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L-Asparaginase Enzyme Production by *Aspergillus Pseudodeflectus* using Submerged Fermentation Under the Influence of Optimization Conditions, Purification and Characterization

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Abstract: Asparaginase converts L-asparagine to L-aspartic acid and has received considerable attention in the recent years for its anti-carcinogenic potential. L-asparaginase is a drug that is used to treat lymphoblastic cancer. The present work examined the capacity of a novel strain to generate extracellular L-asparaginase. The study looked at how L-asparaginase was made using MCDB which had the most L-asparaginase action. This study focuses on the optimization, synthesis, purification, and characterization of the L-Asparaginase enzyme derived from Aspergillus pseudodeflectus. The L-asparaginase was made by first removing the crude enzyme then precipitating it with ammonium sulphate, filtering it through a Sephadex column, and finally making it even purer through ion exchange chromatography. Different sources and concentrations of carbon, nitrogen and amino acid inducers (nutritional factors) as well as pH and temperature (physical parameters) were optimized to achieve enhanced L-asparaginase production. The study findings indicated that the most favourable conditions for enzyme production were a pH of 8.0 at 37°C. The carbon and nitrogen sources that demonstrated the highest efficacy were Lactose (0.5%) and L-asparagine (0.5%), respectively. The yield was also high in vitamin (1%), sodium acetate (1%), citric acid (1%), Tryptophan (1%), and sodium dihydrogen phosphate (1%). The production of L-asparaginase on a large scale can be achieved through continuous fermentation using the medium composition outlined in the study.

Keywords: L-asparaginase, fungi, Characterization, Optimization, Purification, Modified Czapek Dox broth.

Introduction

Enzymes are biological catalytic molecules that are widely exploited in industry to produce different essential products. Enzyme technology has enabled scientists to utilize, modify, and improve the efficacy of enzymes in order to achieve maximal functionality such as food industry, microbiology and in medicine L-asparaginase (EC 3.5.1.1), a chemotherapeutic medication, is used to speed up the conversion of L-asparagine to L-aspartic acid and ammonia. The ability of this substance to prevent the development of cancer has been under more and more scrutiny lately. (Osama et al., 2023; Mana et al., 1995). Cancerous cells typically require asparagine because they have lost the ability to express the gene encoding asparagine synthetase (Ekpenyong et al.,2021).Thus these cells depend on external sources

of L-asparagine for their persistence. Therefore, the usage of L-asparaginase as an intravenous injection will reduce L-asparagine levels and in turn prevents the growth of cancerous cells. AS a result, in the treatment of children with acute lymphoblastic leukaemia (ALL), L-asparaginase is considered as the most effective drug (Nunes et al.,2020).Furthermore, L-asparaginase is widely used to treat other types of cancer such as lymphosarcoma, Hodgkin's disease, reticulo sarcoma as well as different types of leukaemia (EI-Naggar et al.,2018).The enzyme is the cause of the decrease in L-asparagine levels. Because cancer cells cannot synthesize this amino acid, they are selectively eliminated. Several bacteria, such as Enterobacter aerogenes (Mukherjee., 2000), Serratia marcescens (Bernard and Howard,1969), and Enterobacter cloacae (Nawaz et al., 1998), produce the enzyme. The current treatment for acute lymphoblastic leukemia involves the use of enzymes derived from Escherichia coli and Erwinia carotovora(Dhevagi and Poorani, 2006). Nevertheless, long-term human use of L-asparaginase leads to the production of antibodies that may result in anaphylactic shock or make the medication useless. Therefore, searching for strains that are specifically and significantly capable of producing large amounts of L-asparaginase requires ongoing attention to recently discovered organisms. To determine whether Streptomyces can produce L-asparaginase, researchers have examined a variety of Streptomyces species, including *S. karnatakensis, S. venezualae, S. longsporus flavus,* and a marine Streptomyces sp. PDK2. (Narayana et al., 2007). The enzyme is produced worldwide using both solid-state and submerged cultures. There are various reasons why extracellular asparaginases are superior to intracellular asparaginases. Under typical circumstances, they can be produced in great quantities in the culture broth and inexpensively cleaned. Enzymes that break down amino acids play a crucial role in the treatment of certain types of cancer through medication. Research by (Sahu et al.,2007). indicates that asparaginase is effective in treating acute lymphocytic leukemia. Because tumour cells are unable to synthesize L-asparagine, they must absorb it from the blood or other body fluids. Chemotherapeutic medicines containing L-asparaginase enzymes can degrade the circulation L-asparagine. Tumor cells may be indirectly starved of nutrition as a result of the process, which will ultimately cause them to die. Microbial enzymes are more effective than those made by plants or animals because they are easier to modify, develop, and purify and less expensive to create. According to (Savitri et al.,2003), enzymes derived from microbes have greater stability when compared to those derived from plants or animals. The utilization of microbial systems to produce L-asparaginase is gaining popularity due to its cost-effectiveness and environmental friendliness. Microorganisms that can produce this enzyme include filamentous mushrooms, yeasts, and bacteria (Verma et al.,2007). For almost thirty years, asparaginase, derived from the bacteria Escherichia coli and Erwinia chrysanthemi, has been utilized as a medicinal agent (Aghaiypour et al.,2001; Krasotkina et al., 2004). Anaphylaxis and allergic responses are occasionally caused by bacterial L-asparaginases (Keating et al., 1993; Sarquis et al., 2004).Investigating asparaginase from different sources, such as eukaryotic bacteria, may yield an enzyme with fewer deleterious effects. Numerous investigations have demonstrated that filamentous fungus and yeast are capable of producing L-asparaginase. Researchers have found *Aspergillus terreus* and *Aspergillus tamari* to be trustworthy sources of this enzyme. There are few reports on fungi producing L-asparaginase. There are several papers

on the synthesis of L-asparaginase by *Aspergillus pseudodeflectus* species, but none include the entire process of purification and optimization. On the other hand, no information exist about the characterization of the *Aspergillus sp.* enzyme. Additionally, the food processing and therapeutic applications sectors have high need for L-asparaginase. Choosing a suitable strain and employing a good collection technique are critical elements in the production of enzymes for commercialization. The appropriate source species and enzyme concentration are equally crucial for clinical research. To address this issue, alternative sources of enzymes must be investigated. We thus report on the purification and process parameter optimization for L-asparaginase synthesis in this work.

Materials and Methods

Used fungal strains

Fungal samples from the Regional Plant Resource Centre Microbiology Laboratory that showed potential for L-asparaginase production underwent further testing.

Identification and Characteristics

The isolates were classified according to their molecular features. The fungal cultures were identified at the Microbial Type Culture Collection (MTCC) in Chandigarh.

Preparation of pure cultures and inoculum

To keep the pure cultures at 4 °C, they were grown on Sabouraud Dextrose agar slants and subculture regularly. The inoculum was made up of cultures that had been grown on Sabouraud-Dextrose agar plates for 5 to 6 days.

Media used

A 4.5-pH broth containing glucose and asparaginase was formulated, and 20 ml portions were transferred into 100 ml containers. The containers were sterilized for 15 to 20 minutes at 121°C.

Inoculation

A single 5 mm disc of inoculum derived from the culture plates was inoculated into the flask containing broth. The culture flasks were incubated at 30°C for 6 days in static conditions.

Assay for Asparaginase

Qualitative analysis

This is a modified Czapek Dox medium that has glucose (2 grams per liter), L-asparaginase, L-glutaminase, KH2PO41.52 grams per liter, KCl 0.52 grams per liter, MgSO4.7H2O 0.52 grams per liter, small amounts of Fe SO4.7H2O, ZnSO4.7H2O, and $CuNO_3.7H_2O$, and agar (18 grams per liter). For the phenol red dye, the pH of the medium was changed to 6.2. Another step was to put the medium in an autoclave. It was used to make a stock solution of 2.5%

phenol red dye. Then, 0.009% phenol red dye was added to the MCD medium that had been cleaned. The mixture was then put onto plates that had already been sterilized. There was a pink zone around the fungal colonies that showed that L-asparaginase-producing fungi were growing.

Quantitative analysis

The ammonia measurement procedure was carried out by conducting an enzyme assay using a Modified Czapek Dox medium broth following the Nesslerization method as described by Imada. To make the enzyme test mixture, 0.5ml of 0.04M L-asparagine was mixed with 0.5ml of Tris HCl buffer (pH 8.6). Then 0.5ml of enzyme and 0.5ml of distilled water were added. Subsequently, the combination was subjected to incubation for 30 minutes at a temperature of 37^0C degrees Celsius. Following the incubation period, the reaction was halted by introducing 0.5ml of 30% TCA. To estimate the enzyme activity, 7ml of distilled water was combined with 0.5ml of enzyme mixture followed by the addition of 1ml of Nesseler's Reagent. Next, the optical density was measured at a wavelength of 480nm using a spectrophotometer to estimate the enzyme activity.The quantity of ammonia emitted by the test sample was evaluated by comparing it to the reference graph. The measurement of the enzymatic activity of L-asparaginase was conducted using International Units (IU). An International Unit (IU) of L-asparaginase activity is the amount of enzyme required to produce one micromole of ammonia per milliliter per minute at a temperature of 37°C and a pH of 8.6.

Gene sequence

FP-7: New : ITS5f+ITS4R : 100% sequence similarities with Aspergillus pseudodeflectus

>GCAGGTCTGCCCCCGGGCAGGCCTAACCTCCCACCCGTGAATACCTGACCAACGTTGC
TTCGGCGGTGCGCCCCCCCGGGGGTAGCCGCCGGAGACCACACCGAACCTCCTGTCTTT
AGTGTTGTCTGAGCTTGATAGCAAACCTATTAAAACTTTCAACAATGGATCTCTTGGTTCCGG
CATCGATGAAGAACGCAGCGAACTGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA
TCGAGTCTTTGAACGCACATTGCGCCCCCTGGCATTCCGGGGGGGCATGCCTGTCCGAGC
GTCATTGCTGCCCTTCAAGCCCGGCTTGTGTGTTGGGTCGTCGTCCCCCCCGGGGGACG
GGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCAC
CCGCTCGATTAGGGCCGGCCGGGCGCCAGCCGGCGTCTCCAACCTTCTATTTTACCAGGT
TGACCTCGGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAA<

Optimising process parameters for the production of L-asparaginase

The dynamics influencing the L-asparaginase were assessed and the optimizations were carried out by varying one parameter at a time keeping the other parameters unaltered for the maximal production of asparaginase. The optimization of L-asparaginase by *Aspergillus pseudodeflectus* depends on several factors such as incubation period, temperature and pH. The effects of different carbon sources, nitrogen sources, and different inducers are important factors when optimizing the medium to improve L-asparaginase production. They are also essential for the development of organisms by synthesizing key nutrients in a liquid medium.The nutritional parameters were optimized by OFAT(Jenila and Gnanadoss, 2018).

Effect of carbon sources

To examine the influence of different carbon sources on the production of asparaginase the following carbon sources were used: fructose, galactose, lactose, maltose, glucose, mannitol, sorbose, xylose, Arabinose, Raffinose, starch and Inositol. These carbon sources were added to modified Czapek's-Dox medium (MCD) broth at equivalent weight. Various carbon sources were introduced into the medium at a concentration of 1% (W/V) with pH 6.2. Two flasks were set up for each carbon source tested one no. of selected organism Aspergillus pseudodeflectus was inoculated and incubated at 30°C for six days. All environmental conditions remained same for the enhancement of enzyme production. after which the enzyme activity was assessed using the supernatant. The estimation method was same as above.

Effect of Nitrogen Sources

To evaluate the impact of nitrogen sources on the production of asparaginase, both organic and inorganic nitrogen sources were added to the fermentation fluid. The nitrogen sources used include urea, ammonium fluoride, ammonium persulphate, ammonium chloride, cobaltous nitrate, ammonium sulphate, sodium nitrate and potassium nitrate. The nitrogen source was added to the medium at a concentration of 1% (W/V) and incubated for six days and the enzyme activity was determined using the supernatant.

Effect of pH

To ascertain the ideal pH for L-asparaginase production, the fungal culture was inoculated into a series of varying pH solutions (4.5, 5.5, 6.5). The pH of the solutions was subsequently modified by combining 1N NaoH with 1N HCl. Before undergoing autoclaving, the organism underwent inoculation. The supernatant from the six-day incubation period of the flasks was utilized to quantify the asparaginase activity.

Effect of different enhancers to enhance the production of L-asparagine enzyme activity

To assess the impact of various vitamins (C, B1, B6, B2, M) on the formation of asparaginase, the presence of plant oils- peppermint oil and olive oil was examined. Tween-20, Tween-40, and Tween-80 are types of surfactants. Some of the ionic salts that were used were potassium acetate, sodium nitrate, calcium chloride, tricalcium phosphate, sodium thiosulfate, sodium trisulfate, sodium chloride, ammonium sulfate, ferrous sulfate, zinc sulfate, ferric chloride, glycine, calcium phosphate, sodium dihydrogen phosphate, and dipotassium hydrogen phosphate. Different ionic salts were added to the medium at a concentration of 0.5% (W/V). Selected fungi Fusarium proliferative was inoculated. The mixture was then left to grow for 6 days at 30°c, and the supernatant was used to measure the enzyme activity. The estimation method was the same as above. All experimental conditions were maintained constant.

Effect of Aminoacids

The impact of amino acids on the optimization of L-asparaginase production was assessed by introducing a range of amino acids (Threonine, Tryptophan, Proline, Aspartic acid, Phenylalanine, Cysteine, Methionine, Tyrosine, Arginine, Histidine, Citric acid, Oxalic acid,

Yeast extract) at a concentration of 0.5% (W/V) into the medium. The medium was then incubated for 6 days, and the resulting supernatant was utilized to measure the enzyme activity.

Purification studies

Mass production, classification and extraction, purification

The organisms were introduced into the previously specified broth media and kept in a stationary environment at a temperature of 30°C for 6 days. The samples were collected following the appropriate incubation period. The biomass was quantified by weighing it and then subjected to treatment with a 0.05 M Tris-HCL buffer at a pH of 8.5. The sample was centrifuged at a speed of 600 rpm for 20 minutes at a temperature of 4^0C which led to the formation of a liquid. The statement described above was considered to be the basic composition of the enzyme. The enzyme activity and protein content were quantified and thereafter, the sample underwent the initial purification phase using ammonium sulfate precipitation.

Ammonium sulphate precipitation

The initial solution underwent precipitation by adding finely powdered ammonium sulfate until it reached a saturation level of 80%. The temperature was kept constant at 4°C. The material underwent centrifugation at a speed of 6000 rpm for 10 minutes, continuing overnight. Afterward, the pellet that was retrieved was dissolved in a Tris-HCl buffer solution with a pH of 8.5, which had a concentration of 0.05 M (Suchita et al.,2010).

Sephadex G-100 gel filtration

The samples that underwent precipitation with ammonium sulfate were assessed for their enzymatic activity and protein concentration. Afterward, they were subjected to gel filtration using Sephadex G-100 (Patro and Gupta,2012).

Gel-column preparation and sample application

A glass tubing chromatography column measuring 60 cm in height and 2.2 cm in diameter was utilized. The eluent was 0.05 M Tris-HCL buffer with a pH of 8.5. To inhibit the formation of bubbles within the gel bed. Then the eluent was transferred from securely sealed brown bottles into containers containing the same temperature. The gel slurry was prepared by dissolving 5 grams of Sephadex in 200 ml of 0.05 M Tris-HCL buffer and allowing it to swell at room temperature for twenty-four hours. The column was secured and closed using Sephadex. Ammonium sulfate was used to precipitate the samples which were then continuously poured onto the column. The resulting fractions were collected in containers with a volume of 5ml. A random assortment of the gathered fractions underwent protein and enzyme activity analysis, and the fractions demonstrating exceptional enzyme activity were combined.

Ion exchange chromatography

The column was constructed by dissolving the necessary quantity of DEAE-cellulose in Tris-HCl buffer overnight. After the packaging procedure, the column was rinsed with 5M KCl to produce the desired weak ion exchange material. This was followed by rinsing with 5M NaOH to remove ionic charges from the ion exchanger. Afterward, the material was washed with distilled water and a 0.05 M Tris-HCl buffer solution at a pH of 8.5. Following the utilization of Sephadex for fractionating the peak fractions, they were next transferred to the ion exchange column where 5 ml of the fractions were gathered. After assessing the protein and enzyme activity of the samples and the fractions comprising the most active enzymes were consolidated and stored in a deep freezer.

Enzyme characterization based on partial purification

Substrate Specificity

The assay mixture was enriched with several substrates, such as L-arginine, L-phenylalanine, L-histidine, L-glutamine, and L-aspartic acid, to evaluate the enzyme's inclination towards a range of substrates. The substrates were present at a concentration of 0.04M, with L-asparagine serving as a control.

PH optima

The pH of the Tris-HCl buffer in the reaction mixture was methodically varied between 3 and 10 to identify the most favorable pH for enzyme activity. The enzymatic activity was measured at different pH values.

Temperature tolerance

The enzyme was maintained at temperatures ranging from 30 to 50°Cbefore its addition to the reaction mixer for the assay.

Incubation period

The enzyme was incubated at varying temperatures of 30, 37, and 50°Cbefore its addition to the reaction mixer for the assay.

Results and Discussions

Optimising the process parameters for the synthesis of L-asparaginase

Generally carbohydrates are used as carbon sources in the fermentation processes. During industrial fermentation process the energy for the growth of microorganism is achieved either from the oxidation of medium components or from light. The growth and maximum enzyme production are derived from Carbon sources which is normally observed in the synthesis of primary metabolites, such as enzymes. The carbon concentration had a positive effect on L-Asparaginase production and high titres can be obtained in a medium rich of carbon source. The present study aimed to evaluate the impact of substituting glucose in the basal medium with alternative carbon sources, namely Glucose, lactose, Galactose, Maltose, Mannitol, Sorbitol, Raffinose, Inositol, Arabinose, Starch, Xylose, and Fructose on the

equimolecular carbon foundation. The results in (Table 1 and Fig.1) demonstrate that the maximum enzyme activity was promoted when fructose used as a carbon source and enzyme activity obtained was 24.4 IU/ml. while the lowest L-Asparaginase production was recorded when using Glucose 20.8 IU/ml.This results are similar with study of Prakasham et al.,(2007) discovered that lactose is the most effective carbon source for the manufacture of L-asparaginase in aerobic conditions. Venil and Lakshamanpalsmy(2009) have documented a decrease in the synthesis of L-asparaginase following the administration of fructose as a supplement. The highest level of active L-asparaginase synthesis was seen in Staphylococcus sp. when it was fed with maltose. According to Akilandeswari et al.,(2012), it has been determined that proline is the most effective carbon source for the formation of L-asparaginase. Baskar and Renganathan reported that glucose was found to be best carbon source for maximum L-Asparaginase production *Aspergillus terrus MTCC 1782*(Baskar & Renganathan,2011).

Table-1- Effect of different Carbon sources utilization on L-asparaginase production *Aspergillus pseudodeflectus*.

Carbon sources	**Enzyme activity (IU/ml)**
Sorbose	1.1±0.00
Xylose	10.8±0.60
Galactose	0.71±0.01
Lactose	6.8±1.70
Arabinose	0.55±0.01
Maltose	1.22±0.03
Glucose	20.8±1.10
Raffinose	4.0±0.01
Starch	0.51±0.00
Inositol	0.33±0.00
Mannitol	0.68±0.06
Fructose	24.4±0.70

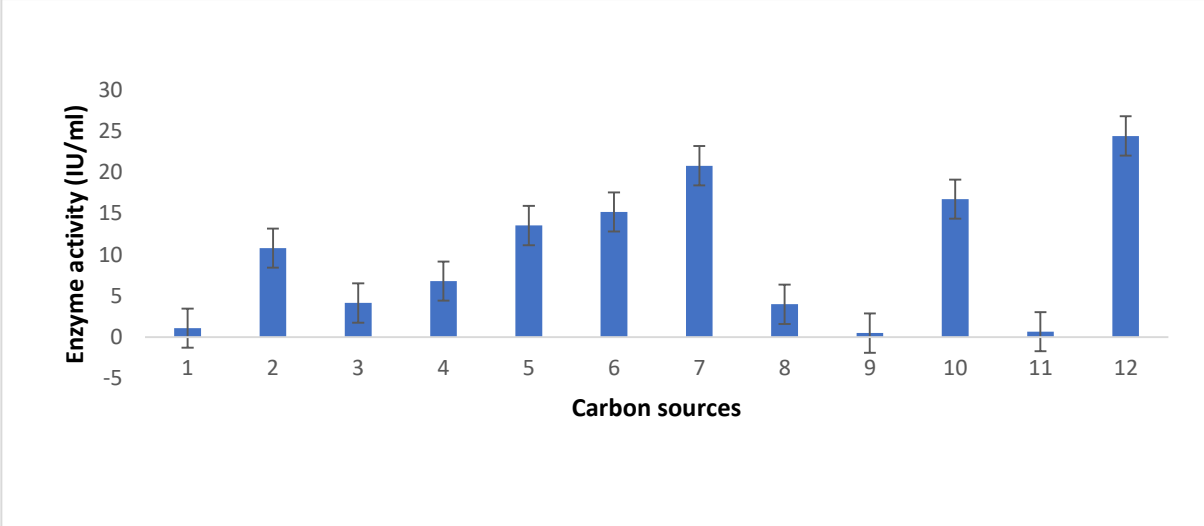

Fig.1- L-asparaginase activity of *Aspergillus pseudodeflectus* with different carbon sources (1%) 1-sorbose 2-Xylose, 3-Galactose, 4-Lactose, 5- arabinose, 6-Maltose, 7- Glucose, 8- raffinose, 9-starch, 10- inositol, 11-Mannitol, 12- Fructose.

Nitrogen sources have been preferred for enhancing the production of L-Asparaginase. The organic form or inorganic form sometimes both, nitrogen source is utilized by most of the industrial enzymes. In most of the industrial fermentation process growth will be faster with supply of organic and inorganic nitrogen source. The results in (Table 2 and Fig.2) demonstrate that the maximum enzyme activity was promoted Ammonium sulphate in the medium enhanced growth of microorganism. and consequently the L-Asparaginase production 32.7 IU/ml. These results are in good agreement with those reported for the production of L-Asparaginase by other microorganisms (Murali, 2011). Kalyanasundaram et al., (2015) used ammonium sulphate as a nitrogen source for the maximum production of L-Asparaginase from A. terreus. Gaffar and Shethna, (1977) observed the positive effect of supplementation of ammonium sulphate in the production of L-asparaginase40. Sreenivasulu et al.,(2009) have reported ammonium sulphate exhibited maximum enzyme production by the isolated fungus VS-26. In contrast, Narayana et al.,(2008) discovered that yeast extract exhibited the highest level of activity and whilst Neelima et al.,(2014) determined that peptone served as the most effective nitrogen source.

Table-2- Effect of different Nitrogen sources utilization on L-asparaginase production by *Aspergillus Pseudodeflectus*.

Nitrogen Sources	**Enzyme activity (IU/ml)**
Ammonium Sulphate	32.7±1.0
Ammonium Fluoride	22.1±0.1
Ammonium Persulphate	0.21±0.03
Ammonium Chloride	0.22±0.04
Sodium Nitrate	0.98±0.06
Ammonium Nitrate	17.1±0.70

Urea	0.13±0.00
potassium Nitrate	16.8±0.81

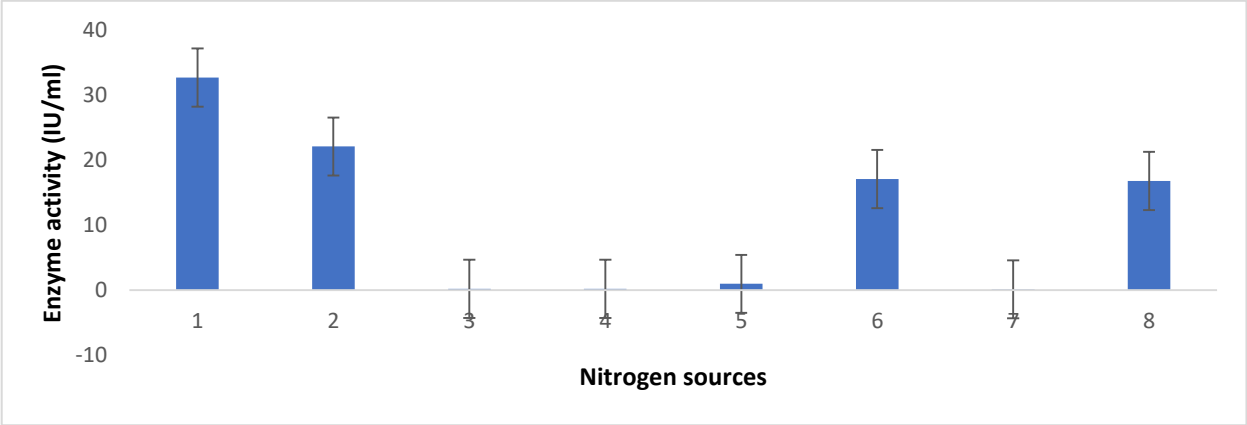

Fig.2- L-asparaginase activity of *Aspergillus pseudodeflectus* with different Nitrogen sources (1%) 1-Ammonium sulphate, 2-Ammonium fluoride, 3-Ammonium persulphate,4-Ammonium chloride, 5- sodium nitrate, 6- Ammonium nitrate, 7-urea, 8- potassium nitrate.

The presence of ionic salts also changes the growth of microorganisms. These salts either speed up or slow down growth depending on how they affect the microorganism's development and work with other metabolic enzyme (Hosamani and Kaliwal.,2011).In this case, vitamin C, vitamin B1, peppermint oil, sodium dihydrogen phosphate, glycine, ferric chloride, zinc sulfate sodium chloride, and sodium acetate all helped the growth and had the highest activity level of 47.6 IU mL−1 at pH 6.5, 6 days of incubation and a concentration of 0.5%. The media used was Lactose (6.0 g/l) and Sodium Nitrate (1.0 g/l). The ions in the prepared media are different, and their effects have been studied and reported, as shown in (Table 3 and Fig 3). When MCD media was mixed with ionic salt, the enzyme activity was the lowest of all the metal ions that were studied.

Table-3- Effect of different Ionic salts utilization on L-asparaginase production by *Aspergillus pseudodeflectus*.

Ionic salts	**Enzyme activity (IU/ml)**
Vitamin-c	19.6±0.60
Vitamin-B1	2.10±0.03
Vitamin-B6	18.0±0.01
Vitamin-B2	0.51±0.01
Vitamin-M	41.2±1.10
Pipermint oil	1.80±0.00
Olive oil	1.68±0.17
Tween-20	1.64±0.00
Tween-40	1.80±0.01
Tween-80	1.52±0.00

Calcium Phosphate	47.6±0.60
Disodium hydrogen orthophosphate	21.52±0.15
Sodium phosphate	2.10±0.11
Glycine	22.12±0.15
Ferric chloride	1.44±0.10
Zinc sulphate	18.86±1.15
Potassium Nitrate	11.80±0.00
Sodium Acetate	2.12±0.01
Ferrous sulphate	1.64±0.00
Ammonium sulphate	1.04±0.00
Sodium chloride	1.78±0.03
Sodium Trisulphate	1.52±0.00
Sodium Thiosulphate	1.64±0.00
Tricalcium phosphate	15.06±0.11
Calcium chloride	1.64±0.00
Sodium Nitrate	1.64±0.00
Potassium Acetate	11.58±0.80

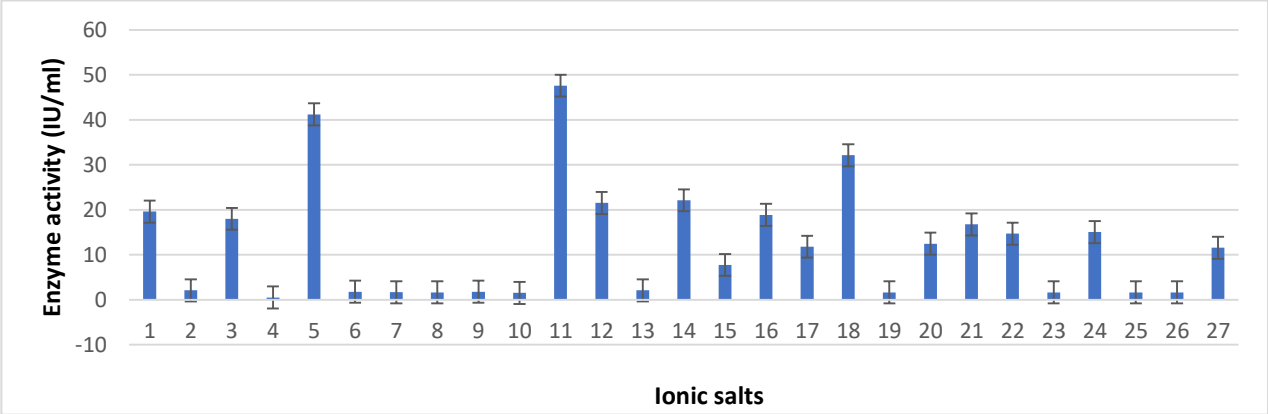

Fig.3- L-asparaginase activity of *Aspergillus pseudodeflectus* with different Ionic salts (0.5%) 1-Vitamin-c, 2-Vitamin-B1, 3-Vitamin-B6, 4-Vitamin-B2, 5-Vitamin-M, 6-pipermint oil, 7-olive oil,8-Tween-20, 9-Tween-40, 10- Tween-80, 11- Calcium phosphate, 12-Disodium hydrogen orthophosphate, 13- Sodium phosphate, 14-glycine, 15-Ferric chloride, 16-Zinc sulphate, 17-Potassium Nitrate, 18-Sodium Nitrate, 19-Ferrous sulphate, 20-ammonium sulphate, 21-Sodium chloride, 22-Sodium Trisulphate, 23-Sodium Thiosulphate, 24-Tricalcium phosphate, 25-Calcium chloride, 26-Sodium Nitrate, 27-Potassium Acetate.

The enzyme activity can be either enhanced or inhibited depending on the change in the pH and hence can influence the growth of microorganisms (Jalgaonwala and Mahajan.,2011).Optimal pH levels in the growing medium are crucial for maximizing L-asparaginase synthesis. It looked at how different starting pH levels (5.5 and 6.5) affected

the production of L-asparaginase. The data for the fungi (Table 4 and Fig 4) showed that pH 6.5 was best for L-asparaginase activity 52 IU/ml. At different pH levels, the test fungus can grow and make intracellular L-asparaginase. This result was similar to that deduced by Thirunavukkarasu et al.,(2011) who worked on the effect of pH on asparaginase reported pH of 6.2 as optimum for asparaginase production by Fusarium sp. but Moorthy et al.,(2010) had found pH 7 was the best for asparaginase activity. G.Thirumurugan et al.,reported an optimum asparaginase production at pH 8.0 by *Aspergillus Terreus* (Thirumurugan et al.,2011).

Table-4- Effect of different pH on L-asparaginase production by *Aspergillus pseudodeflectus.*

Carbon and Nitrogen sources (%)	**Enzyme activity IU/ml (pH 5.5)**	**Enzyme activity IU/ml (pH 6.5)**
Fructose 2.0 + Ammonium sulphate 1.0	3.70±0.41	19.52±0.30
Fructose 2.0 + Ammonium sulphate 2.0	2.85±0.21	11.23±0.23
Fructose 2.0 + Ammonium sulphate 3.0	1.44±0.17	9.28±0.40
Fructose 4.0 + Ammonium sulphate 1.0	8.37±0.11	31.49±0.55
Fructose 4.0 + Ammonium sulphate2.0	13.38±0.15	33.25±0.37
Fructose 4.0 + Ammonium sulphate 3.0	12.13±0.34	37.24±1.01
Fructose6.0 + Ammonium sulphate 1.0	31.56±0.70	52.0± 0.76
Fructose 6.0 + Ammonium sulphate 2.0	29.02±1.06	44.98±1.41
Fructose 6.0 + Ammonium sulphate3.0	24.50±0.66	40.66±0.53

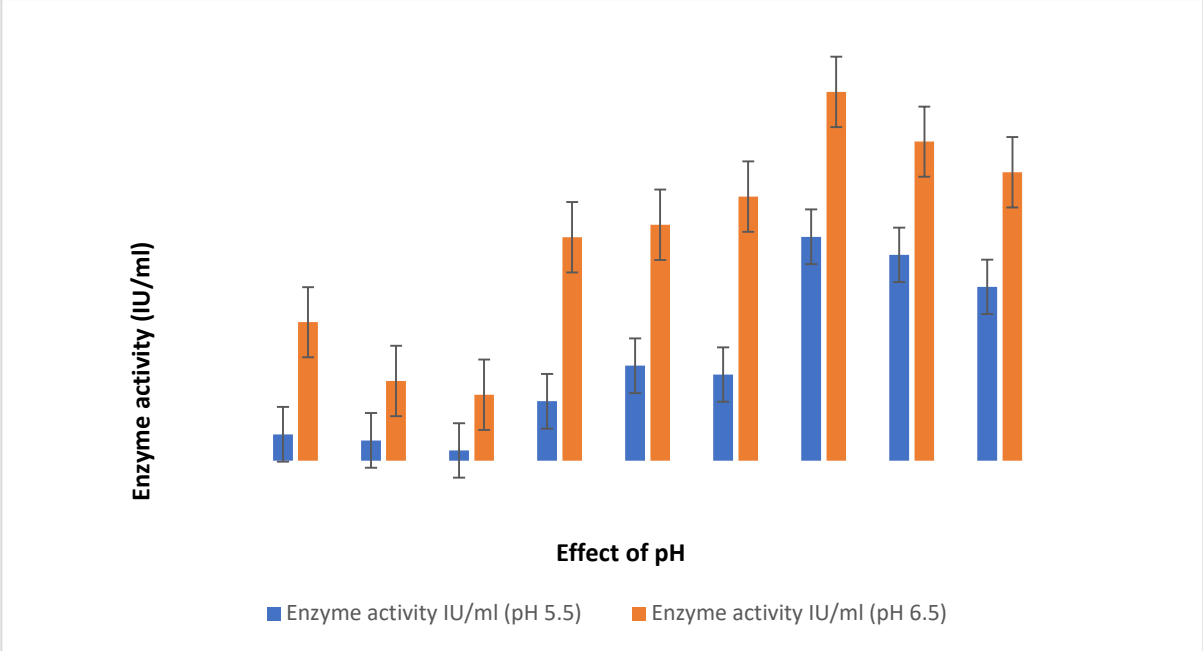

Fig.4- L-asparaginase activity of *Aspergillus pseudodeflectus* with different initial pH values on L-asparaginase production .

The presence of amino acids, which act as co-factors for several biosynthetic enzymes and enhance or inhibit growth depending on their effect on the development of the microorganism (Hosamani and Kaliwal., 2011). The present study demonstrates that citric acid has a beneficial impact on growth with its maximal activity at a pH of 6.5 over a six-day incubation period with 0.5% citric acid concentration and Lactose-6.0g/l and sodium nitrate-1.0g/l media. However, the prepared media contain a variety of amino acids and the impacts of these ions have been examined and documented as illustrated in (Table 5 and Fig 5). The enzyme activity exhibited the lowest value among the amino acids that were tested when MCD media was supplemented with amino acids.

Table-5- Effect of different amino acids utilization on L-asparaginase production by *Aspergillus pseudodeflectus*.

Amino acids	**Enzyme activity (IU/ml)**
Threonine	32.64±1.21
Tryptophan	19.34±1.12
Proline	50.6±1.4
Aspartic acid	1.58±0.14
Phenylalanine	17.6±0.0
Cysteine	0.20±0.01
Methionine	13.31±0.90
Tyrosine	0.44±0.02

Arginine	1.58±0.08
Histidine	11.43±0.89
Citric acid	22.46±1.31
Oxalic acid	0.52±0.01
Yeast Extract	20.04±1.13

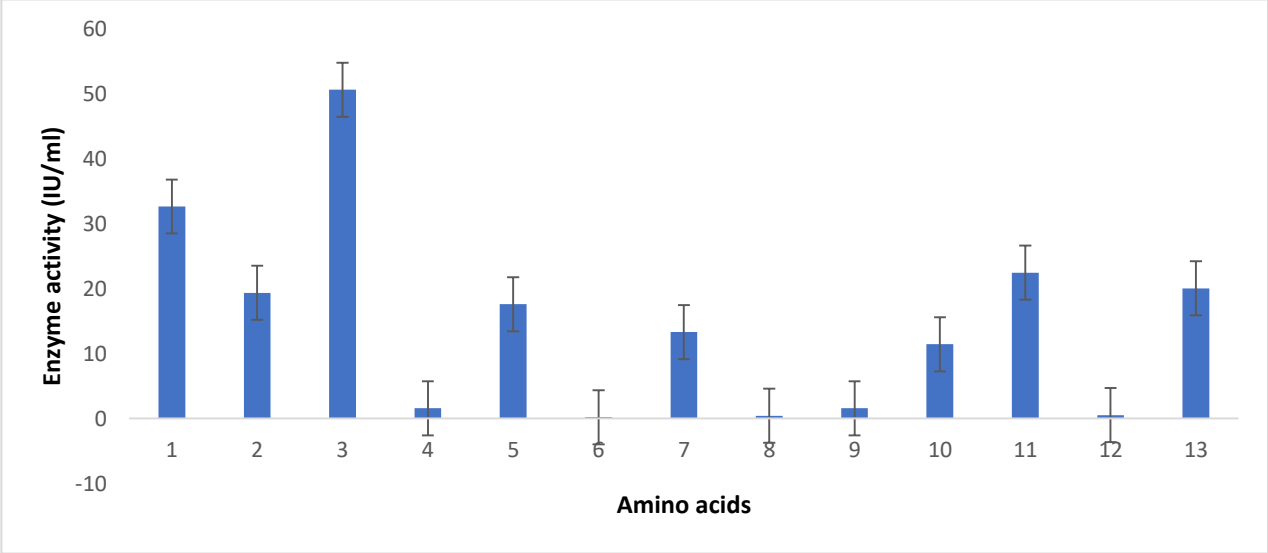

Fig.5- L-asparaginase activity of *Aspergillus pseudodeflectus* with different Amino acids (0.5%) 1-Threonine, 2-Tryptophan, 3-Proline, 4-Aspartic acid, 5-Phenyl alanine, 6-Cysteine, 7-Methionine, 8-Tyrosine, 9-Arginine, 10-Histidine, 11-Citric acid, 12-Oxalic acid, 13-Yeast extract.

Purification studies

The enzyme L-asparaginase was purified from the culture filtrate of *Aspergillus pseudodeflectus* using ammonium sulphate precipitation, ion exchange chromatography followed by gel filtration.The purification procedure summerized in (Table-6). The first step of purification by ammonium sulphate precipitation achieved 1.84 fold purification with 212.24% enzyme recovery. The second purification step was performed by gel filtration using Sephadex G-100 column.. This step showed 1.58 fold increase in enzyme activity with 228.57% enzyme recovery. The final step of purification was done by ion exchange chromatography using DEAE cellulose The fractions showing L-asparaginase activity in this step were collected and pooled. The final step of purification resulted in 1.56 fold increase in enzyme activity with yield of 259.183% enzyme recovery. L-asparaginase from various fungal species have been purified and characterized and reported earlier. L-asparaginase from *Penicillium brevicompactumNRC 829* was purified to 151.12 fold with a specific activity of 574.24 U/mg and yield of 39.90% (Eishafei et al.,2012) and L-asparaginase purified from Mucor hiemalis exhibited a specific activity 69 U/mg with 18.46% recovery and 4.59 purification fold (Monica et al.,2013). Interestingly, L-asparaginase from *Cladosporium sp.* was purified to 867.7 fold with a specific activity of 83.8 U/mg (Kumar and Manonmani,2013). Although, the purification steps followed by various researchers are almost similar for

different fungal species, the purification fold and yield varies. This could be due to the interference of different proteins present in the culture filtrate. *Debaryomyces sp. CECT* 11815 was acquired by employing ammonium sulphate treatment subsequently followed by anion exchange chromatography and gel filtration as recorded by (Dura et al.,2002). Dura found that the asparaginase showed notable instability especially during the gel-filtration phase.

Table-6- Purification profile of L-asparaginase from *Aspergillus Pseudodeflectus*.

Steps	**Collected volume(ml)**	**Total activity (IU)**	**Total protein(mg)**	**Specific activity (IU/mg)**	**purification fold**	**Yield (%)**
culture filtrate	3320	98	520	0.188	0	100
Ammonium sulfate precipitation	150	208	600	0.346	1.84	212.24
Gel filtration	180	224	750	0.298	1.58	228.57
Ion exchange chromatography	220	254	860	0.295	1.56	259.183

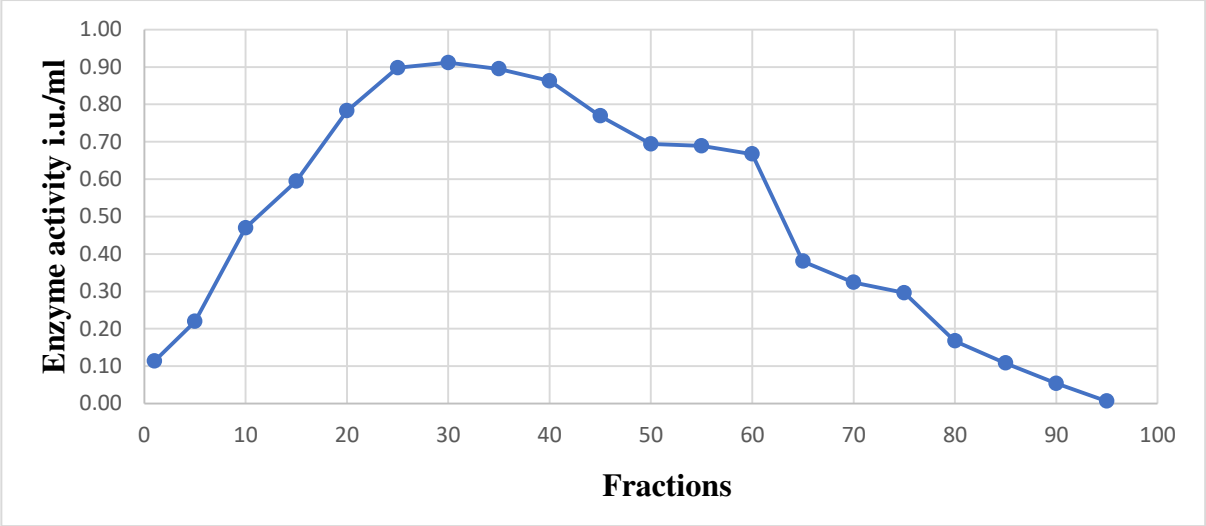

Fig.6- Purification of L-asparaginase from *Aspergillus Pseudodeflectus* theGel filtration column chromatography (Sephadex G-100) of the fractions collected from ammonium sulfate precipitation fractions

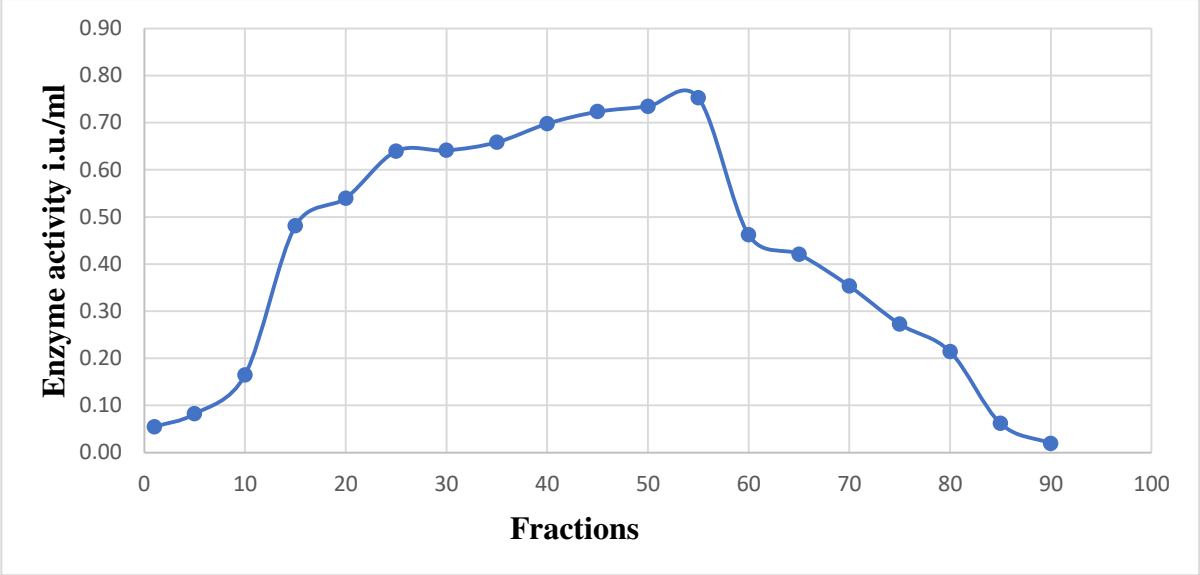

Fig.7- Purification of L-asparaginase from the *Aspergillus pseudodeflectus* ion exchange column chromatography fractions.

Characterization of purified Enzyme

The investigation of the enzyme's pH optimization involved the use of a Tris-HCl buffer with a concentration of 0.05 M. The pH values ranged from 3 to 10. Table 7 indicates that the enzyme exhibited its highest level of activity at a pH of 7. The enzyme exhibited its maximum activity at a temperature of 37°C. The examination of substrate specificity revealed that the enzyme functions as a catalyst for L-asparagine, specifically acting on it as its substrate. Our findings were similar to those of Prakasham et al.,2017 for *Staphylococcus sp*, *Pseudomonas stutzeri*, and *Erwinia carotovora*. produced L-asparaginase at a temperature of 30°C using Serratia marcescens. Narayana et al., (2008) utilized Streptomyces albidoflavus to produce L-asparaginase at a temperature of 35°C. Roberts employed Achromobacteriacae to produce L-asparaginase at a temperature of 20°C. The study also investigated the optimal incubation period for an enzyme and substrate to achieve maximum enzyme activity and the most rapid substrate degradation rate. This was done by incubating the enzyme with the substrate for different amounts of time under the best conditions. It was found out what the enzyme activity was after 10, 20, 30, and 40 minutes. The results show that the asparaginase activity peaked after 10 minutes of incubation at 37° C and pH 7.0. The enzyme activity decreased as the incubation time went up above 10 minutes. As (Moorthy et al.,2010) mentioned, these results were comparable to what was found. However, the highest activity of Streptomyces albidoflavus was observed at pH 8(Abdel-Fattah and Olama,2002).

Table -7- Effect of pH, Temperature, and substrate on partially purified L-asparaginase.

Parameter	**Enzyme activity (IU/ml)**
Temperature Effect	
30	31.08

37	35.00
50	11.40
Incubation Period Effect	
10 min	30.11
20 min	33.20
30 min	20.53
40 min	17.01
Effect of PH	
3	28.19
4	28.58
5	29.02
6	31.00
7	35.00
8	34.81
9	28.85
10	28.15
Effect of Substrate Specificity	
L-arginine	19.04
L-phenylalanine	22.28
L-Histidine	24.44
L-Glutamine	21.10
L-aspartic	35.04
L-asparagine	37.18

Conclusion

The microbial-derived L-asparaginase is highly valued due to its importance in the pharmaceutical and food sectors. Thus, in the current investigation, L-asparaginase activity was achieved using the Imada assay method. The powerful isolates underwent optimization investigations by submerged fermentation. A comprehensive analysis was conducted to optimize manufacturing factors including carbon, nitrogen, amino acids, temperature, pH, and ionic salts. The use of asparagine agar medium is advantageous for achieving optimal enzyme activity. Consequently, the remaining fermentation parameters were conducted using the identical media. According to the experimental data, Fructose (0.5%) was determined to be the most efficient carbon source while Ammonium sulphate (0.5%) was identified as the most effective nitrogen source. The concentration of vitamin was 1%, while the concentrations of sodium acetate, citric acid, Tryptophan, and sodium dihydrogen phosphate were also 1% each. The study results showed that the optimal conditions for enzyme production were a pH of 8.0 and a temperature of 37°C. The L-asparaginase was further purified using gel filtration chromatography with a Sephadex G-100 column. The final overall yield recovery was 259.183%, and the purification resulted in a fold increase of 1.58. Research on the physical factors affecting the effectiveness of pure L-asparaginase has shown that it remains functional

throughout a wide pH range of 8 and at a temperature of 37°C. The enzyme demonstrates a high level of selectivity towards its natural substrate, L-asparagine and demonstrates a high level of stability at a pH of 7.

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References

1. Abbas Ahmed MM, Nageh Abo Dahab F, Taher Taha M, Fareed Hassan SM. (2015) J MicrobBiochem Technol. 7:165-172.

2. Abdel-Fattah YR and Olama ZA. (2002). L-asparaginase production by Pseudomonas aeruginosa in solid-state culture: Evaluation and optimization of culture conditions using factorial designs. Process Biochem.38:115-122.
3. Aghaiypour K, Wlodawer A, Lubkowski J. (2001). Structural basis for the activity and substrate specificity of Erwinia chrysanthemi L-asparaginase. Biochemistry.40(19):5655-64.
4. Akilandeswari K, Kavitha K, Vijayalakshmi M.(2012). Production of bioactive enzyme L-asparaginase from fungal isolates of water sample through submerged fermentation. Int J Pharm Pharm Sci.4(4):363-366.
5. Ashok A, Doriya K, Rao JV, Qureshi A, Tiwari AK, Kumar DS. (2019). Microbes producing L-asparaginase free of glutaminase and urease isolated from extreme locations of Antarctic soil and moss. Scientific reports.9(1):1423.
6. Baskar G. and Renganathan S. (2011).Design of experiments andartificial neural network linked genetic algorithm formodelling and optimization of L-asparaginaseproduction by Aspergillus terreus MTCC 1782. Biotechnology and Bioprocess Engineering. 16(1), 50-58.
7. Dange V. and Peshwe S. (2015). Purification and biochemical characterization of L-asparaginase from Aspergillus niger and evaluation of its antineoplastic activity. Int. J. Sci. Res. 4(2), 564-569.
8. Dhevagi P, Poorani E. (2006). Isolation and characterization of L-asparaginase from marine actinomycetes.
9. Dunlop PC, Roon RJ. (1975). L-Asparaginase of Saccharomyces cerevisiae: an extracellular Enzyme. Journal of bacteriology. 122(3):1017-24.
10. Dura M.A, Flores, M, and Toldra, F. (2022). Purification and Characterization of L-Glutaminase from Debaryomyces spp. Int. J. Food. Microbial.76: 117-126.

11. Elizabeth, T.M., Rai, V., Ali, S.S.(1991). purification and characterization of an L-asparaginase from Fusarium solani. Indian j. Microbiol.Ecol.2:61-70.
12. El-Sabbagh SM, El-Batanony NH, Salem TA. (2013). L-Asparaginase produced by Streptomyces strain isolated from Egyptian soil: Purification, characterization, and evaluation of its anti-tumor. Afr J MicrobiolRes.7(50):5677-86.
13. Elshafei AM, Hassan MM, AbouZeid MA, Mahmoud DA, ElghonemyDH.(2012). Purification, characterization and antitumor activityof L-asparaginase from Penicillium brevicompactum NRC 829. BrMicrobiol Res.2(3): 158-174.
14. Gaffar S.A. and Shethna Y.I. (1977).Purification and Some Biological Properties of Asparaginase from Azotobactervinelandii. Appl Environ Microbiol.33(3),508-514.
15. Gurubasappa SK, Kattimani L.(2010). Screening and optimization of L-asparaginase-A tumor inhibitor from Aspergillus terreus through solid state fermentation. Journal of Advanced Scientific Research.1(01):55-60.
16. Hall, T. A.(1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. In Nucleic acids symposium series.41: 95-98.
17. Heinemann B, Howard AJ. (1969). Production of tumor-inhibitory L-asparaginase by submerged growth of Serratia marcescens. Applied Microbiology.18(4):550-554.
18. Hosamani R, KaliwalBB.(2011). L-asparaginase is an anti-tumor agent produced by Fusarium equiseti using solid-state fermentation. Int J Drug Discov.3(2):88-99.
19. Imada A, Igarashi S, Nakahama K, Isono M. (1973). Asparaginase and glutaminase activities of micro-organisms. Microbiology.76(1):85-99.
20. Jalgaonwala R.E. and Mahajan R.T. (2014). A review: bacterial endophytes and their bioprospecting. J Pharm Res. 4, 795-799.
21. Kalyanasundaram I., Nagamuthu J., Srinivasan B., Pachayappan A. and Muthukumarasamy S (2015).Production, purification and characterization of extracellular L-asparaginase from salt marsh fungi endophytes. World J. Pharma. Sci.4(3), 663-667.
22. Katoh, K., J. Rozewicki and K.D.Yamada. (2019).MAFFT online service: Multiple sequence alignment, interactive sequence choice, and visualization. Brief. Bioinform.20:1160–1166.
23. Keating MJ, Holmes R, Lerner S, Ho DH.(1993). L-asparaginase and PEG asparaginase Leukemia& lymphoma.10(1):153-157.
24. Kim K, Jeong JH, Lim D, Hong Y, Lim HJ, Kim GJ, Shin SR, Lee JJ, Yun M, Harris RA, Min JJ. (2015). L-Asparaginase delivered by Salmonella typhimurium suppresses solid tumors. Molecular Therapy-Oncolytics.2.
25. Krasotkina J, Borisova AA, Gervaziev YV, Sokolov NN. (2004).One-step purification and kinetic properties of the recombinant l-asparaginase from Erwinia carotovora. Biotechnology and Applied Biochemistry.39(2):215-21.
26. Krishna Raju Patro and Nibha Gupta. (2012). Extraction, Purification, and characterization of L-asparaginase from penicillium sp. By submerged fermentation. International Journal for Biotechnology and Molecular Biology Research.3(3):30-34.

27. Kumar NSM, Manonmani HK. (2013).Purification, characterization and kinetic properties of extracellular L-asparaginase produced by Cladosporium sp. World J MicrobiolBiotechnol.29(4): 577-587.
28. Manna S, Sinha A, Sadhukhan R, Chakrabarty SL. (1995).Purification, characterization, and antitumor activity of L-asparaginase isolated from Pseudomonas stutzeri MB-405. Current microbiology.30:291-298.
29. Mohapatra BR, Sani RK, Banerjee UC.(1995). Characterization of L-asparaginase from Bacillus sp. isolated from an intertidal marine alga (Sargassum sp.). Letters in applied microbiology.21(6):380.
30. Moorthy V, Ramalingam A, Sumantha A, Shankaranaya RT. (2010). Production, purification, and characterization of extracellular L-asparaginase from a soil isolate of Bacillus sp. African Journal of Microbiology Research.4(18):1862-7.
31. Mukherjee J, Majumdar S, Scheper T.(2000). Studies on nutritional and oxygen requirements for the production of L-asparaginase by Enterobacter aerogenes. Applied Microbiology and Biotechnology.53:180-184.
32. Murali T.S. L-Asparaginase from marine derived fungal endophytes. Mycosphere, 2(2), 147-155.
33. Mushtaq MS, Siddalingeshwara KG, Karthic J, Sunil DPLNSN, Naveen M, Prathiba KS., (2012). Int J Res Pharmacol Pharm. 1:147-150.
34. Narayana KJ, Kumar KG, Vijayalakshmi M. (2008). L-asparaginase production by Streptomyces albidoflavus. Indian Journal of Microbiology.48:331-6.
35. Nawaz MS, Zhang D, Khan AA, Cerniglia CE. (1998). Isolation and characterization of Enterobacter cloacae capable of metabolizing asparagine. Applied Microbiology and Biotechnology. 50:568-572.
36. Neelima Deshpande, Prachi Choubey, Manasi Agashe. (2014). Studies on optimization of growth parameters for L-asparaginase production by Streptomyces ginsengisoli. The Scientific World Journal.
37. Osama S, El-Sherei MM, Al-Mahdy DA, Bishr M, Salama O, Raafat MM. (2023). Optimization and characterization of antileukemic L-asparaginase produced by Fusarium solani endophyte. AMB Express.13(1):96.
38. Prakasham RS, Rao CS, Rao RS, Lakshmi GS, Sarma PN.(2007). l-asparaginase production by isolated Staphylococcus sp.–6A: design of experiment considering interaction effect for process parameter optimization. Journal of Applied Microbiology.102(5):1382-1391.
39. Roberts J, Holcenberg IS and Dolwy WC. (1972). Crystallization and properties of Achromobacteriacae glutaminase, asparaginase with antitumor activity. J Biol Chem.247:84-90.
40. Sahu MK, Poorani E, Sivakumar K, Thangaradjou T, Kannan L. (2007). Partial purification and anti-leukemic activity of L-asparaginase enzyme of the actinomycete strain LA-29 isolated from the estuarine fish, Mugil cephalus (Linn.). Journal of Environmental Biology.28(3):645.

41. Sangita Ghosh, Sonam Murthy, Sharmila Govindasamy and Muthukumaran Chandrasekaran. (2013).Optimization of L-asparaginase production by Serratia marcescens (NCIM 2919) under solid state fermentation using coconut oil cake sustainable chemical.
42. Sarquis MI, Oliveira EM, Santos AS, Costa GL. (2004). Production of L-asparaginase by filamentous fungi. Memorias do Instituto Oswaldo Cruz. 99:489-92.
43. .Sreenivasulu V., Jayaveera K.N. and Mallikarjuna Rao. (2009). Optimization of process parameters for the production of Lasparaginasefrom an isolated fungus. Research J.Pharmacognosy and Phytochemistry.1(1), 30-2009.
44. Suchita C, Warangkar and Chadrahas N Khobragade. (2010). Purification, characterization, and effect of Thiol compounds on Activity of the Erwinia carotovora L-asparaginase.Enzyme research.
45. Theantana T, Hyde KD, Lumyong S. (2009).Asparaginase production by endophytic fungi from Thai medicinal plants: cytotoxicity properties. International Journal of Integrative Biology.7(1):1-8.
46. Thirumurugan G., Moses Jeyakumar Rajesh, Leelavathy Rajesh, Vanapalli VSV and Rajarammohan Sivasubramanian. (2011).Effect of Inducers and Physical Parameters on the Production of L-Asparaginase Using Aspergillus Terreus. J Bioprocess Biotechniq. 1, 1-6.
47. Venil CK, Nanthakumar K, Karthikeyan K, Lakshmanaperumalsamy P. (2009). Production of L-asparaginase by Serratia marcescens SB08: Optimization by response surface methodology.:10-18.
48. Verma N, Kumar K, Kaur G, Anand S.(2007). L-asparaginase: a promising chemotherapeutic agent. Critical reviews in biotechnology.27(1):45-62.
49. Warangkar SC, Khobragade CN. (2010). Purification, characterization, and effect of thiol compounds on the activity of the Erwinia carotovora L-asparaginase. Enzyme research.