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Original article

Production, purification, characterization, antioxidant and antiproliferative activities of extracellular L-asparaginase produced by *Fusarium equiseti* AHMF4

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ABSTRACT

L-Asparaginase is an antileukemic agent that depletes L-asparagine "an important nutrient for cancer cells" through the hydrolysis of L-asparagine into L-aspartic acid and ammonia leading to leukemia cell starvation and apoptosis in susceptible leukemic cell populations. Moreover currently, bacterial L-asparaginase has been limited by problems of lower productivity, stability, selectivity and a number of toxicities along with the resistance towards bacterial L-asparaginase. Then the current work aimed to provide pure L-asparaginase with in-vitro efficacy against various human carcinomas without adverse effects related to current L-asparaginase formulations. Submerged fermentation (SMF) bioprocess was applied and improved to maximize L-asparaginase production from *Fusarium equiseti* AHMF4 as alternative sources of bacteria. The enzyme production in SMF was maximized to reach 40.78 U mL^{-1} at the 7th day of fermentation with initial pH 7.0, incubation temperature 30 °C, 1.0% glucose as carbon source, 0.2% asparagine as nitrogen source, 0.1% alanine as amino acid supplement and 0.1% KH_2PO_4. The purification of AHMF4 L-asparaginase yielded 2.67-fold purification and 48% recovery with final specific activity of 488.1 U mg^{-1} of protein. Purified L-asparaginase was characterized as serine protease enzyme with molecular weight of 45.7 kDa beside stability at neutral pH and between 20 and 40 °C. Interestingly, purified L-asparaginase showed promising DPPH radical scavenging activity (IC_{50} 69.12 µg mL^{-1}) and anti-proliferative activity against cervical epitheloid carcinoma (Hela), epidermoid larynx carcinoma (Hep-2), hepatocellular carcinoma (HepG-2), Colorectal carcinoma (HCT-116), and breast adenocarcinoma (MCF-7) with IC_{50} equal to 2.0, 5.0, 12.40, 8.26 and 22.8 µg mL^{-1}, respectively. The enzyme showed higher activity, selectivity and anti-proliferative activity against cancerous cells along with tiny cytotoxicity toward normal cells (WI-38) which indicates that it has selective toxicity and it could be applied as a less toxic alternative to the current formulations.

1. Introduction

The use of enzymes in cancer therapy is the most important therapeutic applications of enzymes. Dissimilar to normal cells, cancer cells incapable to produce L-asparagine synthetase hence rely on exogenous resource of this amino acid for survival (Muneer et al., 2020; Pallem, 2019a,b). L-Asparaginase (EC3.5.1.1) has many applications in the pharmaceutical, drugs and nutrition industries (Bedaiwy, 2019). This enzyme used to treat acute lymphocytic leukemia and other related types of blood cancers. It is an enzyme selectively breaks down the amino acid L-asparagine into L-aspartate and ammonia, which leads to nutritional

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deficiency, inhibition of protein and lymphocyte death by apoptosis (Ashok et al., 2019; El-Gendy et al., 2017). There are varieties of organisms like bacteria, fungi, algae, and plants that produce L-asparaginase while the physiochemical characters and kinetic parameters of the enzyme depend on the source of the enzyme. Commercially, it is currently produced from two main sources of bacteria first from recombinant *Escherichia coli*, and second from *Erwinia chrysanthemi* (Muneer et al., 2020). Bacterial L-asparaginases are used for pharmacological purposes but raise suspicion because of a number of cytotoxicity, comprising thrombosis, pancreatitis, hyperglycemia, and hepatotoxicity along with cancerous cells resistant against L-asparaginase preparations of bacterial origin, which is an additional main difficulty throughout cancer management (Baruchel et al., 2020). This position attracted researchers' attention towards the search for a new L-asparaginase that differs from the existing (more selectively with less toxicity) through finding alternative sources of L-asparaginase (Muneer et al., 2020). Fungal L-asparaginase does not possess any unfavorable conditions (Prihanto et al., 2019; Sreejai et al., 2019). *Aspergillus*, *Penicillium*, *Fusarium* and other fungal species gave L-asparaginase without side effect (Bchamin et al., 2019; El-Gendy et al., 2018). The impact of this enzyme is evidenced by the high global demand, which reached $ 380 million in 2017 and is expected to increase up to $ 420 million by 2025 (Alam et al., 2019). Towards attaining this vital importance, it is necessary to improve production conditions including cultural conditions and neutral supplements (Ashok et al., 2019; El-Gendy et al., 2017; El-Naggar and El-Shweihy, 2020). Submerged fermentation (SMF) supported by many authors as the preferable fermentation method for fungal L-asparaginase synthesis from *Trichoderma viride*, *Aspergillus aculeatus*, *Cladosporium* sp., *Rhizomucor miehei*, *Penicillium digitatum*, *Penicillium* sp., *Penicillium brevicompactum* (Cachumba et al., 2016; Costa-Silva et al., 2019) at varied process parameters. The present study has been undertaken to find fungus able to produce L-asparaginase efficiently as an alternative sources for bacteria with higher productivity, stability and efficiency, purification and characterization of enzyme in addition to assessment of its antioxidant and anti-proliferative activities against Hela (cervical epitheloid carcinoma), Hep-2 (epidermoid larynx carcinoma), HCT-116 (colorectal carcinoma), HepG-2 (hepatocellular carcinoma) and MCF-7 (breast adenocarcinoma) cancerous cells compared to the normal cell line (WI-38).

2. Material and methods

2.1. Isolation of producing fungi

Each sample obtained from the garden of the faculty of science, Al-Azhar University, Assuit was brought to the laboratory in sterile polyethylene bags and kept at 4 °C until processing. Soil-plate technique was applied for the isolation of fungal strains according to Warcup (1950). A serial dilution up to 10^{-5} of each soil suspension was cultured on modified Czapek's Dox agar medium (CDA). Inoculated plates were incubated at 28 °C for 96 h and each fungal colony appeared was sub-cultured, purified and tested for their L-asparaginase productivity.

2.2. Qualitative and quantitative analysis for L-asparaginase productivity

The ability of such strains to produce L-asparaginase during its progress was determined by plate assay method on modified CDA medium accompanied with pH 6.2 and indicator phenol red. The fungal isolates that exhibited pink color around the colonies were characterized as L-asparaginase producers (El-Gendy et al., 2015, 2017). L-Asparaginase productivity was measured in AHMF1, AHMF2, AHMF3, AHMF4 and AHMF5 isolates (that produced the highest diameter of the pink zones around the colony). One milliliter spore suspension (10^6 spore mL^{-1}) of each fungus cultivated on potato dextrose medium at 28 °C for 5 days was inoculated individually into Erlenmeyer flasks containing 50 mL of modified Czapek's Dox medium and then incubated under submerged fermentation (SMF) at pH 6.2, 28 °C, 180 rpm for 7 days and then each filtered under vacuum to obtain clear supernatants for analysis.. One L-asparaginase unit (U) is defined as the amount of enzyme that releases one μmole of ammonia per min using nesslerization technique (Mohan Kumar and Manonmani, 2013).

2.3. Morphology, chemotaxonomy and 18S rDNA region sequence analysis of hyper L-asparaginase producer strain AHMF4

AHMF4 strain was identified according to previous taxonomic protocols (Leslie and Summerell, 2006; Dugan, 2006; El-Bondkly, 2012; El-Gendy et al., 2018; Pierozzi et al., 2020; Prakash et al., 2017; Salah et al., 2015; Watanabe, 2010). DNA was extracted, purified and amplified of the nearly complete 18S rDNA using NS1 5′ (GTAGTCATATGCTTGTCTC) 3′ and NS8 5′ (TCCGCAGGTTCACCTACGGA) 3′ primers. The PCR reaction was achieved with 20 ng of genomic DNA in 30 μL reaction mixture. PCR conditions were initial denaturation (5 min at 94 °C) next 35 cycles of primer annealing (1 min at 55 °C), primer extension (1 min at 72 °C), denaturation (1 min at 94 °C) and final extension stage (5 min at 72 °C). The PCR products were tested for true length on a 1.0% agarose gel purified and sequenced using a PRISM BigDye Terminator v3.1 Cycle Sequencing Kit. The DNA sample containing the extension product was added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The combination was incubated at 95 °C for 5 min, after that 5 min on ice and analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA) (Lücking et al., 2020). Sequence data were edited and next relations were confirmed in comparison with 18S rDNA in NCBI GenBank database by BLAST (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov, Altschul et al., 1990) to generate a matrix by MEGA6 program (Tamura et al., 2011). The tree topologies were assessed and phylogenetic trees were deduced by the neighbor-joining tree system. The complete 18S rDNA sequence of AHMF4 strain has been submitted to GenBank database.

2.4. Optimization of L-asparaginase productivity by Fusarium equiseti AHMF4 under submerged fermentation

Submerged fermentation for L-asparaginase was performed using Erlenmeyer flask comprising 100 mL of modified Czapek's Dox medium inoculated with selected fungus. To optimize cultural conditions and nutrient supplementations for L-asparaginase biosynthesis, its productivity was evaluated at different cultural conditions include incubation periods (1, 4, 7, 10 and 13 days), incubation temperature (20, 27, 30 and 37 °C) and initial pH ranged from 5.0 to 10.0 as well as different nutritional requirements include various carbon sources (starch, sorbitol, lactose, fructose, sucrose and glucose), nitrogen sources (tryptone, yeast extract, peptone, beef extract, malt extract and $NaNO_3$), metal supplementation ($MgCl_2$, $CaCl_2$, K_2HPO_4, $BaCl_2$ and NaCl) and amino acid (asparagine, glycine, alanine and methionine) at concentrations of 1.0%, 0.2%, 0.1%, and 0.2%, respectively. Each optimum parameter determined for enzyme productivity was applied in the next experiments.

2.5. Purification of F. equiseti AHMF4 L-asparaginase

All purification strides were done at 4 °C. Culture of *F. equiseti* AHMF4 after seven days of incubation was filtered under vacuum.

The culture broth was centrifuged under cooling at 4000 rpm for 20 min and then clear supernatant was precipitated by ammonium sulphate (80%) with mild stirring, stand for 1 h followed by centrifugation (10,000 rpm for 30 min), suspending the precipitate in phosphate buffer (0.2 M, pH 6.0) and dialyzing against the same buffer. The dialyzed sample with L-asparaginase activity was subjected to QFF column chromatography (16/10 cm, Pharmacia, Sweden) that eluted with phosphate buffer (50 mM, pH 7.0) having a linear gradient of sodium chloride (0.0–1.0 M) at flow rate of 0.5 mL min^{-1}. Active fractions were pooled, dialyzed against phosphate buffer (50 mM, pH 7.0), concentrated and applied to gel filtration sephacryl-200 that equilibrated and eluted with Tris-HCl buffer (0.01 M at pH 8.0) at flow rate of mL min^{-1}. All fractions were evaluated for L-asparaginase activity and protein contents. Fractions with L-asparaginase activity were gathered, dialyzed, concerted and lyophilized for further determinations.

2.6. Protein evaluation and electrophoresis

Protein was estimated based on Lowry et al. (1951). Polyacrylamide gel electrophoresis (SDS-PAGE) was done according to Laemmli (1970). The marker proteins for molecular weight evaluation were Prime-Step™ prestained broad range protein ladder (BioLegend).

2.7. Characterization of the purified L-asparaginase

For purified L-asparaginase activity, optimum temperature was determined using numerous temperatures (10–70 °C) and the thermo-stability was estimated after the pre-incubation of enzyme with each temperature for one hour afore assay. Optimum pH was performed at optimum temperature using appropriate buffer systems at 0.25 M (sodium acetate; pH 3.0–5.0, sodium phosphate; pH 6.0–8.0 and glycine; pH 9.0–10.0 buffers) and the pH stability and residual activity were determined after incubation with these pH values for 24 h before assay. Also, remaining L-asparaginase activity was determined after pre-incubating the enzyme with several types of chemicals, separately including diverse metal ions (50 mM of K^+, Na^+, Mg^{2+} and Ca^{2+} as well as 10 mM of Ba^{2+}, Cu^{2+}, Mn^{2+} and Fe^{3+}) for 1 h at optimum temperature and pH. To characterize the type of L-asparaginase enzyme under study depending on its catalytic site as serine, cysteine, aspartic or metallo-proteases, the enzyme activity was estimated after treatment with different proteases inhibitors including aspartic protease inhibitor (pepstatin A at concentrations of 5, 10 and 15 mM), serine protease inhibitors (phenylmethylsulfonyl fluoride, PMSF, Benzamidine and Nα-Tosyl-L-lysine chloromethylketone at concentrations of 5, 10, and 15 mM), metallo protease inhibitors (ethylenediaminetetraacetic acid, EDTA, ethyleneglycol-bis-(2-aminoethyl)-tetraacetic acid, EGTA and 1, 10-phenanthroline at concentrations of 5, 10 and 50 mM) and cysteine protease inhibitors [(dibenzyl-1-[(R)-biotin-6-aminohexa noyl-aziridine]-2,3-(S,S + R,R)-dicarboxylate), E-64 (N-(*trans*-Epoxy succinyl)-L-leucine-4-guanidinobutylamide) and 2-iodoacetamide at concentrations of 5, 10 and 15 mM] in comparison to the control (with no chemical additives that refer to 100% activity).

2.8. Cytotoxic activity of F. equiseti AHMF4 L-asparaginase against human carcinomas

The cytotoxicity of pure enzyme at various concentrations (ranged between 5 and 100 μg mL^{-1}) was estimated against colorectal carcinoma (HCT-116), hepatocellular carcinoma (HepG-2), breast adenocarcinoma (MCF-7), cervical epitheloid carcinoma (HeLa), epidermoid larynx carcinoma (Hep-2) cell lines, and the normal cell line (WI-38). Cell viability was estimated using 3-(4, 5-dimethythiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay as [(sample - blank)/(control - blank) × 100%] (El-Gendy et al., 2018; Mosmann, 1983). The amount of enzyme which inhibited 50% of viability (IC_{50}) was calculated. The cell viability in presence of DMSO instead of enzyme was measured as a control.

2.9. DPPH radical scavenging activity

The antioxidant activity of L-asparaginase at varied concentrations include 25, 50, 100, 150, 200, and 250 μg mL^{-1} was determined compared to the control (the sample without enzyme) by radical scavenging activity using 2, 2 diphenyl-1-picrylhydrazyl (DPPH) (Rani et al., 2011). DPPH scavenging activity (%) = A control - A test/control × 100. Ascorbic acid (25–250 μg mL^{-1}) was considered as a standard.

3. Results and discussion

3.1. Screening of soil fungal isolates for extracellular L-asparaginase production

Among all isolates in the qualitative assay for extracellular fungal L-asparaginase, fungi under the isolation number AHMF1, AHMF2, AHMF3, AHMF4 and AHMF5 showed pink zones around the colony which refer to their ability to produce L-asparaginase and then their productivities were quantitatively analyzed in SMF, they produced 7.37, 8.04, 9.48, 11.34 and 7.23 U mL^{-1}, respectively. Then AHMF4 isolate was selected as the hyper L-asparaginase producer qualitatively (formed prominent pink zone Fig. 1a) and quantitatively (11.34 U mL^{-1}) for the further studies. The anti-proliferative enzyme, L-asparaginase is dispersed in many fungal species like *Aspergillus, Trichoderma*, *Chaetomium*, *Alternaria, Curvularia*, *Pleurotus*, *Fusarium*, *Pestalotiopsis*, *Penicillium*, *Phaeotrichoconis, Drechslera, Cladosporium*, *Phoma* and *Phomopsis* species (Bedaiwy, 2019; El-Gendy et al., 2015, 2017, 2018; Pallem, 2019a,b; Prihanto et al., 2019; Sreejai et al., 2019).

3.2. Identification of L-asparaginase producer strain AHMF4

Data in Table 1 and Fig. 1b and showed the colony morphology and reproductive structure of AHMF4 isolate after 7 days on potato dextrose agar (PDA). Aerial mycelium of isolate AHMF4 appeared white turned to brownish white, reverse pale, colonies growing with diameter 5.1 cm on PDA after five days, sporodochia present, branched conidiophores scattered in the aerial mycelium, macroconidia are abundant (formed after 5 days from conidiophores, three-septate, 29–46 × 3–5 μm, cylindrical, curved with foot cell). Microconidia are abundant (ellipsoidal, one celled, curved and formed from long lateral phialides, 9.0 × 3.0 μm). Chlamydospores were hyaline, globose, rough walled, borne in pairs on short lateral hyphae with size estimated to be 5–8 μm. During two weeks of submerged fermentation, cellular lipid contents were improved with increasing growth period from 4 to 10 days and then declined due to increase growth period can force the fungal strains to consume their fat reserves as previously observed through findings of Ruiz et al. (2007). The predominant fatty acids 18:2 followed by 18:1, 18:0, 16:0, 18:3 and 18:2 ω6c in the selected strain, AHMF4 were increased from 16.77 ± 0.30, 10.00 ± 1.05, 8.10 ± 0.10, 5.51 ± 0.21, 4.18 ± 0.12 and 3.51 ± 0.0% at the 4th day of incubation to 28.15 ± 1.18, 22.86 ± 1.10, 14.00 ± 0.22, 8.00 ± 0.40, 6.01 ± 0.25 and 7.75 ± 0.02%, respectively after growth period 10 days and then decreased (Table 1). Dugan (2006), El-Gendy et al. (2017, 2018), Leslie and Summerell (2006), Pierozzi et al. (2020), Prakash et al. (2017), Ruiz et al. (2007), Salah et al. (2015) and Watanabe (2010) supported morphological characteristics and fatty acid patterns as a reliable method to distinguish fungi.

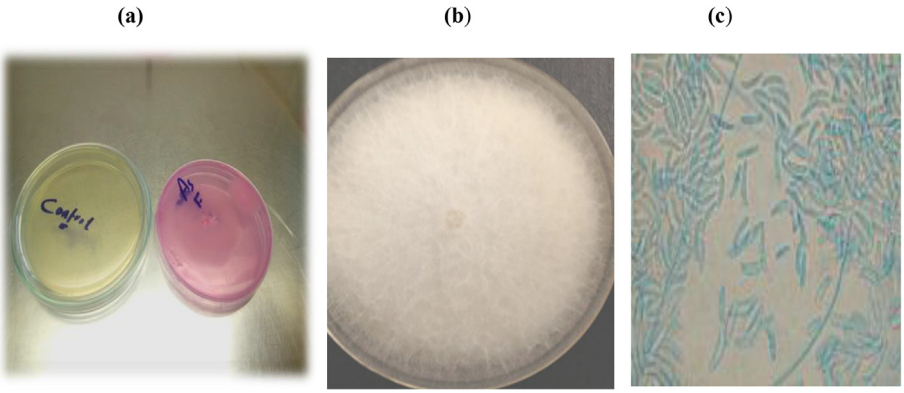

Fig. 1. Qualitative production of L-asparaginase enzyme (a), cultural (b) and microscopic characteristics of AHMF4 isolate (c) on modified Czapex's Dox agar medium.

Table 1
Chemotypic and phenotypic characteristics of AHMF4 isolate.

Chemotypic characteristics/growth period (day)					Macro/microscopic characteristics
Fatty acid composition (%, w/w)	4	7	10	14	
14:0	-	-	0.32 ± 0.01	0.47 ± 0.02	Colonies growing with diameter 5.1 cm on PDA after five days, aerial mycelium appeared white turned to brownish white, reverse pale, sporodochia present, branched conidiophores scattered in the aerial mycelium. Macroconidia are formed from conidiophores, three-septate, 29–46 × 3–5 μm, cylindrical, curved, with foot cell. Microconidia are abundant, ellipsoidal, one celled, curved, and formed from long lateral phialides, 9 × 3.0 μm. Chlamydo spores were hyaline, globose, rough-walled, borne in pairs on short lateral hyphal branched or intercalary, 5–8 μm.
15:0	0.20 ± 0.13	0.92 ± 0.14	1.16 ± 0. 12	0.88 ± 0.14	
16:0	5.51 ± 0.21	6.11± 0.30	8.00 ± 0.40	2.41 ± 0.45	
16:1	-	1.51± 0.02	2.19 ± 0.02	1.21 ± 0.01	
16:2	0.11 ± 0.0	0.30 ± 0.00	0.71 ± 0.01	-	
17:0	0.80 ± 0.01	1.93 ± 0.01	2.06 ± 0.02	-	
17:2	0.16 ± 0.01	1.59 ± 0.05	1.90 ± 0.06	1.00 ± 0.04	
18:0	8.10 ± 0.1	10.60 ± 0.18	14.00 ± 0.22	10.42 ± 0.40	
18:1	10.00 ±1.05	19.53 ±1.08	22.86 ± 1.10	18.71 ± 1.06	
18:2	16.77 ± 0.30	22.04 ± 0.8	28.15 ± 1.18	18.06 ±1.09	
18:3	4.18 ± 0.12	5.15 ± 0.18	6.01 ± 0.25	4.91± 0.21	
20:4	-	-	0.52 ± 0.01	0.50 ± 0.01	
22:5	0.19 ± 0.1	0.74 ± 0.09	0.77 ± 0.13	0.33 ± 0.11	
22:6	-	1.12 ± 0.2	1.00 ± 0.1	-	
24:0	0.25 ± 0.01	0.29 ± 0.01	1.00 ± 0.02	-	
26:0	-	0.50 ± 0.00	0.10 ± 0.00	-	
18:1 ω9c	-	1.31 ± 0.02	1.40 ± 0.03	1.00 ± 0.02	
18:2 ω6c	3.51 ± 0.00	7.75 ± 0.01	7.75 ± 0.02	6.44 ± 0.01	

18S rDNA region sequence from isolate AHMF4 was amplified (with primers NS1 and NS8) and resulted in 1636 bp of fragment as well as sequenced and submitted to GenBank (accession number: MN094111). The achieved sequence of strain AHMF4 had a significant identity to family *Nectriaceae* and genus *Fusarium.* By comparing the producing fungi AHMF4 to the reference members of *Fusarium* sequences enclosed in GenBank, it showed a similarity equal to 99.94% with *Fusarium equiseti* strain Salicorn 8. Fig. 2 clarifies the phylogenetic analysis attained through the neighbor-joining protocol. Morphological and chemotaxonomy characteristics along with phylogenetic analysis of strain AHMF4 were agreed well with the description of *Fusarium* species and then it was identified as *Fusarium equiseti* AHMF4 according to a previous taxonomic protocols (Dugan, 2006; El-Bondkly, 2012; El-Gendy et al., 2017, 2018; Leslie and Summerell, 2006; Pierozzi et al., 2020; Prakash et al., 2017; Ruiz et al., 2007; Salah et al., 2015; Watanabe, 2010).

3.3. Improvement of extracellular L-asparaginase production process parameters under submerged fermentation by F. equiseti AHMF4

L-Asparaginase formation was gradually improved with increasing the growth period to reach a peak (12.57 U mL^{-1}) after a week and then reduced to 8.77 and 6.68 U mL^{-1} after 10 and 13 days of fermentation, respectively (Table 2). Our results are in line with Bedaiwy (2019) on optimizing L-asparaginase of *Pleurotus ostreatus* but the preeminent L-asparaginase productivity of *Fusarium oxysporum* and *Fusarium solani* AUMC 8615 was occurred at the 5th day of incubation period (Isaac and Abu-Tahon, 2016; Yadav and Sarkar, 2014) but *Trichosporon asahii* IBBLA1 secreted the maximum yield of enzyme (20.57 U mL^{-1}) after 60 h (Ashok et al., 2019).

The incubation temperature affects growth, metabolic activity and production of enzyme. When *F. equiseti* AHMF4 was inoculated at different temperatures (20, 27, 30, and 37 °C) in Czapek's Dox medium, it showed maximum yield of extracellular L-asparaginase (18.95 U mL^{-1}) at 30 °C and then decreased to 7.52 U mL^{-1} at 37 °C (Table 2) that may be attributed to incorrect conformation of enzyme molecules owing to the denaturation of mesophilic enzymes at higher temperatures (Ashok et al., 2019). In line with our results *F. culmorum*, *F. solani* AUMC 8615, *F. brachygibbosum*, *Penicillium* sp., *Aspergillus terreus*, *Mucor hiemalis* and *Emericella nidulans*, *T. asahii* IBBLA1 showed maximum activity of L- asparaginase at 30 °C (Ashok et al., 2019; Isaac and Abu-Tahon, 2016; Meghavarnam and Janakiraman, 2015; Prihanto et al., 2019). Conversely, maximum L-asparaginase yields from *Aspergillus fumigatus* and *A. terreus* CCT 7693 were observed at 40 °C and 34.6 °C, respectively (Bchamin et al., 2019; Costa-Silva et al., 2019).

As shown in Table 2, the initial pH values affected the peak of L-asparaginase productivity. The highest yield of enzyme (19.25 U

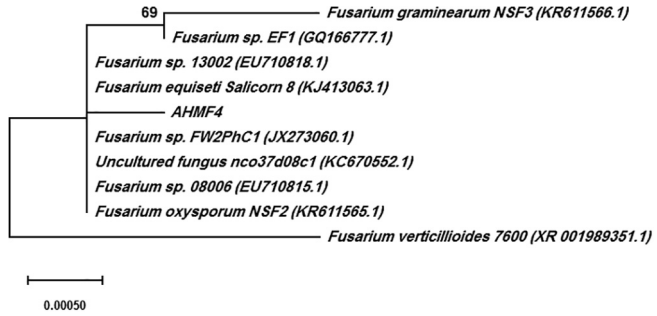

Fig. 2. Phylogenetic tree of 18S rDNA sequences performed by the neighbor joining system for fungal strain AHMF4 and similar fungi.

mL^{-1}) was found at pH 7.0 but in acidity equal to pH 5.0 or alkalinity equivalent to pH 10.0, it decreased to 8.15 and 6.78 U mL^{-1}, respectively. pH is the most essential parameter control the enzyme secretions due to microbes are sensitive to the concentrations of hydrogen ions present in the fermentation process (El-Bondkly and El-Gendy, 2012; El-Gendy, 2010, 2012; El-Gendy and El-Bondkly, 2014; El-Gendy et al., 2015, 2017, 2018). L-Asparaginase from *A. terreus* CCT 7693, *A. fumigatus*, *F. solani* AUMC 8615, *T. asahii* IBBLA1, *F. culmorum*, *F. brachygibbosum* and *F. oxysporum* exhibited the highest enzyme production (13.81, 23.83, 20.57, 0.40, 0.34, and 105.0 U mL^{-1}) at pH 9.49, 8.0, 7.0, 7.5, 6.0, and 5.0 (Ashok et al., 2019; Bchamin et al., 2019; Costa-Silva et al., 2019; Isaac and Abu-Tahon, 2016; Meghavarnam and Janakiraman, 2015). Presumably, extreme pH cause irreversible changes in enzyme through damaging specific amino acid at the active sites at higher pH in addition to hydrolysis of peptide bonds at lower pH (El-Gendy et al., 2015; Pallem, 2019b).

According to data in Table 2 better yields of extracellular L-asparaginase were achieved with the addition of glucose followed by sucrose and fructose (22.54, 19.76 and 15.74 U mL^{-1}, respectively) but starch repressed L-asparaginase production (7.89 U mL^{-1}). However, glucose served as the favorable carbon source for *A. terreus* CCT 7693 L-asparaginase (Costa-Silva et al., 2019), *F. equiseti*, *Fusarium semitectum* and *A. niger* (Muhammad et al., 2013), *Aspergillus* sp. ALAA-2000 (El-Gendy et al., 2015), *F. solani* AUMC 8615 (Isaac and Abu-Tahon, 2016) and *T. asahii* IBBLA1 (Ashok et al., 2019) that could be attributed to the fungi prefer simple sugars rather than complex sugars for growth and production of many metabolites but Yadav and Sarkar (2014) supported sucrose as the best inducer for production by *F. oxysporum* after that dextrose but lactose supported intermediate activity and corn husk was the potent inducer for L-asparaginase synthesis by *F. oxysporum* (Pallem, 2019b).

Data in Table 2 presented the influence of nitrogen sources include sodium nitrate, tryptone, yeast extract, peptone, malt extract and beef extract on enzyme productivity. They resulted in yielding 19.54, 15.73, 14.07, 13.52, 13.33 and 12.08 U mL^{-1} of extracellular L-asparaginase, respectively. In line with our results L-asparaginases production by *Aspergillus* sp. ALAA-2000, *A. fumigatus* and endophytic *F. oxysporum* was improved with asparagine as sole nitrogen source (El-Gendy et al., 2015; Prihanto et al., 2019; Sreejai et al., 2019). Interestingly, the highest enzyme productivity by *T. asahii* IBBLA1 (20.57 U mL^{-1}) was achieved with asparagine at a concentration of 1.0% (Ashok et al., 2019). However, study of Yadav and Sarkar (2014) demonstrated no substantial variance between the type of nitrogen sources and L-asparaginase yield obtained from *F. oxyporum* but ammonium sulphate and sodium nitrate were the best inducers for its production by *F. oxysporum*, *F. semitectum*, *F. equiseti* and *Penicillium* sp. (Hosamani and Kaliwal, 2011; Meghavarnam and Janakiraman, 2015).

Table 2
Optimization of L-asparaginase production process parameters under submerged fermentation by *Fusarium equiseti* AHMF4.

Process parameter	L-Asparaginase activity (U mL^{-1})
Incubation period (day)	
1	2.26
4	8.73
7	12.57
10	8.77
13	6.68
Incubation temperature (°C)	
20	8.57
27	12.11
30	18.95
37	7.52
pH Value	
5	8.15
6	14.05
7	19.25
8	13.38
9	8.44
10	6.78
Carbon source (1.0%)	
Starch	7.89
Lactose	14.22
Sorbitol	14.22
Fructose	15.74
Sucrose	19.76
Glucose	22.54
Without carbon source	0.31
Nitrogen source (0.2%)	
Asparagine (control)	31.01
Malt extract	12.08
Beef extract	13.33
Peptone	13.52
Yeast extract	14.07
Sodium nitrate	19.54
Tryptone	15.73
Without nitrogen source	1.00
Elements supplementation (0.1%)	
$BaCl_2$	29.63
NaCl	31.56
$MgCl_2$	31.62
KH_2PO_4	37.52
$CaCl_2$	19.95
Amino acids (0.2%)	
Glycine	34.80
Alanine	40.78
Methionine	29.66

Metal ions involved in the catalytic processes of such enzyme by serving as electron donor or acceptor thus affect the enzyme activity and stabilization. Compared to the control (22.54 U mL^{-1}) the productivity of extracellular L-asparaginase increased 31.46%, 40.03%, 40.27% and 66.46%, respectively in medium supplemented with 0.1% of $BaCl_2$, NaCl, $MgCl_2$ and K_2HPO_4 individually, respectively but $CaCl_2$ reduced its production by 11.51%.

Bchamin et al. (2019) stated that maximum L-asparaginase productivity from *A. fumigates* was noticed in the presence of Fe^{2+} which was 32.26 U mL^{-1} and the lowest activity was noticed in presence of K^+ which was 2.93 U mL^{-1} whereas Meghavarnam and Janakiraman (2015) recorded that $CaCl_2$ improved L-asparaginase productivity by *F. culmorum* to 1.7-fold but $MnSO_4$ and $CuSO_4$ did not show any effect. Moreover, supplementation with (0.5% w/v) ammonium sulphate supported the highest enzyme productivity from *F. solani* AUMC 8615 (Isaac and Abu-Tahon, 2016).

Amino acid supplement alanine followed by glycine and asparagine exhibited favored effect on extracellular L-asparaginase production (40.78, 34.80 and 31.01 U mL^{-1}) but methionine showed negative effect (29.66 U mL^{-1}). In previous studies glutamine and arginine improved the biosynthesis of L-asparaginase from *F. cul-*

morum and *F. brachygibbosum* while both lysine and glycine inhibited its formation in these species (Meghavarnam and Janakiraman, 2015). Moreover L-asparaginase biosynthesis was improved by 15% with arginine but strongly inhibited by 69% and 72% with isoleucine and cysteine, respectively in *Aspergillus* sp. ALAA-2000 (El-Gendy et al., 2015) but its formation by *A. terreus* CCT 7693 was maximized in Czapek's Dox medium with L-proline (10 gL^{-1}) under SMF (Costa-Silva et al., 2019).

3.4. Purification of L-asparaginase from F. equiseti AHMF4 strain

Extracellular L-asparaginase from *F. equiseti* AHMF4 was purified from liquid culture by successive steps including $(NH_4)_2SO_4$ fractionation (80% saturation), QFF chromatography and Sephacryl S-200 chromatography. Purification steps are potted in Table 3. The purification process yielded 2.67-fold purification, 48% L-asparaginase recovery with final specific activity equal to 488.1 (U mg^{-1}). Marine *A. terreus* L-asparaginase was purified by $(NH_4)_2SO_4$ (65%) precipitation, Sephadex G-100 filtration and DEAE-cellulose to yield 11.96 purification fold (Hassan et al., 2018). Moreover, *A. oryzae* CCT 3940 L-asparaginase was purified to 28.6 purification folds, 6% yield and 282 IU mL^{-1} specific activity after sulfate fractionation, Q Sepharose™, SP Sepharose™ and CM Sepharose™ column chromatography (Dias et al., 2016). The molecular weight of pure enzyme based on SDS polyacrylamide gel electrophoresis determined to be 45.7 kDa (Fig. 3). L-asparaginase obtained using *F. solani* AUMC 8615, *F. culmorum* ASP-87, *A. niger*, *M. hiemalis*, *Aspergillus* sp. ALAA-2000 (AYA-1 and AYA-2), *A. oryzae* CCT 3940 and *A. terreus* was found to have molecular weights (subunits of molecular weights 70 and 80 kDa), 90, 33, 96.32, (25 and 31 kDa), 115, 50.6 and 85 kDa, respectively according to SDS-PAGE analysis (Isaac and Abu-Tahon, 2016; Meghavarnam and Janakiraman, 2015; OM et al., 2014; El-Gendy et al., 2015; Dias et al., 2016; Hassan et al., 2018).

3.5. Characterization of purified enzyme

Whereas AHMF4 L-asparaginase was highly active at 30 °C and retained its total activity (100%) at temperature ranged from 20 to 40 °C for 60 min, its activity intensely lost by 38%, 45% and 71% at 50, 60 and 70 °C, correspondingly (Fig. 4). In earlier reports, the highest L-asparaginase activity along with stability were estimated at 30 °C, 30 to 50 °C, 30 to 70 °C, 37 °C, 40 to 70 °C, 45 to 50 °C from A. *niger* (Dange and Peshwe, 2015), *Aspergillus* sp. ALAA-2000 (El-Gendy et al., 2015), *M. hiemalis* (Thakur et al., 2014), *A. terreus* (Hassan et al., 2018) and *A. oryzae* CCT 3940 (Dias et al., 2016).

Furthermore, purified AHMF4 L-asparaginase exerted the best activity and pH stability (100%) neutral pH 7.0 and then at higher or lower pHs the enzyme activity and stability were steadily reduced (Fig. 5). In the same way, the optimal activity of *M. hiemalis* and AYA 20-1 fusant was obtained at pH 7.0 (El-Gendy et al., 2017; Thakur et al., 2014). *Aspergillus oryzae* CCT 3940 L-asparaginase was more active with pH 7.5–8.0 (Dias et al., 2016) but the suitable pH for the purified enzyme from AYA 20-1 fusant, *A. terreus* and *Aspergillus* sp. ALAA-2000 was estimated to be pH 5.0–8.0 (El-Gendy et al., 2015, 2017). Moreover Isaac and Abu-Tahon (2016) reported that 37 °C was the optimum reaction temperature for enzyme activity produced by *F. solani* AUMC 8615 but its stability was decreases to 35% after storing the enzyme for 2 h at 37 °C but optimum pH value was equal to pH 8.0. L-Asparaginase activity after incubation with K^+ and Mg^{2+} at a concentration of 50 mM was increased by 5.8% and 43.3%, respectively but Ca^{2+} and Na^+ at 50 mM reduced its activity to 77.5% and 99.1%, respectively (Table 4). Furthermore Ba^{2+} at a concentration of 10 mM resulted in enhancement enzyme activity via 22.5% but Mn^{2+} and Cu^{2+} exhibited no effect. Previous reports showed that L-asparaginase acted in a different ways with chemical substances. For instant, Cu^{2+} and Mg^{2+} inhibited L-asparaginase activity achieved from *Aspergillus aculeatus* (Dange and Peshwe, 2015). Conversely, 10 mM of Ba^{2+} repressed enzymatic activity of the fusing strain AYA 20-1 while Mn^{2+}, Cu^{2+} and Na^+ improved the activity to 103%, 106% and 122%, respectively (El-Gendy et al., 2017). Moreover, $MgSO_4$ and $MnSO_4$ at 5 mmolL^{-1} increased *Aspergillus oryzae* L-asparaginase activity by 33% and 49%, respectively but Na_2SO_4, $(NH_4)_2SO_4$, KCl, $CaCO_3$ and NaCl (5 mmol L^{-1}) showed no effect on the purified enzyme (Dias et al., 2016). Also in disagreement with our results, the presence of NaCl highly enhanced L-asparaginase activity from *A. terreus* (Hassan et al., 2018). However, enzymes are categorized into four modes of actions: (1) aspartic protease; (2) serine protease; (3) cysteine protease and (4) metalloprotease. Depending on data in Table 4 aspartic proteases inhibitor (pepstatin A), metallo proteases inhibitor (EDTA; EGTA and 1, 10-phenanthroline) at a concentration of 5 mM increased L-asparaginase activity by 21.92%, 30.42%, 23.71% and 16%. Cysteine protease inhibitor at a concentration of 1, 5 and 15 mM include bADA, E-64 and 2-iodoacetamide had no effect on AHMF4 L-asparaginase activity. However, it was serine protease enzyme due to its activity totally inhibited by serine proteases inhibitor (phenylmethylsulfonyl fluoride, PMSF; Benzamidine at a concentration of 10 to 15 mM and Nα-Tosyl-L-lysine chloromethylketone, TLCK) at concentration of 5.0–15.0 mM. Likewise, L-asparaginase of *Aspergillus* sp. ALAA-2000, fusant AYA 20-1 and *M. hiemalis,* were not metaloproteases (El-Gendy et al., 2015, 2017; Thakur et al., 2014). Conversely *A. oryzae* CCT 3940 L-asparaginase repressed by 53% and 45%, respectively with iodoacetamide and p-chloromercuribenzoate (Dias et al., 2016).

3.6. Assessment of anti-proliferative activity of L-asparaginase against some human carcinomas cell lines

Totally inhibition in the proliferation of Hela, Hep-2, HepG-2 and HCT-116, along with stimulation of 100% death was noticed when they treated with 10, 20, 60 and 80 μg mL^{-1} of L-asparaginase with IC_{50} equal to 2.0, 5.0, 12.40 and 8.26 μg mL^{-1}, respectively (Fig. 6). Moreover breast carcinoma cells (MCF-7) were more resist to the cytotoxic power of L-asparaginase treatments. It retained 91%, 80%, 55%, 42%, 30%, 27% and 25% of its viability after treatment with 5, 10, 20, 40, 60, 80 and 100 μg mL^{-1} of L-asparaginase, respectively (IC_{50} 22.8). Interestingly L-asparaginase showed weak cytotoxicity against the human normal cells WI-38, which indicates that it has selective toxicity (Fig. 6). In a previous studies, fungal L-asparaginase enzyme repressed different malignant cell lines like HepG-2, MCF-7, HCT-116 and A-549 (Bchamin et al., 2019; El-Gendy et al., 2017, 2018). Our findings

Table 3
Purification stages of L-asparaginase yielded from *Fusarium equiseti* AHMF4 strain.

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U mg^{-1})	Yield (%)	Purification fold
Crude extract	104	19,000	182.6	100	1.00
$(NH4)_2SO_4$ precipitation (80%)	53	14,300	269.8	75	1.47
Anion exchange QFF	32	12,100	378.1	63	2.07
Gel filtration sephacryl-200	19	9275	488.1	48	2.67

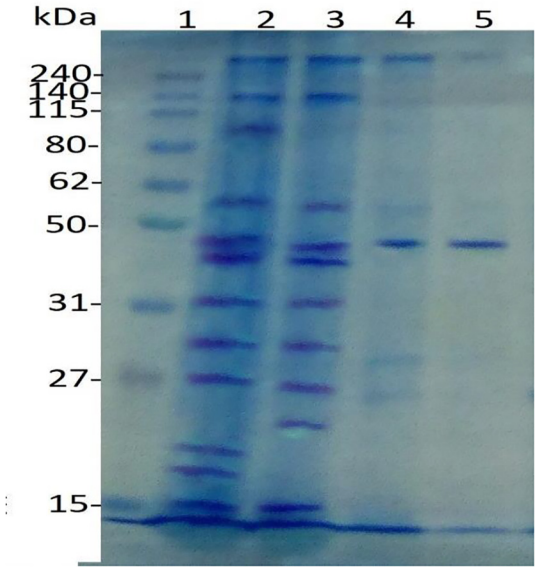

Fig. 3. SDS-PAGE analysis of L-asparaginase during the purification stages. lane 1, molecular weight marker; lane 2, crude enzyme; lane 3, $(NH_4)_2SO_4$ precipitation; lane 4, ion exchange chromatography on anion QFF; lane 5, purified L-asparaginase on sephadex G-200.

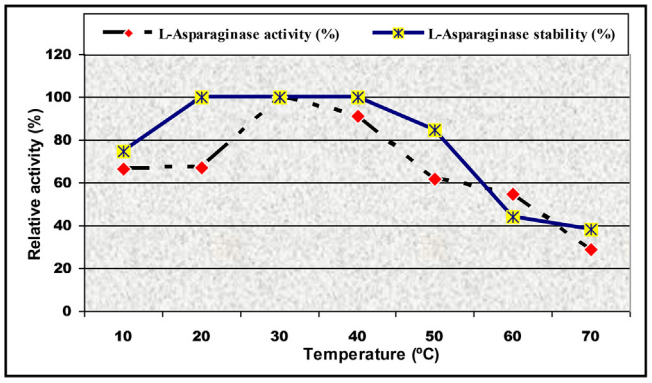

Fig. 4. Effect of temperature on the activity and stability of purified L-asparaginase from *Fusarium equiseti* AHMF4.

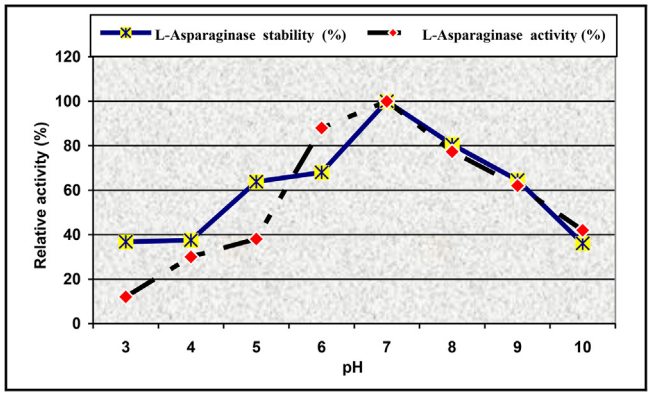

Fig. 5. Effect of pH on the activity and stability of purified L-asparaginase from *Fusarium equiseti* AHMF4.

were in agreement with El-Gendy et al. (2017), who proed that, HCT-116 and HepG-2 viabilities were repressed by 80% and 100% after treatment with purified L-asparaginase from AYA 20-1 fusant

Table 4
Effect of metal ions and enzyme inhibitors on purified L-asparaginase activity.

Metal ion and enzyme inhibitor	Concentration (mM)	L-Asparaginase relative activity (%)*
Control	–	100.0
K^+	50	105.8
Na^+	50	99.1
Mg^{2+}	50	143.3
Ca^{2+}	50	77.5
Ba^{2+}	10	122.5
Cu^{2+}	10	100.0
Mn^{2+}	10	100.0
Fe^{3+}	10	100.0
Aspartic proteases inhibitor		
Pepstatin A	5	121.9
	10	107.1
	15	100.0
Serine proteases inhibitor		
PMSF	5	20.1
	10	0.0
	15	0.0
Benzamidine	5	12.15
	10	0.0
	15	0.0
TLCK	5	0.0
	10	0.0
	15	0.0
Metallo proteases inhibitor		
EDTA	5	130.4
	10	121.8
	50	102.4
EGTA	5	123.7
	10	119.0
	50	104.4
1, 10-Phenanthroline	5	116.5
	10	107.9
	50	100.0
Cysteine protease inhibitor		
bADA	5	100.0
	10	100.0
	15	100.0
E-64	5	100.0
	10	100.0
	15	100.0
2-Iodoacetamide	5	100.0
	10	100.0
	15	100.0

*The activity of L-asparaginase without any chemical additives defined as 100%.

at 20 μg mL^{-1} and Hassan et al. (2018) who mentioned *A. terreus* L-asparaginase gave antitumor capability toward HCT-116, HepG-2 and MCF-7 cells with IC_{50} reached 3.79–12.6 μg mL^{-1}. Interestingly the pure *A. oryzae* CCT 3940 L-asparaginase wholly repressed the cell propagation of a wide range of tumor cells include UACC-62 (melanoma), 786-0 (kidney), NCI-H40 (lung), PC-3 (prostate), NCI-ADR/RES (ovary) and K562 (leukemia) cells (Dias et al., 2016). Moreover, L-asparaginase of *A. fumigatus* exhibited potent anti-proliferative ability toward breast carcinoma, MDA-MB-231, with cell death equal to 71%, 87.7% and 96.5% when 5, 10 and 20 U of L-asparaginase used respectively (Bchamin et al., 2019).

3.7. DPPH scavenging activity of L-asparaginase

Regardless of secondary metabolites obtained from fungi are valued sources of antioxidants, insufficient attentions objected their enzymes like L-asparaginase as antioxidant agents. Data in Fig. 7 indicated that L- asparaginase has shown effective dose-dependent DPPH radical scavenging activity. The IC_{50} of L-ascorbic acid (standard) and L-asparaginase were 36.69 and

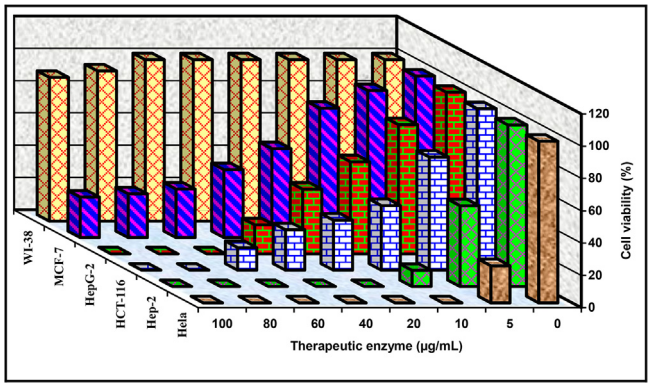

Fig. 6. Effect of purified L-asparaginase from *Fusarium equiseti* AHMF4 on colorectal carcinoma (HCT-116), liver carcinoma (HepG-2), breast carcinoma (MCF-7), cervical epitheloid carcinoma (HeLa) and epidermoid larynx carcinoma (Hep-2) compared with normal cells (WI-38).

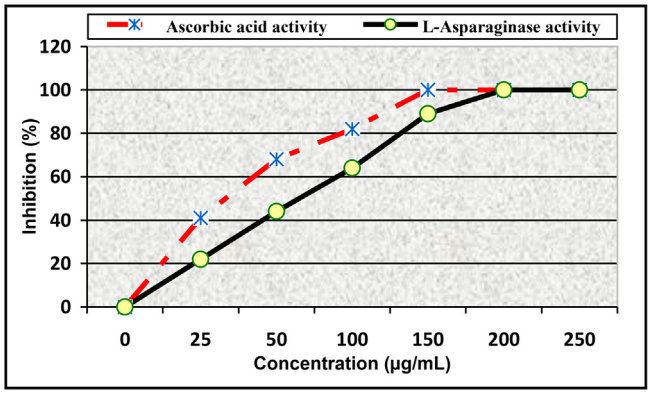

Fig. 7. DPPH scavenging activity of the purified L-asparaginase from *Fusarium equiseti* AHMF4 compared to ascorbic acid.

69.12 µg mL^{-1}, respectively. Also, L-asparaginase from fungal strains include *A. fumigatus, A. flavus* (KUFS 20), *M. hiemalis, T. viride*, *A. tamari,* AYA 20-1 fusant and mangrove *Aspergillus* sp. demonstrated respectable antioxidant/scavenging activity in dose-dependent manner (El-Gendy et al., 2017; Prihanto et al., 2019; Rani et al., 2011; Sreejai et al., 2019; Thakur et al., 2014).

4. Conclusion

Soil fungus, *F. equiseti* AHMF4 proved to be a fruitful producer for L-asparaginase under SMF. The enzyme production in SMF was maximized to reach 40.78 U mL^{-1} at the 7th day of fermentation with initial pH 7.0, incubation temperature 30 °C, 1.0% glucose as carbon source, 0.2% asparagine as nitrogen source, 0.1% alanine as amino acid supplement and 0.1% KH_2PO_4. The purification of AHMF4 L-asparaginase yielded 2.67-fold purification and 48% recovery with final specific activity of 488.1 U mg^{-1} of protein. Pure L-asparaginase was characterized as serine protease enzyme with molecular weight of 45.7 kDa beside stability at neutral pH and between 20 and 40 °C. *Fusarium equiseti* AHMF4 L-asparaginase exhibited powerful antioxidant activity in dose-dependent manner with IC_{50} 69.12 µg mL^{-1}. Total inhibition in the proliferation of Hela, Hep-2, HepG-2 and HCT-116 was noticed when they treated with 10, 20, 60 and 80 µg mL^{-1} of L-asparaginase with IC_{50} equal to 2.0, 5.0, 12.40 and 8.26 µg mL^{-1}, respectively. Breast carcinoma cells (MCF-7) were more resist to the cytotoxic power of L-asparaginase treatments. It retained 25% of its viability after treatment with 100 µg mL^{-1} of L-asparaginase but IC_{50} was 22.8 µg mL^{-1}. Interestingly L-asparaginase showed weak cytotoxicity against the human normal cells WI-38, which indicates that it has selective toxicity. Then *Fusarium equiseti* AHMF4 could be alternative L-asparaginase source for bacteria to solve the problems of lower productivity along with high toxicity, lower selectivity and stability.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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