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Research Article

PURIFICATION, CHARACTERIZATION AND EVALUATION OF L-ASPARAGINASE FROM ENDOPHYTIC FUNGI *FUSARIUM* SP. LCJ273 FOR ITS ANTICANCER PROPERTY

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ABSTRACT

L-Asparaginase (L-asparagine amido-hydrolase, EC 3.5.1.1) is an industrially and pharmaceutically important enzyme that catalyses the hydrolysis of asparagine into aspartic acid and ammonia. In this study, L-asparaginase was produced from *Fusarium* sp. LCJ273. The crude L-asparaginase produced was precipitated using ammonium sulphate precipitation followed by purification using ion exchange chromatography. The molecular weight of L-asparaginase was found to be approximately 66 kDa by SDS-PAGE. The culture filtrate, ammonium sulphate precipitate and partially purified L-asparaginase exhibited optimum activity in pH 7.0 at 30°C. The partially purified L-asparaginase from *Fusarium* sp. LCJ273 showed 100% relative activity towards the natural substrate L-asparagine. Further, the kinetics studies of partially purified L-asparaginase revealed that K_m value was 24.2 mM and V_{max} value was 125 mM min^{-1}. The *in vitro* anticancer activity of partially purified L-asparaginase was investigated. Different concentration of partially purified enzyme was tested on three different cell lines namely MCF-7 (breast cancer cell line), Hep-2 (human laryngeal carcinoma cells) and vero cell lines by MTT assay (3-4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide). Purified L-asparaginase reduced cell viability of MCF-7 (16.61%), Hep-2 (8.12%) and vero cell line (88.73%) after 72 hours with IC_{50} value of 33.21, 9.28 and 622.99 µg/mL respectively and showed better toxicity on the cell lines at a concentration of 120 µg/mL.

Keywords: Endophytic fungi, L-Asparaginase, SDS-PAGE, DEAE-cellulose, *Fusarium* sp. LCJ273

1. INTRODUCTION

L-Asparaginase is one of the potentially important therapeutic enzymes of interest which accounts for about 40% of the total worldwide enzymes are sold as antileukemic agents [1-3]. L-Asparaginase has great demand in medical application and food industries to decrease the formation of carcinogenic acrylamides in potato chips and biscuits [4-6]. Therapeutic L-asparaginase demand is increasing every year. L-Asparaginase catalyses the hydrolysis of L-asparagine into aspartic acid and ammonia [7]. Tumor cells require L-asparagine for their development. L-Asparaginase reduces the L-asparagine present in blood and secondarily in the starving cancer cells resulting in significant lymphatic cell death [8, 9]. L-Asparaginase is widely obtained from animal, plant and microorganisms such as bacteria, filamentous fungi, yeast and actinomycetes [10, 11]. However, L-asparaginase production from microorganisms is preferred because they can be easily cultured and conditions for maximizing enzyme production can be easily optimized. In addition, purified enzyme can be obtained in large volumes [12, 13]. L-Asparaginase production from bacteria exhibited minimum enzyme activity and also showed difficulty in extraction. There also had allergic effects due to long-term administration resulting in anaphylaxis, allergic reactions, hypersensitivity and other toxic reactions [14]. L-Asparaginase from fungi such as *Aspergillus terreus* [15], *Aspergillus niger* [16], *Aspergillus oryzae* [17], *Cladosporium* sp. [18], *Penicillium* and *Fusarium* [19-22] is found to be an alternative source and also show higher activity. Purification of L-asparaginase is very important to study its functional properties. In addition, enzyme characterization is essential to know the properties of enzymes such as pH, temperature, substrate specificity and kinetic parameters [23-25]. The purification, characterization of purified L-asparaginase, kinetic properties of L-asparaginase and antitumor activity of purified L-asparaginase from *Fusarium* sp. LCJ273 on MCF-7, Hep-2 and vero cell line for further

pharmaceutical applications is reported in this paper.

2. MATERIAL AND METHODS

2.1. L-Asparaginase production under submerged fermentation

Modified Czapek Dox Broth (MCDB) was used for the production of L-asparaginase. A 5mm disc of actively growing *Fusarium* sp. LCJ273 was added to a 100 mL of MCDB. The constituents of MCDB were (g/L) Dextrose-2.0, L-asparagine-10.0, KH_2PO_4-1.52, KCl-0.52, $MgSO_4.7H_2O$-0.52, $CuNO_3.3H_2O$-trace, $ZnSO_4.7H_2O$-trace and $FeSO_4.7H_2O$-trace. The mixture was incubated under shaking conditions at 30°C and 120 rpm for 5 days. After incubation the broth was collected and centrifuged to remove the mycelia. Broth supernatant was used to determine the activity of L-asparaginase [26].

2.2. L-Asparaginase purification

Five-day old culture of *Fusarium* sp. LCJ273 was cultured under optimized conditions for production of L-asparaginase. The culture was filtered through cheese cloth to remove the mycelium. 1000 mL culture filtrate was then centrifuged (10,000 rpm for 15 min) at 4°C and again filtered with Whatman No. 1 filter paper.

2.3. Ammonium sulphate precipitation

The *Fusarium* sp. LCJ273 culture was centrifuged and the supernatant was used as crude enzyme source for purification of the enzyme. L-Asparaginase from crude extracts was exposed to ammonium sulphate precipitation at 80% saturation was allowed to stand overnight. The precipitate was collected by centrifugation at 10,000 rpm for 15 min and again resuspended in 50 mM Tris-HC1 buffer (pH 8.5). Dialyzed extensively against same buffer and kept at 4°C overnight.

2.4. Ion exchange column Chromatography

The dialyzed enzyme was loaded on to a DEAE-Cellulose ion exchange column, which was pre-equilibrated, washed with 50 mM Tris-HCl buffer (pH 8.5) and unbound proteins were washed repeatedly. After 30 minutes, sample was eluted with using 0.2 M, 0.4 M, 0.6 M, 0.8 M and 1.0 M NaCl in the same buffer at regular time intervals at a flow rate of 1mL/min. 5 mL fractions were collected and evaluated for L-asparaginase activity. The active fractions of the L-asparaginase were pooled, concentrated and stored in freezer for further characterization. The eluted sample was used for the characterization of protein by SDS PAGE. The total specific activity, yield and purification fold was calculated based on the method described by Nelson and Cox [27].

2.5. Molecular mass determination of L-asparaginase

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out following the method described by Laemmli [28] using a 10% w/v resolving gel and 5% w/v stacking gel. After electrophoresis, Coomassie Brilliant Blue (CBB) R250 was used to stain the protein bands. The stained gel was destained with destaining solution (Methanol 50 mL, Glacial acetic acid 7.0 mL and Distilled water 43 mL) and washed with distilled water. The proteins fractionated into bands were observed and their molecular weight was determined using standard protein marker.

2.6. Influence of physico-chemical properties on L-asparaginase activity

2.6.1. Influence of pH on L-asparaginase activity

The effect of pH on partially purified L-asparaginase, ammonium sulphate precipitate and crude filtrate was investigated by maintaining different pH ranging from (4.0-10). The partially purified L-asparaginase (10 µg) was pre-incubated for 30 minutes in various buffer system such as sodium phosphate citrate buffer (50 mM; pH 4.0 - 6.0), Tris-HCl buffer (50 mM; pH 7.0 - 9.0) and glycine-NaOH buffer (50 mM; pH 10.0). In the reaction mixture substrate was added and evaluated for L-asparaginase activity. Similarly, L-asparaginase present in ammonium sulphate precipitate and culture filtrate were also assayed at different pH.

2.6.2. Influence of temperature on L-asparaginase activity and thermal stability

The optimum temperature and stability pattern of the L-asparaginase activity was determined by dissolving the partially purified L-asparaginase (10 µg) in 50 mM Tris-HCl buffer (7.0) for *Fusarium* sp. LCJ273 was studied at various temperature ranging from 20°C to 45°C for 30 minutes and activity of L-asparaginase was determined. Ammonium sulphate precipitate and culture filtrate were also examined for L-asparaginase activity. The thermal stability of the enzyme was determined by incubating the partially purified enzyme for 1 h at various temperatures (20°C to 45°C). The L-asparaginase activity was estimated by performing the standard assay conditions.

2.7. Substrate specificity

Substrate specificity of the partially purified L-asparaginase enzyme, ammonium sulphate precipitate and culture filtrate were studied using different substrates such as L-asparagine, glutamine and aspartic acid at a concentration of 5.0 mM. The substrates mixed with 10 µg of partially purified enzyme, ammonium sulphate precipitate and culture filtrate. The reaction mixture was incubated for 30 minutes at 30°C and L-asparaginase activity was determined. The results were expressed as the relative percentage of the enzyme activity observed.

2.8. Kinetic constants of partially purified L-asparaginase

Effect of substrate concentration and determination of K_m and V_{max} value of partially purified L-asparaginase enzyme were calculated by the method of Lineweaver and Burk, (1934) [29] with various concentrations (1.0 mM-5.0 mM) of L-asparagine. L-Asparagine was dissolved in 0.05 M Tris-HCl buffer (pH 7.0). The relationship between the substrate concentration and activity of L-asparaginase was examined by the curve expressing the Michaelis-Menten pattern. L-Asparaginase was estimated by standard assay conditions. Microsoft Excel was used to calculate linear regression for estimating V_{max} and K_m. The maximum velocity, Michaelis constant was calculated based on the method described by Nelson and Cox. The formula used is mentioned below:

$$1/V = (K_m/V_{max})\ 1/S + (1/V_{max})$$

V_{max} (Maximum velocity); The reciprocal of the intercept on the positive Y-axis is the V_{max} (mM minute^{-1}); K_m (Michaelis constant); The reciprocal of the intercept on the negative X-axis is the K_m (mM).

2.9. Anti-cancer activity of L-asparaginase by MTT assay

The anti-proliferative activity of the purified L-asparaginase enzyme on human cell lines such as MCF-7 (breast cancer cell line), Hep-2 (human laryngeal cells), and vero cell line was assessed by the reduction of yellow MTT [3- (4, 5-dimethyl thiazol-2-yl) -2, 5-diphenyl tetrazolium bromide] to purple formazan. The cells were maintained in respective medium supplemented with 10% Fetal bovine serum, Penicillin (100 U/mL) and Streptomycin (100 µg/mL) in a humidified atmosphere of 5% CO_2 at 37°C. The cells were plated in 100 µl of medium in 96-well plates, the cell line such as MCF-7, Hep-2 and vero were exposed to different concentration of L-asparaginase ranging from 20, 40, 60, 80, 100 and 120 µg/mL. After incubation for 72 hours, the culture medium was removed and 150 µl of fresh culture medium and 50 µl of MTT were added to all the wells. The pellets in aluminium foil were kept at 37°C for 4 hours, after which plates were rapidly inverted with a firm flick to remove the culture medium and suspend the formazan crystal by adding 200 µg/mL of Dimethyl sulfoxide (DMSO). Glycine buffer (pH 6.0) was added for adjusting the pH and the absorbance was measured at 570nm using ELISA reader. Control wells contained only cells without compound. All concentration was done in triplicates.

3. RESULTS AND DISCUSSION

The L-asparaginase from *Fusarium* sp. LCJ273 was purified using ammonium sulphate precipitate (80%) and DEAE-cellulose column chromatography which has been widely used for purification of endophytic L-asparaginase [30,31]. Acetone also used for the precipitation of fungal L-asparaginase [32]. Ammonium sulphate precipitation was employed. At the end of the purification process the L-asparaginase activity was increased to 3.7 fold.

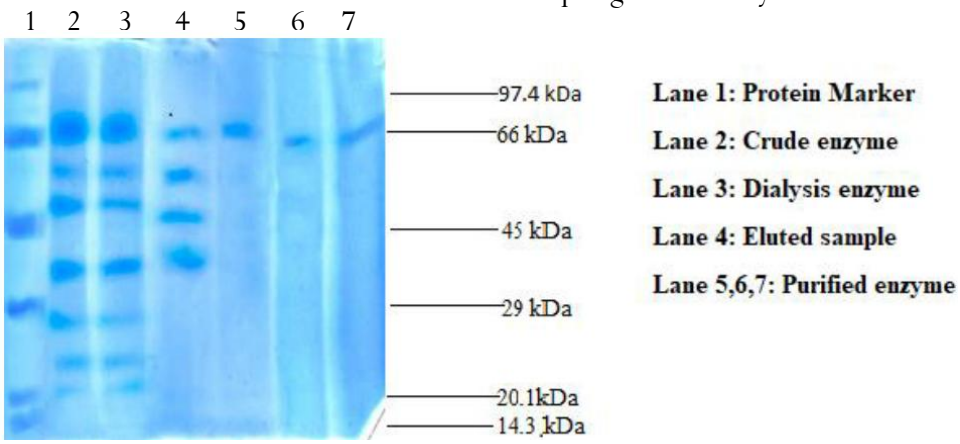

Fig. 1: Molecular weight determination of purified L-asparaginase from *Fusarium* sp. LCJ273 on SDS-PAGE

The molecular mass of the partially purified L-asparaginase from *Fusarium* sp. LCJ273 was determined by SDS-PAGE. The partially purified L-asparaginase was subjected to SDS-PAGE showed a single homogenous band stained with Coomassie Brilliant Blue (CBB R250). The molecular weight of the partially purified L-asparaginase from *Fusarium* sp. LCJ273 was found to be ~66 kDa (Fig. 1). Earlier studies show that the molecular weight of L-asparaginase isolated from *Penicillium* sp. was found to be 66 kDa [1, 8]. L-Asparaginase from other microbes has been characterized based on their molecular weight reported in earlier: *Streptomyces albidoflavus* with 112 kDa [31], *Cladosporium* sp. with 117 kDa [18], *Aspergillus flavus* with 100 kDa [33], *Aspergillus niger* with 48 kDa [34], *Trichoderma viridae* with 99 kDa [35]. L-Asparaginase from *Fusarium* sp. having molecular weight 44.7 kDa was also reported [36]. The molecular weight of L-asparaginase therefore could differ from one organism to other organism; it may be inferable to its genetic diversities.

3.1. Physico-chemical properties of partially purified L-asparaginase

3.1.1. Influence of pH on L-asparaginase activity

The effect of partially purified L-asparaginase, ammonium sulphate precipitate and culture filtrate from *Fusarium* sp. LCJ273 were assessed under different pH conditions ranging from 4 to 10. The partially purified L-asparaginase from *Fusarium* sp. LCJ273 showed 100% of relative activity at pH 7, when L-asparagine was used as the substrate (Fig. 2).

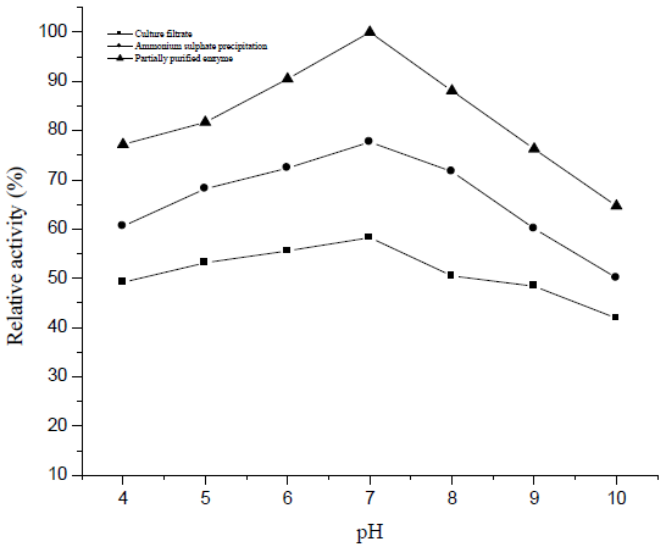

Fig. 2: Relative activity of L-asparaginase enzyme at different pH from *Fusarium* sp. LCJ273

Ammonium sulphate precipitate with 77.7% relative activity and culture filtrate with 58.3% relative activity were also detected at pH 7.0. Similar results were reported by earlier researchers in *Mucor hiemalis* [12], *Rhizomucor miehei* [37] and *Trichoderma viride* [35]. L-Asparaginase from *Cylindrocarpon obtusisporum* MB-10 and *Staphylococcal* was reported that the optimum pH of L-asparaginase activity was found to be 7.4 and 8.8 [25, 38]. However, significant relative activity was detected between pH 4–6 with relative activity ranging from 77%-90% for partially purified L-asparaginase. Low relative activity was observed at pH 10 with relative activity of 64%. Further increase or decrease in pH resulted in reduced L-asparaginase activity and pH is an important factor to be used as a therapeutic agent.

3.1.2. Effect of different temperature and stability on L-asparaginase activity

The influence of various temperatures on the partially purified L-asparaginase, ammonium sulphate precipitates and culture filtrate from *Fusarium* sp. LCJ273 was assessed under different temperature varies from 20°C to 45°C. The optimum temperature for partially purified enzyme was found to be 30°C with 100% relative activity (Fig. 3).

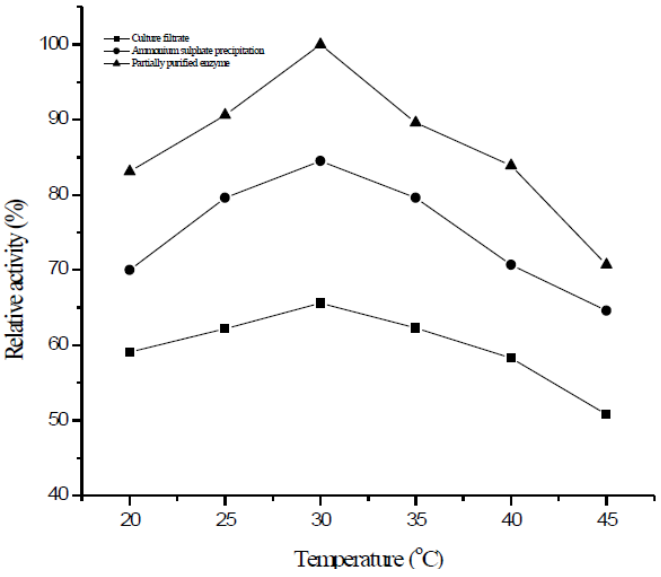

Fig. 3: Relative activity of L-asparaginase enzyme at different temperatures from *Fusarium* sp. LCJ273

The L-asparaginase activity of culture filtrate and ammonium sulphate precipitate was 65% and 84% at 30°C for *Fusarium* sp. LCJ273. On the contrary, optimum activity of L-asparaginase from *Erwinia*

carotovora [39] and *Pseudomonas aeruginosa* 50071 [40] have been detected at 35°C and 37°C respectively.

Thermal stability of the partially purified L-asparaginase, ammonium sulphate precipitate and culture filtrate from *Fusarium* sp. LCJ273 was measured by incubating the enzyme-substrate mixtures for 1 h at different temperatures (20°C-45°C). In *Fusarium* sp. LCJ273 results showed that the optimum temperature was 30°C and it was stable up to 60 min with 100% relative activity. Above and below the range of optimum temperature, the activity of L-asparaginase was decrease gradually. The purified L-asparaginase was 100% stable at 30°C and above 90% stability was observed at 25°C and 35°C. Loss of partially purified L-asparaginase activity was observed 83% at 40°C and 70% activity at 45°C for *Fusarium* sp. LCJ273. The stability of L-asparaginase decreased gradually when temperature increased to 40°C and above. At temperature below 40°C the L-asparaginase was stable. The statistical analysis further confirmed that 30°C was significant for partially purified enzyme, ammonium sulphate precipitate and culture filtrate when compared to other temperatures. Several researchers have noticed various temperature for optimum L-asparaginase activity 37°C [35], 40°C [32].

3.2. Substrate specificity

The substrate specificity of partially purified L-asparaginase, ammonium sulphate precipitate and culture filtrate from *Fusarium* sp. LCJ273 was studied using different substrates such as L-asparagine, L-aspartic acid and L-glutamine presented in Fig. 4. Partially purified L-asparaginase from *Fusarium* sp. LCJ273 showed maximum activity towards original substrate L-asparagine [41, 42], very low activity towards L-aspartic acid and no specificity towards L-glutamine. Partially purified L-asparaginase revealed maximum oxidization activity 100% in culture. 74% with the culture filtrate and 83% with the ammonium sulphate precipitate from *Fusarium* sp. LCJ273. Relative specificity of L-asparaginase towards the L-asparagine was maximum among all the substrates used. Huang *et al.* (2014) [37] reported L-asparaginase from *Rhizomucor miehei* having low glutaminase. Raha *et al.* (1990) [38] and Nagarajan *et al.* (2014) [43] isolated glutaminase free L-asparaginase from *Cylindrocarpon obtusisporum* MB-10 and *Alternaria* sp. respectively. Some bacterial species have also been reported to produce L-asparaginase [31, 44].

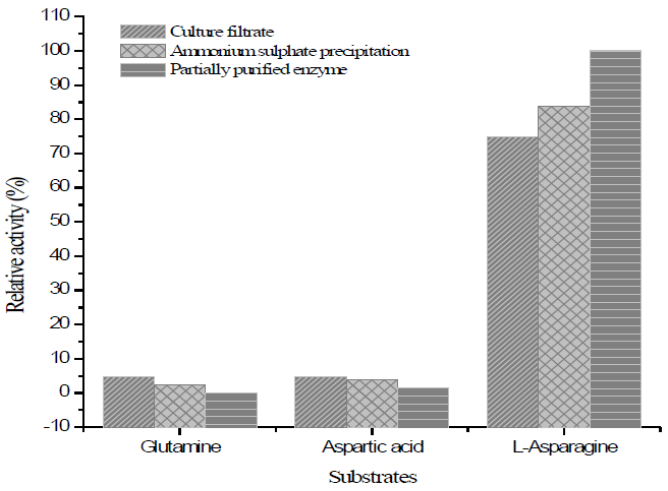

Fig. 4: Substrate specificity of L-asparaginase enzyme from *Fusarium* sp. LCJ273

3.3. Kinetic constants of partially purified L-asparaginase

The kinetic constants of partially purified L-asparaginase from *Fusarium* sp. LCJ273 was determined using L-asparagine. The K_m and V_{max} values for the partially purified L-asparaginase was calculated from the Lineweaver-Burk plot and presented in Fig. 5. The K_m values of *Fusarium* sp. LCJ273 were 24.2 mM for L-asparagine and the V_{max} were 125 mM min^{-1} are summarized in Table 1. The affinity of L-asparaginase from *Fusarium* sp. LCJ273 towards L-asparagine was higher than L-asparaginase from *Aspergillus aculeatus* [45]. Lower K_m value was attained for L-asparaginase from *Erwinia chrysanthemi* 3937, *Mucor hiemalis, E. coli* and *Penicillium* sp. [8, 12, 46-47]. The partially purified L-asparaginase from *Fusarium* sp. LCJ273 showed stronger affinity towards its original substrate L-asparagine.

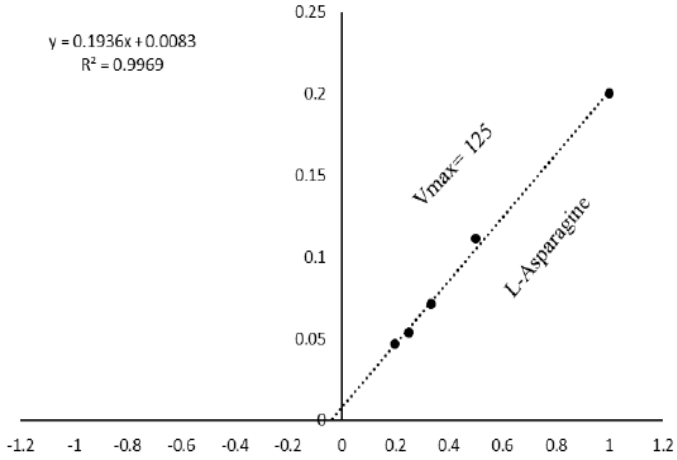

Fig. 5: Lineweaver-Burk plot of partially purified L-asparaginase from *Fusarium* sp. LCJ273

Table 1: Kinetic constant of partially purified L-asparaginase from *Fusarium* sp. LCJ273

Substrate	Wavelength (nm)	V_{max} (mM)	K_m (mM min^{-1})
L-asparagine	450	125	24.2

3.4. Anticancer activity of L-asparaginase

The cell lines namely MCF-7, Hep-2 and vero cell lines were treated with different concentrations ranging from 20, 40, 60, 80, 100 and 120 µg/mL of L-asparaginase produced by *Fusarium* sp. LCJ273, for 72 hours measured by MTT assay. Incubation of cancer cells with the different concentration of L-asparaginase enzyme significantly reduce the viability of tumour cells and cell inhibition percentage was significantly increased with high concentration of L-asparaginase. Purified L-asparaginase from *Fusarium* sp. LCJ273 was found to be highly effective against the tested cancer cells such as MCF-7, Hep-2 and vero cell line were dose dependent, increased the dose of L-asparaginase resulted in the inhibition of cell growth. The purified L-asparaginase was selectively inhibited tumour cells reproduction without any cytotoxic effect towards the non-carcinogenic normal cells at the different concentrations studied. Morphological changes in the tumour cell lines after treatment with purified fungal L-asparaginase were evaluated under an inverted microscope [48, 49]. The cells showed some characteristics like shrinkage, loss of cell adhesion, cellular rounding and cells detachment from surface of the wells (Fig. 6). Purified L-asparaginase from *Fusarium* sp. LCJ273 was found to be highly effective against the MCF-7 (16.61%), Hep-2 (8.12%) and vero cell line (88.73%) where IC_{50} value was recorded in the range of 33.21, 9.28 and 622.99 µg/mL respectively (Fig. 7).

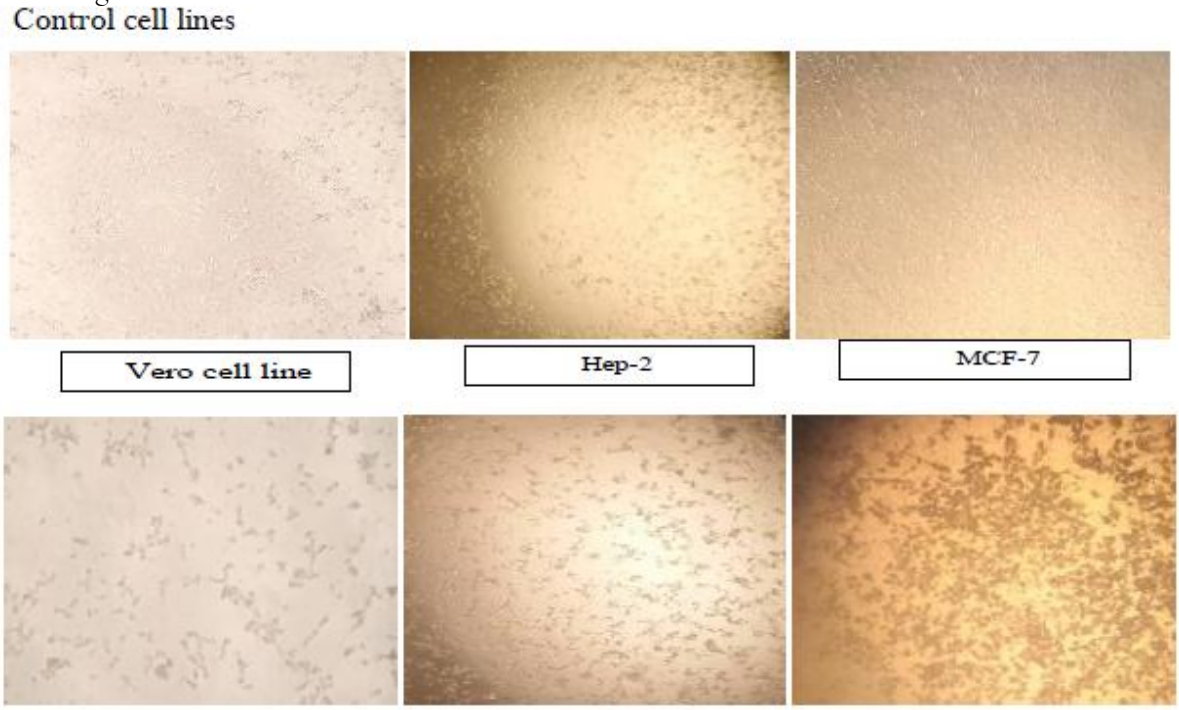

Fig. 6: *In vitro* cell cytotoxicity of partially purified L-asparaginase produced by *Fusarium* sp. LCJ273

These result were compared with other studies where L-asparaginase from *Bacillus licheniformis* and marine *Aspergillus terreus* strain showed the maximum toxicity effects against the cancer cell lines such as MCF-7, Jurkat clone E6-1, K-562 (IC_{50} value 0.78 IU, 0.22 IU, 0.153 IU) Hep-G2 and HCT-116 (with IC_{50} value of 3.79-12.6 µg/ml) [50, 51]. L-Asparaginase from *Penicillium cyclopium* inhibit the growth of human cell lines such as Hep-G2 (Hepatocellular carcinoma), PC3 (Prostate carcinoma) and MCF-7 (Breast carcinoma) with IC_{50} value of 14ìg/ml, 37ìg/ml and 12.5ìg/ml respectively [52]. El-Naggar *et al*. (2015) [53] studied the L-asparaginase activity against Hep2, CACO2, HEPG 2 and IC_{50} value was 2-4 U/mL. L-Asparaginase from bacteria like *E. coli* and *Erwinia carotovora* develop toxicity and hypersensitivity to the drug [54]. Purified L-asparaginase enzyme from *Fusarium* sp. LCJ273 showed non-toxic effects to normal vero cell line.

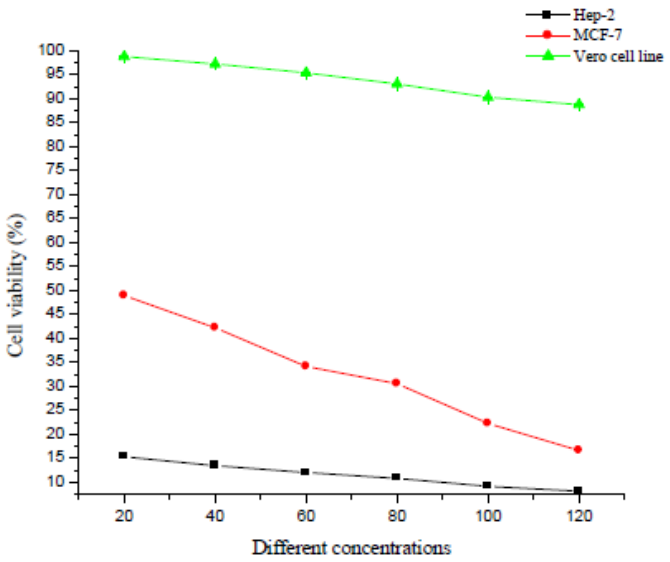

Fig. 7: Cytotoxic effect of L-asparaginase obtained from *Fusarium* sp. LCJ273 on Hep-2, MCF-7 cells, vero cell line and treated with different concentrations

4. CONCLUSION

L-Asparaginase from endophytic fungi finds broad application in food and pharmaceutical industries. L-Asparaginase was purified by ammonium sulphate precipitation and DEAE cellulose ion exchange chromatography. The molecular weight of L-asparaginase from *Fusarium* sp. LCJ273 was found to be 66 kDa. The partially purified L-asparaginase had an optimum pH 7.0 and temperature 30°C, showed high specificity towards the L-asparagine. L-Asparagine used as a natural substrate for L-asparaginase. The results of the present study show that L-asparaginase from *Fusarium* sp. LCJ273 can be used in the development of anti-tumour drugs.

Conflict of interest statement

We declare that we have no conflict of interest.

5. ACKNOWLEDGEMENT

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6. REFERENCES

1. Elshafei MA, Mohamed HM, Abd-Elmontasr AM, Mahmoud DA, Elghonemy DH. *Methodology*, 2012; **2:**158-174.
2. Manna S, Sinha A, Sadhukhan R, Chakrabarty SL. *Current Microbiology*, 1995; **30:**291-298.
3. Warangkar SC, Khobragade CN, Dawane BS, Bhosale RB. *International Journal of Biotechnology Applications*, 2009; **1:**5-13.
4. Pedreschi R, Hertog M, Robben J, Noben JP, Nicolaï B. *Postharvest Biology and Technology*, 2008; **50:**110-116.
5. Mohan Kumar NS, Manonmani HK. *Asian Journal of Microbiology, Biotechnology and Environmental Sciences*, 2012; **14:**149-155.
6. Gurunathan B, Sahadevan R. *Biotechnology and Bioprocess Engineering*, 2011; **16:**50-58.
7. Meena B, Anburajan L, Dheenan PS, Begum M, Vinithkumar NV, Dharani G, Kirubagaran R. *Bioprocess and Biosystems Engineering*, 2015; **38:**373-388.
8. Patro KR, Gupta N. *International Journal of Biotechnology and Molecular Biology Research*, 2012; **3:**30-34.
9. Dhanam JG, Kannan S. l*Advanced BioTech*, 2013; **13:**01-05.
10. Peterson RE, Ciegler A. *Applied Microbiology*, 1969; **17**:929.
11. Savitri, Astana N, Azmi W. *Indian Journal of Biotechnology*, 2003; **2:**184-194.
12. Monica T, Lincoln L, Niyonzima FN, Sunil SM. *Journal of Biocatalysis and Biotransformation*, 2013; **9:**12-14.
13. Baskar G, Renganathan S. *Chiang Mai Journal of Science*, 2011; **38:**270-279.
14. Narta UK, Kanwar SS, Azmi W. *Critical Reviews in Oncology/Hematology*, 2007; **61:**208-221.
15. Balasubramanian K, Ambikapathy V, Panneerselvam A. *International Journal of Advances in Pharmaceutical Research,* 2012; **3:**778-783.
16. Laan VD, Stor MC, Lange DI, Mohrmann L. *Aspergillus niger* asparaginase variants and their commercial uses. 2018; *US Patent No.* WO2008128974.
17. Matsui T, Friis EP, Yamagishi A. *Design of thermostable asparaginases and their use for reduction of acrylamide in foods*. 2008; WO 2008110513.
18. Kumar NM, Manonmani HK. *World Journal of Microbiology and Biotechnology*, 2013; **29:**577-587.
19. Curran MP, Daniel RM., Guy GR, Morgan HW. *Archives of Biochemistry and Biophysics*, 1985; **241:**571-576.
20. Gulati R, Saxena RK, Gupta R. *Letters in Applied Microbiology*, 1997; **24:**23-26.

21. Gallagher MP, Marshall RD, Wilson R. *Essays in Biochemistry*, 1989; **24:**1.
22. Sarquis MIDM, Oliveira EMM, Santos AS, Costa GLD. *Memorias do Instituto Oswaldo Cruz*, 2004; **99:**489-492.
23. Meghavarnam AK, Janakiraman S. *Journal of Anesthesia and Critical Care Open Access*, 2015; **2:**00064.
24. Amena S, Vishalakshi N, Prabhakar M, Dayanand A, Lingappa K. *Brazilian Journal of Microbiology*, 2010; **41:**173-178.
25. Sobis M, Mikucki J. *Acta Microbiologica Polonica*. 1991; **40:**143-152.
26. Jenila AV, Gnanadoss JJ. *Biosciences Biotechnology Research Asia*, 2018; **15:**887-898.
27. Nelson DL, Cox MM. Lehninger principles of biochemistry. 4th ed *Freeman WH and Co*, New York, USA, 2004; p. 1100.
28. Laemmli UK. *Nature,* 1970; **227:**680-685.
29. Lineweaver H, Burk D. *Journal of the American Chemical Society*, 1934; **56:**658-666.
30. Emmanuell E, Nzelibe HC, Onyike E. *Journal of Microbial and Biochemical Technology*, 2015; **7:**404-409.
31. Narayana KJP, Kumar KG, Vijayalakshmi M. *Indian Journal of Microbiology*, 2008; **48:**331-336.
32. Mahajan RV, Saran S, Kameswaran K, Kumar V, Saxena RK. *Bioresource Technology*, 2012; **125:**11-16.
33. Loureiro CB, Borges KS, Andrade AF, Tone LG, Said S. *Advances in Microbiology*, 2012; **2:**138.
34. Akilandeswari K, Kavitha K, Vijayalakshmi M. *International Journal of Pharmacy and Pharmaceutical Sciences*, 2012; **4:**363-366.
35. Lincoln L, Niyonzima FN, More SS. *The Journal of Microbiology, Biotechnology and Food Sciences*, 2015; **4:**310.
36. Asha A, Pallavi B. *Journal of Advance Researches in Biological Sciences*, 2012; **4:**293-301.
37. Huang L, Liu Y, Sun Y, Yan Q, Jiang Z. *Applied and Environmental. Microbiology,* 2014; **80**:1561-1569.
38. Raha SK, Roy SK, Dey SK, Chakrabarty SL. *Biochemistry International*, 1990; **21:**987-1000.
39. Kamble VP, Rao RS, Borkar PS. *Indian Journal of Biochemistry Biophysics*, 2006; **43:**391-394.
40. El-Bessoumy AA, Sarhan M, Mansour J. *Journal of Biochemistry and Molecular Biology*, 2004; **37:**387-393.
41. Devi SARITA, Azmi WAMIK. *International Journal of Life Science and Pharma Research,* 2012; **2:**56-61.
42. Muslim SN. *International Journal of Current Engineering and Technology,* 2014; **4**.
43. Nagarajan A, Thirunavukkarasu N, Suryanarayanan TS, Gummadi SN. *Research Journal of Microbiology,* 2014; **9:**163-176.
44. Husain I, Sharma A, Kumar S, Malik F. *PLoS One*, 2016; **11:**e0148877.
45. Dange VU, Peshwe SA. *Bionano Frontier*, 2011; **4:**162-167.
46. Kotzia GA, Labrou NE*Journal of Biotechnology*, 2007; **127:**657-669.
47. Willis RC, Woolfolk R. *Journal of Bacteriology*, 1974; **118**:231-232
48. Rani SA, Sundaram LA, Vasantha BP. *Asian Journal of Pharmaceutical and Clinical Research*, 2011; **4:**174-177.
49. Alrumman SA, Mostafa YS, Al-Izran KA, Alfaifi MY, Taha TH, Elbehairi SE. *Scientific Reports*, 2019; **9:**3756.
50. Mahajan RV, Kumar V, Rajendran V, Saran S, Ghosh PC, Saxena RK. *PLoS One*, 2014; **9:**e99037.
51. Hassan SW, Farag AM, Beltagy EA. *Journal of Pure and Applied Microbiology*, 2018; **12:**1845-1855.
52. Shafei MS, El-Refai HA, Mostafa H, El-Refai AMH, El-Beih FM, Easa SM, Gomaa, SK. *Current Trends in Biotechnology and Pharmacy*, 2015; **9:**132-140.
53. El-Naggar NE, Moawad H, El-Shweihy NM, El-Ewasy SM. *BioMed Research International*, 2015; **2015:**1-17.
54. Dhanam JG, Kannan S. *Advanced BioTech*, 2013; **13**:01-05.