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Production of L-Asparaginase using Soil Isolate of *Cladosporium sp.* and Evaluation of its Antitumor and Antioxidant Activities

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Author's contribution

The sole author designed, analysed, interpreted and prepared the manuscript.

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ABSTRACT

The enzyme L-asparaginase is given prime attention in healthcare settings owing to its significant therapeutic applications in oncology. The present study isolated five different species of fungi belonging to the genera Aspergillus (23.8 and 9.5%), Cladosporium (19%), Fusarium (14.3%) and Penicillium (19%) from soil samples. Screening on Czapek Dox's medium indicated that the isolate *Cladosporium sp.* ASP4 exhibit higher potential of synthesizing L-asparaginase than other fungi by means of causing a halo zone measuring 22.3 mm dia. Fermentation of standard medium ingredients along with the solid substrate soya bean meal by the potential fungal isolate resulted in the production of 20.53 IU/mL of L-asparaginase. Optimization studies deciphered the carbon source sucrose, supplementary nitrogen ammonium nitrate, pH ≥8.0 and the temperature 50°C as

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favorable process parameters for the enzyme production. Purified L-asparaginase of the present study demonstrated moderate antineoplastic activity against the A549 lung cancer cells with the IC_{50} values of ≥50 IU/mL. The enzyme displayed better antioxidant property compared to those of previous studies by way of scavenging the radicals with the SC_{50} values of ≥250 mg/mL. These findings encourage carrying out further elaborate studies for prospective biotechnological and pharmaceutical applications of the purified enzyme.

Keywords: L-asparaginase; Cladosporium sp.; solid state fermentation; antitumor; antioxidant.

1. INTRODUCTION

Asparaginase (L-asparigine amidohydrolase) is an enzyme that catalyzes the hydrolysis of asparagine to aspartic acid. L-asparagine is a non-essential amino acid normally involved in the metabolic control of cell functions in nerve and brain tissues. Asparaginases are naturally occurring enzymes expressed and produced by microorganisms, plants, and animals. In plants, asparaginase is used for nitrogen fixation, storage and transport. Highly purified asparaginase has a molecular weight of 36.8-160 kDa and is reported to be stabilized by addition of buffer salts or extra protein (Dharmsthiti & Luechai, 2010). Asparaginase has tremendous applications in various fields such as medical, pharmaceutical, food processing and biotechnology.

L-asparaginase is one of the known drugs in the treatment of cancer, especially acute lymphoblastic leukemia and other malignant neoplasms including myelocytic leukemia, Hodgkin disease, reticulosarcoma, melanosarcoma and so on (Stecher et al., 1999). It has also been used for treatment of lymphosarcoma, lymphoproliferative disorders and in many other clinical experiments relating to tumor therapy in combination with the chemotherapy. In recent years, several bio-conjugation protocols have been developed to improve the pharmacokinetic and immunological properties of the anti-leukemic enzyme L-asparaginase.

Besides its application in medicine, asparaginase is used as a food processing aid to reduce the formation of acrylamide, a suspected carcinogen, in starchy food products. By adding asparaginase before baking or frying the food, asparagine is converted into another common amino acid, aspartic acid, and ammonium. As a result, asparagine cannot take part in the Maillard reaction, and therefore the formation of acrylamide is significantly reduced. It plays an important role in the biogeocycling of carbon and nitrogen in natural waters and sediments (Pedreschi et al., 2008).

Since the discovery of L-asparaginase from the bacteria *Escherichia coli* in 1963, researchers have recognized its oncological importance and continued to focus on many microbial sources to explore its extraction. Among the bacteria, *E. coli*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Bacillus circulans* and *Photobacterium leigonathi* have been reported to produce asparaginase (Abdel-Fattah & Olama, 2002; Prakasham et al., 2009). Fungal species of importance in this field are *Aspergillus tamari*, *A. niger, Penicillium*, *Fusarium*, and *Cladosporium cladosporioides* (Sarquis et al., 2004; Ali et al., 1994; Chakraborty & Shivakumar, 2021). Besides, the actinomycetes such as *Streptomyces sp*., *Nocardia sp*. and the yeasts *Rhodotorula sp.* and *Rhodosporidium* (Basha et al., 2009; Amena et al., 2010; El-Naggar & El-Shweihy, 2020) have also been explored for asparaginase production.

The pharmacological potential of L-asparaginase is greatly influenced by its structural, chemical and kinetic properties. However, the structure-function relationships of L-asparaginase synthesized by different microorganisms vary among themselves and hence some of the enzymes are contraindicated with adverse side-effects in clinical applications (El-Naggar & El-Shweihy, 2020). In recent years, there are increasing reports on the complications such as allergic reactions and anaphylaxis caused by the L-asparaginase of several bacterial sources in the treatment of human neoplastic diseases (Sarquis et al., 2004; Chow & Ting, 2015; Bungtongdee et al., 2019).

In order to avoid any undesirable clinical complications, selection of L-asparaginase with safe and *in vivo* compatible properties is critical for oncological applications. This necessitated the research on exploring the production of this enzyme by other eukaryotic microorganisms viz., yeast and filamentous fungi with minimal hostile

effects (Osama et al., 2023). The fungi are considered to be a special group of microorganisms endowed with excellent survival capacities in extreme environments and synthesis of wide array of therapeutic enzymes. Therefore, the present study was aimed for *in vitro* production of L-asparaginase enzyme using soil-borne fungi and to assess its antitumor and antioxidant activities.

2. MATERIALS AND METHODS

2.1 Sample Collection

For the isolation of fungi, wet soil samples were collected from five different areas of Northern Border University, Arar, Saudi Arabia. A total of 25 samples each weighing 10g from a depth of 30cm were collected over a period of three months from February-April, 2024. Samples were collected using sterile spatula in sterile polythene bags and transported to the laboratory of Faculty of Applied Medical Sciences and stored at 4^{o}C until further processing.

2.2 Isolation and Identification of L-Asparaginase Producing Fungi

Soil samples were subjected to isolation of fungi by standard spread plate method on potato dextrose agar (PDA) medium. Briefly, one gram of the soil sample was serially diluted up to 10^{-7} dilution using sterile distilled water. Aliquots of 0.1 ml of samples from the dilutions 10^{-4} to 10^{-7} were transferred onto PDA plates and spread using sterile L- rod. All the plates were incubated at room temperature and the growth of fungi was observed after 3-6 days.

The isolates of fungi grown on PDA were screened for asparaginase production by a screening method prescribed by Sarquis et al. (2004). Sterile plates of modified Czapek Dox's (MCD) agar medium were prepared and each plate was inoculated with a loopful of well grown mycelium of fungi in the center. The plates were incubated at 28°C for 3-4 days. Since the production of L-asparaginase causes the increase in pH due to the release of ammonia, the enzyme producing fungus was identified by the zone formation around the colony indicating the change of color of phenol red indicator of the medium. The diameter (cm) of halo zone produced by each fungal colony was measured and recorded for comparative study. Mycelial colony which facilitated highest enzyme activity by way of causing larger zone was designated as a potential fungal isolate.

The selected fungal isolated was further subjected to identification studies by standard techniques for the determination of phenotypic, cultural and microscopic characteristics.

2.3 Production of L-asparaginase

For the production of L-asparaginase by the potential isolate, the method of solid state fermentation (Vimal & Kumar, 2022) was followed. The inoculum was prepared by suspending the spores of the fungus grown on PDA in 5mL of 0.01% of tween-80 solution. Soya bean meal collected from a local market was ground to obtain coarse particles (0.4-0.8 cm) to serve as the solid substrate for the fermentation. Ten gram (w/w) of this substrate taken in a 250mL conical flask was mixed with 20mL of mineral salt solution. The culture medium included the following composition (g/L): Glucose (2.0), L-asparagine (10.0), KH_2PO_4 (1.52), KCl (0.52) and trace amounts of $MgSO_4.7H_2O$, $CuNO_3$, $ZnSO_4$ and $FeSO_4$. This autoclaved medium was inoculated with 5mL of spore suspension, mixed thoroughly and incubated at room temperature for 7 days. Subsequently, the fermentation medium was suspended in 20mL of 0.05M Tris HCl buffer (pH 8.6) and agitated for 30 min. Then, it was filtered through cheese-cloth with squeezing and the filtrate was centrifuged at 2500 rpm for 10 min. The supernatant containing crude enzyme was collected in a sterile vial and stored at 4^{o}C until use.

2.4 Assay of Enzyme Activity

The assay of L-asparaginase activity was determined in terms of quantitative measure of NH_3 released by the enzyme (Freitas et al., 2021). Briefly, the reaction mixture contained 0.2mL of 0.05M Tris-HCl buffer (pH 8.6), 1.7mL of 0.01M L-asparagine and 0.1mL of crude enzyme. The reaction was carried out at 37°C for 30 min. The reaction was stopped by adding 0.1 mL of 1.5M trichloroacetic acid. This reaction mixture was added with the reagent grade water to make up a final volume of 7mL and added with 1mL of Nessler's reagent. The mixture was left at room temperature for 10 min and the color development was measured using UV-vis spectrophotometer at 450 nm. Estimation of release of ammonia was calculated by the standard curve graphical method. The enzyme yield was expressed as IU per milliliter (IU/mL) based on the concentration of the enzyme. One unit of L-asparaginase activity was defined as the amount of enzyme that released 1 µmol NH_3 per min under standard conditions of the assay.

2.5 Optimization of Process Parameters

In order to achieve enhanced production of L-asparaginase, optimization studies were conducted to determine the influential parameters of the fermentation (El-Naggar & El-Shweihy, 2020; Sisay et al., 2024). Carbon sources such as glucose, sucrose, maltose and lactose were tested for their augmenting efficiency of the enzyme production. Supplementary nitrogen sources assessed for their impact on fermentation were, ammonium chloride, ammonium nitrate, ammonium sulfate and peptone. Similarly, the pH of the fermentation medium was set at different ranges (6-8 and 9) and evaluated for their influence on fungal growth and enzyme production. The effect of different incubation temperatures (30, 40, 50 and 60^{o}C) on the fermentation process was also tested for enzyme production. Best parameters were selected based on the maximum enzyme production as determined by the method mentioned before.

2.6 Purification of L-asparaginase

The purification of enzyme from the crude extract was carried out at 4^{o}C according to the modified method of El-Bessoumy et al. (2004). The crude enzyme was concentrated by precipitation using ammonium sulfate to obtain 80% saturation. The precipitate was suspended and dialyzed against 0.01M phosphate buffer (pH 8.5). Separation and elution of crude enzyme was performed using CM-Sephadex G-50 ion-exchange chromatography adjusted with the flow rate of 1mL/min. The active fractions were collected and concentrated using ammonium sulfate and dialysis. Further purification of the enzyme was carried out by gel filtration on Sephadex G-100 column pre-equilibrated with 0.01M phosphate buffer (pH 8.5). Each fraction collected at a flow rate of 1mL/min was stored in a separate microfuge tube containing 2mL of equilibration buffer.

For the purpose of determining the molecular weight of the enzyme produced by the fermentation process, the purified fraction of L-asparaginase was separated on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) along with standard protein markers.

2.7 Assay of Anti-tumor Activity

The antitumor activity of the purified L-asparginase, in terms of its cytotoxic effect, against A549 cell line of human lung cancer was estimated by adopting the 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay suggested by Prakasham et al. (2009). The reduction of tetrazolium salt of MTT to insoluble formazan crystals by the mitochondrial dehydrogenases in metabolically active cells was indicated by change of color from yellow to purple. Formazan crystals were further solubilized by the addition of detergent solution and the concentration was estimated. Monolayer of A549 cells prepared in a fetal bovine serum free medium were maintained in a 96-well micro titration plate (5 x 10^4 cells/well). Various concentrations of the enzyme (5, 10, 25, 50, 75 and 100 IU/mL) were added to the wells and incubated at 37^{o}C for 48 h. Separate wells of cell culture devoid of the enzyme sample served as control cells. The absorbance of the treated cell suspensions was measured at 570 nm by ELISA plate reader (Awareness Tech, USA) at regular intervals. The relative cell viability was estimated with reference to the amount of insoluble formazan salt. The mean percentage of viable cells were determined in comparison with that of control culture. The cytotoxic effects of the enzyme aliquots were calculated from the linear equation of the dose–dependent curve and expressed as inhibitory concentration IC_{50} values. Further confirmation of cytotoxic effect of the enzyme was accomplished by visualization using inverted tissue culture microscope.

2.8 Evaluation of Antioxidant Activity

The antioxidant activity of L-asparaginase samples was determined by DPPH (1,1-diphenyl-2-picryl-hydrazyl) scavenging assay according to Chua et al. (2023). Under oxidized conditions, the DPPH remains as a stable radical owing to the presence of its unpaired electron. The enzyme L-asparaginase, which is an antioxidant radical scavenger, upon addition, donates an electron to DPPH and converts it into a non-radical. This causes the bleaching i.e., change of color of DPPH from violet to pale yellow. Different concentrations of the enzyme (10, 50, 100, 250, 500 and 1000µg/mL) were prepared in dimethyl sulfoxide and aliquots of 150µL were loaded in a 96-well micro titration plate. To each well, 150µL of 0.004% DPPH solution prepared in methanol was added and the plate was incubated at RT for 30 min in the dark. Subsequently, the absorbance in the reaction mixture, formed due to the bleaching of DPPH, was monitored by UV-vis Spectrophotometer at 515 nm. Parallel set up using an efficient

scavenger (10mM ascorbic acid) was also maintained for the comparative study of tested enzyme aliquots. The percentage of half maximal scavenging concentration (SC_{50}) was calculated by the following equation:

$$Antioxidant\ capacity\ (\%) = \frac{SC_{50} of\ Ascorbic\ acid}{SC_{50} of\ the\ sample} x\ 10^3$$

2.9 Statistical Analysis

The assays such as screening of fungal isolates for L-asparaginase synthesis, antitumor and antioxidant activities were conducted in triplicates unless and otherwise stated. Statistical analysis of the results was done using two-tailed Student's *t* test to obtain appropriate mean and standard deviation (SD) values. Data in tables and figures represent the means, with standard error of ±1 (halo zone measurement) ±2 (percentages of cell viability and radical scavenging activities).

3. RESULTS

3.1 Isolation and Identification of Soil Fungi

Studies on isolation of fungi from the soil samples yielded the growth of six different types of colonies. Further to the standard macroscopic and microscopic studies, the presence of 21 isolates of different fungi were noted. The different species of fungi and their percentages of prevalence in the soil were as follows: *Aspergillus niger* (23.8%), *Fusarium sp.* (14.3%), *Cladosporium sp.* (19%), *Penicillium sp.* (19%), *Microsporum sp.* (14.3%) and *Aspergillus fumigatus* (9.5%). The assay of L-asparaginase production by the soil fungi indicated that the isolates cause the halo zones ranging in sizes of 5.1-22.3 mm diameter (Table 1). The isolate *Cladosporium sp.* ASP4 was observed to synthesize higher amount of enzyme than the others by way of producing largest halo zone. Upon additional phenotypic, cultural (olivaceous green to blackish green, powdery colonies) and microscopic characteristics (chains of conidia closest conidiophores shield shaped chain branches), the identity of L-asparaginase synthesizing *Cladosporium sp.* was confirmed (Fig. 1).

3.2 Enzyme Production and Optimization of Process Parameters

The fermentation studies indicated that, with the standardized medium composition and incubation conditions, the yield of L-asparaginase by the potential isolate *Cladosporium sp.* ASP4 is 20.53 IU/mL. Determination of enzyme activity and protein concentration estimation further confirmed the production of L-asparaginase by the fungal strain. The SDS-PAGE study inferred that the purified enzyme possesses a molecular weight 90 kDa.

The evaluation of parameters including the essential constituents of fermentation medium and extrinsic factors was carried out by manipulating them in different setups of the fermentation process. These studies revealed the best components that favor the maximum production of the enzyme. Among the different carbon sources tested, sucrose was found to be the most suitable substance as it caused the production of 18.72 IU/mL of L-asparaginase compared to other sugars (Fig. 2). The supplementary nitrogen source ammonium nitrate showed better influencing property on the enzyme yield (17.86 IU/mL) than its counterparts (Fig. 3). The fermentation medium set at a pH of 8.0 favored higher production of the enzyme (20.36 IU/mL) than those with other pH ranges (Fig. 4). Studies on optimization of different incubation temperatures deciphered that the fermentation carried out at 50°C facilitates a production of 19.73 IU/mL of the enzyme which is much higher than those with other temperature ranges (Fig. 5).

Table 1. Results of screening of soil fungi for enzyme activity

Isolate no.	Soil fungi and halo zone diameter (mm)					
	AN	***FS***	***CS***	***PS***	***MS***	***AF***
1	16.3±0.5	10.5±0.7	17.3±0.4	18.1±0.5	8.1±0.7	9.6±0.3
2	15.1±0.2	6.7±0.3	19.1±0.8	13.3±0.3	5.1±0.2	7.1±0.7
3	17.4±0.6	8.8±0.5	18.2±0.5	12.7±0.6	7.6±0.4	NA
4	7.6±0.4	NA	22.3±0.2	9.4±0.4	NA	NA
5	8.9±0.2	NA	NA	NA	NA	NA

AN, *Aspergillus niger*; FS, *Fusarium sp.*; CS, *Cladosporium sp.*; PS, *Penicillium sp.*; MS, *Microsporum sp.*; AF, *A. fumigatus*; NA, not available

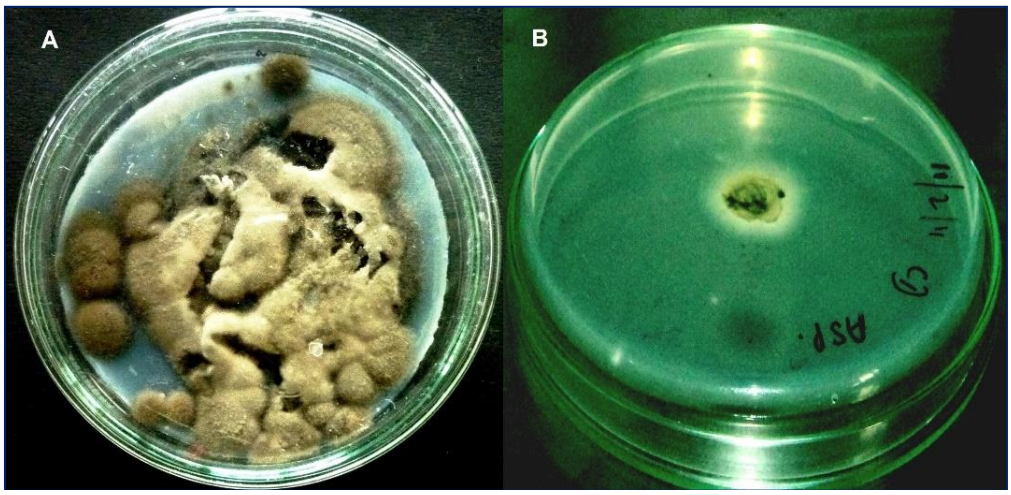

Fig. 1. Mycelial growth (A) and enzyme activity (B) of *Cladosporium sp.* ASP4

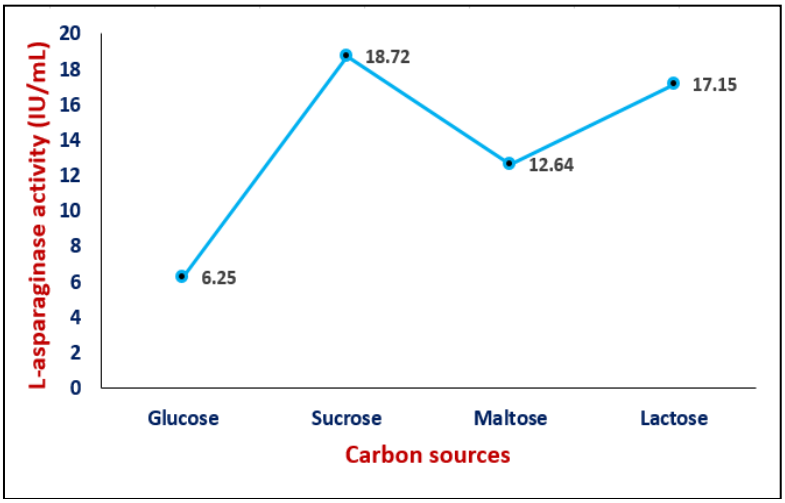

Fig. 2. Effect of carbon sources on enzyme activity

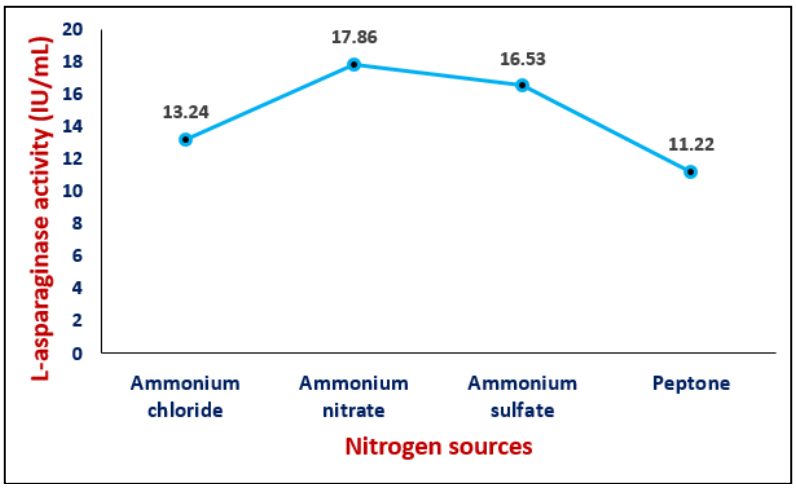

Fig. 3. Effect of nitrogen sources on enzyme activity

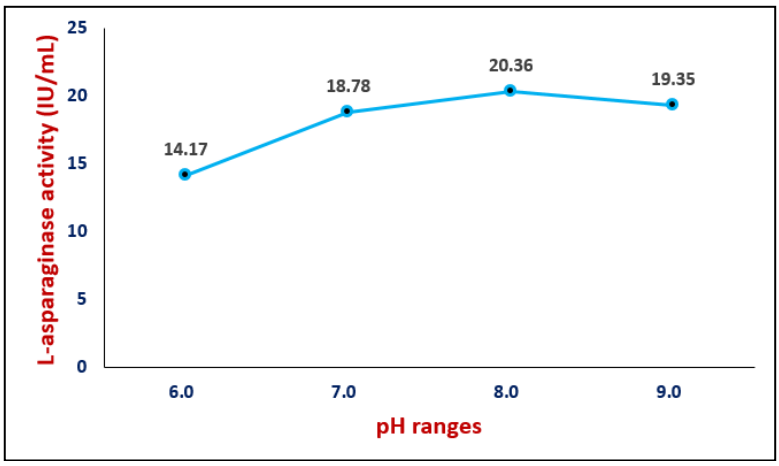

Fig. 4. Enzyme activity at different pH ranges

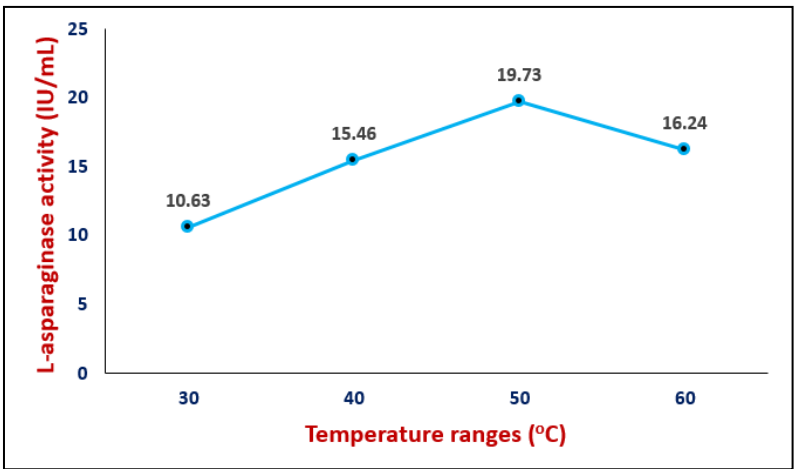

Fig. 5. Enzyme activity at different temperature ranges

Table 2. Results of assay of cytotoxicity of L-asparaginase against A549 cells

Concentration of L-asparaginase (IU/ml)	Viability (%) of cells and incubation time				
	10 h	15 h	24 h	36 h	48 h
5	97±1.5	93±1.2	88±1.6	83±1.7	80±1.9
10	91±1.0	86±0.9	81±1.2	74±1.3	69±1.2
25	87±0.5	83±1.3	76±0.9	68±0.5	60±1.7
50	88±1.3	82±1.7	68±0.8	59±1.1	45±1.3
75	84±0.8	72±0.8	61±1.0	44±0.2	38±0.9
100	76±0.5	62±0.4	45±1.2	33±1.0	24±0.7

3.3 Antitumor Activity of L-asparaginase

The studies on the antineoplastic activity of the L-asparaginase produced by the fungal strain *Cladosporium sp*. ASP4 against the A549 tumor cell lines revealed its cytotoxic potential. Subsequent to the treatment of tumor cells with the enzyme, the viabilities of the cells were recorded in terms of absorbance values at intervals of 10, 15, 24, 36 and 48 h. Different concentrations of the enzyme exhibited varying degrees of cytotoxicity depending on the duration of treatment. The cytotoxic effect was observed to be directly proportional to the concentration of the enzyme as well as the treatment time (Table 2). The lower concentrations of the enzyme did

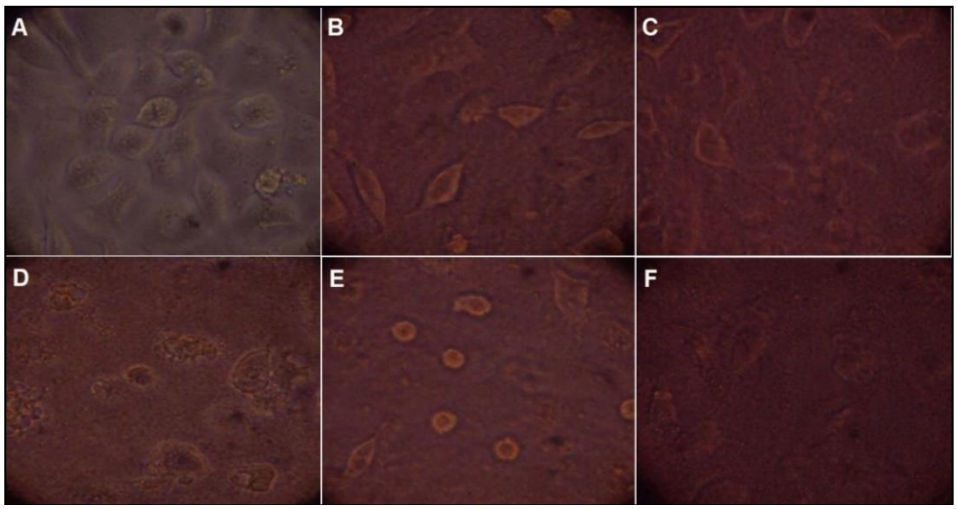

Fig. 6. Assay of cytotoxicity of L-asparaginase against A549 cells
A, Control cells and Cells treated for B, 10h; C, 15h; D, 24h; E, 36h; F, 48h

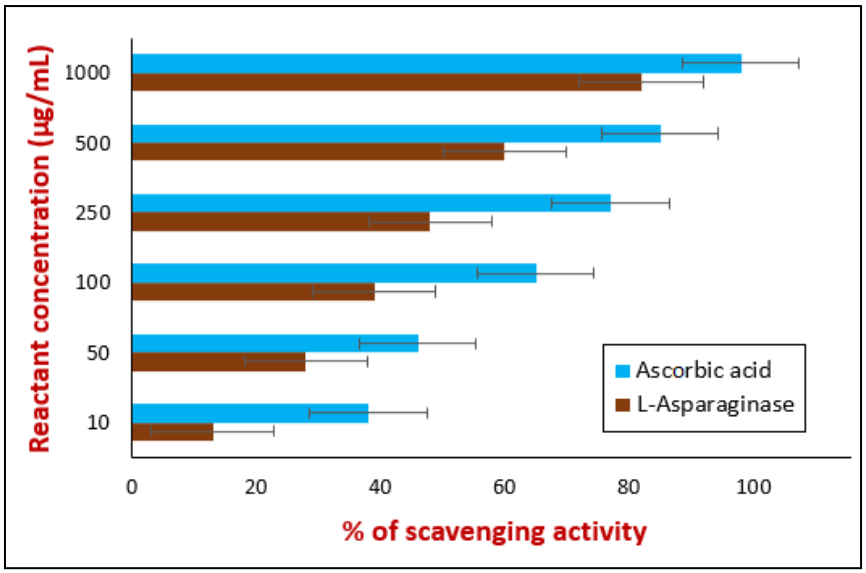

Fig. 7. Results of DPPH assay of L-asparaginase for antioxidant activity

not attain the IC_{50} values even after 48 h of treatment. The L-asparaginase concentrations ≥50 IU/mL displayed IC_{50} values commencing from the 24th h of treatment. The microscopic study indicated the cytotoxic effects of the enzyme on the morphology of A549 tumor cells such as segregation, shrinkage, rounding and anomaly of nuclear architecture (Fig. 6).

3.4 Antioxidant Activity assay of L-asparaginase

The DPPH assay conducted to evaluate the antioxidant activity the enzyme demonstrated the radical scavenging activity of L-asparaginase in comparison with that of the standard reactant ascorbic acid (Fig. 7). The standard antioxidant displayed superior scavenging activity even with a low SC_{50} value of 50µg/mL. The enzyme L-asparaginase produced in the present study presented a moderate scavenging activity requiring 250µg/mL as its SC_{50} value.

4. DISCUSSION

Cancer is a major public health problem and the second most leading cause of deaths worldwide. Leukemia is a life threatening disease of hematological malignancies characterized by

differentiation and uncontrolled proliferation of leukocytes in the bone marrow and peripheral blood. Complications due to leukemia are usually high and it mainly afflicts the older adults (Siegel et al., 2024).

Among the different options available for chemotherapy of leukemia, the L-asparaginase based drugs are considered as cornerstone of treatment protocols by virtue of their robust action on tumor cells (Maese & Rau, 2022). The therapeutic action of L-asparaginase is due to a specific mechanism which negatively affects the metabolism of tumor cells. Generally, the growth of both normal cells and malignant cells require L-asparagine amino acid. In order to establish metastasis, the tumor cells need substantial amount of asparagine for protein synthesis. While the normal cells are able to synthesize their L-asparagine, the tumorous cells, due to the low expression or lack of L-asparagine synthetase, depend on extracellular source of this amino acid. The enzyme L-asparaginase, when introduced into the body fluids, causes catalysis and depletion of L-asparagine circulating in the extracellular pool. As a result, the tumor cells are deprived of the essential factor for their malignant growth. Consequently, the cancerous cells undergo starvation and eventual death (Chand et al., 2020).

The present study has been conducted with an objective of exploring common fungi for the production of L-asparaginase endowed with pharmaceutical properties. For the purpose of isolating L-asparaginase producing fungi, the natural source i.e., the soil was utilized. Since the soil is frequently impacted by the environmental changes, the microorganisms, notably fungi, present in it develop suitable adaptations for their survival in challenging conditions. The metabolites and the enzymes produced by these fungi possess more efficient therapeutic potentials compared to that of other microbes (Udayan et al., 2023). Similar to our study, Siddalingeshwara & Lingappa (2011) and Sisay et al. (2024) have utilized soil source for isolating the L-asparaginase synthesizing fungi. Studies conducted elsewhere on L-asparaginase production have isolated fungi from different sources such as animal feed (Dharmsthiti & Luechai, 2010), plant endophytes (Chow & Ting, 2015; Chua et al., 2023; Hatamzadeh et al., 2020), marine environment (Vimal & Kumar, 2022) and so on.

In the present study, fungi belonging to six different species such as *A. niger*, *Fusarium sp.*, *Cladosporium sp.*, *Penicillium sp.*, *Microsporum sp.* and *A. fumigatus* were isolated from the soil samples (Table 1). Study of prevalence of these fungi indicated that the molds *A. niger* and *A. fumigatus* occur with high and low frequencies respectively in the soil. The fungal species *Cladosporium sp.* and *Penicillium sp.* were isolated in moderate numbers. The results pertaining to the isolation of soil borne fungi in our research are in conform to the previous studies (Vimal & Kumar, 2022; Hatamzadeh et al., 2020). In contrast, a recent study conducted by Sisay et al. (2024) isolated the L-asparaginase producing fungi such as *Trichosporon asahii* (mold), *Candida palmioleophila* and *C. manassasensis* (yeasts) from the soil samples and no isolates of the genus Aspergillus, Fusarium, and Penicillium were obtained. It may be explained that the differences of the environmental conditions in the geographical areas of the present research (Saudi Arabia) and that of the previous study (Kenya) influence the existence of microbes in the soil.

The screening of soil fungi for the selection of L-asparaginase producing isolate was carried out on a modified Czapek Dox's (MCD) agar medium. The production of ammonia indicated by the consequent zone formation due to the change of pH of the medium enabled the identification of L-asparaginase producing fungus. Many research studies have utilized this medium for screening the L-asparaginase synthesizing fungi (Chua et al., 2023; Hatamzadeh et al., 2020; Baskar & Renganathan, 2009). Alternatively, other earlier studies have employed media such as modified M-9 medium (Prakasham et al., 2009; Basha et al., 2009; Vimal & Kumar, 2022), Potato dextrose agar (Siddalingeshwara & Lingappa, 2011). However, the MCD medium is considered suitable for screening of L-asparaginase producing fungi owing to its composition which favors both the growth of the organism and enzyme production (Sisay et al., 2024).

The screening study revealed that all the fungal isolates were capable of synthesizing L-asparaginase by causing halo zones with the diameter ranging in sizes of 5.1-22.3 mm (Table 1). However, the mold *Cladosporium sp.* demonstrated superior property for L-asparaginase production. In consonance with our finding, Hatamzadeh et al. (2020) have demonstrated the L-asparaginase synthesizing potential of *Cladosporium sp.* Studies conducted

previously have explored the molds such as *Fusarium spp.* (Chua et al., 2023), *A. niger* (Dharmsthiti & Luechai, 2010), *A. tamarii* (Sarquis et al., 2004), *A. terreus* (Siddalingeshwara & Lingappa, 2011; Baskar & Renganathan, 2009) and *Penicillium* (Vimal & Kumar, 2022) for extraction of the enzyme L-asparaginase.

For the purpose of production of L-asparaginase using the potential isolate *Cladosporium sp.* ASP4, the method of solid state fermentation was carried out in the present study. Our study is in agreement with the contemporary studies which advocate the solid state fermentation as it comprises of slow growth of the mold and stable production of the enzyme (Dharmsthiti & Luechai, 2010; Basha et al., 2009; El-Naggar & El-Shweihy, 2020; El-Bessoumy et al., 2004; Siddalingeshwara & Lingappa, 2011; da Silva Menezes et al., 2017; Mohan Kumar & Manonmani, 2013). In contrast, various other studies have produced the enzyme by submerged fermentation (Sarquis et al., 2004; Basha et al., 2009; Amena et al., 2010; Vimal & Kumar, 2022; Chua et al., 2023; Baskar & Renganathan, 2009; Moharam et al., 2010; Moorthy et al., 2010). The solid substrate utilized in our study was soya bean meal, which reportedly contains substantial protein to offer sufficient nitrogen required for the fermentation process. Similarly, many research studies have utilized the soya bean meal for L-asparaginase production by marine actinomycetes (Basha et al., 2009), *Streptomyces rochei* (El-Naggar & El-Shweihy, 2020), *P. aeruginosa* (El-Bessoumy et al., 2004), *A. niger* (Dharmsthiti & Luechai, 2010) and so on. Some studies have employed groundnut oil cake powder (Amena et al., 2010; Baskar & Renganathan, 2009), carob pod (Siddalingeshwara & Lingappa, 2011) and wheat bran (Vimal & Kumar, 2022; Mohan Kumar & Manonmani, 2013) as a solid substrate for L-asparaginase production.

Review of literature inferred that there is no specific medium that can be used for different microorganisms for the enhanced production of L-asparaginase (Osama et al., 2023). Generally, the microbes differ among themselves by requiring specific nutrients and environmental conditions for enzyme production. Accordingly, there is a need to optimize the parameters of the fermentation process suiting to the growth characteristics of the microbe to achieve maximum production of the enzyme (El-Naggar & El-Shweihy, 2020). Sisay et al. (2024) have explained that the carbon and nitrogen sources are the critical factors which influence the physiology and metabolism of fungi to produce the enzyme. Siddalingeshwara & Lingappa (2011) have explained that the physiological pH is an important requisite for the tumoricidal activity of L-asparaginase.

Optimization of carbon source in the present study identified sucrose as the best option for higher production of L-asparaginase by *Cladosporium sp.* ASP4 (Fig. 2). Comparable works carried out on yeasts demonstrated that the sucrose and fructose have augmenting effect on L-asparaginase production (Sisay et al., 2024; Moguel et al., 2022). Other independent studies with *P. lilacinum* (Vimal & Kumar, 2022) and *S. rochei* (El-Naggar & El-Shweihy, 2020) have reported dextrose as the optimal carbon source for L-asparaginase production. Another striking observation of our study is the lowest production of L-asparaginase (6.25 IU/mL) when glucose was used as a carbon source. This confirms view that the glucose act as the repressor for production of L-asparaginase (Moharam et al., 2010).

Among the supplementary nitrogen sources, the ammonium nitrate displayed the superior influencing property on the enzyme production followed by ammonium sulfate and ammonium chloride (Fig. 3). In contrast to our finding, Dharmsthiti & Luechai (2010) have observed the negative effect of ammonium chloride on the production of L-asparaginase production by the fungus *A. niger*. However, Baskar & Renganathan (2009) have advocated ammonium chloride as the optimal nitrogen source for promoting *A. terreus* mediated synthesis of L-asparginase.

Optimization studies on the hydrogen ion concentration indicated the pH 8.0 as the promoting factor for L-asparaginase production (Fig. 4). In consistent with our observation, Siddalingeshwara & Lingappa (2011) have recorded a stable L-asparaginase production by *A. terreus* at the alkaline pH of 8.0 and the diminishing activity of the enzyme below this level. Many other studies on the production of fungal L-asparaginase have demonstrated that pH ranges of 7-9 are optimal for the enzyme synthesis by the mold *A. niger* (Dharmsthiti & Luechai, 2010), yeasts *T. asahii* and *C. palmioleophila* (Sisay et al., 2024) and the actinomycetes *S. gulbargensis* (Amena et al., 2010) and *S. rochei* (El-Naggar & El-Shweihy, 2020).

Previous studies on the optimization of incubation temperatures have demonstrated the temperature ranges 30-40^{o}C as optimal for enzyme production by fungi (Dharmsthiti & Luechai, 2010; Sisay et al., 2024; Siddalingeshwara & Lingappa, 2011) and actinomycetes (Amena et al., 2010). The potential isolate of the present study produced L-asparaginase at temperature ranges of 30-60^{o}C with a peak at 50^{o}C (Fig. 5). The high temperature requirement by *Cladosporium sp.* ASP4 may be attributed to its adaptation to the tropical climatic conditions of Saudi Arabia from where it was isolated.

The assay on the antitumor activity of the L-asparaginase inferred that the IC_{50} value is directly proportional to the enzyme concentration (Table 2). The enzyme failed to cause the half maximum cytotoxicity at lower concentrations (5-25 IU/mL) throughout the assay duration. The highest test concentration of the enzyme consumed 24h of treatment to attain its IC_{50} value. This infers that the enzyme concentrations ≥100 IU/mL would display efficient tumoricidal activity in a relatively shorter time against the lung cancer cells A549. Our study resonates with the findings of Prakasham et al. (2009) who demonstrated that IC_{50} values corresponding to 100-200 IU/mL are required for L-asparginase to cause lethality against the human T lymphocytes tumor cells CCRF-CEM. Comparable estimate (112 µg/mL) has been made by Moharam et al. (2010) from their studies with the liver cancer cells Hep-G2. Another study with the L-asparaginase of *S. rochei* recorded astoundingly low IC_{50} values (≤10 IU/mL) against the tumor cells of HeLa (epitheliod carcinoma), epidermoid larynx carcinoma (Hep2), breast carcinoma (MCF-7), Colorectal adenocarcinoma (Caco2) and hepatocellular carcinoma (HepG2) (El-Naggar & El-Shweihy, 2020). It may be explained that, by virtue of excellent adaptive capacities, the actinomycetes produce L-asparaginase enzymes with efficient cytotoxic properties.

The enzyme L-asparaginase produced in the current study displayed a moderate radical scavenging activity compared to the standard antioxidant ascorbic acid (Fig. 7). While the ascorbic acid exhibited the half maximum radical scavenging (SC_{50}) even at the lowest test concentration, the enzyme needed relatively higher concentrations (≥250 µg/mL) to achieve its effect. Proximal to our finding, Moharam et al. (2010) have abstracted high SC_{50} values (325 µg/mL) for the L-asparaginase produced in their study. However, a recent study established a substantial SC_{50} value of 6 mg/mL for the L-asparaginase produced by the mold *F. fujikuroi* (Chua et al., 2023). These estimates signify that the L-asparaginase produced by the *Cladosporium sp.* ASP4 of our study comprise a more efficient antioxidant property than those reported by the previous studies.

Results obtained in this study are encouraging to carry out further insightful studies with the synthesized L-asparaginase for exploring the opportunities of its promising biotechnological and pharmaceutical applications in the future.

5. CONCLUSIONS

The enzymes produced by soil borne fungi, besides rendering survival characteristics, possess efficient therapeutic potentials. Most of the soil fungi are capable of synthesizing L-asparaginase enzyme which can be detected by screening on Czapek Dox's agar medium. The mold *Cladosporium sp.* ASP4, despite its moderate prevalence in soil, displays superior property of L-asparaginase production. The solid state fermentation using soya bean meal renders better production of L-asparaginase by the fungi. The carbon sources glucose and sucrose act respectively as repressor and promotor for the production of the enzyme. Addition of the supplementary nitrogen source enhances the synthesis of L-asparaginase by the potential fungal isolate. The pH of 8.0 and temperature of 50^{o}C serve as optimal parameters for *Cladosporium sp.* ASP4 to produce the enzyme. The L-asparaginase produced in the present study displays a moderate antitumor activity against A549 cells with the IC_{50} values of ≥50 IU/mL. Assay of antioxidant property indicate that the synthesized enzyme demonstrates better radical scavenging activity in comparison with those of previous studies.

DISCLAIMER (ARTIFICIAL INTELLIGENCE)

The author hereby declares that NO generative AI technologies such as Large Language Models (ChatGPT, COPILOT, etc.) and text-to-image generators have been used during writing or editing of this manuscript.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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