asparaginase productivity.

Effect of carbon source concentrations

Glucose and sucrose were the most suitable carbon sources for l- asparaginase production by *A. niger* and *A. quadrilineatus*. These sugars were assessed at concentrations (0.5,1.0,1.5,2, 2.5, and 3%).

Effect of pH

Fermentation media were adjusted at pH values of (2, 3, 4, 5, 6, 7, and 8) and l-asparaginase was assessed.

Effect of nitrogen source

Fermentation medium were supplemented with (l-asparagine, peptone, yeast extract, sodium nitrate, and ammonium sulfate) and l-asparaginase was measured.

Effect of l-asparagine concentrations

Asparagine was the most suitable nitrogen source for l-asparaginase production,, and various concentrations from 0.2% to 1.2% were therefore examined for their effect.

Effect of temperature

Isolates were incubated at temperatures ranging from 20°C to 45°C to determine the optimum temperature for l-asparaginase production.

Effect of agitation rate on l-asparaginase production

Both static conditions and agitation were assessed their impact on asparaginase production. Previously optimized conditions were tested using different shaking speeds (100, 120, 160, and 200rpm).

Effect of phosphorus concentration

Media were supplemented with different concentrations of KH_2PO_4 (0.02%, 0.05%, 0.1%, 0.15%, and 0.2%) under the previously identified optimum conditions.

Purification of l-asparaginase

Ammonium sulfate precipitation

Ammonium sulfate was adopted for enzyme precipitation considering high solubility, pH versatility, and low heat of solution. The method used was previously described (Soniyamby et al., 2011) to partially purify l-asparaginase.

Dialysis

Dialysis step was used to remove residual ammonium sulfate and concentrate the crude enzyme preparation.

Fractionation of the extract using Sephadex G-200 column

The concentrated dialysate was further purified on a Sephadex G-200 column (Burda et al., 2005).

SDS-PAGE

Purified enzyme identity and molecular weight were examined using polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970). Molecular weight was evaluated by comparison to the molecular weights of standard protein markers (17-175kDa).

Anticancer activity

Mammalian cell lines

Purified l-asparaginase from *A. niger* showed the highest specific activity and was evaluated for cytotoxicity against breast carcinoma (MCF-7), colon adenocarcinoma (HCT-116), and hepatocellular carcinoma (HepG-2) cells. Cell lines were kindly provided by the VACSERA Tissue Culture Unit, Egypt. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) complemented with 10% heat-inactivated fetal bovine serum, 50 µg/ml gentamycin, HEPES buffer and1% L-glutamine was used for cells proliferation. All cells were preserved at 37ºC in a moistened atmosphere with 5% CO_2 and were subcultured twice per week.

Cytotoxicity

Purified l-asparaginase from *A. niger* was dissolved in HPLC grade DMSO at concentrations of 1.95 to 1000µg/mL. Cytotoxicity was evaluated using the colorimetric crystal violet staining method (Mosmann, 1983; Gomha et al., 2015).

Antibacterial activity

Purified l-asparaginase from *A. quadrilineatus* was evaluated for inhibition of *Escherichia coli* and *Staphylococcus aureus* (MRSA) growth. One hundred µL of purified l-asparaginase was added to the wells in agar plates inoculated with bacteria. Plates were incubated at 37°C for 24h. Inhibition diameter was measured using a ruler (Elamary & Salem, 2020).

Biosynthesis using solid-state fermentation (SSF)

Three substrates were used; onion peels and safflower plant wastes were chosen since these plants were cultivated in the soils where producers were found. Pomegranate peel was chosen simply as a common and available waste. The fermentation was performed as previously described (Aparna & Raju, 2015).

Statistical analysis

All experiments were prepared in triplicate. Differences between mean values were evaluated by one-way analysis of variance (ANOVA) with a significance level of 5%.

Results

L-asparaginase producing isolates

Our obtained results showed that twenty-four isolates (77.4%) from the tested 31 fungal

strains had the ability to produce extracellular l-asparaginase enzyme as evidenced by forming pink zone on modified Czapek's dox agar medium containing phenol red (Table 1). Pink zone diameters in the range from 12±0.2 to 52±8.718mm. The maximum diameter (52±8.718mm) was produced by *A. terreus*-21 and the minimum (12±0.2mm) was associated with *E. nidulans*-17 as illustrated in Table 1.

TABLE 1. Qualitative and quantitative estimation of L-asparaginase by the tested fungal isolates

Fungal isolates	Mean pink zone diameter (mm) ± SD	Source soil of isolation cultivated with plant	Mean L-asparaginase activity (U/mL) ± SD
AN- 1	-	Olive	-
AN- 2	19±0.2	Onion	9.808± 0.18930**
AN- 3	16±0.529	Olive	0.2164±0.00167
AN- 4	22.3±1.868	Guava	3.108±0.00998**
AN- 5	16±0.916	Pomegranate	5.952±0.05794**
AN- 6	16±2.5	Common fig	5.496±0.11558**
AN- 7	18±2.291	Sugarcane	6.624 ± 0.28044**
AN- 8	-	Common fig	-
AN- 9	-	Broad bean	-
AN- 10	21±4.583	Olive	6.724± 0.02059**
AN- 11	-	Common fig	-
AN- 12	-	Olive	-
AN- 13	-	Palm	-
AN- 14	-	Olive	-
AQ- 15	23±2	Safflower	7.348± 0.12328**
EN - 16	17.5±2.689	Guava	4.976± 0.00866**
EN - 17	12±0.2	Olive	0.568± 0.00436*
EN- 18	17±3.041	Common fig	3.388±0.00755**
EN- 19	12.5±1.323	Olive	0.520±0.01605
AT- 20	41±9	Broad bean	4.916±0.01368**
AT- 21	52±8.718	Olive	4.788± 0.03401**
AT- 22	34±5.291	Sugarcane	6.012±0.01255**
AT- 23	49±1.5	Spinach	6.364± 0.02939**
AT- 24	29±5	Common fig	2.652± 0.01838**
AF- 25	39±3.606	Onion	5.220± 0.00201**
AF- 26	30±0	Spinach	4.040 ± 0.01167**
AF- 27	37±5	Broad bean	4.716± 0.02194**
AF- 28	27±1.5	Safflower	4.296±0.01133**
AF- 29	29±2.646	Sugarcane	1.956±0.01298**
AF- 30	35±0	Common fig	3.928±0.03649**
FS-31	44±2	Lemon	4.232± 0.00708**

AN: *Aspergillusniger*, AQ: *Aspergillus quadrilineatus*, EN: *Emericella nidulans*, AT: *A. terreus*, AF: *A. flavus*, FS: *Fusarium solani*
*: Means values are significant compared with control, **: Means values are highly significant compared with control.

L-asparaginase production by submerged fermentation (SMF)

Yields of l-asparaginase were assessed by cultivation on modified Czapek's dox liquid medium. L-asparaginase Table 1 activity ranged from 0.2164±0.00167 to 9.808± 0.18930U/mL, with the maximum record in *A. niger*-2 and the lowest in *A. niger*-3 (Table 1).

Optimizing of l-asparaginase production

Incubation period

Enzyme levels varied with time (Figs. 1A & 2A). The highest production for *A. niger* and *A. quadrilineatus* isolates (16.1 and 13.5U/ mL, respectively) was observed at the end of the third day (72h). Further incubation led to a decrease in l-asparaginase activity.

Temperature

Isolates grew and produced l-asparaginase over temperatures from 20°C to 45°C. The optimal temperature for l-asparaginase was found to be at 30 °C with the activities of 14.3 and 13.9U/mL for *A. niger* and *A. quadrilineatus*, respectively (Figs. 1A & 2A).

Carbon source

Not all carbon sources exhibited the same impact on enzyme productivity. The highest activity produced by *A. niger* and *A. quadrilineatus* (15.9 and 14U/mL, respectively) was observed in glucose and sucrose containing media, respectively (Fig. 1B & 2B). The lowest production by *A. niger* was noted with lactose (2.3U/mL). Maltose was the least effective source for *A. quadrilineatus* (5.5U/mL).

Results in Fig. 1B revealed that the highest activity of *A. niger* l-asparaginase (16.2U/ mL) was estimated in 2% glucose. However, gradually decreasing l- asparaginase activity was observed with higher glucose levels. The optimum activity of *A. quadrilineatus* (13.8 U/ mL) was seen with 1.5% sucrose (Fig. 2B) .

pH

L-asparaginase production by highest producing isolates showed that the initial pH of the fermentation medium was an important factor for l-asparaginase production. Maximum activity 15.7 and 14U/mL by *A. niger* and *A. quadrilineatus*, respectively, was obtained at pH 6. Decreasing the initial pH to 2.0 reduced l-asparaginase (Figs. 1A & 2A).

Nitrogen source effect

Enzyme production was ubstantially affected nitrogen source present in the fermented medium. Maximum l-asparaginase activity 15.8 and 14.5U/mL for *A. niger* and *A. quadrilineatus*, respectively, was detected in growth media containing l-asparagine. Ammonium sulfate least supported l-asparaginase production. Enzyme production is affected by both organic and inorganic nitrogen in the culture medium.

L-asparagine (1%) was the best nitrogen source, supporting activities of 14.5 and 13.8 U/ml for *A. niger* and *A. quadrilineatus*, respectively (Figs. 1B & 2B).

Agitation rate

L-asparaginase activity increased with rate from 100 to 160rpm. Maximum production was 15.86 and 13.9 U/ml for *A. niger* and *A. quadrilineatus*, respectively, at 160rpm (Figs. 1A & 2A).

Phosphorus concentration

L-asparaginase production was phosphorus dependent. Maximum yield, which was 15.2 and 13.9U/mL for *A. niger* and *A. quadrilineatus*, respectively), was seen with 0.1% KH_2PO_4 (Figs. 1B & 2B).

Enzyme purification

Enzymes were purified from the highest producing isolates of *A. niger* and *A. quadrilineatus*. Initial l-asparaginase activity of crude filtrates were 16 and 13.9U/mL with specific activity 21.33 and 15.6U/mg, respectively (Table 2). When the enzyme was concentrated by 80% ammonium sulfate, enzyme activity increased to be 20 and 17.6 U/ mL with specific activity of 29.8 and 22.5U/ mg, respectively. Dialysis led to an increase in the specific activity of to 33.23 and 27.7U/mg, respectively (Table 2). Further purification on a Sephadex G-200 column chromatography. The dialyzed partially purified enzyme was loaded on sephadex G-200 column produced about 30 fractions(5mL for each). The specific activity of *A. niger* and *A. quadrilineatus* preparations after gel filtration was 50.4 and 37.4U/mg, respectively.

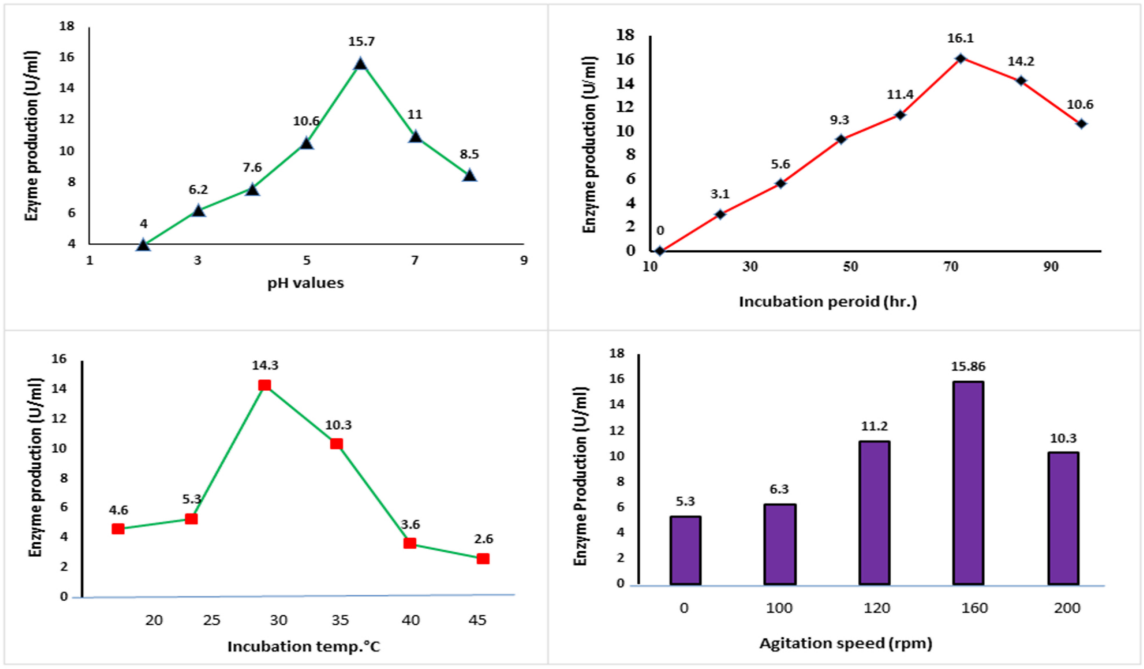

Fig. 1A. Optimum conditions for L- asparaginase production by *A. niger*

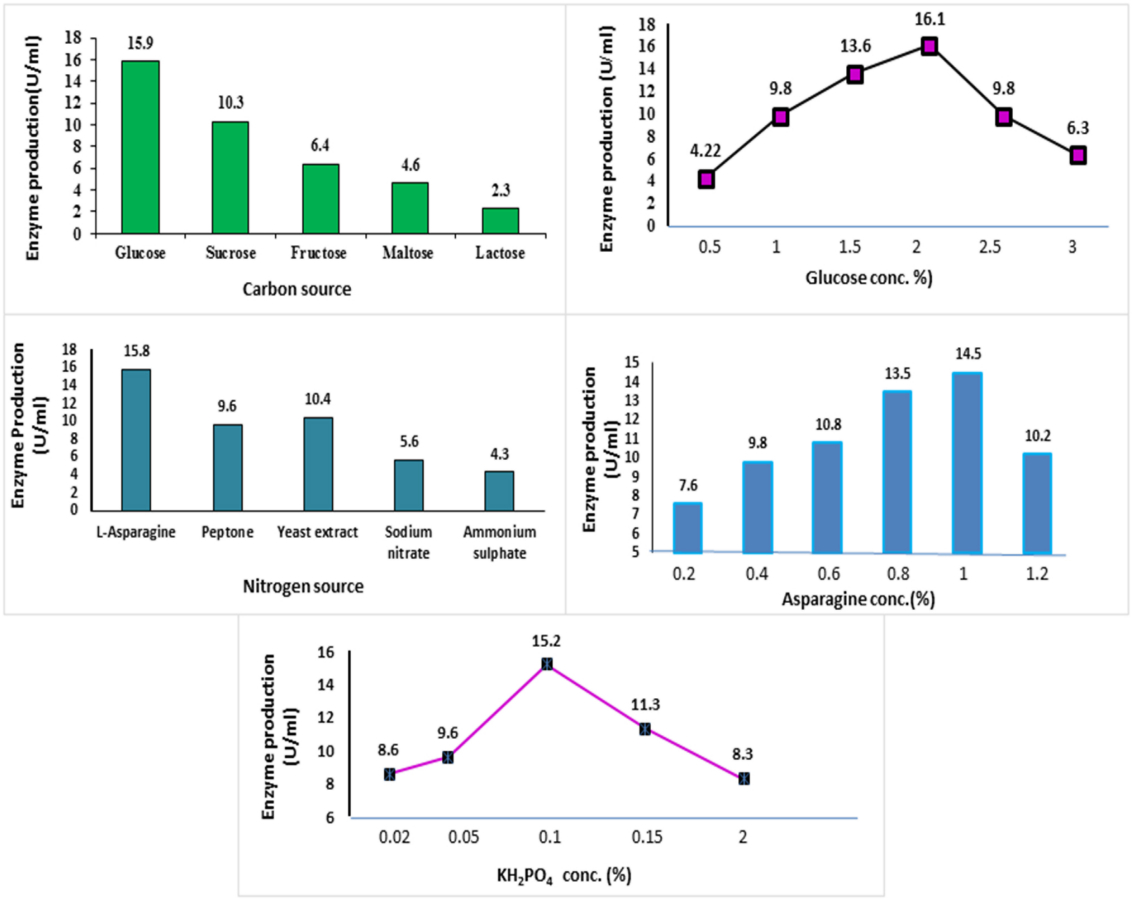

Fig. 1B. Optimum conditions for L- asparaginase production by *A. niger*

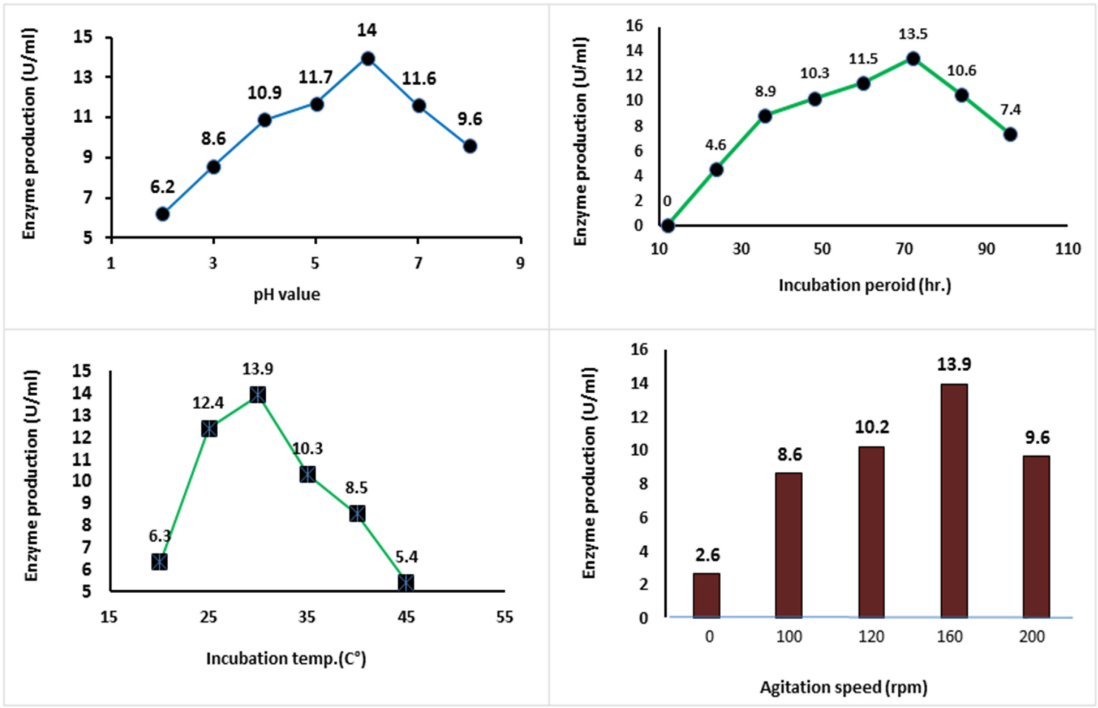

Fig. 2A. Optimum conditions for L-asparaginase production by *A. quadrilineatus*

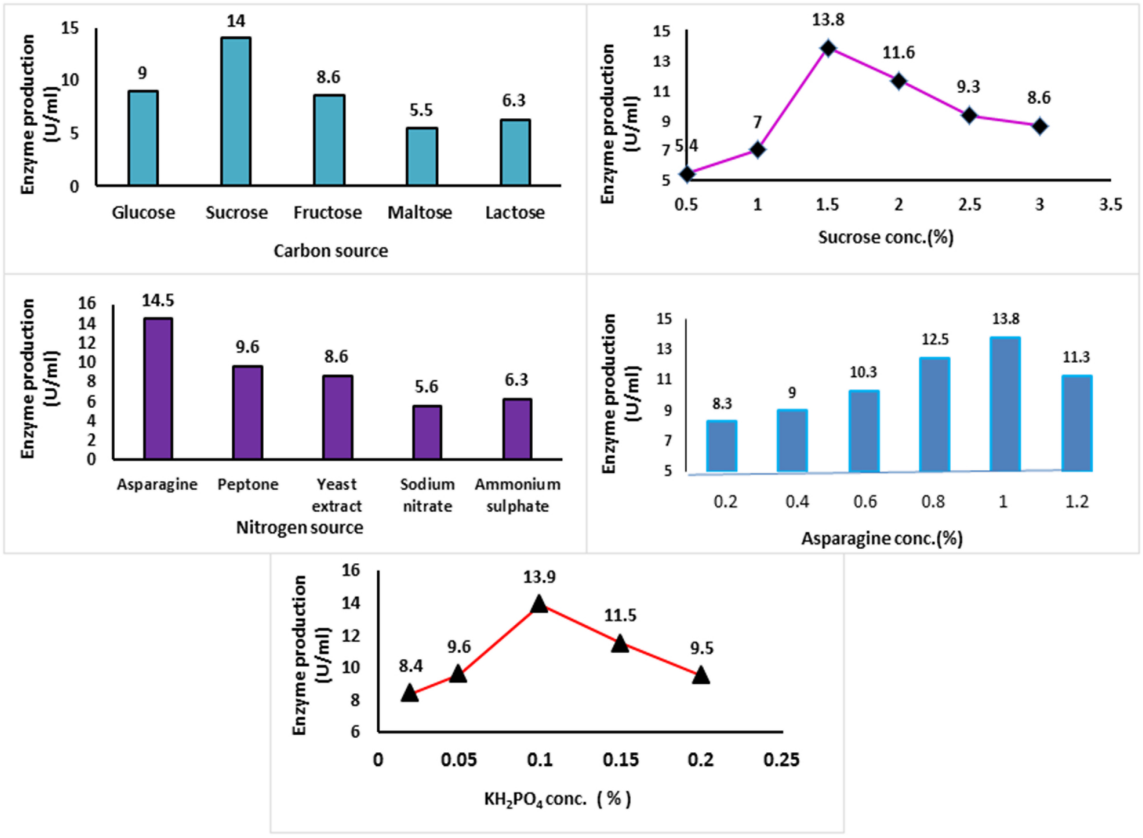

Fig. 2B. Optimum conditions for L-asparaginase production by *A. quadrilineatus*

TABLE 2. Purification profile of L-asparaginase obtained from *A. niger* and *A. quadrilineatus*

Fungi	Purification step	Total volume (mL)	Protein (mg/mL)	Total protein (mg)	Enzyme activity (U/mL)	Total activity (U)	Specific activity (U/mg)
A. niger	Crude	200	0.757	150	16.0	3200	21.13
	Ammonium sulfatee	20	0.670	13.4	20.0	400	29.8
	Dialysis	20	0.662	13.2	22.0	440	33.23
	Sephadex G-200 F(16)	15	0.603	9.0	30.4	450	50.4
A. quadrilineatus	Crude	200	0.890	178	13.9	2780	15.6
	Ammonium Sulfatee	20	0.780	15.6	17.6	352	22.5
	Dialysis	20	0.740	14.8	20.5	410	27.7
	Sephadex G-200 F(15)	15	0.690	10.3	25.8	387	37.4

SDS-PAGE and molecular weight determination

Protein bands after electrophoresis exhibited a single protein band for each isolate, indicating the high enzyme purity. Hence, molecular weights for individual bands were 27.8 KDa for *A. quadrilineatus* and 50.4kDa for *A. niger* (Figs. 3& 4).

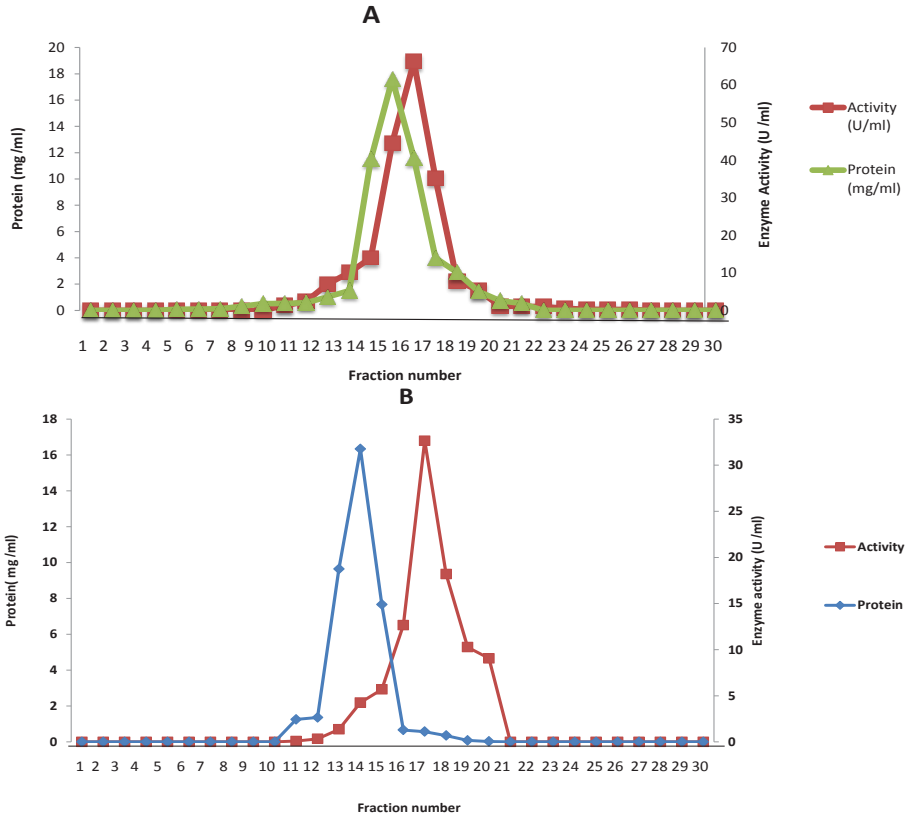

Fig. 3. Elution profile of *A. niger* (A) and *A. quadrilineatus* (B) on Sphedax G-200 illustrating enzyme activity (U/mL) and protein concentration (mg/mL)

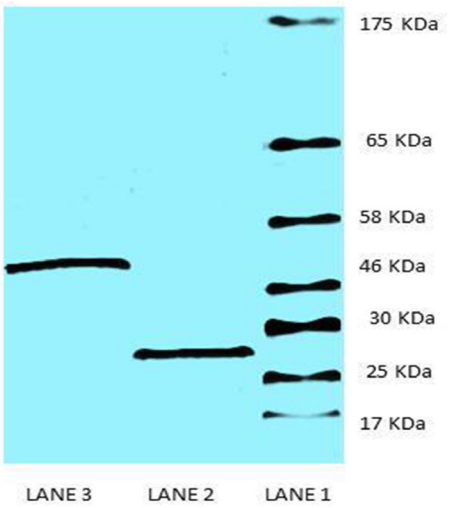

Fig. 4. SDS-PAGE of L-asparaginase profile for *A. niger* and *A. quadrilineatus* [Lane 1: Molecular weight of protein markers, Lane 2: L-asparaginase purified from *A. quadrilineatus* and Lane 3: L-asparaginase purified from *A. niger*]

Antitumor efficacy of purified l-asparaginase from A. niger

The inhibitory activity of purified l-asparaginase from *A. niger* (AsE) at a concentrations of 1.95 to 1000µg/mL against colon adenocarcinoma (HCT-116), breast carcinoma (MCF-7), and hepatocellular carcinoma (HepG-2) cells suggested IC_{50} values of 28.9µg/mL, 36.1, and 82.6µg/mL, respectively (Figs. 5 A, B, C).

Antibacterial efficacy

Purified l-asparaginase from *A. quadrilineatus* did not display antibacterial efficiency against two pathogenic bacterial strains.

Biosynthesis of l-asparaginase by using agro-industrial wastes

L-asparaginase production by two high producing isolates was assessed using only agro-industrial wastes as inexpensive carbon and nitrogen sources. Fungi produced l-asparaginase during cultivation on tested substrates with variable efficiency (Table 3). The highest activity for *A. niger* (23.548±0.00000U/g) was observed after culture on a mixture of onion and pomegranate peels (50%: 50% w/w). This medium increased yield 4.46 and 5 fold in l-asparaginase compared to individual onion and pomegranate peel powders, respectively (Table 3). A synergistic effect of binary formulations composed of pomegranate peels and safflower wastes (50%: 50% w/w) showed increases of 0.69 and 2.92 fold in l-asparaginase compared with individual substrates, respectively. A binary mixture of safflower wastes and onion peels increased enzyme production 0.36 fold, similar to the increase seen with safflower wastes alone, but less than the obtained yield using onion peels alone. The fusion of onion, pomegranate, and safflower wastes with equal proportions (1/3:1/3:1/3 w/w) produced elevations 0.4, 0.54, and 2.58 fold compared to individual substrates, respectively. The lowest yield of l-asparaginase was observed using safflower plant wastes (1.688±0.00038U/g). The highest yield (23.548±0.00000U/g) by *A. quadrilineatus* was observed using pomegranate peels alone. Interestingly, safflower plant wastes supported the lowest yield (1.704±0.02621U/g) (Table 3). Binary mixtures of pomegranate with either onion or safflower (50%:50% w/w) produced higher yields than those obtained by using a ternary mixture of the three substrates in equal proportions (6.724±0.01544U/g). A combination of pomegranate and safflower wastes produced a yield of 9.192±0.01494U/g, a 4.4 fold increase compared safflower wastes alone. A binary mixture of onion and pomegranate (50%:50% w/w) yielded 8.036±0.01019U/g with an increase in enzyme yield of 0.37 fold relative to onion peels. L- asparaginase level after cultivation of *A. quadrilineatus* on a binary mixture of onion and safflower was 1.7208±0.00899U/g, a slight increase from the yield obtained using safflower plant wastes alone (Table 3).

TABLE 3. Enhancement of L-asparaginase activity of the highest producer isolates by using agro-industrial wastes

Sample	Mean L-asparaginase activity (U/mL) ± SD
A. niger O	4.324 ± 0.01289**
A. niger P	3.924 ± 0.07243**
A. niger S	1.688 ± 0.00038**
A. niger O+P	23.548 ± 0.00000**
A. niger O+S	2.288 ±0.00819**
A. niger P+S	6.612 ± 0.02171**
A. niger Mix3	6.048 ±0.04580**
A. quadrilineatus O	5.856 ± 0.01384**
A. quadrilineatus P	23.548 ± 0.00000**
A. quadrilineatus S	1.704 ± 0.02621**
A. quadrilineatus O+P	8.036 ± 0.01019**
A. quadrilineatus O+S	1.7208 ± 0.00899**
A. quadrilineatus P+S	9.192 ± 0.01494**
A. quadrilineatus Mix3	6.724 ± 0.01544**

O: Onion, P: Pomegranate, S: Safflower, Mix 3: Mixture of the 3 substrates.

**: Means values are highly significant compared with control.

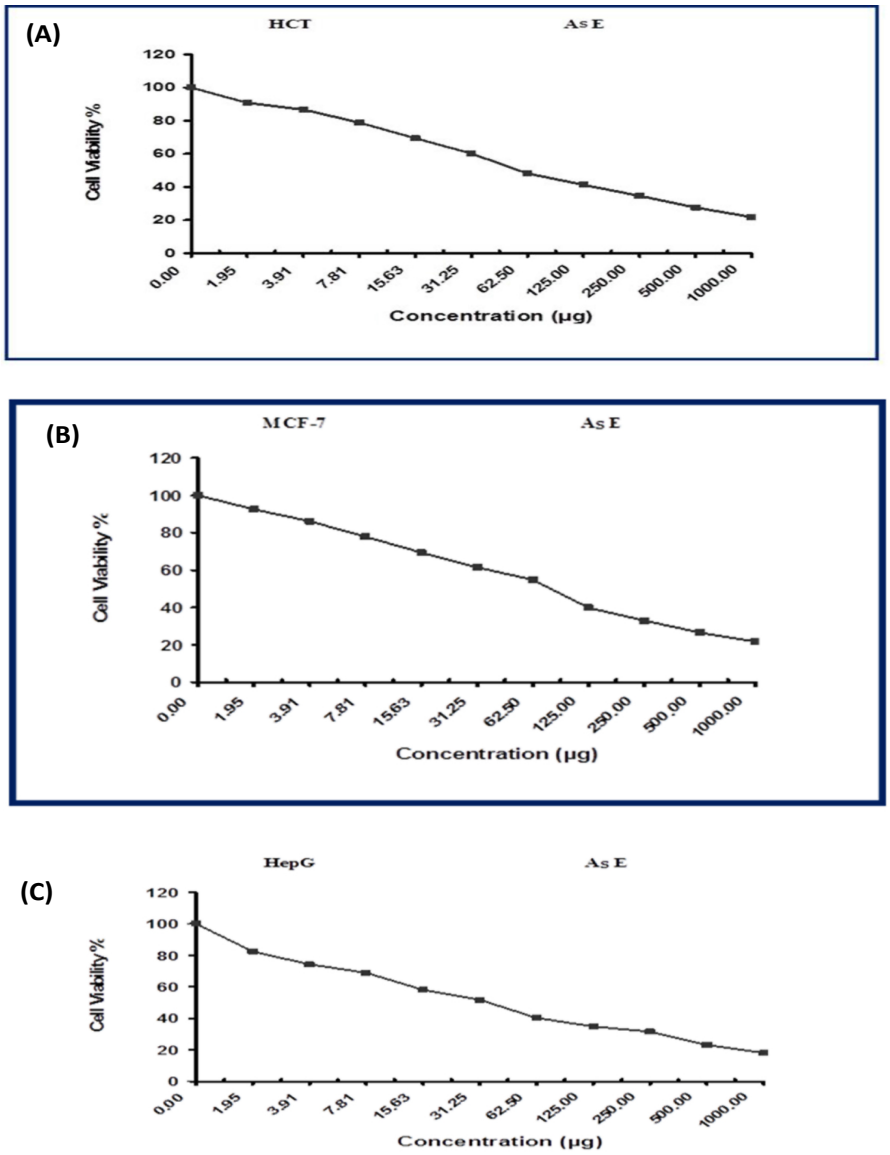

Fig. 5. Antitumor potential of purified L-asparaginase from *A. niger* against colon adenocarcinoma (A), breast carcinoma (B), and hepatocellular carcinoma(C)

Discussion

Fungal asparaginase enzyme is an important anti-carcinoma agent with minimal side effects. In our study, twenty-four isolates (77.4%) from 31 fungal strains were found to be l-asparaginase producers by forming pink zone with variable degrees (Table 1). An indicator color change from yellow to pink signifies the production of l-asparaginase enzyme that hydrolyzes asparagine to aspartic acid, liberating ammonia and increasing pH (Gulati et al., 1997). The same results were previously obtained by El-Said et al. (2016) who confirmed that the endophytic *Aspergillus niger* SVUAn1 was the highest L-asparaginase producer. In a past study by Ali et al. (2018) found that *Fusarium oxysporum*, *F. incarnatum*, *A. terreus*, and *A. sydowii* recorded the highest pink zone. Twenty-one of the 43 fungal isolates obtained from rhizosphere soils in Egypt produced l-asparaginase (El-Hefnawy et al., 2015). Conversely**,** Balbool & Abdel-Azeem (2020) reported that only seven isolates were l-asparaginase producers from the tested 25 endophytic isolates. The present study noted no correlation between pink zone diameter and enzyme activity and this in-agreement with El-Hefnawy et al. (2015) and Ali et al. (2018). Variations in enzyme levels may be due to either the nature of the isolate or fermentation conditions (Bedaiwy

et al., 2016). *A. niger* exhibited the highest l-asparaginase activity (9.808±0.18930U/m) and *A. quadrilineatus* also produced substantial enzyme (7.348±0.12328U/m). These species were the highest producers from three genera and were chosen for further studies. L- asparaginase production by *A. niger* and *E. nidulans* by submerged and solid- state fermentation was previously described by Mishra (2006), Jayaramu et al. (2010) and Dange & Peshwe (2015). Jenila & Gnanadoss (2018) indicated that *Fusarium* sp. LCJ273 produced 9.18U/mL. The maximum l-asparaginase formed by *A. terreus* was 10.97U/mg (Farag et al., 2015). Doriya & Kumar (2016) reported a maximum of 34.45U/mL in *A. terreus*. Maximum l-asparaginase activity produced by *A. sydowii* and *F. oxysporum* was 3.98 and 3.91U/mL (Ali et al., 2018).

The optimum l-asparaginase activity of the highest producing isolates was recorded after 72 hr. of incubation at 30°C (Fig. 1A & 2A). Consistently, Thakur et al. (2014) reported that a short incubation time reduces L-asparaginase decomposition by protein hydrolysis enzymes while the prolonged incubation time led to a decrease in l-asparaginase activity and this may be explained by exhaustion of some medium constituents or the production of toxic metabolites. El-Hefnawy et al. (2015) confirmed that the optimum incubation period for l-asparaginase production by *Penicillium oxalicum* was 72 h. Previous studies mentioned that the optimum l-asparaginase activity by *Aspergillus* species was also reported after 72 h of incubation (Siddalingeshwara & Lingappa, 2010; Gurunathan & Sahadevan, 2011; Balasubramanian et al., 2012; Mushtaq et al., 2012). L-asparaginase activity decreased with increasing temperature this may be attributed to that soil fungi are mesophilic and their metabolic activities decrease at higher temperatures. Similarly, Dange & Peshwe (2015) reported that optimum l-asparaginase productivity by *A. niger* at 30°C. Several other fungi also showed maximum l-asparaginase activity at 30°C such as *Penicillium* sp. (Kotra et al., 2013), *Aspergillus terreus* (Balasubramanian et al., 2012), and *Mucor hiemalis* (Monica et al., 2013).

Maximum l-asparaginase productivity was observed by supplementing fermentation media with 2% glucose or 1.5% sucrose for *A. niger* and *A. quadrilineatus*, respectively (Figs. 1B & 2B). Carbon is the essential nutrient for fungal growth and enzyme production. El-Said et al. (2016) also found that glucose is the greatest carbon source for l- asparaginase production by *A. niger*. Similarly, earlier studies confirmed that glucose was the best carbon source for l-asparaginase production by various fungi (Thakur et al., 2014; Kalyanasundaram et al., 2015; Issac & Abu-Tahon, 2016; Ali et al., 2018). Fermentation media containing sucrose produced maximum l-asparaginase activity by *Penicillium oxalicum* (El-Hefnawy et al., 2015), and 3% and 5% glucose were preferred concentrations for l-asparaginase production by endophytic PBL13 *Penicillium simplicissimum* and *Fusarium oxysporum* MKS1 (Chow & Ting, 2017). Conversely, El-Hefnawy et al. (2015) reported that 0.5% sucrose was the best concentration for l-asparaginase productivity by *Penicillium oxalicum*.

In our study, pH 6 supported the highest l-asparaginase production (Fig. 1A & 2A). Consitently, Jayaramu et al. (2010) confirmed that the optimum l-asparaginase production by *E. nidulans* was observed at pH 6. An initial pH of 6 was also optimum for l-asparaginase production by *Penicillium* sp. (Mushtaq et al., 2012). Several previous studies confirmed an optimum pH range of 6 to 6.3 for asparaginase production (Baskar & Renganathan, 2009; Baskar et al., 2010; Gurunathan & Sahadevan, 2011, 2012; El-Refai et al., 2014; Farag et al., 2015).

Further, the highest l-asparaginase levels were seen with 1% l-asparagine as a nitrogen source (Fig. 1B & 2B). L-asparagine also had a positive influence in previous studies (Baskar & Renganathan, 2009, 2012; Gurunathan & Sahadevan, 2011; El-Refai et al., 2014). Doriya & Kumar (2016) identified l-asparaginase as an effective supplement for *Aspergillus* sp. C7 cultured in MCD medium and l-asparagine was the best nitrogen source for asparaginase production by marine *A. terreus* (Farag et al., 2015). In contrast, ammonium sulfate (0.5%) was most effective for *F. solani* (El-Hefnawy, et al., 2015), and Kalyanasundaram et al. (2015) also reported maximum production by *A. terreus* was detected in a medium containing ammonium sulfate. Our obtained data in completely agreement with Gurunathan & Sahadevan (2011) who confirmed that maximum l-asparaginase formation by *Aspergillus terreus* MTCC 1782 using 1% (w/v) l-asparagine and maximum l-asparaginase activity for *Trichoderma viride*

was noted in growth medium supplemented with 0.5% asparagine (Lincoln & More, 2014).

The maximum production of l-asparaginase was observed at an agitation rate 160rpm (Figs. 1A & 2A). Similarly, the highest yield for *Streptomyces brollosae* NEAE-115 was seen at 150rpm (El-Naggar et al., 2019). In contrast, Sundaramoorthi & Dharamsi (2019) found an optimum for thermophilic *Penicillium notatum* at 200rpm.

Growth in 0.1% KH_2PO_4 induced the highest l-asparaginase activity in select isolates (Figs. 1B & 2B). KH_2PO_4 supplementation has a significant impact on l-asparaginase production (Kumar et al., 2009). Phosphate is required for growth and metabolism and plays a main role in regulating enzymes and the synthesis of primary and secondary metabolites (Weinberg, 1974; El-Naggar et al., 2017). In a past study by Baskar & Renganathan (2009) reported that K_2HPO_4 has an important role in l-asparaginase production.

Enzymes were purified by ammonium sulfate precipitation, dialysis, and Sephadex G-200 (Table 2, Fig. 3). Fewer purification stages are desirable since of about 10% enzyme activity is realized is lost after each purification step (Bora & Bora, 2012). Molecular weight of purified l-asparaginase from *A. niger* was approximately 50.4kDa and 27.8kDa for *A. quadrilineatus* (Fig.4). Molecular weight estimates for asparaginase vary with source and genetic variation (Monica et al., 2013; El-Naggar et al., 2018). In a past study by Akilandeswari et al. (2012) reported an MW of purified l-asparaginase from *A. niger* of 48kDa. *A. niger* asparaginase is defined in the DSM's dossier as a glycoprotein with MW of about 50kDa (DSM, 2007). Endophytic *Aspergillus* sp. ALAA-2000 produced enzymes with MWs of 25kDa (AYA-1) and 31kDa (AYA-2) (Abbas Ahmed et al., 2015). Purified l-asparaginase from *Penicillium citrinum* and *A. fumigatus* WL002 showed MW of 30kDa (Patro & Gupta, 2014; Dutta et al., 2015). Purified l-asparaginase significantly inhibited the proliferation of HCT-116, HePG-2, and MCF-7cells with IC_{50} concentrations of 28.9, 36.1, and 82.6µg/mL, respectively (Fig. 5). L-asparaginase hydrolyzes asparagine without affecting normal cells. Conversely, the enzyme can cause tumor cell death (Shakambari et al., 2019). Ali et al. (2018) confirmed that crude asparaginase from *A. sydowii* and *F. oxysporum* displayed anti-leukemic activity against murine RAW 264.7 leukemia cells with IC_{50} of 50.0 and 62.5U/mL, respectively. Also, anti-cancer activity was observed in human colon carcinoma, and cell death reached 70 and 73% *A. sydowii* and *F. oxysporum* enzyme, respectively. Higher anti-cancer activity (up to 80%) was observed in liver and breast carcinoma cell. L-asparaginase from *A. fumigatus* exhibited antitumor potential against human breast cancer MDA-MB-231 cells; 20U of enzyme caused 96.5% of cell death (Benchamin et al., 2019). Reduction in the leukemic cell (A431) viability by 50% after exposure to 250µg $mL^{-1}$of purified l-asparaginase from *A. niger* for 72h (Dange & Peshwe, 2015). Elshafei et al. (2012) noted that purified l-asparaginase from *Penicillium brevicompactum* showed antitumor efficacy against HepG-2 cells (hepatocellular carcinoma) with IC50 of 43.3µg/mL. L-asparaginase from *Aspergillus oryzae* exhibited a significant inhibition for the growth of HeLa and HepG-2 cells (Sudarkodi & Sundar, 2018). D'Souza & Asha (2019) showed significant cytotoxic activity of l- asparaginase from *Bacillus* sp. in MCF-7, HeLa, HepG-2, and 3T3L1 cells.

Antibacterial efficacy of purified l-asparaginase was not detected. In harmony with our obtained data, El-Naggar et al. (2020) reported that asparaginase from *Streptomyces rochei* NEAE-K had no antimicrobial activity against several microorganisms, including *E. coli and S. aureus.* No evidence currently exists that purified l-asparaginase shows antibacterial efficacy.

Selected isolates produced l-asparaginase during cultivation with agro-industrial wastes without additional nutrients. Maximum yield (23.548 ± 0.00000 U/ml) was obtained with *A. niger* in a mixture of onion and pomegranate peel powders (50%:50% w/w) and cultivation *A. quadrilineatus* on pomegranate peels only (Table 3). The diversity in l-asparaginase levels may be specific to the micro-organism and dependent on the concentration of asparagine in agro-industrial wastes. Ratios of substrates may also affect enzyme production. Similarly, Aparna & Raju (2015) found that corn ear and cauliflower stalk highly promoted l-asparaginase production by *A. terreus* MTCC 1782 and the ratios of these substrates exhibited a synergistic interaction. Dias et al. (2015) reported that the maximum l-asparaginase yield by *A. niger* LBA02 was 89.22U/g obtained using a ternary mixture of

soybean meal, cottonseed meal, and wheat bran in equal proportion. Increases in yields of 13.53, 13.53, and 71.53 fold, respectively, were observed after 96 h of fermentation compared to individual feedstocks. whereas the addition of orange peel had no or negative effects on enzyme production. Further, *A. niger* LBA02 l-asparaginase activity reached 2380.11U/gds using Passion fruit peel flour as a substrate (da Cunha et al., 2018). Maximum l-asparaginase yield for *A. niger* was supported using the bran of *Glycine max* (39.9±3.92U/gds), *Phaseolus mungo* (30.7±3.69U/gds) and *Cajanus cajan* (26.14± 3.67U/gds) (Mishra, 2006). L-asparaginase production by *Pseudomonas plecoglossicida* RS1 was increased twofold by onion peel extract and garlic peel extract supplemented with (0.3% w/w) l-asparagine (Shakambari et al., 2017). Several studies examined the asparagine content of wastes used as substrates. In this respect, Fredotovic et al. (2020) discussed the amino acid content of common onion, *Allium cepa* L., and found a large amounts of aspartic acid (6.100±0.083mg/g DW). Further, pomegranate peel powder contains asparate in a ratio of 0.3g/100g (Rowayshed et al., 2013). Al Surmi et al. (2016) found that Egyptian safflower seeds contain 2.59g of aspartic acid per 100g of protein.

Conclusions

Twenty-four fungal isolates obtained from rhizosphere soil were l-asparaginase producers to various degrees. L-asparaginase purified from *A. niger* significantly inhibited the proliferation of human breast adenocarcinoma (MCF-7), liver cancer (HepG-2), and colon adenocarcinoma (HCT-116) cells. Purified l-asparaginase from *A. quadrilineatus* did not exhibit antibacterial efficiency. The highest producing isolates generated enzyme during cultivation on agro-industrial wastes without additional nutrients. Further, studies are necessary to determine optimum conditions for l-asparaginase production using agro-industrial wastes. Furthermore, work is needed for the purification and characterization of enzyme for use in acrylamide mitigation and inhibition of biofilm formation by pathogenic bacteria.

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فعالية انزيم الاسباراجيناز الخارجى المنقى من فطر الاسبرجيللس نيجر ضد الخلايا السرطانية وتعزيز انتاجه باستخدام المخلفات الزراعية الصناعية

أسماء صبرى يسين محمد، أمانى عطا الشهير
قسم النبات والميكروبيولوجى- كلية العلوم- جامعة جنوب الوادى- قنا 83523 - مصر.

تهدف الدراسة الحالية الى معرفة قدرة 31 عزلة فطرية معزولة من تربة الجذور على افراز انزيم الاسباراجيناز. 24 عزلة فطرية كانت لهم المقدرة على انتاج الانزيم. الاسبرجيللس نيجر-2 والاسبرجيللس كوادريلانتس سجلوا اعلى انتاجية 9,808± 0,18930 و 7,348± 0,12328 (وحدة/ملى), على التوالي. كانت الظروف المثلى لإنتاج الإنزيم عند 30 درجة مئوية لمدة 72 ساعة، ودرجة الحموضة 6 عند 160 دورة في الدقيقة، و 0,1٪ من البوتاسيوم داى هيدروجين فوسفات في وجود 2٪ جلوكوز و1,5٪ سكروز كمصدر للكربون و1٪ ال- اسباراجين بواسطة الاسبرجيللس نيجر والاسبرجيللس كوادريلانتس، على التوالي. تم إجراء ترسيب الانزيم باستخدام كبريتات الأمونيوم واتبع ذلك عملية التنقية عن طريق الفصل الغشائى باستخدام السيفادكس جى-200مع نشاط نوعى للانزيم 50,4 و37,4 وحدة/مليجرام بواسطة الاسبرجيللس نيجر والاسبرجيللس كوادريلانتس، على التوالي. واظهرت نتائج تحليل البروتين بتقنية التفريد الكهربى ان الوزن الجزيئى لانزيم الاسباراجيناز المنقى من الاسبرجيللس نيجر حوالي 50,36 كيلو دالتون و 27,8 كيلو دالتون من الاسبرجيللس كوادريلانتس. منع الاسباراجيناز المنقى بشكل كبير تكاثر خلايا القولون السرطانية, خلايا سرطان الكبد و خلايا سرطان الثدي وكانت التركيزات المثبطة لنمو 50% من تلك الخلايا السرطانية هى 28,9, 36,1 و 82,1 ميكروجرام/ مل، على التوالي ولم يظهر الانزيم المنقى أي فعالية مضادة للبكتريا. تم تعزيز إنتاج الأسباراجيناز باستخدام المخلفات الزراعية والصناعية. تم الحصول على أقصى إنتاجية (23,548 ± 0.00000 وحدة / مل) بزراعة الاسبرجيللس نيجر على خليط من مسحوق قشر البصل والرمان (50٪: 50٪ وزن / وزن) وزراعة الاسبرجيللس كوادريلانتس على قشر الرمان فقط.