Study of Fungal L-Asparaginase and Unlocking its Potential in Food Products

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DECLARATION BY STUDENT

I hereby declare that the data presented in this Dissertation report entitled, "Study of L-Asparaginase and Unlocking its Potential in Food Products" is based on the results of investigations carried out by me in the Microbiology Programme at the School of Biological Science and Biotechnology, Goa University under the supervision of Dr. Lakshangy Charya and the same has not been submitted elsewhere for the award of a degree or diploma by me. Further, I understand that Goa University or its authorities will not be responsible for the correctness of observations/ experimental or other findings given in the dissertation.

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COMPLETION CERTIFICATE

This is to certify that the dissertation **report "Study of Fungal L-Asparaginase and Unlocking its Potential in Food Products"** is a bonafide work carried out by **Ms. Isha Kumar Vaze** under my supervision in partial fulfillment of the requirements for the award of the degree of Master of Science in Microbiology, in the Discipline Microbiology, at the School of Biological Sciences and Biotechnology, Goa University.

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PREFACE

The motive behind the research on fungal asparaginase is to mitigate the formation of acrylamide in food products. Acrylamide is a potential carcinogen formed during high-temperature cooking processes, particularly in starchy foods like potatoes and grains. Asparagine, an amino acid present in these foods is a precursor to acrylamide formation.

Fungal asparaginase enzymes were developed to address this issue by breaking down asparagine into aspartic acid and ammonia, thus reducing acrylamide levels in food products.

The genesis of fungal asparaginase lies in biotechnological research aimed at finding safer, more effective and economically viable ways to mitigate acrylamide formation in food processing.

In the food industry, fungal asparaginase has the potential to revolutionize food processing by significantly reducing acrylamide levels in a variety of products including potato-based food, bakery items, and coffee. By incorporating fungal asparaginase into food processing protocols, manufacturers can improve food safety and meet regulatory requirements regarding acrylamide levels, thus enhancing consumer confidence in the safety and quality of their products.

Additionally, fungal asparaginase offers a natural and environmentally friendly solution to acrylamide reduction compared to chemical additives.

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ABBREVIATIONS USED

Abbreviation	Expansion
AEI	Acyl-enzyme intermediate
ALL	Acute Lymphoblastic Leukemia
CDA	Czapek's Dox Agar
CDB	Czapek's Dox Broth
PDA	Potato Dextrose Agar
Rf	Resolving factor

ABSTRACT

L-asparaginase is an enzyme that catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia. This enzyme is usually derived from the *E.coli* and *Erwinia sp.,* for commercial production. L-asparaginase enzymes have been widely used in the medical field for the treatment of ALL and non-Hodgkin lymphoma. It is also used in the reduction of the L-asparagine in the foods which is the precursor for the acrylamide formation. However, the enzymes obtained from bacterial sources have side effects on the human system. Therefore fungal sources like *Aspergillus sp., and Penicillium sp.,* have been used for the extraction of enzymes that help in overcoming the drawbacks caused by bacterial L-asparaginases. Submerged fermentation of the fungal isolates in the modified Czapek's Dox Broth was performed for the extraction of the fungal L-asparaginase enzyme. Salting out method of protein precipitation, Nessler's test for the determination of enzyme activity, and Protein estimation by Folin Lowry's method were the various tests that were employed to study fungal L- asparaginase enzyme.

Keywords: L asparaginase, acrylamide, submerged fermentation, enzyme assay

CHAPTER 1

INTRODUCTION

1.1BACKGROUND

L-asparaginase is an enzyme that catalyzes the hydrolysis of asparagine into L- aspartic acid and ammonia. This enzyme is commercially derived from microbial sources like *E.coli* and *Erwinia* species for its application in industries (Doriya *et al.,* 2016). L- asparaginase is largely used in medicine for the treatment of Acute Lymphoblastic Leukemia (ALL) which is a type of cancer that majorly affects the white blood cells and also for the treatment of non-Hodgkin lymphoma(van den Berg, 2011). L-asparaginase is also widely used in the food industry to reduce the levels of acrylamide in certain foods (Muneer *et al*., 2020; Qeshmi *et al.,* 2018).

The main role of the L-asparaginase enzyme is to break down the amino acid called L-asparagine (Talluri *et al*., 2014). The L-asparagine, the substrate of the L-asparaginase enzyme, is non-essential for normal human cells and is not affected by the enzyme as it can produce and meet its requirement of the amino acid (Queshmi *et al.,* 2018). Its application in medicine is for cancer treatment as I-asparagine is an essential amino acid for cancer cells which require an external supply of the amino acid for its development, growth, and survival (Talluri *et al*., 2014).

The use of L-asparaginase in the food industry is by virtue of the enzyme reduces the occurrence of Maillard reaction in starchy foods when heated at high temperatures. Starchy food such as potatoes and grains when heated at high temperatures leads to the formation of a chemical compound, acrylamide, which is a known potent carcinogen. Maillard reaction is a reaction of sugars with amino acids. In this reaction, the reducing sugars react with the asparagine and form acrylamide (Muneer *et al.,*2020; Queshmi *et al.,* 2018). L-asparaginase enzyme helps in minimizing the levels of a precursor molecule called asparagine thereby reducing the chances of the formation of acrylamide in foods when heated at high temperatures (Da Cunha *et al.,* 2019).

L-asparaginase can be obtained from various sources like bacteria, actinomycetes, yeast, and fungi. Bacteria such as *E. coli* and *Erwinia sp.,* have been widely used for the commercial production of L-asparaginase (Batool *et al.,*2015; Talluri *et al., 2*014). *Aspergillus sp., Penicillium,* and *Fusarium sp.* are the fungal sources that are used to extract the enzyme on an industrial scale (Doriya *et al.,* 2016; Farag *et al.,* 2015).

Fungal asparaginases offer various advantages over bacterial asparaginases some of which are listed below:

- The L-asparaginase derived from bacteria produces allergic reactions, changes in liver dysfunction, and hypersensitivity in the mammalian system.
- Enzymes derived from fungus are more stable and have higher specificity in breaking down the asparagine (Batool *et al.,*2015; Wang *et al.,* 2021).
- Fungal asparaginase has a longer half-life therefore results in the need for fewer dosages (Krishnapura *et al.,* 2015; El-Nagga *et al.,* 2014).
- In the food industry, the L-asparaginases used are derived from the fungus as they are considered GRAS (Generally recognized as safe).
- Also fungal enzymes are easy to extract as they are produced extracellularly and can be carried out by fermentation.

Although the majority of applications for L-asparaginase are in the medical field, there has been less research done on its usage in the food industry. The majority of L-asparaginases that are employed are obtained from bacterial sources. Therefore, in this study, we are focussing on the L-asparaginase derived from the fungal sources.

1.2 AIMS AND OBJECTIVES

The purpose of this research is to isolate, identify and test the various fungi for the presence of the enzyme L asparaginase. Since fungal asparaginases are produced extracellularly and fungal growth is inexpensive, they are straightforward to extract enzyme. Additionally, the studies revealed that fungal asparaginases have fewer negative effects.

The objectives are as follows:

- To isolate fungi and screen them for the production of L Asparaginase enzyme
- Identification of the fungal isolates producing asparaginase enzyme
- To determine the enzyme activity
- To evaluate the ability of fungal asparaginase to effectively degrade asparagine in food products.

1.3 HYPOTHESIS

- The use of fungal asparaginase in food products will effectively reduce the levels of asparagine thereby leading to decreased acrylamide formation during food processing.
- Acrylamide reduction in food treated with fungal asparaginase enzyme will lower the risk of cancer.

1.4 SCOPE

Fungal asparaginase enzyme is utilized in the food industry to reduce the formation of acrylamide, a potential carcinogen, during food processing. It breaks down the precursor amino acid, asparagine thereby minimizing acrylamide levels in foods like potato products, snacks, bakery items, and coffee. This enzyme enhances food safety and quality, making it valuable for various food applications.

Also using fungi for the production of enzymes rather than bacteria is advantageous. As fungi have a broader range of metabolic capabilities compared to bacteria. Fungi can also thrive in harsher environmental conditions including extremes of pH, temperature, and salinity making them more resilient for industrial applications where stability and resilience are essential.

CHAPTER 2

LITERATURE REVIEW

2.1 L-asparaginase enzyme

L-asparaginase (L-asparagine aminohydrolase EC 3.5.1.1) is an enzyme that catalyzes the hydrolysis of asparagine into L- aspartic acid and ammonia (Batool *et al.,* 2015).

Two isozymes of this particular enzyme have been identified, Type I and Type II. Both are classified based on their enzymatic activities for both L- asparagine and L- glutamine. Type I L-asparaginase are cytosolic enzyme that shows low affinity towards L-asparagine and high affinity towards L-glutamine. Whereas, Type II isozymes are periplasmic enzymes that have high affinity towards L-asparagine and low or negligible specificity against L-glutamine (Izadpanah Qeshmi *et al.,* 2018). The L-asparaginase enzyme is made up of homotetramer which is composed of two dimers, with two protomers which make up the tight dimer. Fig 2.1.1 (A) shows two quaternary states of Type I tetramers. The tight dimers are colored green/wheat and red/pink, respectively and (B) shows Type II Asparaginase which is formed by two tightly bound dimers (green and yellow color) or blue cyan (Lubkowski & Wlodawer, 2021).

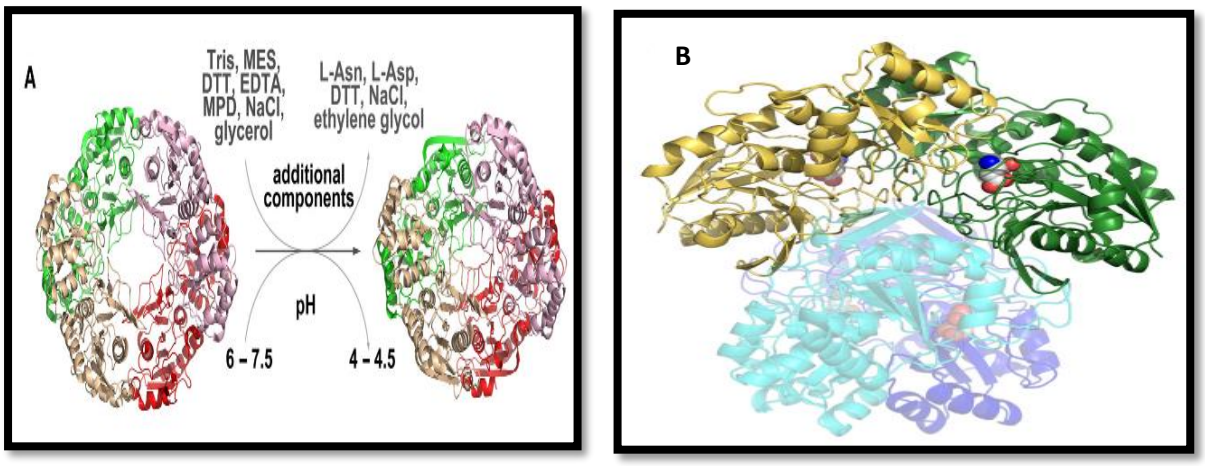


Figure 2.1.1 Structures of Type I (A) and Type II (B) l-asparaginase enzymes from *E.coli* (Lubkowski & Wlodawer, 2021).

Commercially this enzyme is derived from *E. coli* and *Erwinia* species. Enzymes derived from the *Erwinia carotovora* consist of two tetramers (ABCD and EFGH) which are made up of four identical monomers (A to H) each. The active site of the enzyme consists of amino acids between the two adjacent monomers (Lubkowski & Wlodawer, 2021).

Most of the type II L-asparaginases are derived from the mesophilic sources whereas Type I L-asparaginases are derived from the thermophiles. Both these enzymes can be obtained from the *E.coli*. Only Type II isozyme shows the anti-tumor activity which may vary with the strains or culture conditions of the microorganisms (Izadpanah Qeshmi *et al.,* 2018).

Asparaginase enzyme is freely soluble in water and is insoluble in methanol, acetone, and chloroform. The L-asparaginase enzyme has a wide range of pH stability which ranges from 4.5 to 11.5. with the optimum of 8.6. The enzyme is stable at temperatures ranging from 30-40^0 C for the enzymes obtained from the mesophiles whereas the temperature ranges from 70-95^0 C and optimum at 65^0 C for those which are obtained from the thermophilic sources (Dumina & Zhgun, 2023). The L-asparaginase enzyme is produced in microorganisms and plants, however, humans do not produce this enzyme (Whitecar *et al.*, 1970; Batool *et al.,* 2015; Muneer *et al*., 2020; Stecher *et al.,* 1999).

2.2 Mode of action

L-asparaginase enzyme catalyzes the hydrolysis of the asparagine into aspartic acid and ammonia (Talluri *et al.*, 2014).

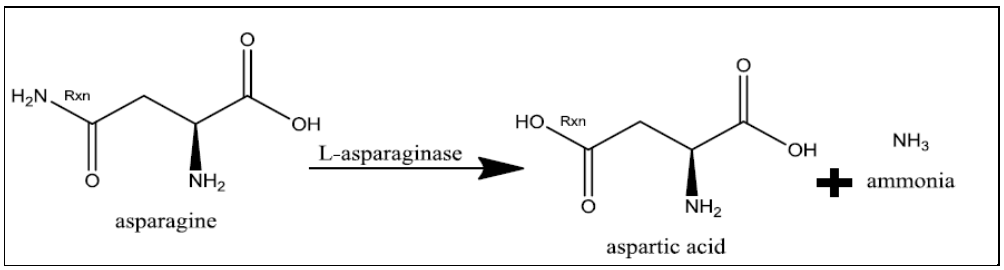


Figure 2.2.1 Enzymatic reaction of L-asparaginase enzyme (Talluri *et al.,* 2014)

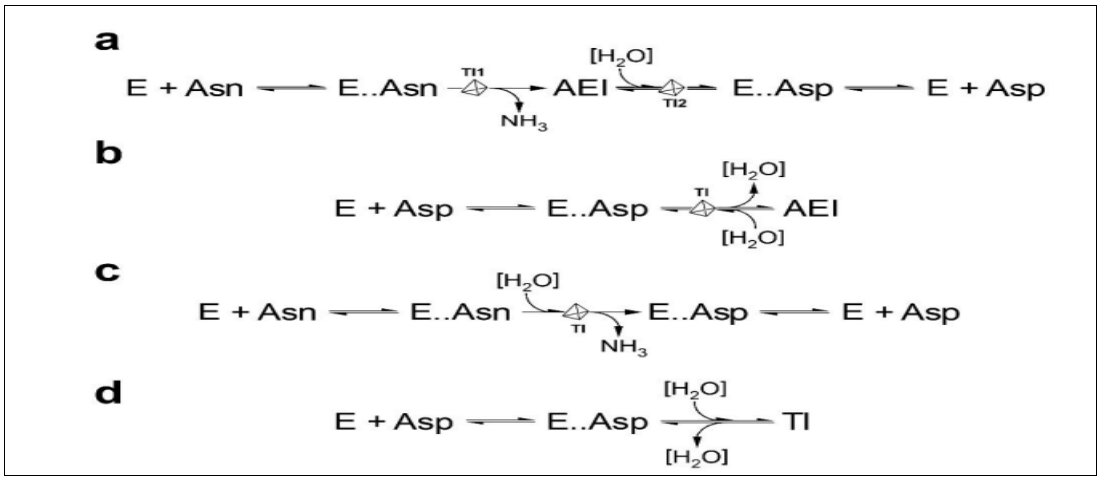


Figure 2.2.2 Mechanism of action of L-asparaginase enzyme (a) and (b) showing double displacement reaction and (c) and (d) showing direct displacement reaction (Lubkowski *et al.*, 2020)

Generally, the mode of action of the L-asparaginase takes place in the two reactions, the double displacement reaction shown in Fig 2.2.2 a and b and the direct displacement reaction (Fig 2.2.2 c and d). In the first mechanism (Fig 2.2.2 a and b) an intermediate AEI (covalent acyl-enzyme intermediate) is formed after the nucleophilic attack by an enzyme residue on

carboxamide carbon of L- asparagine which results in the release of ammonia. This step is irreversible as it releases ammonia from the active site. Water molecule carries out the second nucleophilic and reversible reaction which results in the formation of L-aspartic acid which completes the catalytic cycle. Each catalytic cycle produces TI1 and TI2 (two tetrahedral intermediates). These two nucleophilic substitutions are referred to as the double displacement reactions. Direct displacement reaction (Fig 2.2.2 c) also takes place which is carried out by the water molecule. In this reaction, aspartic acid and ammonia are released simultaneously (Lubkowski *et al.*, 2020).

2.3 Sources of L-asparaginase

Enzyme L-asparaginase is produced by microbial forms such as bacteria, algae, yeast, actinomycetes, archaea, fungi, and also plants but the enzyme is absent in humans. Currently, these enzymes have been isolated from microbial sources namely *E. coli* and *Erwinia* species (Talluri *et al.*, 2014; Doriya *et al.*, 2016).

2.3.1 Bacterial sources

Many bacterial sources are known to produce L-asparaginase which includes *E. coli, Staphylococcus species, Helicobacter species,* etc (Talluri *et al.*, 2014). L-asparaginase produced from various bacterial sources is widely used as they are easy to manipulate (Doriya *et al.*, 2016). Bacterial sources of L-asparaginase enzyme are summarized in Table 2.3.1

Sources with reported L-asparaginase levels include *Escherichia coli* and *Erwinia aroideae*. Among bacterial genera that produce the enzyme, gram-negative bacteria like *Thermus thermophilus* and *Vibrio succinogenes* have been also used for L-asparaginase production. Since marine microorganisms are thought to be a significant source of bioactive enzymes, L-asparaginase from them has also been examined (Izadpanah Qeshmi *et al.*, 2018). *E.coli* and

Erwinia species are extensively used in the treatment of ALL. However, they are known to cause allergic reactions to human health. Therefore, various approaches are made to find other microbial sources that can reduce these drawbacks caused by the L-asparaginases derived from the bacteria and have less toxic effects (Doriya *et al.*, 2016).

2.3.2 Yeast

L-asparaginase isolated from yeast has been known to have fewer side effects in the human system as compared to bacterial sources. Current sources of L-asparaginases include a variety of yeast species, most notably *Saccharomyces cerevisiae,* whose ASP3 gene is involved in enzyme synthesis. Other yeast species include *Candida sp., Rhodosporidium sp.,*(Talluri *et al.,* 2014). Additionally, L-asparaginase was also extracted from the *Candida utilis* cell culture broth (Kil *et al.,* 1995). *Pichia polymorpha*, which was isolated from Egyptian soils via the enrichment approach, has also been found to produce L-asparaginase enzyme (Foda *et al.,* 1980) and others that have been tabulated below in Table 2.3.1.

2.3.3 L-asparaginase from Actinomycetes

Actinomycetes are prokaryotic organisms that play an essential role in the environment. *Streptomyces* species are known to produce the L-asparaginase enzyme that has been tabulated below in Table 2.3.1 (Talluri *et al.,* 2014). Sahu *et al.* isolated L-asparaginase from the actinomycete strain LA-29 source from the stomach contents of the fish *Mugil cephalus* in the Vellar estuary. This enzyme was found to exhibit significant enzyme activity (Talluri *et al.,* 2014). L-asparaginases isolated from the marine actinomycetes have shown a cytotoxic effect on acute T-cell leukemia and also myelogenous leukemia (Doriya *et al.,* 2016; Batool *et al.,* 2015).

2.3.4 Archaea

Thermococcus zilligii produces thermostable l-asparaginase, which was found to be useful in lowering the amount of acrylamide in French fries. The enzyme's potential for industrial applications where temperature stability is critical was highlighted by its excellent activity at high temperatures and its ability to preserve significant activity even after prolonged heat exposure (Zuo *et al*., 2015). Also, another hyperthermophilic archaeon called *T. sibiricus* is known to produce the L-asparaginase enzyme. Till date, only these two archaea have been reported for the production of the L-asparaginase enzyme (Dobryakova *et al.,* 2024).

2.3.5 L-asparaginase from Fungi

Numerous fungal strains are effective L-asparaginase producers. Despite a great deal of research on bacterial L-asparaginase, this enzyme can occasionally cause hypersensitivity reactions like anaphylactic shock. L-asparaginase from *Aspergillus terreus*, a filamentous fungus, exhibited a stronger carcinostatic impact on static tumors (Farag *et al.,* 2015). This fungus was isolated from the decomposing vegetable waste by Ali *et al.,*1994. Fungi are the ideal option to obtain L-asparaginase enzyme with lesser harmful effects (Doriya *et al.,* 2016). Other fungal sources are listed in Table 2.3.1.

Table 2.3.1 List of Microbial sources producing L-asparaginase enzyme

Microbial sources	Reference
Bacteria	
E. coli	(Talluri *et al.,* 2014 ;Cedar & Schwartz, 1968; Aly *et al.,* 2020; Doriya *et al.*, 2016)
Staphylococcus capitis *Staphylococcus aureus*	(Talluri *et al.*, 2014) (Youssef *et al.,* 2022; Hassan *et al.,* 2022)
Bacillus licheniformis *Bacillus sonorencis*	(Talluri *et al.,* 2014; Abdelrazek *et al.,* 2019; Alrumman *et al.,* 2019) (Aly *et al.,* 2020)
Streptococcus albus	(Talluri *et al.,* 2014)
Helicobacter pylori	(Cappelletti *et al.,* 2008; Ghasemian *et al.,* 2019; Muneer *et al.,* 2020)
Erwinia chrysanthemi *Erwinia aroideae* *Erwinia carotovora*	(Kotzia & Labrou, 2007; Izadpanah Qeshmi *et al.,* 2018) (Peterson & Ciegler, 1969; Doriya *et al.,* 2016) (Howard & Carpenter, 1972)
Halomonas elongata	(Ghasemi *et al.,* 2017; Ebrahiminezhad *et al.,* 2011)
Salinivibrio sp	(Ebrahiminezhad *et al.,* 2011)
Citrobacter	(Davidson *et al.,* 1977; Shah *et al.,* 2010)
Yeast	
Yeast *Saccharomyces cerevisiae*	 (Batool *et al.,* 2015; Dunlop & Roon, 1975);
Candia utilis *Candida glaebosa*	(Batool *et al.,* 2015; Kil *et al.,* 1995; Talluri *et al.,* 2014) (Correa *et al*., 2020)
Psychrotolerant yeast *Leucosporidium scottii* L115	(Sánchez-Moguel *et al.,* 2023)
Rhodosporidium toruloides	(Talluri *et al.,* 2014)

Actinomycetes	
Streptomyces karnatakensis, S. venezuelae	(Talluri *et al.*, 2014; Mostafa, 1979)
Streptomyces albidoflavus	(Narayana *et al.*, 2008)
Streptomyces parvulus	(USHA *et al.*, 2011)
Streptomyces gulbargensis	(Amena *et al.*, 2010; Talluri *et al.*, 2014)
Fungi	
Aspergillus terreus	(Doriya *et al.*, 2016; Farag *et al.*, 2015; Loureiro *et al.*, 2012)
Aspergillus niger	(Doriya *et al.*, 2016; Mishra, 2006; Da Cunha *et al.*, 2019)
Aspergillus oryzae	(Moubasher *et al.*, 2022; Da Cunha *et al.*, 2019; Dias *et al.*, 2016)
Fusarium trincictum	(Doriya *et al.*, 2016)
Fusarium equiseti	(Doriya *et al.*, 2016; El-Gendy *et al.*, 2021)
Cladosporium sp	(Kumar *et al.*, 2013; Doriya *et al.*, 2016)
Endophytic fungi *Lasiodiplodia theobromae*	(Ba *et al.*, 2018; Moubasher *et al.*, 2022)

2.4 Applications of l-asparaginase

2.4.1 Pharmaceutical applications

L-asparaginase enzyme is commercially available for the treatment of Acute Lymphoblastic Leukemia and also non-Hodgkin lymphoma (van den Berg, 2011). Transaminase enzymes are used up by the normal cells to synthesize L-asparagine, but neoplastic cells are deficient in this enzyme and must obtain it externally. As a result of the L-asparaginase enzyme depleting all the available asparagine, cancer cells starve to death (Batool *et al.*,2015).

Fundamentally, L-asparaginases are produced on an industrial scale mainly from E. *coli* and *Erwinia chrysanthemi* which are listed below:

L-asparaginase from *E. coli*

In children and young people aged one month to twenty-one, this medication is used in combination with other medications to treat acute lymphoblastic leukemia. It is an enzyme that plays a key role in L-asparagine metabolism. Additionally, it makes oxaloacetate generation easier, which is necessary for overall cellular metabolism.

It can be injected intramuscularly or intravenously. Therapeutic L-asparaginase derived from *Escherichia coli* induces apoptosis by reducing the amounts of non-essential amino acid, asparagine, in lymphoblastic leukemic cells (Batool *et al.,* 2015; Wang *et al*., 2021).

PEGylated form of native *E. coli* L-ASNase

The main method of administration for PEG-asparaginase has been intramuscular injection at a dose of 2500 IU/m 2. The development of anti-PEG-asparaginase antibodies is necessary for PEG-asparaginase activity. The asparaginase activity is neutralized by these anti-PEG-asparaginase antibodies, which accelerates plasma clearance and shortens the duration of the drug's impact. The production of anti-PEG-asparaginase antibodies is enhanced by previous exposure to native *E. coli* asparaginase (Fu & Sakamoto, 2007; Muneer *et al*., 2020).

These forms of enzymes derived from *E.coli* cause adverse effects in the human system therefore to lessen the adverse effects L-asparaginase from *E. chrysanthemi* has been developed.

L-asparaginase from *E. chrysanthemi*

It is an inhibitor of protein synthesis used to treat acute lymphoblastic leukemia (ALL) in people intolerant to the *E. coli* bacterium's asparaginase. It functions by reducing the amounts of asparagine, a crucial amino acid needed for DNA synthesis and the survival of cancerous

cells, which results in cell death. It is injected intramuscularly and used in conjunction with other anticancer drugs (Keating, 2013).

2.4.2 Biosensors

Leukemic patients can now be diagnosed with leukemia using biosensors based on l-asparaginase. These biosensors can be used to measure asparagine levels. By accurately assessing the asparagine levels in normal and leukemic blood serum samples, these biosensors have demonstrated positive outcomes (Muneer *et al*., 2020).

Since ASNase catalyzes the hydrolysis of L-asparagine, which releases L-aspartic acid and ammonium ions and promotes a medium pH value change followed by color variation, ASNase-based biosensors are an ensuring and innovative technology. It is primarily based on colorimetric detection (Nunes *et al*., 2021).

2.4.3 Food industry

The enzymatic hydrolysis of L-asparagine to aspartic acid by L-asparaginase allows it to effectively limit the development of acrylamide in fried potato products. When certain foods, such as cereals and potatoes, are heated to high temperatures and contain a lot of asparagine, Maillard processes involving the reduction of sugars and amino acids produce acrylamide. Acrylamide has been categorized as "probably carcinogenic to humans" by the International Agency for Research on Cancer. After infrequent exposure, this substance has also been linked to peripheral neuropathy in humans. Therefore, acrylamide's prevalence in staple foods poses a risk to public health. The use of L-asparaginase can selectively reduce the asparagine content thereby preventing the formation of acrylamide in the foods when heated at high temperatures (Muneer *et al*., 2020; Qeshmi *et al.,* 2018).

L-asparaginase is thought to be inert and to have no influence on the overall synthesis of Maillard products, but it is beneficial for mitigating acrylamide. One of the essential acrylamide precursors can be specifically eliminated by L-asparaginase through its hydrolytic activity (Onishi *et al.*, 2015).

Asparaginase application reduced acrylamide in dry potato powder by 90%. Steamed green beans in coffee can mitigate acrylamide by lowering the caffeine level by 55-74% using a water or solvent partition technique (Xu *et al.*, 2016).

The reaction involved in the acrylamide formation in foods is shown in Fig 2.4.1

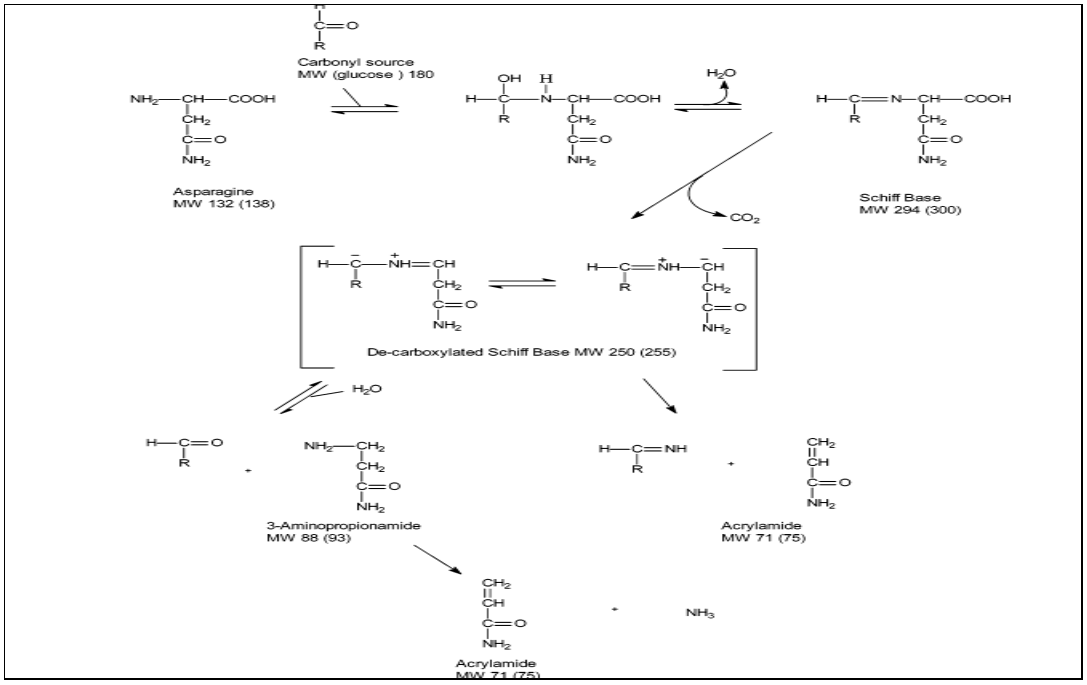


Figure 2.4.1 Acrylamide formation in foods (Zyzak *et al.*, 2003)

During the Maillard reaction, a Schiff base is created when the R-amino group of free asparagine combines with a carbonyl source. The Schiff base decarboxylates when heated creating a product that can react in one of two ways. It can hydrolyze to produce 3-

aminopropionamide, which, when heated, can further deteriorate by eliminating ammonia to produce acrylamide. Alternatively, by removing an imine, the decarboxylated Schiff base might break down straight to produce acrylamide (Zyzak *et al.,* 2003).

The enzymes obtained from the fungal sources have been reported as GRAS (generally regarded as Safe) by the FDA. The L-asparaginase obtained from fungal sources like *Aspergillus oryzae* has been widely used in the food industry. However, the details about whether type I or type II L-asparaginase are not clear (Cachumba *et al.,* 2016).

2.5 Advantages of using fungal L-asparaginase over bacterial L-asparaginase

In the medical field, bacterial asparaginase has been used widely. However, the mammalian system tends to have adverse effects from the bacterial L-asparaginases. Hence, it is challenging to get a more effective and less harmful enzyme. Therefore research is going on to produce this enzyme from the other microbial sources.

Fungal asparaginase often possesses a lower level of immunogenicity. This implies that it is less likely to cause an immunological response in the patients, which lowers the possibility of allergic responses and the development of neutralizing antibodies, both of which can eventually make the treatment useless.

In contrast to bacterial asparaginase, fungal asparaginase frequently has a longer half-life in the bloodstream. This could result in the need for fewer dosages, which would lower the frequency of administration and possibly increase patient compliance.

When it comes to temperature stability, fungal asparaginase surpasses bacterial asparaginase.

Fermentation techniques can be used to manufacture fungal asparaginase, which may have advantages over bacterial production methods in terms of cost-effectiveness and scalability.

When bacterial asparaginase is not available or is not recommended because of adverse responses, fungal asparaginase probably could be used as a substitute for bacterial asparaginases (Krishnapura *et al*., 2015; El-Nagga *et al*., 2014).

CHAPTER 3

METHODOLOGY

3.1 Sample collection

In this attempt to isolate fungi, the soil sample was collected from various places. Three samples were collected:

Sample 1: Compost soil

Sample 2: Garden soil (Shiroda)

Sample 3: Garden soil (Siolim)

3.2 Isolation and Screening of Fungi Using Selective Media

1g of soil from each sample was weighed and added to the test tubes containing 9 ml of sterile saline. The sample tube 10^0 from each sample was vortexed and then the samples were serially diluted up to 10^{-3}. The dilutions (0.1 ml) were spread-plated on the modified Czapek's Dox Agars plates. The modified Czapek's Dox contains 0.009% phenol red as a pH indicator and 0.1% L-Asparagine as a sole nitrogen source (Gulati *et al.,* 1997; Soniyamby *et al.,* 2011). The plates were then incubated at room temperature for 4-5 days.

The fungal isolates showing a pink-colored zone around the colony were positive for L asparaginase enzyme production.

3.3 Purification of the L-Asparaginase-positive fungi

The fungal colonies that showed a pink color around them were purified on Sabouraud's Dextrose Agar and Potato Dextrose Agar plates. The fungal isolates were regularly checked for pigment production.

The isolates that did not show any pigment production were again checked for enzyme production by spot-inoculating them on the modified Czapek's Dox Agar plates. (Soniyamby *et al.,* 2011)

3.4 Identification of fungi

Lactophenol cotton Blue staining is widely used for the preparation of wet mount of the fungal isolates. Lactophenol cotton blue stain was prepared by using 70% ethanol. A clean grease-free slide was taken and a few drops of the lactophenol cotton blue stain (mounting medium) was put on the slide. With the help of the sterile needle and the forceps, the fungal mycelia was transferred onto the mounting medium. Coverslip was put carefully and the fungus was viewed under 10x and then 40x (Leck, 1999).

3.5 Preparation of spore suspension

The fungal isolates were streaked on the PDA slants and incubated at room temperature for 5 days. Sterile saline containing Tween 80 was added to the test tube. The fungal growth was then scrapped with a sterile nichrome loop and the fungal mass was filtered using sterile muslin cloth. The filtrate was diluted and used as inoculum for the submerged fermentation (Zhang *et al.,* 2013).

3.6 Submerged fermentation

1ml of the last dilution of the L-Asparaginase positive isolates was inoculated in modified Czapek's Dox Broth. The inoculation was done in two ways (Soniyamby *et al.,* 2011):

Flasks containing 20ml of the Modified Czapek's Dox broth with phenol red and without phenol red (for enzyme assay and protein estimation).

3.7 Dry weight

The fungal isolates positive for L-asparaginase production were inoculated in the modified CDB without phenol red. After every 24 hours, the flask was removed and the fungal biomass was filtered by using the sterile muslin cloth. The biomass was collected in pre-weighed

aluminium cup. Then the cup was placed in the oven for drying. The dry weight was checked every after 2hours.

3.8 **Enzyme assay and Protein estimation (**Indira *et al.,* 2015; IMADA *et al.,* 1973; Peterson, 1979)

Enzyme assay was carried out by the method given by Imada *et al.,* and protein estimation by Lowry's method given by Peterson (Annexure III). The L-asparaginase-positive fungi were inoculated in 20ml of the Modified Czapek's dox broth without phenol red indicator in quadruplicates and one flask as a control. The flasks were then incubated on a shaker incubator at 160rpm. After the regular interval (24hrs, 48hrs, 72hrs, 96hrs) one flask of each sample was taken. The culture broth was filtered using sterile muslin cloth. The fungal biomass was used to estimate dry weight and the filtrate was used to carry out enzyme assay by the modified method of Wriston (Nessler's test) and the protein estimation by Lowry's method. The protein concentration was calculated by using the formula y= 0.0017x+0.1131 obtained from the standard graph.

The enzyme activity was carried out at three different temperatures, room temperature, 37^0 C and at 50^0 C.

3.9 **Paper chromatography to confirm hydrolysis of L-Asparagine** (Moffatt & Lytle, 1959; DeMott *et al.,* 2024)

The ammonia released was detected by the Nessler's test. To further confirm the reduction of the asparagine content the paper chromatography was carried out.

Ninhydrin reacts with the alpha-amino group colored complex. Ninhydrin reacts with the free amino group of the amino acid and gives a purple-colored complex called Ruhemann's purple. The brown color is produced when amide reacts with ninhydrin. The intensity of the color

developed is directly related to the concentration of the amino acid present. L-aspartic acid produces a purple spot due to the presence of the free amino group where as L-asparagine reacts with the ninhydrin and produces a brown-colored spot due to the presence of the amide group.

To carry out this experiment, the fungal isolates that tested positive for L-asparaginase were cultured in 20 milliliters of modified Czapek's dox broth that contained 0.1% l-asparagine as substrate and without phenol red indicator. The flasks containing the fungus were incubated on a shaker incubator at 160rpm. The flasks were inoculated in quadruplets. After every 24hrs one flask was taken for testing. 5ml of the culture broth was centrifuged at 8,000rpm for 15 minutes. Along with amino acid standards of L-aspartic acid (0.1%) and L-asparagine (0.1%), the resulting supernatant was spot-inoculated on Whatman filter paper No. 1. The spots were allowed to dry completely and then the paper chromatogram was placed in a chamber or 500ml beaker containing n-Butanol: acetic acid: water in the ratio 5:4:1 solvent system. After the solvent system reached 2/3rd of the paper, it was removed and the paper chromatogram was allowed to dry completely at room temperature and then sprayed with 2% ninhydrin solution. The chromatogram was allowed to dry for about 15 minutes in the oven at 80^0 C for color development, spots were marked and Rf values were calculated using the formula:

$$R_f = \frac{\text{Distance traveled by solute}}{\text{Distance traveled by solvent}}$$

To check the enzyme activity on treated and untreated potato samples the above procedure was followed.

3.10 Enzyme purification (Indira *et al.,* 2015)

The ammonium sulfate precipitation method was used to partially purify the enzyme. Using this approach, 5% of the spore suspension was added to 200ml of modified Czapek's Dox broth and allowed to grow for 3 days.

The culture broth was filtered using sterile muslin cloth and the filtrate was centrifuged. The filtrate was collected in a beaker.

The filtrate was collected in a beaker and magnetic flee was added. The beaker was then kept on the magnetic stirrer. By using the chart, the amount of ammonium sulfate to be added was calculated. The weighed ammonium sulfate was added slowly to the beaker.

The added ammonium sulfate was allowed to dissolve entirely in the beaker by keeping it on the stirrer. The beaker was kept in the refrigerator overnight.

The fraction was centrifuged at 10,000 rpm for 10 minutes the following day and the supernatant was collected in a beaker. The supernatant was used for the enzyme assay and the pellet was used for the protein estimation. Based on the results obtained for enzyme assay, the supernatant was further used to carry out a higher percentage of ammonium sulfate saturation. In this way, the purification was carried out from 20% to 70%.

3.11 Application of the partially purified enzyme

The potato was cut into slices (10g) and was treated with the purified enzyme. The potato slices were Pre-treatment by drying treatment followed by vacuum treatment, and then freezing treatment. After each treatment enzyme was applied onto the slices and then incubated at 60^0 C for 10mins. The sample extract was prepared by homogenizing the sliced potatoes with 70% ethanol (Ciesarová & Kukurová, 2024).

a. Sample extract preparation

The sliced potatoes (treated and untreated) were homogenized with the blender. Then the homogenized potato extract was added to 70% of the ethanol. The mixture was filtered with the help of the muslin cloth or the filter paper. To the insoluble 5ml of the 70% ethanol was added and was filtered again till a soluble fraction was obtained.

The soluble fraction was again filtered by using a 0.45um membrane. The filtrate obtained was used for paper chromatography.

b. Pre-treatment of the sliced potato

A. Drying treatment

Sliced potatoes were treated at 90^0 C for 20mins

B. Vacuum treatment

It was carried out at room temperature for 10mins by using an aspirator

C. Freezing treatment

Done at -10 to -20^0 C for 20 minutes and then thawed at room temperature.

After each treatment enzyme was spread evenly on the surface of the sliced potato. This is followed by the incubation of the treated potato sample at 60^0 C for 10mins. A sample extract of the treated and untreated potato was prepared (Ciesarová & Kukurová, 2024).

CHAPTER 4

ANALYSIS AND CONCLUSIONS

4.1 Fungal isolation, purification, and screening

In an attempt to isolate L-asparaginase-producing fungus, the soil samples from Shiroda, Siolim, and compost soil were diluted and spread-plated on the PDA plates. Upon incubation, morphologically distinct fungal colonies were obtained only from the Shiroda sample (Fig 4.1.1). The results for the three samples are tabulated in Table 4.1.1.

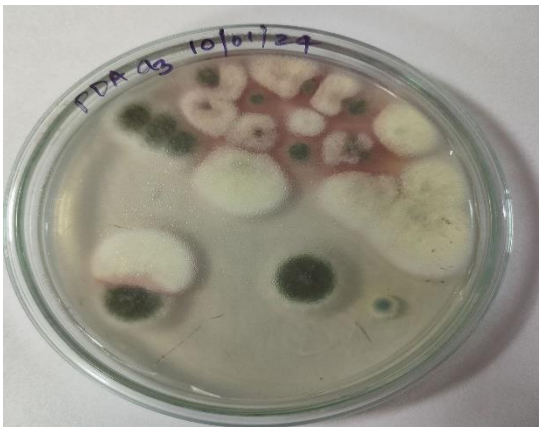


Figure 4.1.1 Fungal isolates from Shiroda soil sample

Table 4.1.1 Table showing no. of fungal isolates positive for L-asparaginase production

Samples	No. of fungal isolates obtained	No. of cultures positive for L-asparaginase enzyme
Compost soil	-	-
Soil sample from Shiroda	3 (a2A5,a2A8,a3A1)	2 (a2A5,a2A8)
Soil sample from Siolim	2 (a1, a4)	-

The soil sample from Shiroda gave three fungal isolates out of which 2 were positive for L-asparaginase enzyme production when screened on the modified CDA plates.

The fungal cultures that showed a pink zone around them were purified (Fig 4.1.2a, 4.1.2b, and 4.1.2c) on the Potato Dextrose Agar plates and then incubated for 4-5 days at room temperature. During the incubation period, the plates were regularly checked for pigment production.

(a) (b) (c)

Figure 4.1.2 (a) a2A5, (b) a2A8 and (c) a3A1 isolates put for purification on PDA plates

The colonies that showed pigment production were discarded and the non-pigment-producing colonies were spot-inoculated on the modified Czapek's Dox agar plates and then incubated for 4-5 days at room temperature. The colonies were then checked for pink-colored zones around them.

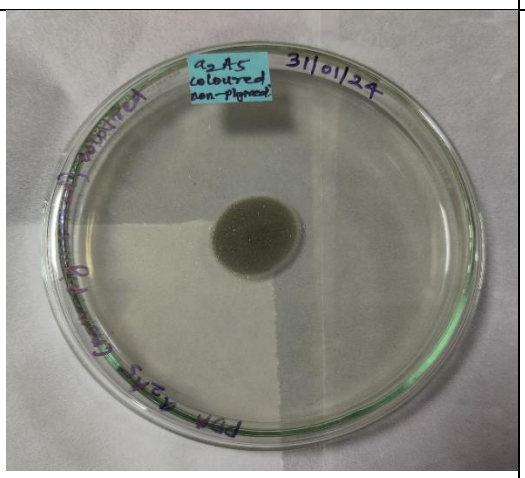
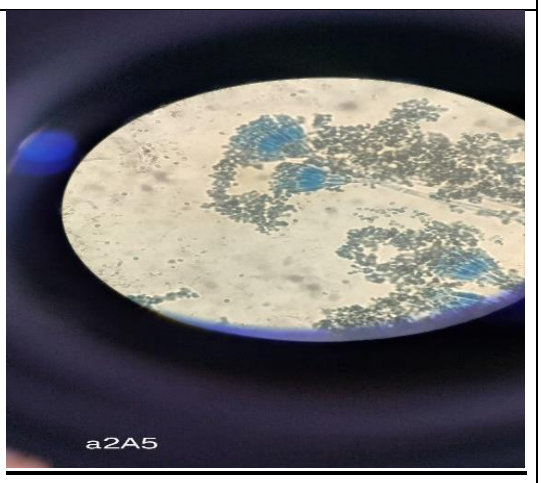
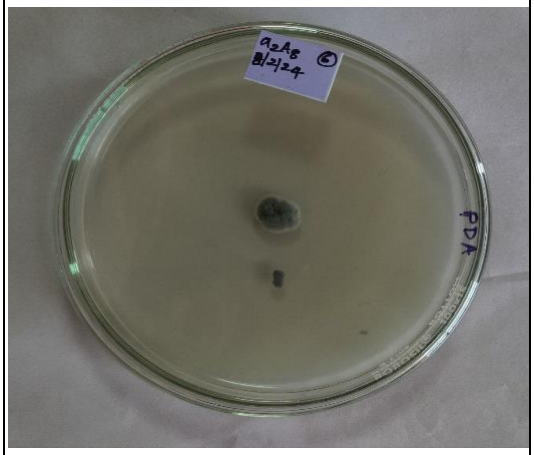
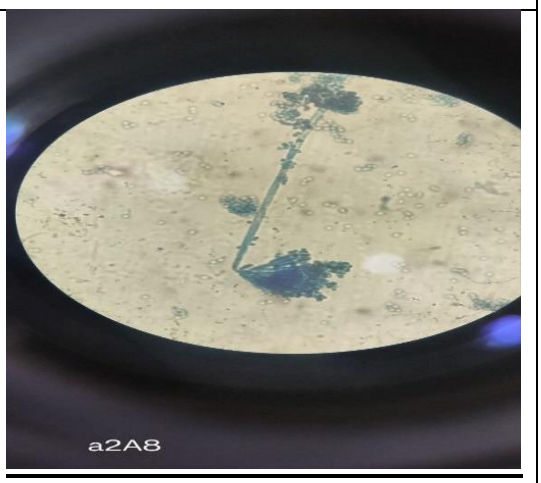
Figure 4.1.3 a2A5, a2A8, and a3A1 purified fungal isolates were spot inoculated on the modified CDA plates.

Out of the 3 isolates, only 2 showed a pink zone around the colony. The a2A5 culture showed the maximum zone area as compared to the isolate a2A8.

4.2 Identification of the fungal isolates

The fungal cultures a2A5 and a2A8 that were positive for L-asparaginase production were both tentatively identified as *Penicillium sp.,* based on the colony morphological characters and wet mount (Table 4.2.1). The colonies showed white mycelial growth with a green center. When observed under a microscope the hyphae were septate and with monoverticilate conidiophores arising from the metulae giving it a brush-like appearance, a characteristic feature of the genus *Penicillium*.

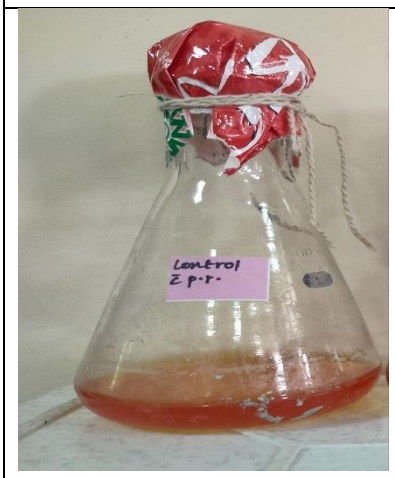
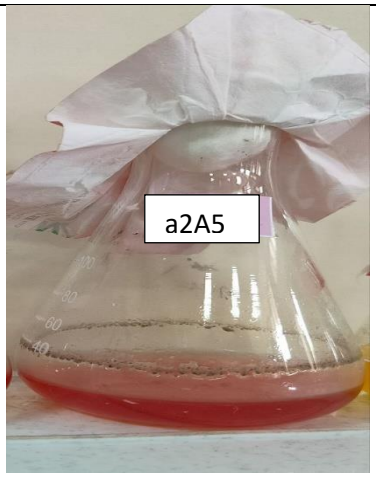
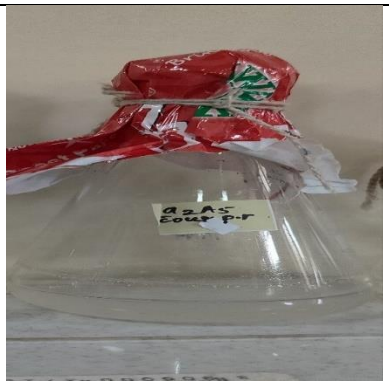
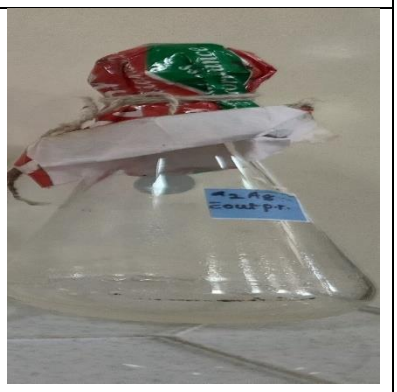
Table 4.2.1 Fungal colonies in PDA plates and wet mount of the isolates a2A5 and a2A8

Isolate	Fungal Colony on PDA	Microscopic mount
a2A5		
a2A8		

4.3 Submerged fermentation

Both the fungal isolates were inoculated in the modified CDB with phenol red and without phenol red for mass multiplication to carry out the enzyme assay (Table 4.3.1). The L-asparaginase-positive cultures were inoculated in triplicates. One flask each (broth without phenol red indicator) was checked for the enzyme assay by Nessler's method every 24 hours.

Table 4.3.1 Submerged fermentation of the fungal isolates(A) with phenol red and (B) without phenol red

Control media	Isolate a2A5	Isolate a2A8
A		
		
B		
		

The enzyme activity of the two fungal isolates determined upon incubation for three days is summarized in Table 4.3.2. The fungal isolate a2A5 showed the highest enzyme activity of 51.6 IU/ml on Day 3 and hence, this culture was used for further studies.

Table 4.3.2 Enzyme assay of cultures in IU

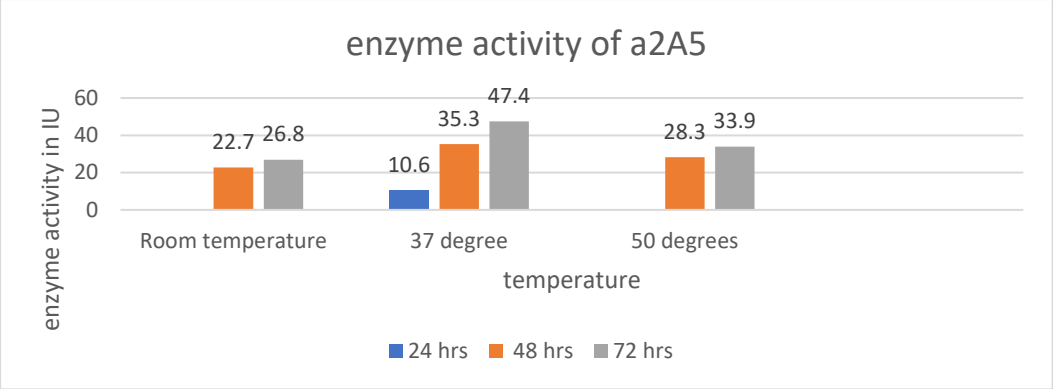
	Enzyme activity in IU/ml	
Culture	**Day2**	**Day3**
a2A5	42.3	51.6
a2A8	25.3	34.7

4.4 Effect of different temperatures on L-asparaginase activity

The enzyme activity of L-asparaginase from the isolate a2A5 was carried out at three different temperatures, room temperature, 37^0 C, and 50^0 C. The highest enzyme activity was obtained at 37^0 C, followed by 50^0 C, and the least at room temperature (Table 4.4.1)

Table 4.4.1 Effect of different temperatures on enzyme activity

Days	**37^0 C**	**Room temperature**	**50^0 C**
	Enzyme activity (IU/ml)		
Day1	10.6	-	-
Day2	35.3	22.7	28.3
Day3	47.4	26.8	33.9



4.5 Growth and enzyme activity of the fungal isolate a2A5

Growth in terms of dry weight and L-asparaginase production in terms of enzyme activity and protein content, of the fungal isolate a2A5 was determined over a period of 4 days. The enzyme assay of isolate a2A5 was carried out by using Nessler's test and protein estimation by Lowry's method. The dry weight of the a2A5 fungal isolate was checked every 2 hours until the constant weight was noted (Table 4.5.1). The dry weight of the isolate increased with the incubation period. The enzyme activity was carried out by using Nessler's test. Enzyme activity and the protein content of the culture filtrate increased with the increasing incubation period. The L-asparaginase activity was 48.4 IU/ml and protein content was 297 µg/ml on day 4 of incubation at room temperature.

Table 4.5.1 Growth and enzyme activity of the fungal isolate a2A5.

Days	Dry Weight/20 ml (gms)	Enzyme Activity (IU/ml)	Protein Estimation (ug/ml)
Day1	0.0108	11.3	8.764
Day2	0.0192	39.7	100.529
Day3	0.2085	48.4	130.529
Day4	0.3052	64.6	297

4.6 Partial Purification of L-asparaginase enzyme by ammonium sulfate precipitation.

The lipase enzyme from the *Penicillium expansum* showed partial purification at 50% of the ammonium sulfate saturation (Stöcklein et al., 1993). Also, the xylanase enzyme from *Penicillium chrysogenum* was partially purified by ammonium sulfate precipitation at 60% saturation (Haas et al., 1992).

Therefore, the partial purification of the enzyme was carried out from 20% to 70% saturation. The enzyme activity was checked at each saturation level (Table 4.6.1) and it was maximum at 70% saturation indicating enzyme precipitation at 70% saturation of ammonium sulfate. The results obtained are similar to that of the endophytic fungus *Talaromyces pinophilus* which showed partial purification at 60-70 % saturation (Krishnapura & Belur,2016). The partial purification of fungal ligninolytic enzymes from Bhadra Wildlife Sanctuary was carried out from 20-905 saturation level (Banakar & Thippeswamy, 2014). The partial purification of the xylanase-producing fungi isolated from the fouled soil was carried out from 0-50% saturation (Sakthiselvan et al., 2014).

Table 4.6.1 Enzyme assay and protein estimation of the partially purified enzyme

Saturation	**Enzyme activity (IU/ml)**	**Protein estimation (µg/ml)**
20%	-	9.941
30%	-	514.647
40%	-	976.411
50%	16.6	832.882

60%	47.3	766.411
70%	52.3	718.764

4.7 Application of the partially purified L-asparaginase

The application of partially purified L-asparaginase enzyme from the fungal isolate a2A5 was checked on the potato. The untreated and treated potato samples with partially purified enzymes were analyzed for L-aspargine breakdown. The Ninhydrin reagent was added to the test tubes containing the treated and untreated potato sample extracts (Table 4.7.1). The absorbance of the sample extracts (treated and untreated) was taken at 340nm for the detection of the L-aspartic acid (Table 4.7.2).

Table 4.7.1 Untreated and treated potato sample extracts

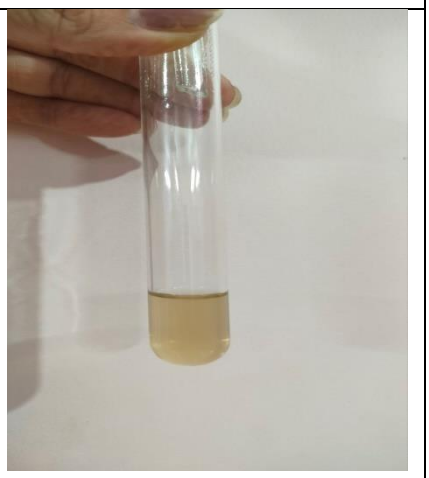
Untreated potato sample extract	Treated potato sample extract
	

Table 4.7.2 OD of the sample extracts

Sample extract	**OD at 340nm**	
	Set 1	**Set II**
Untreated sample	0.363	0.352
Treated sample	0.176	0.125

The OD of the untreated potato sample was higher as compared to that of the treated sample which indicated that the L-asparagine had been hydrolyzed by the applied partially purified enzyme.

4.8 Confirmation of reduction of L-asparagine by paper chromatography

The ammonia released was detected by the Nessler's test. To further confirm the reduction of the asparagine content the paper chromatography was carried out.

The samples were applied on the Whatman filter paper no. 1 and placed in the beaker containing the solvent system. After the solvent system reached the 2/3rd of the chromatogram it was removed, dried, and sprayed with the Ninhydrin solution. The Rf values of the spots were calculated (Table 4.9.1). On the chromatogram of the untreated potato sample, the spot (sample) obtained was darker as compared with the spot obtained for the treated potato sample extract which indicated that there was a reduction in the L-asparagine present in the sample. The Rf value of the standard L-aspartic acid and the Rf value for the treated potato sample extract was the same i.e., 0.674, which also confirms that there was a reduction in the L-asparagine content.

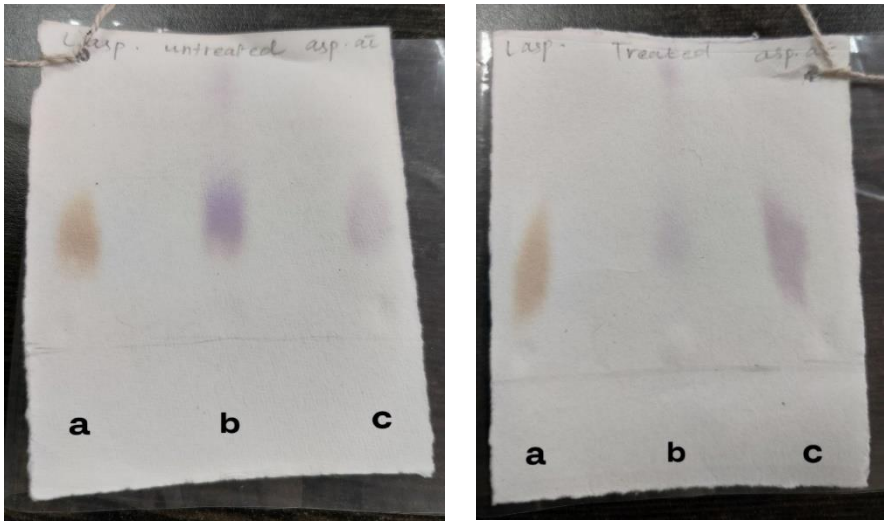


Figure 4.8.1 Chromatogram of untreated and treated potato sample (a: standard L-asparagine, b: potato sample extract, c: standard L-aspartic acid)

The Rf values of the spot obtained are tabulated in Table 4.8.1

Table 4.8.1 Rf values of the sample extracts

Sample	Rf value	
	Untreated sample	Treated sample
Standard L-asparagine	0.658	0.662
Standard L-aspartic acid	0.668	0.674
Potato sample extract	0.647	0.674

Conclusion

A total of three soil samples were collected from three different locations. The two fungal isolates (a2A5 and a2A8) obtained from Shiroda soil sample were positive for the production of L-asparaginase production and were tentatively identified as *Penicillium sp.* based on the morphological characters and microscopic details. Based on the qualitative enzyme assay, the fungal isolate a2A5 showed more enzyme production as compared to the a2A8 isolate.

The isolates were then checked for quantitative enzyme activity by Nessler's method. The isolate a2A5 showed the highest enzyme activity. The same isolate was then checked for enzyme activity at different temperatures (37^0 C, RT, and 50^0 C) and the activity was maximum at 37^0 C. The Partial purification of the enzyme was achieved at 70% saturation. The partially purified enzyme was then applied to the sliced potato with various pre-treatments (drying treatment, vacuum, and freezing treatment). The treated potato sample exhibited a reduction in the L-asparagine content as compared to the untreated sample and showed a higher OD as compared to the treated potato sample extract which indicated that there is a reduction in the L-asparagine content.

Hence the fungal l-asparaginases can be used in the reduction of L-asparagine content in the food thereby reducing the chances of acrylamide formation in the food when heated at high temperatures.

REFERENCES

1. Abdelrazek, N. A., Elkhatib, W. F., Raafat, M. M., & Aboulwafa, M. (2019, March 21). *Experimental and bioinformatics study for production of l-asparaginase from Bacillus licheniformis: a promising enzyme for medical application*. AMB Express.
2. Alrumman, S. A., Mostafa, Y. S., Al-Izran, K. A., Alfaifi, M. Y., Taha, T. H., & Elbehairi, S. E. I. (2019, March 6). *Production and Anticancer Activity of an L-Asparaginase from Bacillus licheniformis Isolated from the Red Sea, Saudi Arabia*. Scientific Reports.
3. Aly, N., El-Ahwany, A., Ataya, F. S., & Saeed, H. (2020, October 26). Bacillus sonorensis L. Asparaginase: Cloning, Expression in E. coli and Characterization. *The Protein Journal*, *39*(6), 717–729.
4. Amena, S., Vishalakshi, N., Prabhakar, M. C., Agsar, D., & Lingappa, K. (2010, January 1). *Production, purification and characterization of l-asparaginase from streptomyces gulbargensis.* DOAJ (DOAJ: Directory of Open Access Journals).
5. Amena, S., Vishalakshi, N., Prabhakar, M., Dayanand, A., & Lingappa, K. (2010, March 1). *Production, purification and characterization of L-asparaginase from Streptomyces gulbargensis*. Brazilian Journal of Microbiology.
6. Amena, S., Vishalakshi, N., Prabhakar, M., Dayanand, A., & Lingappa, K. (2010, March 1). *Production, purification and characterization of L-asparaginase from Streptomyces gulbargensis*. Brazilian Journal of Microbiology.
7. Ba, B., Abdel-Azeem, A. M., Mh, M., & Ea, H. (2018, December 31). *Production of L-Asparaginase (L-ASN) from endophytic Lasiodiplodia theobromae hosted Teucrium polium in Egypt*. Microbial Biosystems Journal.

8. Baky, H. H. A. E., & Baroty, G. S. E. (2016, January 1). *Optimization of Growth Conditions for Purification and Production of L-Asparaginase bySpirulina maxima*. Evidence-based Complementary and Alternative Medicine.
9. Banakar, S. P., & Thippeswamy, B. (2014, July 7). Isolation and partial purification of fungal ligninolytic enzymes from the forest soil fungi isolated from Bhadra Wildlife Sanctuary. *Frontiers in Biology*, *9*(4), 291–299.
10. Batool, T., Makky, E. A., Jalal, M., & Yusoff, M. M. (2015, November 7). *A Comprehensive Review on l-Asparaginase and Its Applications*. Applied Biochemistry and Biotechnology.
11. Batool, T., Makky, E. A., Jalal, M., & Yusoff, M. M. (2015, November 7). A Comprehensive Review on l-Asparaginase and Its Applications. *Applied Biochemistry and Biotechnology*, *178*(5), 900–923.
12. Broome, J. D. (1968, September). Factors which may influence the effectiveness of L-asparaginases as tumor inhibitors. *British Journal of Cancer*, *22*(3), 595–602.
13. Cachumba, J. J. M., Antunes, F. A. F., Peres, G. F. D., Brumano, L. P., Santos, J. C. D., & Da Silva, S. S. (2016, December). Current applications and different approaches for microbial l-asparaginase production. *Brazilian Journal of Microbiology*, *47*, 77–85.
14. Capizzi, R. L., Bertino, J. R., & Handschumacher, R. E. (1970, February). L-Asparaginase. *Annual Review of Medicine*, *21*(1), 433–444.
15. Cappelletti, D., Chiarelli, L. R., Pasquetto, M. V., Stivala, S., Valentini, G., & Scotti, C. (2008, December 1). *Helicobacter pyloril-asparaginase: A promising chemotherapeutic agent*. Biochemical and Biophysical Research Communications.
16. Carta de-Angeli, L., Pocchiari, F., Russi, S., Tonolo, A., Zurita, V. E., Ciaranfi, E., & Perin, A. (1970). Effect of L-asparaginase from Aspergillus terreus on ascites sarcoma in the rat. *Nature*, *225*(5232), 549–550.

17. Cedar, H., & Schwartz, J. H. (1968, December). Production of l -Asparaginase II by Escherichia coli. *Journal of Bacteriology*, *96*(6), 2043–2048.
18. Ciesarová, Z., & Kukurová, K. (2024). Impact of l-asparaginase on acrylamide content in fried potato and bakery products. *Acrylamide in Food*, 473–491.
19. Correa, H. T., Vieira, W. F., Pinheiro, T. M. A., Cardoso, V. L., Silveira, E., Sette, L. D., Pessoa, A., & Filho, U. C. (2020, April 1). *L-asparaginase and Biosurfactants Produced by Extremophile Yeasts from Antarctic Environments*. Industrial Biotechnology (New Rochelle, N.Y.).
20. Da Cunha, M. C., Aguilar, J. G. D. S., De Melo, R. R., Nagamatsu, S. T., Ali, F., De Castro, R. J. S., & Sato, H. H. (2019, December 1). *Fungal L-asparaginase: Strategies for production and food applications*. Food Research International.
21. Da Cunha, M. C., Aguilar, J. G. D. S., De Melo, R. R., Nagamatsu, S. T., Ali, F., De Castro, R. J. S., & Sato, H. H. (2019, December 1). *Fungal L-asparaginase: Strategies for production and food applications*. Food Research International.
22. Da Cunha, M. C., Aguilar, J. G. D. S., De Melo, R. R., Nagamatsu, S. T., Ali, F., De Castro, R. J. S., & Sato, H. H. (2019, December 1). *Fungal L-asparaginase: Strategies for production and food applications*. Food Research International.
23. Davidson, L., Burkom, M., Ahn, S. Y., Chang, L. C., & Kitto, B. (1977, January 1). *l-Asparaginases from Citrobacter freundii*. Biochimica Et Biophysica Acta. Proteins and Proteomics.
24. De Moura Sarquis, M. I., Oliveira, E. M. M., Santos, A. S., & Da Costa, G. L. (2004, August 1). *Production of L-asparaginase by filamentous fungi*. Memorias Do Instituto Oswaldo Cruz.

25. DeMott, J. E., Hilvano, Z., Cann, H., Baldwin-Zurek, I., Kershisnik, W., & Hartwell, S. K. (2024, January 1). *Novel Design of Low Volume Mobile Phase Paper Chromatography*.

26. Dias, F. F. G., Ruiz, A. L. T. G., Della Torre, A., & Sato, H. H. (2016, September 1). *Purification, characterization and antiproliferative activity of l-asparaginase from Aspergillus oryzae CCT 3940 with no glutaminase activity*. Asian Pacific Journal of Tropical Biomedicine/Asian Pacific Journal of Tropical Biomedicine.

27. Dobryakova, N., Dumina, M., Zhgun, A. A., Pokrovskaya, M., Aleksandrova, S. S., Zhdanov, D. D., & Kudryashova, E. (2024, January 1). *L-Asparaginase Conjugates from the Hyperthermophilic Archaea Thermococcus sibiricus with Improved Biocatalytic Properties*. MDPI.

28. Doriya, K., & Kumar, D. S. (2016, November 12). Isolation and screening of l-asparaginase free of glutaminase and urease from fungal sp. *3 Biotech*, *6*(2).

29. Doriya, K., Jose, N., Gowda, M., & Kumar, D. (2016). Solid-State Fermentation vs Submerged Fermentation for the Production of l-Asparaginase. *Marine Enzymes Biotechnology: Production and Industrial Applications, Part I - Production of Enzymes*, 115–135.

30. Doriya, K., Jose, N., Gowda, M., & Kumar, D. (2016). Solid-State Fermentation vs Submerged Fermentation for the Production of l-Asparaginase. *Marine Enzymes Biotechnology: Production and Industrial Applications, Part I - Production of Enzymes*, 115–135.

31. Dumina, M., & Zhgun, A. (2023, January 31). Thermo-L-Asparaginases: From the Role in the Viability of Thermophiles and Hyperthermophiles at High Temperatures to a Molecular Understanding of Their Thermoactivity and Thermostability. *International Journal of Molecular Sciences*, *24*(3), 2674.

32. Dunlop, P. C., & Roon, R. J. (1975, June). L-Asparaginase of Saccharomyces cerevisiae: an extracellular Enzyme. *Journal of Bacteriology*, *122*(3), 1017–1024.
33. Ebrahiminezhad, A., Rasoul-Amini, S., & Ghasemi, Y. (2011, February 1). l-Asparaginase Production by Moderate Halophilic Bacteria Isolated from Maharloo Salt Lake. *Indian Journal of Microbiology*, *51*(3), 307–311.
34. El-Gendy, M. M. A. A., Awad, M. F., El-Shenawy, F. S., & El-Bondkly, A. M. A. (2021, April 1). *Production, purification, characterization, antioxidant and antiproliferative activities of extracellular L-asparaginase produced by Fusarium equiseti AHMF4*. ˜ Al-œMi'galaï Al-sa'udiyaï Lī-ulum Al-ḥayaï.
35. El-Nagga, N. E. A., El-Ewasy, S. M., & El-Shweihy, N. M. (2014, May 1). Microbial L-asparaginase as a Potential Therapeutic Agent for the Treatment of Acute Lymphoblastic Leukemia: The Pros and Cons. *International Journal of Pharmacology*, *10*(4), 182–199.
36. Farag, A. M., Hassan, S. W., Beltagy, E. A., & El-Shenawy, M. A. (2015, December 1). *Optimization of production of anti-tumor l-asparaginase by free and immobilized marine Aspergillus terreus*. Egyptian Journal of Aquatic Research/Egyptian Journal of Aquatic Research.
37. Fu, C. H., & Sakamoto, K. M. (2007, August). PEG-asparaginase. *Expert Opinion on Pharmacotherapy*, *8*(12), 1977–1984.
38. Ghasemi, A., Asad, S., Kabiri, M., & Dabirmanesh, B. (2017, August 11). *Cloning and characterization of Halomonas elongata L-asparaginase, a promising chemotherapeutic agent*. Applied Microbiology and Biotechnology.
39. Ghasemian, A., Al-marzoqi, A., Al-abodi, H. R., Alghanimi, Y. K., Kadhum, S. A., Shokouhi Mostafavi, S. K., & Fattahi, A. (2019, April 16). Bacterial l-asparaginases for

cancer therapy: Current knowledge and future perspectives. *Journal of Cellular Physiology*, *234*(11), 19271–19279.

40. Gulati, R., Saxena, R. K., & Gupta, R. (1997). A rapid plate assay for screening L-asparaginase producing micro-organisms. *Letters in applied microbiology*, *24*(1), 23–26.
41. Haas, H., Herfurth, E., Stöffler, G., & Redl, B. (1992, October 27). *Purification, characterization and partial amino acid sequences of a xylanase produced by Penicillium chrysogenum*. Biochimica Et Biophysica Acta. G, General Subjects/Biochimica Et Biophysica Acta. General Subjects (Online).
42. Hassan, T. J., & Hussein, S. I. (2022). DEVELOPMENT OF BIOPROCESSES FOR PRODUCTION AND PURIFICATION OF L-ASPARAGINASE FROM STAPHYLLOCOCCUS AUREUS, AND IN VITRO EFFECACY AGAINST HUMAN BREAST CNCER CELL LINE. *Iraqi Journal of Agricultural Sciences*, *53*(6), 1525-1538.
43. Hosamani, R., & Kaliwal, B. B. (2011). L-asparaginase an anti-tumor agent production by Fusarium equiseti using solid state fermentation. *Int J Drug Discov*, *3*(2), 88-99.
44. Howard, J. B., & Carpenter, F. H. (1972, February 1). *l-Asparaginase from Erwinia carotovora*. Journal of Biological Chemistry/˜ the œJournal of Biological Chemistry.
45. Imada, A., Igarasi, S., Nakahama, K., & Isono, M. (1973). Asparaginase and glutaminase activities of micro-organisms. *Journal of general microbiology*, *76*(1), 85–99.
46. IMADA, A., IGARASI, S., NAKAHAMA, K., & ISONO, M. (1973, May 1). Asparaginase and Glutaminase Activities of Micro-organisms. *Journal of General Microbiology*, *76*(1), 85–99.

47. Indira, Nagamuthu, Balakrishnan, Dr., & Arulmoorthy. (2015, January). *Production, purification and characterization of extracellular L-Asparaginase from salt marsh fungal endophytes*. ResearchGate. Retrieved 2024, from
48. Izadpanah Qeshmi, F., Homaei, A., Fernandes, P., & Javadpour, S. (2018, March). Marine microbial L-asparaginase: Biochemistry, molecular approaches and applications in tumor therapy and in food industry. *Microbiological Research*, *208*, 99–112.
49. Keating, G. M. (2013, June 21). Asparaginase Erwinia chrysanthemi (Erwinaze®): A Guide to Its Use in Acute Lymphoblastic Leukemia in the USA. *BioDrugs*, *27*(4), 413–418.
50. Kil, J. O., Kim, G. N., & Park, I. (1995, January 1). *Extraction of ExtracellularL-Asparaginase fromCandida utilis*. Bioscience, Biotechnology, and Biochemistry.
51. Kotzia, G. A., & Labrou, N. E. (2007, January 1). *l-Asparaginase from Erwinia Chrysanthemi 3937: Cloning, expression and characterization*. Journal of Biotechnology.
52. Krishnapura, P. R., & Belur, P. D. (2016, February 1). *Partial purification and characterization of L-asparaginase from an endophytic Talaromyces pinophilus isolated from the rhizomes of Curcuma amada*. Journal of Molecular Catalysis. B, Enzymatic.
53. Krishnapura, P. R., Belur, P. D., & Subramanya, S. (2015, April 13). A critical review on properties and applications of microbiall-asparaginases. *Critical Reviews in Microbiology*, 1–18.
54. Kumar, N., Ravi, R., & Manonmani, H. K. (2013, May 1). *Production and optimization of l-asparaginase from Cladosporium sp. using agricultural residues in solid state fermentation*. Industrial Crops and Products.

55. Leck A. (1999). Preparation of lactophenol cotton blue slide mounts. *Community eye health*, *12*(30), 24.

56. Loureiro, C. B., Borges, K. S., Andrade, A. F., Tone, L. G., & Said, S. (2012, January 1). *Purification and Biochemical Characterization of Native and Pegylated Form of L-Asparaginase from <i>Aspergillus terreus</i> and Evaluation of Its Antiproliferative Activity*. Advances in Microbiology.

57. Lubkowski, J., & Wlodawer, A. (2021, June 19). Structural and biochemical properties of L-asparaginase. *The FEBS Journal*, *288*(14), 4183–4209.

58. Lubkowski, J., Vanegas, J., Chan, W. K., Lorenzi, P. L., Weinstein, J. N., Sukharev, S., Fushman, D., Rempe, S., Anishkin, A., & Wlodawer, A. (2020, May 4). Mechanism of Catalysis by l-Asparaginase. *Biochemistry*, *59*(20), 1927–1945.

59. Lubkowski, J., Vanegas, J., Chan, W. K., Lorenzi, P. L., Weinstein, J. N., Sukharev, S., Fushman, D., Rempe, S., Anishkin, A., & Wlodawer, A. (2020, May 4). Mechanism of Catalysis by l-Asparaginase. *Biochemistry*, *59*(20), 1927–1945.

60. Mashburn, L. T., & Wriston, J. C. (1963, July). Tumor inhibitory effect of L-asparaginase. *Biochemical and Biophysical Research Communications*, *12*(1), 50–55.

61. Mishra, A. (2006, January 1). *Production of L-Asparaginase, an Anticancer Agent, From Aspergillus niger Using Agricultural Waste in Solid State Fermentation*. Applied Biochemistry and Biotechnology.

62. Moffatt, E. D., & Lytle, R. I. (1959, May 1). Polychromatic Technique for Identification of Amino Acids on Paper Chromatograms. *Analytical Chemistry*, *31*(5), 926–928.

63. Mostafa, S. (1979, January 1). *Production of L-Asparaginase By Streptomyces Karnatakensis and Streptomyces Venezuelae*. Zentralblatt Für Bakteriologie, Parasitenkunde, Infektionskrankheiten Und Hygiene. 2., Naturwissenschaftliche,

Abteilung, Mikrobiologie Der Landwirtschaft, Der Technologie Und Des Umweltschutzes.

64. Moubasher, H. A., Balbool, B. A., Helmy, Y. A., Alsuhaibani, A. M., Atta, A. A., Sheir, D. H., & Abdel-Azeem, A. M. (2022). Insights into Asparaginase from Endophytic Fungus *Lasiodiplodia theobromae*: Purification, Characterization and Antileukemic Activity. *International journal of environmental research and public health*, *19*(2), 680.
65. Moubasher, H., Balbool, B. A., Helmy, Y. A., Alsuhaibani, A. M., Atta, A., Sheir, D. H., & Abdel-Azeem, A. M. (2022, January 7). *Insights into Asparaginase from Endophytic Fungus Lasiodiplodia theobromae: Purification, Characterization and Antileukemic Activity*. International Journal of Environmental Research and Public Health/International Journal of Environmental Research and Public Health.
66. Muneer, F., Siddique, M. H., Azeem, F., Rasul, I., Muzammil, S., Zubair, M., Afzal, M., & Nadeem, H. (2020, February 12). Microbial l-asparaginase: purification, characterization and applications. *Archives of Microbiology*, *202*(5), 967–981.
67. Muneer, F., Siddique, M. H., Azeem, F., Rasul, I., Muzammil, S., Zubaır, M., Afzal, M., & Nadeem, H. (2020, February 12). *Microbial l-asparaginase: purification, characterization and applications*. Archives of Microbiology.
68. Narayana, K., Kumar, K., & Vijayalakshmi, M. (2008, June 12). *L-asparaginase production by Streptomyces albidoflavus*. Indian Journal of Microbiology.
69. Nunes, J. C. F., Cristóvão, R. O., De Carvalho Santos-Ebinuma, V., Faria, J. L., Silva, C. G., Neves, M. C., Freire, M. G., & Tavares, A. P. M. (2021, August 20). *L-Asparaginase-Based Biosensors*. Encyclopedia.

70. Nunes, J. C. F., Cristóvão, R. O., De Carvalho Santos-Ebinuma, V., Faria, J. L., Silva, C. G., Neves, M. C., Freire, M. G., & Tavares, A. P. M. (2021, August 20). *L-Asparaginase-Based Biosensors*. Encyclopedia.

71. Onishi, Y., Prihanto, A. A., Yano, S., Takagi, K., Umekawa, M., & Wakayama, M. (2015, February 4). Effective treatment for suppression of acrylamide formation in fried potato chips using L-asparaginase from Bacillus subtilis. *3 Biotech*, *5*(5), 783–789.

72. Peterson, G. L. (1979, December). Review of the folin phenol protein quantitation method of lowry, rosebrough, farr and randall. *Analytical Biochemistry*, *100*(2), 201–220.

73. Peterson, R. E., & Ciegler, A. (1969, July). L-Asparaginase Production by Erwinia aroideae. *Applied Microbiology*, *18*(1), 64–67.

74. Pokrovskaya, M. V., Pokrovskiy, V. S., Александрова, Anisimova, N., Андрианов, Treschalina, E. M., Пономарев, & Соколов. (2012, April 1). *Recombinant intracellular Rhodospirillum rubrum L-asparaginase with low L-glutaminase activity and antiproliferative effect*. Biochemistry (Moscow). Supplement. Series B, Biomedical Chemistry.

75. Qeshmi, F. I., Homaei, A., Fernandes, P., & Javadpour, S. (2018, March 1). *Marine microbial L-asparaginase: Biochemistry, molecular approaches and applications in tumor therapy and in food industry*. Microbiological Research.

76. Sahoo, A. (2024, January 24). *Penicillium: Occurrence, Structure, Reproduction*. Biology Learner.

77. Saif, F. A., Yaseen, S. A., Alameen, A. S., Mane, S. B., & Undre, P. B. (2020, October 1). Identification of Penicillium Species of Fruits Using Morphology and Spectroscopic Methods. *Journal of Physics: Conference Series*, *1644*(1), 012019.

78. Sakthiselvan, P., Naveena, B., & Partha, N. (2014, December 1). *Molecular characterization of a Xylanase-producing fungus isolated from fouled soil*. Brazilian Journal of Microbiology.

79. Sánchez-Moguel, I., Costa-Silva, T. A., Pillaca-Pullo, O., Flores-Santos, J. C., Freire, R. K. B., Carretero, G., Da Luz Bueno, J., Camacho-Córdova, D. I., Santos, J. H. P. M., Sette, L. D., & Pessoa, A. (2023, June 1). *Antarctic yeasts as a source of L-asparaginase: Characterization of a glutaminase-activity free L-asparaginase from psychrotolerant yeast Leucosporidium scottii L115*. Process Biochemistry.

80. Schrappe, M., Reiter, A., Ludwig, W., Harbott, J., Zimmermann, M., Hiddemann, W., Niemeyer, C. M., Henze, G., Feldges, A., Zintl, F., Kornhuber, B., Ritter, J., Welte, K., Gadner, H., & Riehm, H. (2000, June 1). *Improved outcome in childhood acute lymphoblastic leukemia despite reduced use of anthracyclines and cranial radiotherapy: results of trial ALL-BFM 90. German-Austrian-Swiss ALL-BFM Study Group.* Blood.

81. Shah, A., Karadi, R., & Parekh, P. (2010, June 15). Isolation, Optimization and Production of L-asparaginase from Coliform Bacteria. *Asian Journal of Biotechnology*, *2*(3), 169–177.

82. Stecher, A., De Deus, P. M., Polikarpov, I., & Abrahão-Neto, J. (1999, December 1). *Stability of l-asparaginase: an enzyme used in leukemia treatment*. Pharmaceutica Acta Helvetiae/Pharmaceutica Acta Helvetiae.

83. Stöcklein, W., Sztajer, H., Menge, U., & Schmid, R. D. (1993, June 1). *Purification and properties of a lipase from Penicillium expansum*. Biochimica Et Biophysica Acta. Lipids and Lipid Metabolism/Biochimica Et Biophysica Acta. L, Lipids and Lipid Metabolism.

84. Talluri, V. P., Bhavana, M., Kumar, M. M., & Rajagopal, S. (2014, May). L-Asparaginase: An Ultimate Anti-Neoplastic Enzyme. *International Letters of Natural Sciences*, *15*, 23–35.

85. Tareke, E., Rydberg, P., Karlsson, P., Eriksson, S., & Törnqvist, M. (2000). Acrylamide: a cooking carcinogen?. *Chemical research in toxicology*, *13*(6), 517–522.

86. USHA, R., MALA, K. K., VENIL, C. K., & PALANISWAMY, M. (2011). Screening of Actinomycetes from Mangrove Ecosystem for L-asparaginase Activity and Optimization by Response Surface Methodology. *Polish Journal of Microbiology*, *60*(3), 213–221. https://doi.org/10.33073/pjm-2011-030

87. van den Berg, H. (2011, February). Asparaginase revisited. *Leukemia & Lymphoma*, *52*(2), 168–178.

88. van den Berg, H. (2011, February). Asparaginase revisited. *Leukemia & Lymphoma*, *52*(2), 168–178.

89. Vimal, A., & Kumar, A. (2017, January 2). *Biotechnological production and practical application of L-asparaginase enzyme*. Biotechnology & Genetic Engineering Reviews.

90. Wang, Y., Xu, W., Wu, H., Zhang, W., Guang, C., & Mu, W. (2021, September 1). *Microbial production, molecular modification, and practical application of l-Asparaginase: A review*. International Journal of Biological Macromolecules.

91. Whitecar, J. P., Bodey, G. P., Harris, J. E., & Freireich, E. J. (1970, March 26). L-Asparaginase. *New England Journal of Medicine*, *282*(13), 732–734.

92. Xu, F., Oruna-Concha, M. J., & Elmore, J. S. (2016, November). The use of asparaginase to reduce acrylamide levels in cooked food. *Food Chemistry*, *210*, 163–171.

93. Youssef, M. M., Darwish, D. B. E., Alrdahe, S. S., Al-Awthan, Y. S., Elfaki, I., Alnour, T. M., Darwish, A. M., Saad, E. A., & Habeb, S. (2022, August 2). *Purification, Molecular Characterization, and Anticancer Activities of L- Asparaginase extracted from Staphylococcus aureus*. Egyptian Journal of Chemistry.

94. Zhang, K., Yuan-Ying, S., & Cai, L. (2013, December). An Optimized Protocol of Single Spore Isolation for Fungi. *Cryptogamie, Mycologie*, *34*(4), 349–356.

95. Zuo, S., Zhang, T., Jiang, B., & Mu, W. (2015, June 16). *Reduction of acrylamide level through blanching with treatment by an extremely thermostable l-asparaginase during French fries processing*. Extremophiles.

96. Zuo, S., Zhang, T., Jiang, B., & Mu, W. (2015, June 16). *Reduction of acrylamide level through blanching with treatment by an extremely thermostable l-asparaginase during French fries processing*. Extremophiles.

97. Zyzak, D., Sanders, R. A., Stojanovic, M., Tallmadge, D. H., Ebehart, L., Ewald, D. K., Gruber, D. C., Morsch, T. R., Strothers, M. A., Rizzi, G. P., & Villagran, M. D. (2003). Acrylamide formation mechanism in heated foods. Journal of Agricultural and Food Chemistry, 51, 4782–4787.

Appendices

APPENDIX 1: Media Composition

Modified Czapek's Dox medium without an indicator (g/100ml)

Glucose	0.2g
l-Asparagine	0.1g
KH_2PO_4	0.152g
NaCl	0.052g
$MgSO_4$	0.052g
$ZnSO_4$	0.005g
$FeSO_4$	0.003g
Agar	1.8g
pH	6.2

Modified Czapek's Dox medium with Phenol red indicator (g/100ml)

Glucose	0.2g
l-Asparagine	0.1g
KH_2PO_4	0.152g
NaCl	0.052g
$MgSO_4$	0.052g
$ZnSO_4$	0.005g
$FeSO_4$	0.003g
Agar	1.8g
Phenol red	0.009g

pH	6.2

Potato Dextrose Agar (g/1L)

Potato infusion	200g
Dextrose	20g
Agar	15g
pH	5.6

Sabouraud's Dextrose agar (g/1L)

Peptone	10g
Dextrose Monohydrate	40g
Agar	15g
pH	5.6

Normal saline

NaCl	0.85g
Distilled water	100ml

APPENDIX 2: Reagents

Lactophenol cotton blue

Lactophenol cotton blue stain	5ml
Ethanol	5ml

2% Ninhydrin solution

Ninhydrin	2g
Ethanol	100ml

Solvent system for paper chromatography

n-Butanol	50ml
Acetic acid	40ml
Distilled water	10ml

APPENDIX 3: Methods for quantitative estimation

Nesslerisation of ammonia (Imada *et al.*,1973)

Principle:

Nessler's Reagent (K_2HgI_4) also called Potassium Mercuric Iodide reacts with the ammonia present in the sample and produces a yellow to brown-colored complex under alkaline conditions. The intensity of the color change is directly proportional to the ammonia present in the given sample.

Reagents

Nessler's reagent (g/100ml)

A)	Mercuric chloride	10g
	Potassium iodide	7g
	Distilled water	50ml
B)	NaOH	16g
	Distilled water	50ml
	Mix A and B gently before using	

0.04M L-asparagine (g/20ml)

L-Asparagine	0.1201g
Distilled water	20ml

0.05M Tris HCl buffer

1M Tris HCl	1ml
Distilled water	19ml

1.5M Trichloroacetic acid (1.5M)

Trichloroacetic acid	4.89g
Distilled water	20ml

Ammonium Chloride (100mM working stock) (Indira *et al.*,)

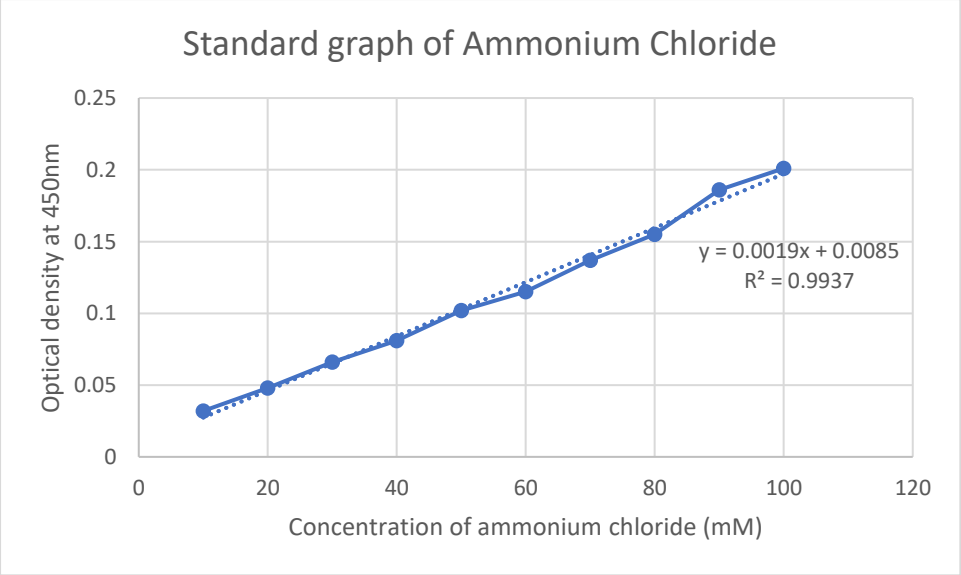
Ammonium chloride	0.2675g
Distilled water	50ml

Using 100mM ammonium chloride stock concentration ranging from 100mM – 0mM was made.

Procedure

A reaction mixture with a total volume of 2.0 mL, comprising 0.5 mL of 0.05 M tris HCl buffer (pH 8.2), 0.5 mL of 0.04 M L-asparagine, and 1 mL of 100 mM ammonium chloride, was incubated for 30 minutes at 37°C. 0.5 mL of 1.5 M trichloroacetic acid (TCA) was added to the mixture to stop the reaction, and then 0.2 mL of Nessler's reagent was added. The reaction mixture was kept at room temperature for 10mins after which absorbance was measured at 450nm.

Standard caliberation curve was prepared using ammonium chloride and graph was plotted by adding concentration of Ammonium Chloride (mM) on X-axis and OD at 450 nm on Y-axis.



Protein estimation by Folin Lowry method (Peterson, 1979)

Principle:

It is a biochemical assay that detects the concentration of the protein in the sample. This process involves the alkaline peptide nitrogen interacting with copper ions, which is followed by the copper-catalyzed oxidation of aromatic acids, which reduces the Folin-Ciocalteau phosphomolybdic phosphotungstic acid to heteropolymolybdenum blue.

Reagents

A)	NaOH	0.1g
	Distilled water	100ml
	Na_2CO_3	2g
B)	Sodium Potassium Tartarate	1g
	Distilled water	100ml
	Copper sulfate	0.5g

Reagent 1

50ml of A + 1ml of B	51ml

Reagent 2

Folin Ciocalteau	5ml
Distilled water	5ml

Bovine Serum albumin (300ug/ml working stock)

Diluent : distilled water

Procedure

Using 300ug stock solution concentration ranging from 300ug – 50ug was prepared. Using the BSA concentration the total volume was made to 1ml by adding distilled water. 5ml of the reagent 1 was added in all tubes and incubated in the dark at room temperature. After 10 minutes of incubation, 0.5ml of reagent 2 was added to all the tubes and kept for 30 minutes of incubation at room temperature. Absorbance was measured at 660nm.

The standard graph was plotted by adding the concentration of BSA in ug on the X-axis and OD at 660nm on the Y-axis.