Isolation and identification of microbial strains producing antineoplastic agent L-asparaginase

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Abstract
L-asparaginase (3.5.1.1) is an amidohydrolase enzyme that provides a catalytic site for converting asparagine into ammonia and aspartate. It is used to treat various types of cancer especially acute lymphoblastic leukemia. Our study aims to screen and identify indigenous fungal strains that produce asparaginase enzymes using plate assay method. In this study we isolated and screened four fungal strains. Out of four strains, one fungal strain was found to have maximum asparaginase production. Further, the fungal strain was subjected to liquid culture media, the maximum production of the enzyme was found on eight days of inoculation.

Keywords: Asparaginase, glutaminase, acute lymphoblastic leukemia (ALL), *Aspergillus Sp.*, antineoplastic agent.

Introduction

L-asparaginase reduces the activation energy of the reaction which involves the conversion of the L-asparagine into L-aspartate and ammonia[5]. The beta-acyl-enzyme intermediate is the consequence of an attack on the carbon atom of l-asparagine amide after the strong base NH_2 activates the enzyme's nucleophilic residue and afterward, a water molecule activates the nucleophile, which attacks the ester carbon one after the other, releasing ammonia and creating l-aspartic acid (Figure 1)[26,28]. ASNase also possesses glutaminase activity which involves conversion of L-glutamine via hydrolyzing it into glutamate and ammonia.

ASNase is a microbial enzyme that can be used as a therapeutic agent for cancer patients, especially in all lymphoproliferative disorders such as acute lymphoblastic leukemia (ALL), Hodgkin lymphoma, acute myelocytic leukemia, reticle sarcoma, melanosarcoma chemotherapy and lymph sarcoma etc.[12] In 1953, Kidd[15] showed that Guinea pig serum could prevent proliferation of cancer cells with the help of successive experiments on mice. He analyzed that mice showed reduction in tumor cells when treated with guinea pig serum injections.

The main reason behind its use as a therapeutic agent is that this enzyme targets an important metabolite asparagine which is a crucial metabolite for tumor cells. Both glutamine and asparagine are non-essential amino acids required for unlimited growth of tumor cells and their depletion can lead to destruction of tumor cells[2,23]. Because of their low asparagine synthetase (ASNS) levels, certain solid and hematological malignancies respond well to L-asparaginase therapy. Healthy cells are capable of producing their asparagine with the help of Asparagine synthetase (ASNS) enzyme but tumor cells rely on normal cells for asparagine[9] (Figure 1).

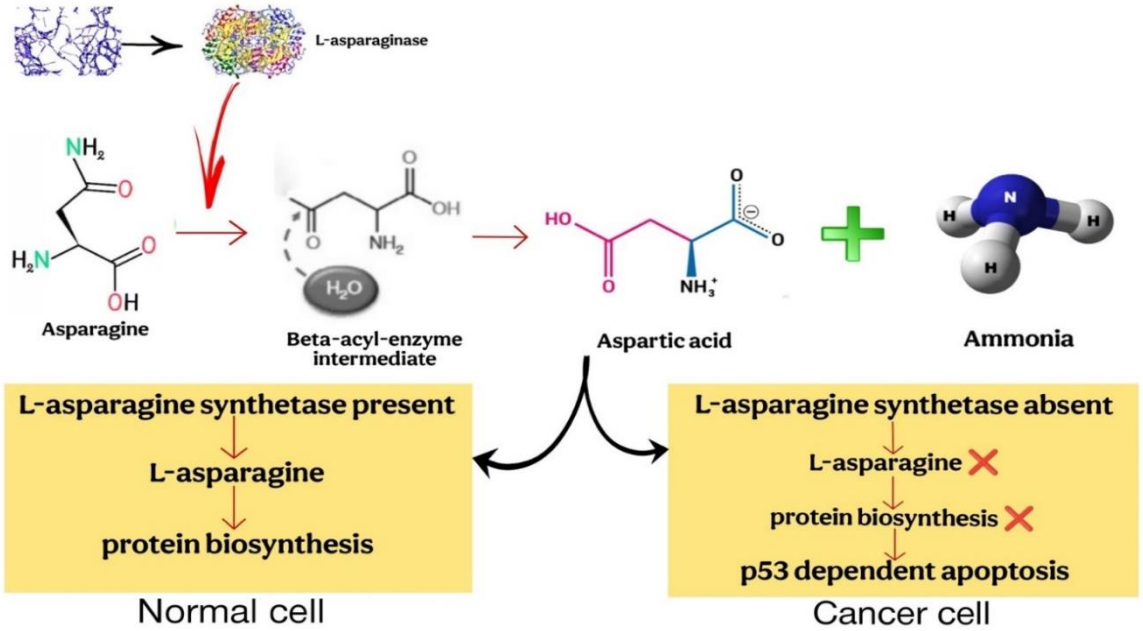

Figure 1: Therapeutic significance of L-asparaginase

Decreased RNA, DNA and protein synthesis, suppression of cell development and finally the activation of apoptotic cell-death processes are the results of insufficient levels of cellular asparagine[23]. Its great biodegradability, lack of toxicity and ease of administration at the local site account for its favored use. Other agents are relatively expensive and are known to cause significant discomfort when given to patients[24]. ASNS gene which is highly regulated at the transcriptional level contains information for expression of an enzyme, asparagine synthetase (ASNS) which uses aspartate and glutamine as a substrate to make asparagine and glutamate as a product, that is why, this enzyme is also known as glutamine hydrolyzing or glutamine dependent asparagine synthetase[9].

ASNS containing cells show less sensitivity towards the L-asparaginase enzyme which promotes reduction of asparagine level, so the ASNS enzyme maintains the asparagine level in case of its exhaustion[8,16]. The development of anti-asparaginase antibodies or the L-glutaminase activity of L-asparaginase, a secondary enzyme activity, is the reason for the adverse effects of *E. coli* L-asparaginase treatment[19,20]. L-asparaginase treatment adverse effects include allergy, abnormal coagulation, thrombosis, pancreatitis, liver failure, hyperglycemia and brain dysfunction, among others. Yeasts and filamentous fungi are the second largest category of deposited protein sequences according to the National Centre for Biotechnology Information[7], opening up new options for diverse sources of enzymes.

The ability to produce humanized L-asparaginase is the primary advantage of employing eukaryotic microorganisms, such as filamentous fungi, in addition to their increased productivity, protein stability and secretion capability[5,13]. L-asparaginase is used as a food processing aid to reduce the formation of acrylamide, a suspected carcinogen and neurotoxin, in starchy products. Some foods include bread and other baked goods. Fried or baked potato products when baked at 120°C, result in the formation of acrylamide because reducing sugars present in these foods combine with asparagine to form acrylamide during Maillard reaction[4]. L-asparaginase also possesses antimicrobial activity[14]. Antifungal and antibacterial activity is also possessed by this enzyme[29] which can be used against various microbes.

Encasing the enzyme L-asparaginase into a biopolymeric chitosan nanoparticle for efficient delivery improved its antibacterial properties[29]. PEG-native *E. coli* L-asparaginase (EcA) is the main treatment for the majority of ALL patients; sadly, many of them suffer from hypersensitivity reactions. This hypersensitivity may be brought on by either a negative immunological response to the enzyme or by the formation of neutralizing antibodies, also known as silent shutdown[22]. Numerous immunological responses such as hypersensitivity, abnormal clotting and allergic reactions, have been connected to bacterial sources of L-asparaginase. A new supply-like administration of asparaginase in combination with new drugs is needed to lessen these allergic and immunological problems[27].

The enzymes produced by fungi are probably less immunogenic than those produced by bacteria because they have evolved more closely with humans[1,10]. L-asparaginase is commercially available with names like Oncaspar, Colaspase, Crasnitin, Kidrolase, Erwinase and Elspar. The two commercially available L-asparaginases, Oncaspar and Erwinase, have asparagine Km and Kcat values of around 0.05 mM and 200–560 s^{-1} respectively[3,6]. A study by Sengupta et al[25] produced EcA variants with decreased immunogenicity, glutaminase activity, the inability to bind to pre-existing antibodies and longer serum half-lives produced as a result of the targeted protein engineering technique.

Material and Methods

Materials: The chemicals used for this study were purchased from the respective suppliers: Sodium Nitrate ($NaNO_3$), Potassium Dihydrogen Phosphate (KH_2PO_4), Magnesium Sulphate ($MgSO_4$), Potassium Chloride (KCl), Ferrous Sulphate ($FeSO_4$), Zinc Sulphate ($ZnSO_4$), Agar Powder and Phenol Red from Himedia, Bromothymol blue from Thomas Baker and L-Asparagine from CDH Ltd.

Isolation and Screening of L-asparaginase producing fungal strains: Soil samples were collected from a paddy field in Pihani village, Hardoi, using sterile bags for microbial isolation. After collection, 1.0 grams of soil was suspended in 10 mL of Milli-Q water and 300 µL of the suspension was inoculated onto Potato Dextrose Agar (PDA) containing Petri plates. The Petri plates were then placed in an incubator set at 25°C. Subsequently, four distinct fungal colonies (black, pink, olive-green and dark brown) were successfully isolated using the repeated streaking method[30].

All the pure colonies were tested for L-asparaginase production on Modified Czapek Dox agar (MCDA) medium which contains 0.09 g phenol red and 0.07 g Bromothymol blue(BTB), 0.03 g $NaNO_3$ (Sodium nitrate), $FeSO_4.7H_2O$ (Ferrous Sulphate), $ZnSo_4.7H_2O$ (Zinc Sulphate),1.52 g KH_2PO_4 (Potassium dihydrogen phosphate), 0.52g KCl (Potassium chloride),10g L-asparagine, 0.52g $MgSO_4 \cdot 7H_2O$ (Magnesium Sulphate) and 20 g agar per liter of Milli Q water. The pH of the MCDAwas adjusted to 5.5 by using 1M sodium hydroxide[11,21]. The medium was autoclaved and transferred into disinfected Petri plates. $NaNO_3$ was used as the only nitrogen source to prepare control plates. Pure colonies of isolated fungal strains were added to each plate individually and the plates were allowed to incubate for 72 hours at 37°C.

Morphological Characterization of L-asparaginase producing Fungal Strains: The most popular and easily prepared method for staining and studying fungus is the

lactophenol cotton blue (LPCB) wet mounted preparations. Three ingredients make up the preparation: cotton blue which colors the chitin in the fungal cell walls; lactic acid which maintains fungal structures and phenol which kills any living things. The concept behind the lactophenol cotton blue (LPCB) staining procedure is to facilitate the identification of the fungal cell walls. Fungal strains were stained with LPCB as described previously[18] and the prepared slides were used for imaging using the light microscope.

Secretion of L-asparaginase in liquid culture medium: The liquid media contain 10 g L-asparagine, 0.52 g $MgSO_4$ (Magnesium Sulphate) and 1.52g KH_2PO_4 (Potassium dihydrogen phosphate) per liter of Milli Q water. The Erlenmeyer flask (250 ml) filled with 80 ml of the liquid culture media was autoclaved and each flask was inoculated with *Aspergillus pink* strain (spore count per mL, ~ 4 X 10^6). The culture was allowed to grow at 25^0C in the BOD incubator. The aliquots of growth media were taken out every 24 hours for the next 14 days. The L-asparaginase activity was determined spectrophotometrically by quantifying the ammonia released using the Nesslerization method at 480 nm as explained earlier by Kumar et al[17]. In simple terms, 50 µL of liquid culture and 750 µL of freshly prepared L-asparagine (20 mM) mixed in 50 mM Tris–HCl buffer (pH 8.0) were incubated at 37°C for 30 minute.

The reaction was then quenched by adding 200 µL of 1.5 M trichloroacetic acid (TCA). The precipitated protein was extracted from the reaction mixture by centrifuging it for five minutes at 10,000 rpm and 4°C. 1.6 mL of pure water, 200 µL of Nessler's reagent and 200 µL of supernatant were mixed together and the absorbance of the resulting mixture was measured at 480 nm. The amount of ammonia released during the reaction was calculated using the ammonium sulfate standard curve. The specific activity of L-asparaginase is expressed in umole/min/ml of liquid culture medium.

Results and Discussion

All four pure colonies were tested for screening on MCDA media. The only nitrogen source employed for L-asparaginase screening on MCDA is L-asparagine. These amidohydrolases break down amine groups, releasing aspartate and ammonia when L-asparaginase is present. According to the study by Mahajan et al[21], the pH of the medium rises as a result of ammonia released in the medium, further reacting with water to form ammonium hydroxide. BTB dye had a distinct color contrast zone that changes from yellow at acidic pH to green at neutral pH and blue at alkaline pH whereas phenol red dye is yellow at acidic pH and turns pink at alkaline pH; this is seen surrounding the colonies on MCDA plates.

After two or three days one strain shows a positive indication towards L-asparaginase production. As we used the dual-indicator approach, we got one pink colony, producing a distinct pink color on MCDA (Figure 2B) plates while the other three colonies produced no color on MCDA plates. Therefore, we have interpreted this colony's ability to produce pink as a sign that L-asparaginase is being produced. Morphological characterization revealed distinct colonial and microscopic features that were recorded. According to morphology, we recognized this L-asparaginase producing colony belonging to *Aspergillus species* and named them according to their morphology as *Aspergillus pink* (Figure 2C).

As we got promising strain for L-asparaginase production, enzyme secretion was observed during the microbial growth in defined broth medium up to 14 days and then via analysis of specific activity data, we analyzed that *Aspergillus pink* is showing highest activity on the 8th day (Figure 3). Activity increases up to the 8th day and starts decreasing after the 8th day likely due to nutrient depletion in broth medium. That shows that L-asparaginase secretion in liquid media is highest around the 8th day.

Conclusion

Our investigation led to the successful isolation and identification of an indigenous *Aspergillus strain* that exhibits significant secretion of L-asparaginase in liquid culture media.

Figure 2: (A) Isolated fungal strain on PDA medium (B) Pink colour formation around the colony due to secretion of L-asparaginase on MCDA plate, (C) Morphological identification of isolated fungal strain by lactophenol cotton blue staining method.

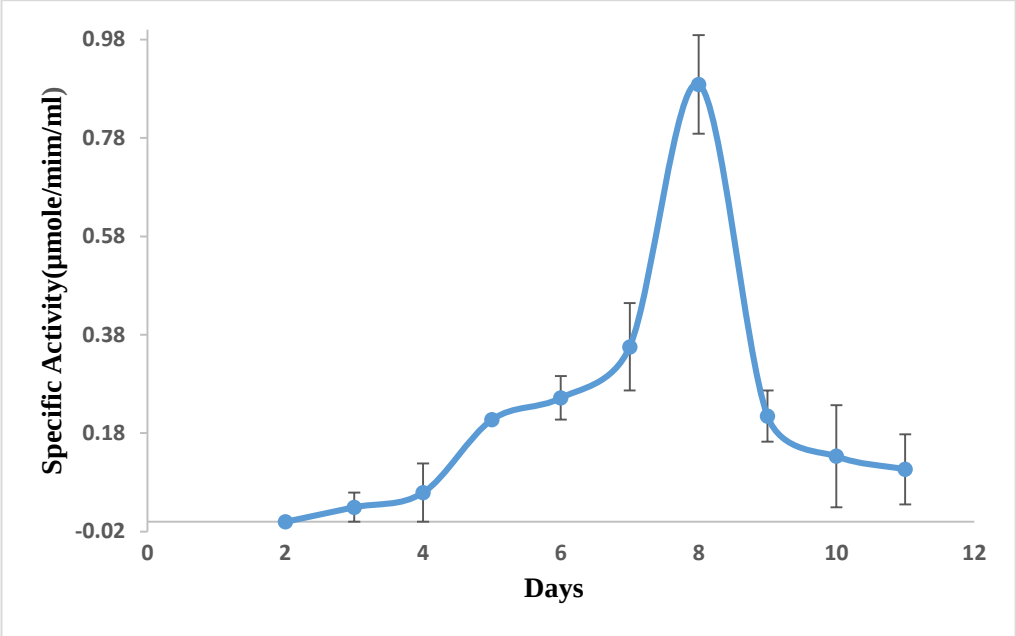

Figure 3: Secretion of L-asparaginase in the liquid culture medium by *Aspergillus sp.* containing 1% (w/v) of L-Asparagine.

Future work involving detailed biochemical and structural characterization of the secreted enzyme will explore its therapeutic potential, particularly in cancer treatment, as well as its possible applications in the food industry. This strain could prove to be a valuable biological resource for both sectors. In the future, this strain can offer safer alternatives and may contribute to the development of more targeted therapies for leukemia.

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