

Effective production and purification of L-asparaginase enzyme from microbial sources isolated from fertile soil and its application as an antimicrobial agent

Submitted in partial fulfillment of the requirements for the award of Master of Science in Medical Biotechnology and Clinical Research

By

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BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **PAVAN KUMAR.E (42101004)** who carried out Project entitled "**Effective production and purification of L-asparaginase enzyme from microbial sources isolated them fertile soil and it's application as an antimicrobial agent**" under the supervision from September 2023 to April 2024.

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DECLARATION

I, **PAVAN KUMAR.E (42101004)** hereby declare that the Project Report entitled "Effective production and purification of L-asparaginase enzyme from microbial sources isolated them fertile soil and it's application as an antimicrobial agent" done by me under the guidance of **Dr. A. DAYANANDAN Professor (Research)** at the Centre for Molecular and Nanomedical Sciences, Sathyabama Institute of Science and Technology, Chennai, is submitted in partial fulfilment of the requirements for the award of Master of Science in Medical Biotechnology and Clinical Research.

DATE: 18/04/2024

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ABSTRACT

L-Asparaginase is an anti-neoplastic drug used in lymphoblastic leukemia chemotherapy. Nowadays, this enzyme derived from bacterial sources, mostly L-asparaginase II from *Escherichia coli* and in lesser amount L-Asparaginase of *Erwinia* sp. has medical utilization. The long-term usage of these agents leads to allergic reactions and new asparaginase with new immunological characteristics is required. Halophilic bacteria might contain L-Asparaginase with novel immunological properties that can be used in hypersensitive patients. In this experiment, we have screened moderate Halophilic bacteria for L-Asparaginase production ability and showed that Halophilic bacteria produce intra- and extracellular L-asparaginase. Pr 4 was found to produce the highest L-Asparaginase (653.64 U/ml supernatant) extracellularly. And it has also shown the antimicrobial activity against the clinical pathogens.

Keywords: L-Asparaginase, Halophile, Bacteria, 16S rRNA

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ABBREVIATIONS

DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
NACL	Sodium Chloride
PMSF	Phenylmethylsulfonyl fluoride
PCR	Polymerase chain reaction
EDTA	Ethylenediamine tetraacetic acid
DTT	Dithiothreitol
GAD	Glutamic acid decarboxylase
LAB	Lactic acid bacteria
TCA	Tricarboxylic acid cycle
CO ₂	Carbon dioxide
DMSO	Dimethyl sulfoxide
DEAE	Diethylaminoethyl
KDa	Kilo Dalton

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CHAPTER 1

1 INTRODUCTION

L-Asparaginase is a crucial enzyme that plays a pivotal role in cancer therapy, particularly in the treatment of acute lymphoblastic leukemia (ALL) and certain other malignancies. This enzyme catalyzes the hydrolysis of the amino acid L-asparagine into aspartic acid and ammonia. By depleting circulating asparagine, L-Asparaginase selectively targets cancer cells that are unable to synthesize their own asparagine due to the lack of the enzyme asparagine synthetase, leading to inhibition of protein synthesis and eventual cell death. The history of L-Asparaginase dates back to the 1950s when its therapeutic potential was first recognized in leukemia treatment. Over the years, various forms of L-Asparaginase have been developed, including those derived from *Escherichia coli* bacteria, *Erwinia chrysanthemi*, and pegylated formulations, each with its own pharmacokinetic and immunological profile. Despite its clinical efficacy, L-Asparaginase therapy is associated with several adverse effects, including hypersensitivity reactions, pancreatitis, coagulopathy, and hepatotoxicity, necessitating careful patient monitoring and management (Capizzi and Frova, 1970).

L-Asparaginase holds a prominent position in the medical field, primarily for its therapeutic applications in cancer treatment, particularly acute lymphoblastic leukemia (ALL). This enzyme's ability to catalyze the conversion of L-asparagine to aspartic acid and ammonia serves as a cornerstone in cancer therapy by depriving malignant cells of a crucial nutrient, ultimately impeding their growth and inducing apoptosis. Over the decades, L-Asparaginase has been extensively studied and utilized in various clinical settings. It is often incorporated into chemotherapy regimens as a component of combination therapy, where it acts synergistically with other anti-cancer agents to enhance treatment efficacy. Furthermore, L-Asparaginase has shown promise in the treatment of other malignancies beyond ALL, including acute myeloid leukemia (AML), non-Hodgkin lymphoma, and some solid tumors. Research efforts continue to explore its potential applications in these contexts, aiming to broaden its therapeutic utility and improve patient outcomes. Despite its clinical benefits, the use of L-Asparaginase is not without challenges. Adverse effects such as hypersensitivity reactions,

hepatotoxicity, and pancreatitis are commonly observed and necessitate careful monitoring and management during treatment (Belén *et al.*, 2020).

Beyond its well-established role in cancer therapy, L-Asparaginase has garnered attention for its potential antimicrobial properties. Studies have suggested that L-asparaginase exhibits inhibitory effects against various microbial pathogens, including bacteria, fungi, and parasites, presenting a promising avenue for combating infectious diseases. Research has indicated that L-Asparaginase exerts its antimicrobial activity through multiple mechanisms. One proposed mechanism involves the depletion of extracellular asparagine, an essential amino acid for microbial growth, thereby inhibiting protein synthesis and impairing microbial viability. Additionally, L-asparaginase has been shown to induce metabolic alterations and disrupt cellular processes in microbial pathogens, further contributing to its antimicrobial effects. The antimicrobial potential of L-Asparaginase extends to a wide range of microorganisms, including both Gram-positive and Gram-negative bacteria, as well as fungal pathogens and parasites. This broad spectrum of activity underscores the versatility of L-asparaginase as a potential therapeutic agent against various infectious diseases. Moreover, L-asparaginase's antimicrobial activity has been investigated in different contexts, including both *in vitro* studies and animal models of infection. These studies have provided valuable insights into the mechanisms of action and potential applications of L-Asparaginase as an antimicrobial agent. While further research is needed to fully elucidate the antimicrobial properties of L-Asparaginase and optimize its therapeutic potential, the existing evidence highlights its promise as a novel approach to combating microbial infections (Tandon *et al.*, 2021).

L-Asparaginase stands as a vital component in the arsenal against cancer, particularly in the treatment of acute lymphoblastic leukemia (ALL) and other malignancies. Its efficacy lies in its ability to degrade circulating L-asparagine, an essential amino acid for cancer cell survival, thereby inducing selective cytotoxicity in malignant cells while sparing normal tissues. The role of L-Asparaginase in cancer therapy was first recognized in the 1950s, and since then, it has become a cornerstone of treatment regimens for ALL, both in pediatric and adult populations. By exploiting the asparagine dependency of cancer cells, L-Asparaginase effectively curtails their growth and proliferation, leading to tumor regression and improved patient outcomes. Beyond all, L-Asparaginase has demonstrated efficacy in other hematologic malignancies,

including acute myeloid leukemia (AML) and non-Hodgkin lymphoma, as well as certain solid tumors. Its versatility in targeting a wide range of cancer types underscores its significance in modern oncology. Furthermore, research efforts have focused on optimizing the therapeutic potential of L-Asparaginase through the development of novel formulations, including pegylated variants, which exhibit improved pharmacokinetic properties and reduced immunogenicity, thus enhancing treatment tolerability and efficacy (Kelo *et al.*, 2002).

CHAPTER 2

2. LITERATURE SURVEY

2.1. HISTORY OF ENZYME RESEARCH

Enzymes have been used by human civilization since times immemorial. A professor of physiology at the University of Heidelberg, Wilhelm Freidrich Kühne first coined the term "enzyme" in 1877 (Gurung *et al.*, 2013). First use of enzyme in cheese making by Egyptians as early as in 400 B.C. was mentioned in Illiad by Homer. The general theory of chemical catalysis, first published in 1835 by J. J. Berzelius, showed an example of what is presently termed as an enzyme –diastase of malt, and it was clearly indicated that starch is hydrolysed more efficiently by the enzyme rather than by sulfuric acid. The first enzyme to be used on an industrial scale was rennet obtained from dried calves 'stomach in 1874 by Danish chemist Christian Hansen. Louis Pasteur in the 18th century recognized that fermentation is catalyzed by enzymes. Later in 1897, Edward Buchner isolated enzymes from yeast cells and demonstrated the synthesis of ethanol from glucose. J.B. Sumner in 1926 isolated urease from jack bean and emphasized on the protein nature of enzymes. However, crystallization studies carried by J. Northrop on pepsin, trypsin and chmyotrypsin proved the basis of protein nature of enzymes. In 1914, trypsin began to be used generously in detergent industries due to its protein degrading property. By 1960, traditional sulfuric acid hydrolysis of starch was almost done away with enzymatic hydrolysis using l-asparaginases. Henceforth, enzymes began to be utilized in different sectors, including pharmaceutical, food, bioengineering, textile industries etc (Jia *et al.*, 2021).

For example, L-Asparaginase is an amidohydrolase with enzyme nomenclature as E.C.3.5.1.1 that hydrolyses the amide bond in L-Asparagine and converts to L-Aspartic acid and ammonia (Verma *et al.*, 2017).

2.1.1 Enzymes for medical applications

- Therapeutic applications (L-Asparaginase for the treatment of leukaemia)
- Digestive enzymes (l-asparaginase, l-asparaginase, lipase).

2.1.2 Diagnostic applications

- Enzyme linked immunosorbent assay (ELISA): (peroxidase, alkaline phosphatase, β -galactosidase)
- Enzyme multiplied immunoassay technique (EMIT): (lysozyme, malate dehydrogenase)
- Enzymatic analysis of blood components: glucose, urea, uric acid, cholesterol, pyruvate, lactate etc.

2.1.3 Food enzymes

- Breakdown of starch (l-asparaginase for production of glucose from starch)
- High fructose corn syrup production (glucose isomerase for fructose production)
- Production of superior quality of bakery products (xylanase and α -l-asparaginase for enhancing loaf volume)
- Dairy product formation (β -galactosidase for lactose-free milk production)
- Extraction and clarification of fruit juice (pectinase for haze removal from juices)
- Alcoholic beverage production like wine, beer (glucanase and xylanase for haze removal from alcohols).

2.1.4 Enzymes for technological applications

- In detergents (lipases)
- Textile conditioning (cellulase)
- Leather processing (l-asparaginase)
- Paper and pulp industry (hemicellulases and xylanases)

2.1.5 Enzymes for animal feedstock

- Enhancing digestibility of feedstock (xylanase)
- Removal of phosphate (phytase)

2.2. INTRACELLULAR AND EXTRACELLULAR ENZYME

Intracellular enzymes are the enzymes that are synthesized and retained within the cell for the internal cellular use. Enzymes that act inside cells are responsible for catalysing millions of reactions that occur in metabolic pathways such as glycolysis in the mitochondria and in the photosynthetic pathway in the chloroplast. The lysosome contains many enzymes that are mainly responsible for destroying old cell.

Extracellular enzymes are ones that an organism secretes outside its body for purposes such as digestion of food substances to then ingest once broken down (or absorb). Some fungi do this using mycelium. Certain bacteria also secrete extracellular enzymes to solubilize its substrate externally.

2.3. FACTORS AFFECTING ENZYMATIC ACTIVITY

pH- every enzyme works best within a specified range of pH and has an optimum pH where the enzyme activity is the greatest. This is due to the fact that any change in pH alters the conformation of the active site of the enzyme molecule because of change in the charge of the functional groups of amino acids present which thereby affects the binding of the substrate to the enzyme at the active site.

Temperature- the rate of enzyme catalysed reaction increases with the increase in temperature. This is because as the temperature increases, kinetic energy possessed by both the enzyme and substrate increases, and rate of intermolecular collisions also increases. The minimum energy required to cross over the activation energy barrier is achieved and hence, reaction rates increase by 4-8% per degree centigrade rise in temperature. Above the optimum temperature, enzyme undergoes denaturation and hence, reaction rate slows down.

Concentration of enzyme- rate of reaction increases with the increase in the concentration of enzyme as more active sites are available for the substrates to bind and form products.

Concentration of substrate- rate of reaction increases with the increase in concentration of substrate as more substrate molecules can bind to the active sites

present on enzyme surface. But higher substrate concentrations cause saturation of the active sites with substrates that cannot adhere to further substrate molecules and thus reaction rate does not increase.

Inhibitors-inhibitors are mainly of three types-competitive, uncompetitive, and noncompetitive inhibitors. Competitive inhibitors are structural analogs of the substrate and directly compete for the active sites on enzyme. This kind of inhibition is a temporary and reversible process which reduces reaction rate. In case of uncompetitive inhibition, the inhibitor combines with the enzyme-substrate (ES) complex, which cannot undergo further reaction to yield normal products. The degree of inhibition increases when the substrate concentration increases. Noncompetitive inhibitors bind with either the free enzyme at its active site or with the ES complex, inhibiting the action of both. The effects are not reversed with increase in substrate concentration.

Ionic strength-ionic strength is a measure of concentration of charges in a solution. Ions affect charges of the amino acids present at the active site and thus alter the stability and solubility of both the enzyme and substrate, subsequently affecting reaction rates.

2.4.L-Asparaginase

2.4.1. Historical background

L-Asparaginase (L-Asparaginase amidohydrolase, E.C.3.5.1.1) is an amidase group enzyme that causes the hydrolysis of the amide bond in L-Asparagine and converts to L-Aspartic acid and ammonia (Verma *et al.*, 2017). L-Asparaginase possess anti-leukemic activity to be studied by different researchers across the globe (Verma *et al.*, 2007). L-Asparaginase activity was first detected by Lang in beef tissues in 1904. The pioneer observation that turned out to be a deciding factor for further research and developmental studies on this enzyme as a potential anti-neoplastic agent was carried out by Clementi (1922) who revealed that L-Asparaginase is abundant in guinea pig serum. Later, Kidd (1953) depicted that guinea pig serum was able to inhibit the growth of lymphoid tumours in rodents and certain spontaneous and radiation induced leukaemia in mice (Kidd, 1970). Later, Neuman and McCoy in 1956 depicted the metabolic differences between malignant and normal cells in vitro in the absence and presence of the amino acid, asparagine (McCoy, Maxwell and Neuman, 1956).

Broome (1963) performed research to analyse the relative contribution of complement and L-Asparaginase in anticancer activity and concluded that L-Asparaginase is a major anticancer agent in guinea pig serum (Broome, 1963). The first clinical trial on L-Asparaginase was performed in 1966 by Dolowy (Dolowy *et al.*, 1967).

2.4.2. L-Asparaginase-its role in normal and cancerous cells

Human cells require L-Asparagine for protein synthesis and its survival. L-Asparagine is not an essential amino acid in normal cells, as it can be synthesized naturally in the body by an enzyme known as L-Asparagine synthetase from Aspartic acid and glutamine or can be taken externally. However, malignant cells are devoid of L-Asparagine synthetase and cannot synthesize L-Asparagine and are dependent on circulating pool of L-Asparagines for their growth and cell proliferation (Asthana and Azmi, 2003). Thus, depletion of this naturally available L-Asparagine reserve will specifically target tumour cells as they are unable to complete protein synthesis. Krall *et al.*, 2016 has shown that cancer cells are dependent on exogenous source of L-Asparagine for their survival (Krall *et al.*, 2016).

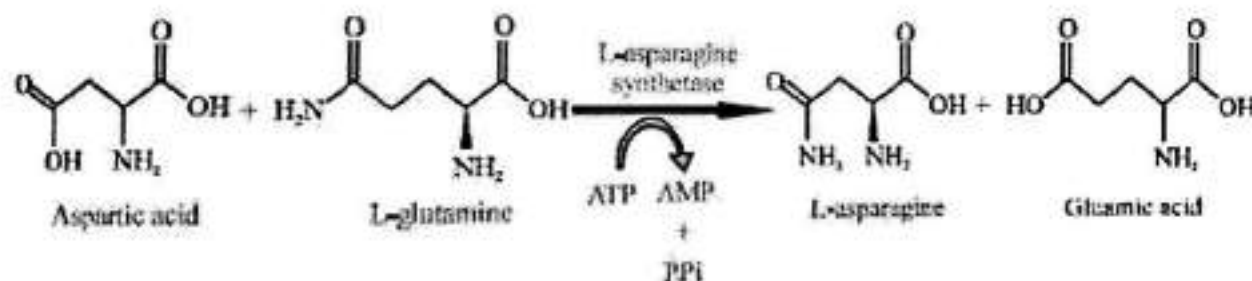


Fig 1: Biosynthesis of L-Asparagine

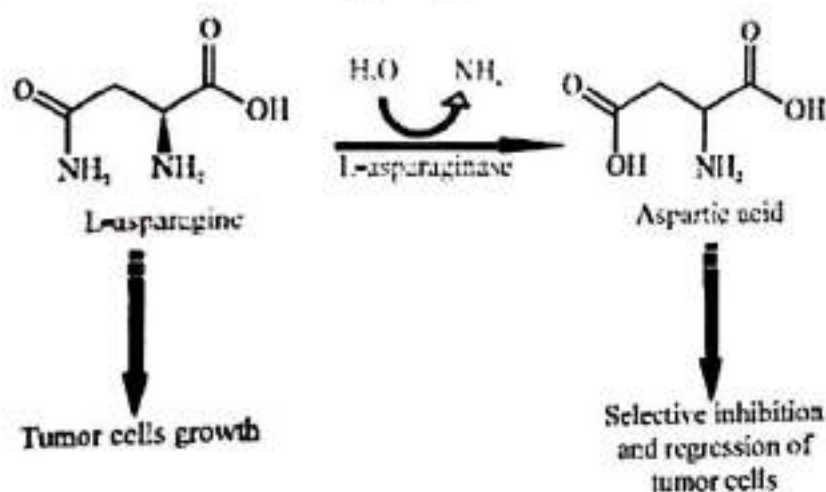
2.4.3. L-Asparaginase-types and structure

There are two types of L-Asparaginase-type I and type II according to the terminology in *E. coli*. Type I L-Asparaginase is a low affinity, constitutive enzyme presents in the cytoplasm whereas type II form is a high affinity, periplasmic form, activated in the cells during anaerobiosis (El-Naggar and Saber, 2022).

Usha *et al.*, 2011 has shown that type II enzyme displays considerable enzyme activity. Type II L-Asparaginase production is triggered by changes in aeration,

carbon source and availability of amino acids (Jennings and Beacham, 1990). The active form of *E. coli* L-Asparaginase II consists of a tetramer of identical subunits presented as A-D (Uma Maheswar Rao *et al.*, 2000). Each monomer comprises about 330 amino acid residues that constitute 14 β strands and 8 α helices placed into two identifiable domains, the larger N-terminal domain and the C-terminal domain connected by a linker composed of 20 amino acid residues (McCoy, Maxwell and Neuman, 1956). Each of the four active sites is located between the N- and C-terminal domains of adjacent monomers. Thus, the L-Asparaginase tetramer can be considered as a dimer of dimers. a, b). Access to the active site is controlled by this loop (Aung *et al.*, 2000; Kozak *et al.*, 2002).

2.4.4. Mechanism of action of L-Asparaginase



Lymphocytic leukemic cells are deprived of L-Asparagine synthetase and are dependent on exogenous pool of L-Asparagines for their cell growth and proliferation. Development of malignant cells is hindered upon treatment with L-Asparaginase which catalyses the breakdown of L-Asparagine to L-Aspartate and ammonia (Dolowy *et al.*, 1967).

Cells require a constant supply of L-Asparagine to synthesize proteins. Normal cells possess the enzyme L-Asparagine synthetase which takes L-Aspartate and adds an amine group forming L-Asparagine. Thus, L-Asparagine is a nonessential amino acid for normal cells. L-Asparaginase on the other hand removes the amine group forming L-Aspartate and ammonia (Krall *et al.*, 2016). L-Aspartate is a precursor of ornithine in the urea cycle and in the

transamination reactions producing oxaloacetate in the gluconeogenic pathway resulting in formation of glucose (SIECIECHOWICZ, IRELAND and JOY, 1988). When introduced into the blood stream, this enzyme will breakdown all the asparagine that it finds resulting in starvation and ultimately death of tumour cells (Graham, 2003). It also obstructs free diffusion of L-Asparagine from the surrounding tissues to the tumour cells. High blood pressure causes the enzyme molecules to penetrate into the intercellular spaces from fine capillaries and hydrolyze L-Asparagine (Pütter, 1970). L-Asparaginase also inhibits glycoprotein synthesis in leukemic cells (Kidd *et al.*, 2015).

CHAPTER 3

3. AIM AND SCOPE OF INVESTIGATION

3.1 AIM

To produce and purify L-Asparaginase enzyme with the help of microbes isolated from the fertile soil.

3.2 OBJECTIVE

- To collect the sample from fertile soil and isolate bacteria.
- To screen the production of extracellular L-asparaginase from isolated cultures.
- To optimize and produce L-asparaginase using liquid fermentation.
- To purify the produced enzyme and increase its efficiency.
- To characterize the produced enzyme for its activity and stability.
- To access its antimicrobial activity against clinical microbes.

CHAPTER 4

4. MATERIALS AND METHODOLOGY

4.1. SAMPLE COLLECTION

Soil samples were collected from four different places, namely: madanapally (Sample 1), beach soil (Sample 2) collected sample from garden (Sample 3), The soil samples were collected in sterile zip-lock polythene bags using sterile gloves and transferred to laboratory. The samples are measured approximately 1 gram and dissolved into the distilled water. The sample were plated to spread plate technique and incubated 37C for 24 hours.

4.2.SCREENING OF L-ASPARAGINASE PRODUCING BACTERIAL STRAINS

The primary screening of L-Asparaginase producers were carried out by streak-plate method. L-Asparaginase producers are identified by their pinkish-red colonies on modified M9 media after 24 hours. Control plates were of modified M9 medium without L-Asparagine, instead NaNO_3 was used as sole source of nitrogen. Isolated colonies on modified M9 medium were aseptically transferred onto nutrient agar slants for further analysis. All the isolated organisms were studied for their quantitative L-Asparaginase producing capabilities. The strain with maximum enzyme productivity was selected for consecutive studies and primary identifications were carried out according to Bergey's Manual of Determinative Bacteriology (1974). Similar studies were also performed by Audipudi *et al.*, (2013) using mangrove soil. Phylogenetic and nucleotide sequencing were performed by GCC Biotech, Kolkata. All media used for screening and identification purposes were procured from HiMedia-India and Merck-Germany.

4.3.GRAM STAINING

A thin bacterial smear was prepared from 24hour old nutrient broth culture of the pure strain on a clean grease free slide. The smear was air dried and heat fixed by passing over a Bunsen flame. The smear was covered with crystal violet as primary stain for one minute and washed under running tap water until water drops run colourless.

Gram's iodine as mordant was added and the slide was kept for one minute. After, this the slide was washed under tap water and 95% ethanol was poured until only a faint violet colour remains. The slide was kept for 3-4 seconds and washed thoroughly under water. The smear was counterstained with safranin for 45 seconds. The slide was washed, excess stain drained off and air dried and observed under 10X, 40X and oil immersion objective.

4.4.COMPOSITION OF FERMENTATION MEDIUM

Modified M9 medium having the composition- $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ -6.0g; KH_2PO_4 -3.0g; NaCl -0.5g; L-Asparagine-10.0g; 1molL-1 $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ -2.0mL; 0.1molL-1 $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ -1.0mL; Glucose-0.2g; Agar-20.0g and Phenol Red-0.005% as indicator per litre of distilled water, pH 7.0 was used for isolation of L-Asparaginase producers and fermentation purposes (Gulati *et al.*, 1997).The sterilization of media components were carried out in an autoclave at 121°C at 15 psig for 15 minutes.

4.5.L-ASPARAGINASE PRODUCTION

Submerged fermentation method was employed for production of L-Asparaginase in laboratory (Talluri *et al.*, 2013).1ml of 24hour inoculum of each of the isolated L-Asparaginase producing organism was inoculated to 50ml of sterilized modified M9 medium in 250 ml Erlenmeyer flask and incubated at 37°C for 48 hours under shaking conditions (120 rpm). Initial pH of the medium was maintained at 7.0. After 48 hours, the fermentation broths were subjected to centrifugation at 5000 rpm for 10 minutes. The cell mass was discarded and the supernatants were used as the sources of crude enzyme.

4.6.OPTIMIZATION:

4.6.1 Impact of incubation duration on L-asparaginase synthesis
100 milliliters of the submerged fermentation broth were infected with one percent of the bacterial strain that produced L-asparaginase. The broth was incubated at 180 rpm and 37 °C. The fermented broth was centrifuged for ten minutes at 4500 rpm after

every 12 hours (12, 24, 36, 48, 60, and 72). The supernatant, devoid of cells, was collected and used in both qualitative and quantitative L-asparaginase tests.

4.6.2 THE EFFECT OF PH ON THE SYNTHESIS OF LASPARGINASE

The L-asparaginase production broth (100 milliliters) was utilized to inoculate 1% of the bacterial strain. The broth was prepared based on the different pH values (3, 4, 5, 6, 7, and 8). The broth that had been infected with the bacterial strain was shaken at 180 rpm at 37°C. The fermented broth was centrifuged for ten minutes at 4500 rpm after the incubation time. Both qualitative and quantitative analyses were conducted using the supernatant, which was collected.

4.6.3 INFLUENCE OF TEMPERATURE ON L-ASPARGINASE STABILITY AND ACTIVITY

100 milliliters of the L-asparaginase-producing broth were employed to inoculate 1% of the bacterial strain. The broth that had been infected with the bacterial strain was stirred at 180 rpm at 37°C. Following the incubation time, the fermented broth was centrifuged for ten minutes at 4500 rpm. The released L-asparaginase enzyme was incubated for 30 minutes at the following temperature ranges: 40°C, 50°C, 60°C, 70°C, 80°C, 90°C, and 100°C. The stability and activity of the L-asparaginase enzyme produced by the bacterial strain were determined by qualitative and quantitative measurements.

4.6.4 SOURCES OF CARBON AND NITROGEN'S IMPACT ON THE SYNTHESIS OF L-ASPARGINASES

The analysis of the effects of simple and complex carbon sources (maltose, xylose, beef extract, starch, sucrose, and galactose) and nitrogen sources (peptone, ammonium sulphate, ammonium chloride, urea, and dextrose) on the synthesis of L-asparaginases. The sources of carbon and nitrogen have concentrations of 1% (w/v) and 0.55% (w/v), respectively. Following a 48-hour incubation period at 37 °C and 180 rpm, the L-asparaginase enzyme production was measured. L-asparaginase production was quantitatively assessed after incubation.

4.7 PARTIAL PURIFICATION OF L-ASPARAGINASE ENZYME

4.7.1 Ammonium sulphate precipitation

The enzyme was partially purified by 70% Ammonium sulfate by precipitation. The purification process should be either performed at 4°C at all times or with (pH-7.0) 0.1M Phosphate buffer (Tandon *et al.*, 2021). The precipitation of the crude enzymes was prepared by slowly adding the 70% Ammonium sulfate into the crude until it reaches saturation. The precipitate was pelleted by centrifugation at 5000 rpm for 10mins mins at 28°C. The separated pellet was mixed with 0.1M Potassium phosphate buffer (pH 7.0).

4.8. ESTIMATION OF L-ASPARAGINASE ACTIVITY

Modified method of Mashburn and Wriston (1963) was used for assaying L-Asparaginase activity. L-Asparaginase catalyzes the hydrolysis of L-Asparagine producing L-Aspartic acid and ammonia. Released ammonia was quantified by direct Nesslerization method wherein ammonia reacts with the Nessler's reagent to form a yellowish-orange compound. The reaction was started by adding 0.25ml of 0.04 M L-asparagine and 0.5ml of 0.025M sodium borate buffer (pH 8.6) to 0.25ml of crude enzyme and incubated for 10 minutes at 37°C. The reaction was stopped by addition of 0.5ml of 0.1(N) Trichloroacetic acid (TCA) solution. After centrifugation at 10000 rpm for 10 minutes, 0.5ml of supernatant was added to 1ml of 1(N) NaOH, 0.25ml of 0.1M EDTA and 5.75ml of sodium borate buffer (pH 8.6). After 2 minutes, 0.5ml Nessler's reagent was added and after 5 minutes, O. D. was taken at 450nm. Amount of released ammonia was determined from a standard curve prepared using $(\text{NH}_4)_2\text{SO}_4$ as a standard. One International Unit (IU) of L-Asparaginase is defined as the amount of enzyme required to liberate 1 μ mole of ammonia per minute under defined assay conditions.

4.9. STANDARD CURVE OF L-ASPARAGINASE

Different concentrations of ammonium sulphate were used to prepare the standard curve. A 10mM stock solution was prepared and several dilutions in the range from 0.25-1.0mM were performed. Then 1ml of each concentration was incubated with

4.75ml of 0.025M sodium-borate buffer (pH 8.6), 0.5 ml of 0.1M Trichloroacetic acid, 1 ml of 1N NaOH and 0.25 ml of 0.1M EDTA for 10 minutes at 30°C. Then 0.5ml of Nessler's Reagent was added and after 5 minutes, the absorbance was measured at 436nm using spectrophotometer.

4.10. SDS- PAGE

The molecular weight of the protein molecules was determined using the SDS-PAGE method. In order to combine the 30% acrylamide-bis acrylamide 2 mL, 4X Tris-HCl buffer (pH 8.8) with the separation gel (12% gel), first prepare the gel (12%). 30% acrylamide-bis acrylamide 2 mL, distilled water 1.75 mL, 10% APS 100 µl, TEMED 10 µl, and Tris – HCl buffer (pH 8.8) 1.25 mL. After mixing, they are put onto the plates with SDS notches. To keep the gel from oxidizing, 1 mL of isopropanol was added. Running water was used to remove the isopropanol from the gel after it had set. Using dry tissue paper, the water is drained. To make the stacking gel, add 30% acrylamide-bis acrylamide 1.25 mL, 4x Tris-HCl 3 mL, distilled water 3 mL, 10% APS 100µl, and TEMED 10µl after producing the separating gel. A combination is present, and a comb is used to secure the separating gel's top layer. Five minutes later, the comb was taken out of the gel. The gel was inserted into the SDS-Page tank and submerged in one running buffer (1x). After mixing 3 µl of loading dye with 20 µl of l-asparaginase sample, the mixture was added to the SDS-Page gel. The electrical circuit was linked to the SDS-Page equipment, which had a voltage setting of 60V.

4.11. ANALYSIS THE ANTI-BACTERIAL ACTIVITY OF THE BACTERIAL STRAIN

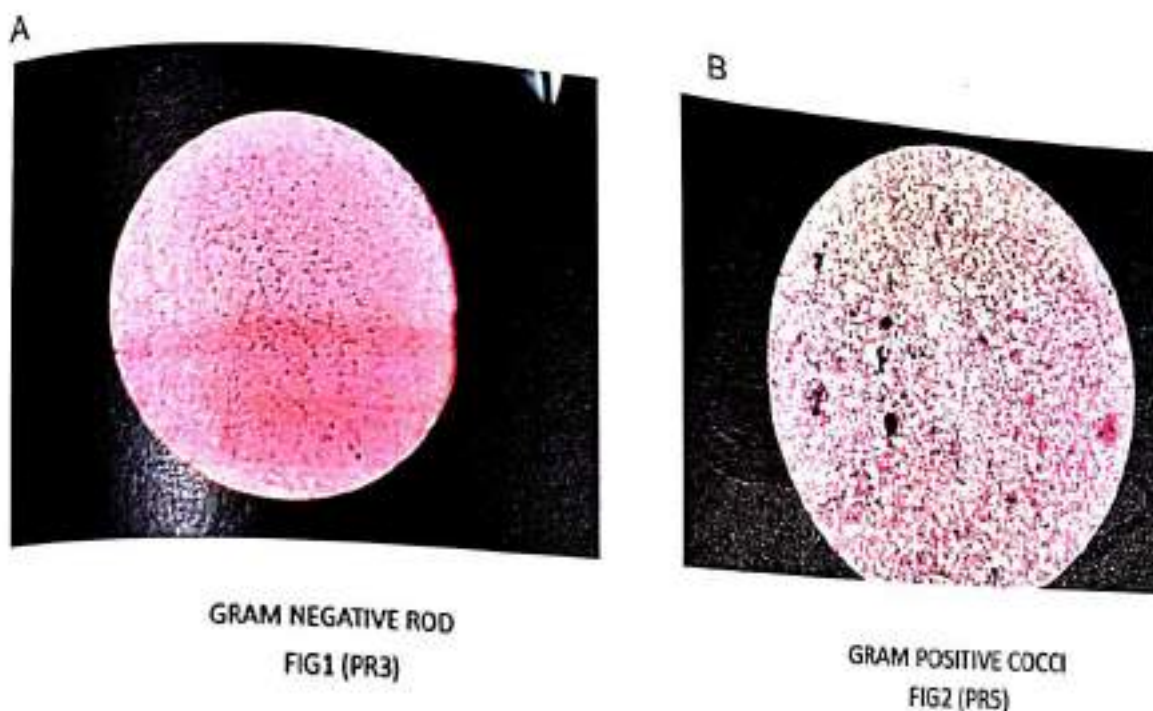
The antimicrobial gradient method combines the principle of dilution methods with that of diffusion methods in order to determine the MIC value. It is based on the antimicrobial agent tested in the agar medium (Balouiri *et al.*, 2015).

- The Mueller Hinton agar plates are prepared and swab the test organism in log phase condition.
- Then, filter paper or discs are placed on the agar plate surface. Add the 25µl and 50µl of the bacterial extract on the discs.

- The positive control (ampicillin) was added on 50µl to the disc. The Petri dishes are incubated under suitable conditions.
- Antimicrobial agent diffuses into the agar and inhibits germination and growth of the test organism and then the diameters of the inhibition growth zone.

4.11. Antimicrobial activity

To check the antimicrobial activity the purified samples were used. And the antimicrobial activity of the enzyme was checked against clinical pathogens. The cultures *E. coli* and *Streptococcus* were cultured from clinical samples. The resistance and susceptibility of the clinical pathogens *Streptococcus*, *E. coli* strain was tested by adding 10 µl of the purified enzyme in MH agar.



Fig;- 5.2. Gram staining results of L-Asparaginase positive bacteria(A, B)

5.3. OPTIMUM CONDITIONS FOR ENZYME ACTIVITY

5.3.1. Temperature

Basal medium was prepared to inoculate with 1 % sample culture (2.2×10^9 CFU/mL, 24 h) and the medium was then incubated at every 5 degrees between 30-65°C at pH 7.0 for 36 h at 120 rpm in a shaker incubator while other factors remained constant at their optimal values. After incubation, the L-Asparaginase was estimated by the DNS method (Riwaldt *et al.*, 2021).

5.3.2. pH

To identify the influence of pH on enzyme production basal medium was prepared with various pH values such as 3, 4, 5, 6, 7, 8, were found to be 60U/ml, 120U/ml, 130U/ml, 140U/ml, 290U/ml, 348U/ml, in the previous studies conducted by the highest pH under the standard assay condition was observed using L-asparaginase as the substrate. Since the optimal pH for standard strain in the present study was around the neutral range, no additional alkaline was needed.

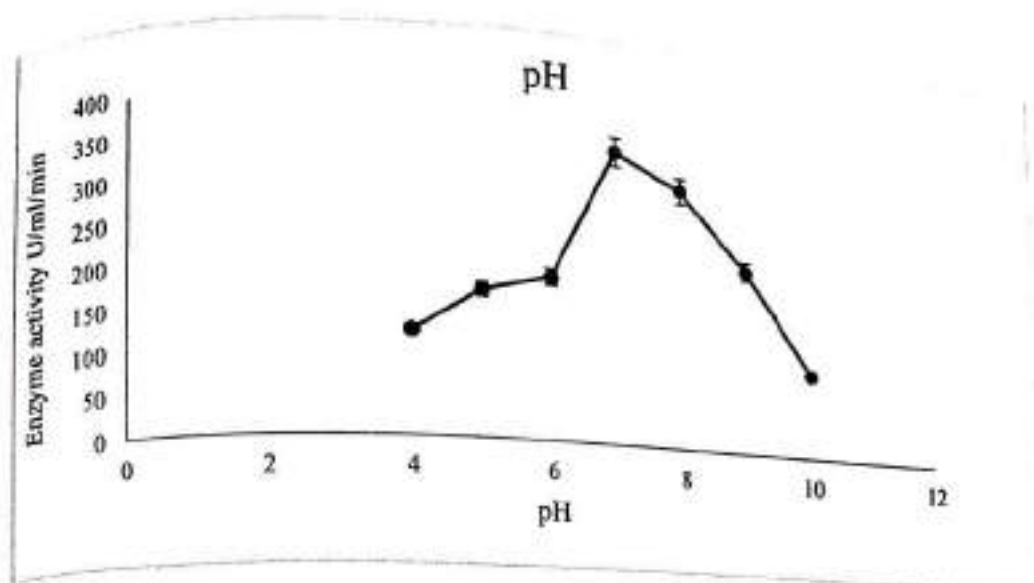


Fig 5.3.2: Effect of pH for the production of L-asparaginase

5.3.3. Incubation Time

The influence of different incubation times in the range 12, 18, 24, 36, 48, 72, and 96 h were performed to optimize the enzyme production. All other parameters remained constant at pH 7.0 and 120 rpm at 38 °C on the basal medium. 1 ml culture medium was taken from the fermentation medium at the incubation times of 12, 18, 24, 36, 48, 72, and 96 h and each sample were centrifuged 4500 rpm for 10 mins. Supernatants were examined for the activity of the α -l-asparaginase enzyme having starch soluble asa substrate (Saeed *et al.*, 2024).

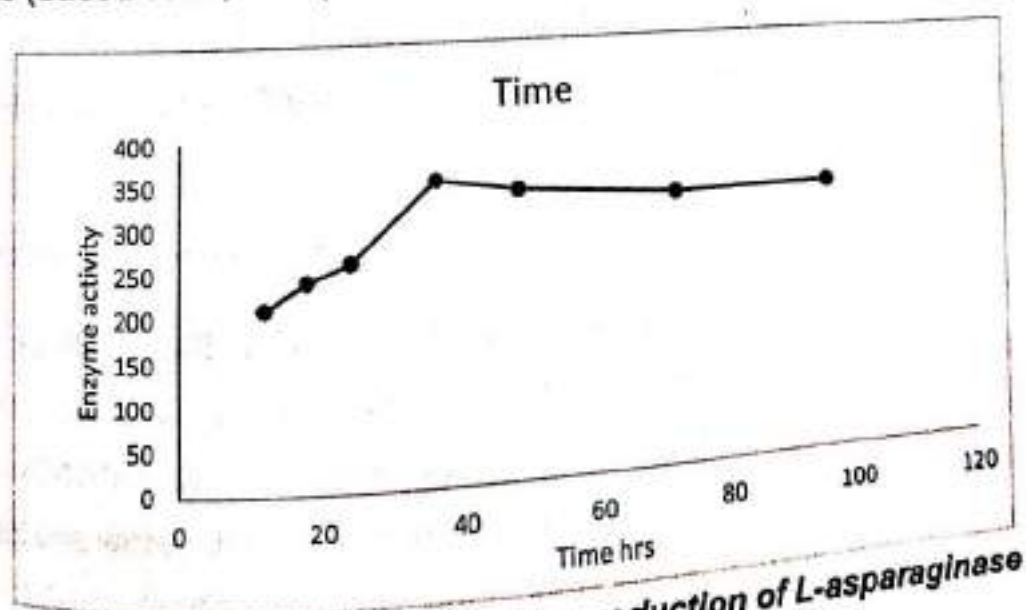


Fig 5.3.3: Effect of time for the production of L-asparaginase

5.3.4. Inoculum size

By varying the inoculum size for the enzyme production, the results were observed for the respective concentrations 0.5%, 1%, 5%, 10% and 20%. All other parameters were remained constant and checked for the enzyme activity.

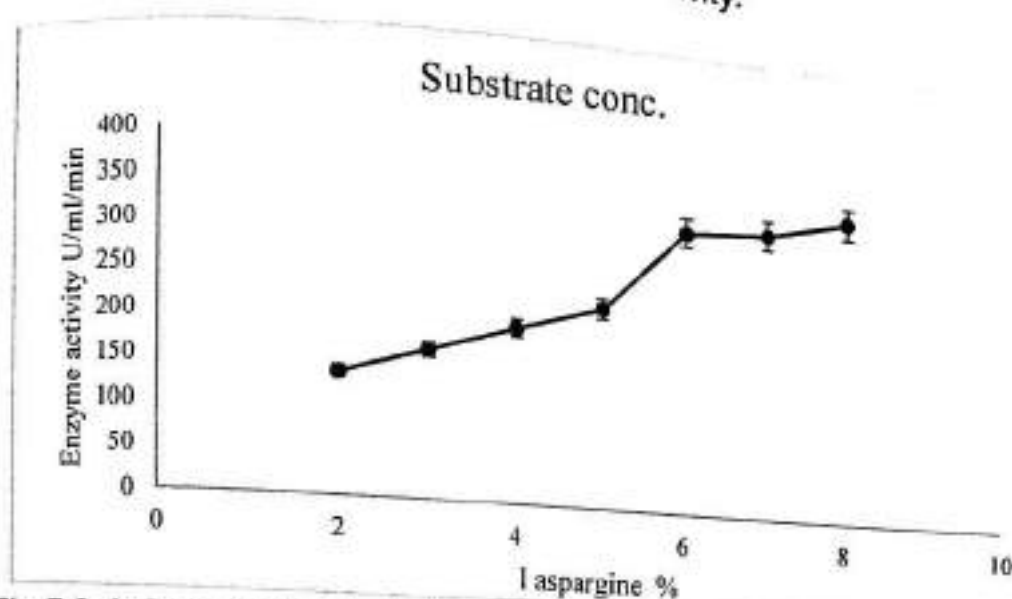


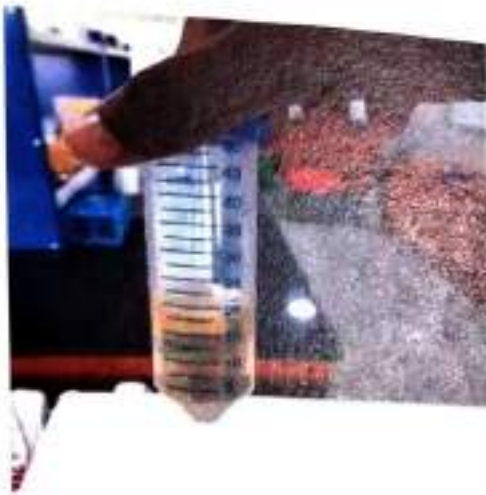
Fig 5.3.4: Effect of substrate concentration for the production of L-asparaginase

5.3.5. Substrate concentration

To optimize the production of L-asparaginase enzyme, bacterial strains were grown on basal medium containing starch as a carbon source. With carbohydrate concentrations of 0%, 0.5%, 0.75%, 1%, 1.25%, 1.5%, 1.75%, 2% & 2.5% media were prepared and cultures were inoculated and then incubated at 35 °C for 2 days with 160 rpm agitation speed. Then the extracellular L-asparaginase produced was examined using Bernfeld method.

5.4. Chromatography Purification

Then to purified further by column chromatography using Sephadex g-100 was performed. While the elution fraction labelled 10,11 and 12 had the highest enzyme activity of 494.1655 U/ml, 647.8962 U/ml and 443.1562 U/ml respectively (Fig 6). These three fractions were mixed to obtain maximum yield. The yield obtained was about 2% and over 18.7 folds of purity level compared to crude. Compared to the another study Padmavathi *et al.*, 2018) in which the specific activity of 88.9 U/mg was obtained for the 55.4% protein yield and also obtained 40.8% purification fold (Padmavathi *et al.*, 2018).



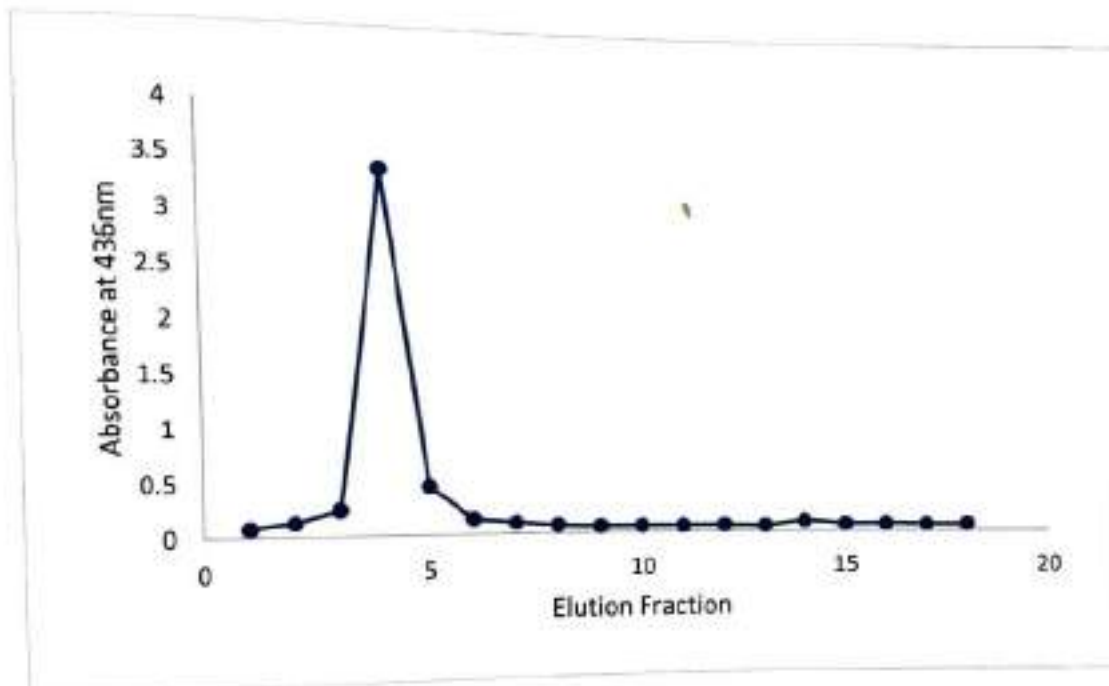
A



B



C



D

Fig;- 5.5: Absorbance range of the elution fractions for the dialysed enzyme(A, B, C, D)

5.5. SDS- PAGE

SDS PAGE was used to determine the molecular weight of α -l-asparaginase enzyme produced. The figure shows that the molecular weight of l-asparaginase enzyme to be 79 kDa with the help of Bio-helix protein marker (broad range 3 kDa to 260 kDa). As resulted in the other article, it was found that the molecular weight of thermostable α -l-asparaginase was to be 63 kDa produced by the organism of *Bacillus licheniformis* is 63 kDa. Where molecular weight of probiotic *Lactobacillus sp* produced α -l-asparaginase enzyme determined was 95 kDa by SDS-PAGE in the study (Padmavathi et al., 2018)

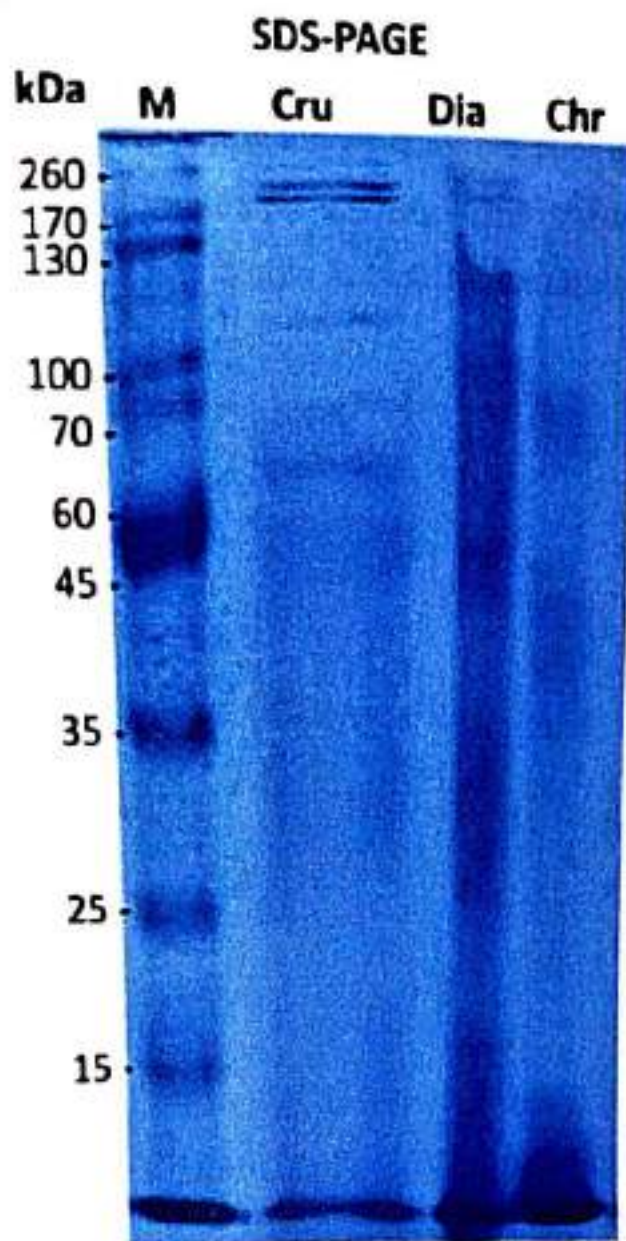


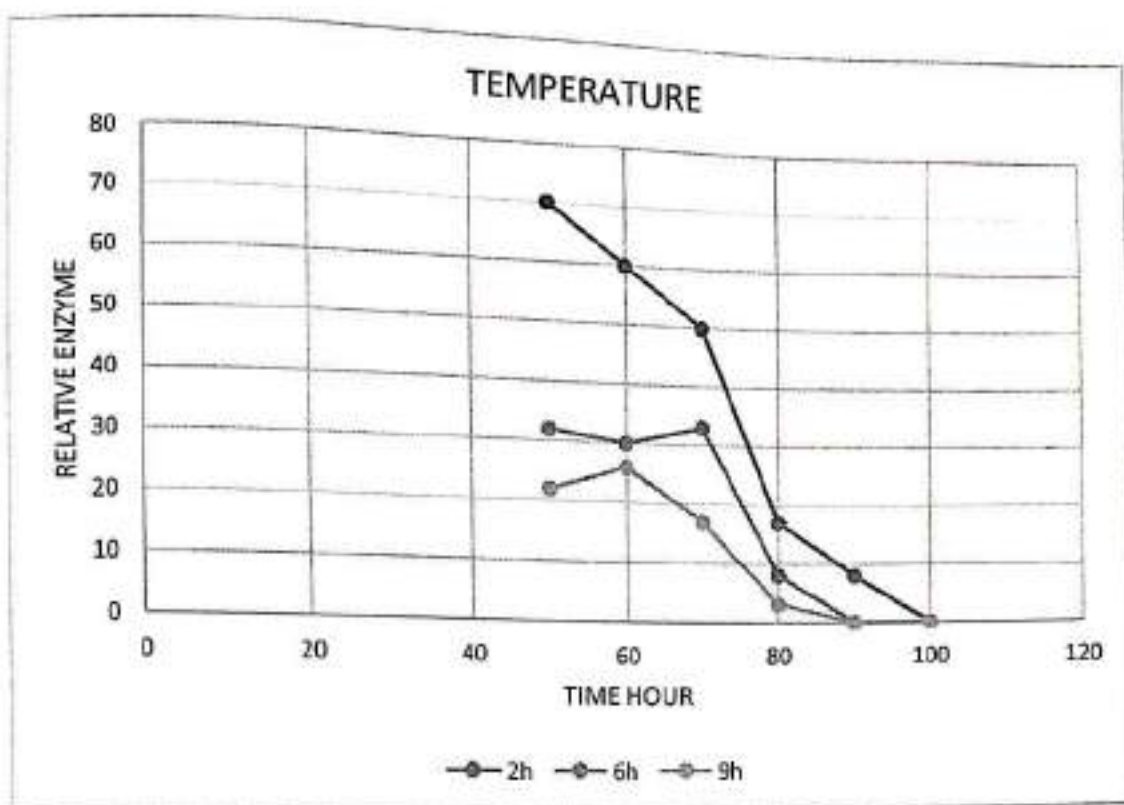
Fig 5.4: SDS- PAGE for the produced enzyme. Lane 1- (Protein Marker), Lane 2- (Crude), Lane 3- (Dialysis), Lane 4- (Chromatography).

5.6. CHARACTERIZATION OF THE PURIFIED

5.6.1. Temperature

And the shown relative activity for the optimum temperature of the purified protein, the activity was determined at different temperatures ranging from 40°C to 100°C. The α -l-asparaginase enzyme retained more than 70% activity at 70°C, 80°C and 90°C which decreased to 25% at 100°C. The optimum temperature for enzyme activity was found to be 50°C. The thermostability of α -l-asparaginase was further increased when the

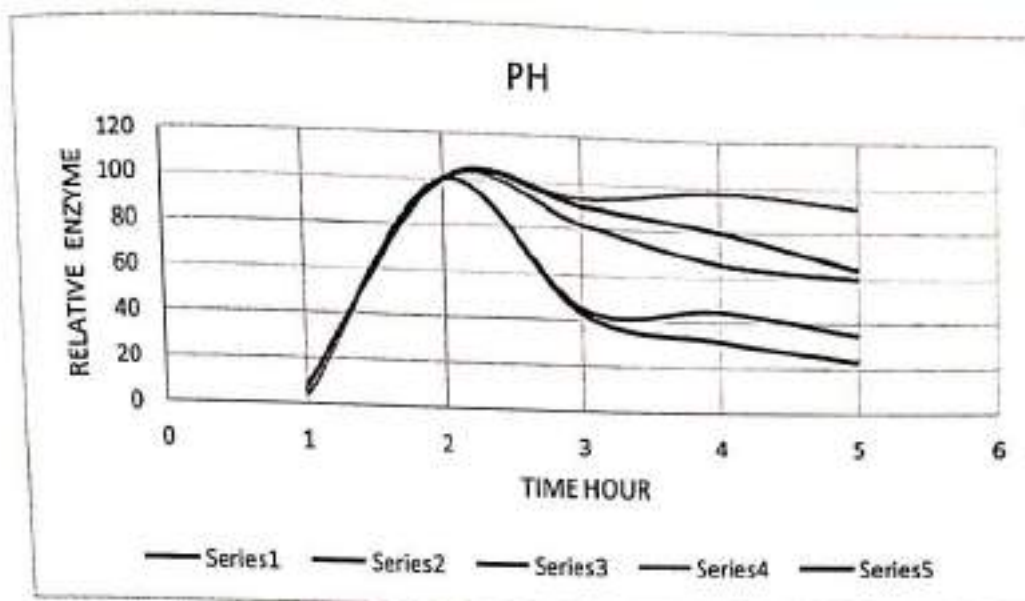
enzyme was incubated in the presence of substrate at 50°C and 60°C. Approximately 40% activity was retained after incubating the enzyme-substrate complex at 60°C for 9 hr and at 70°C for 2 hr. The increased thermostability of α -L-asparaginase in the presence of substrate could be due to the formation of a stable substrate-enzyme complex with higher structural and conformational stability. This showed that the enzyme produced by the *Bacillus subtilis* was stable in high temperatures and have the ability to recover from the influence of heat.



Fig;- 5.6.1: Effect of temperature on the purified L-asparaginase

5.6.2. pH

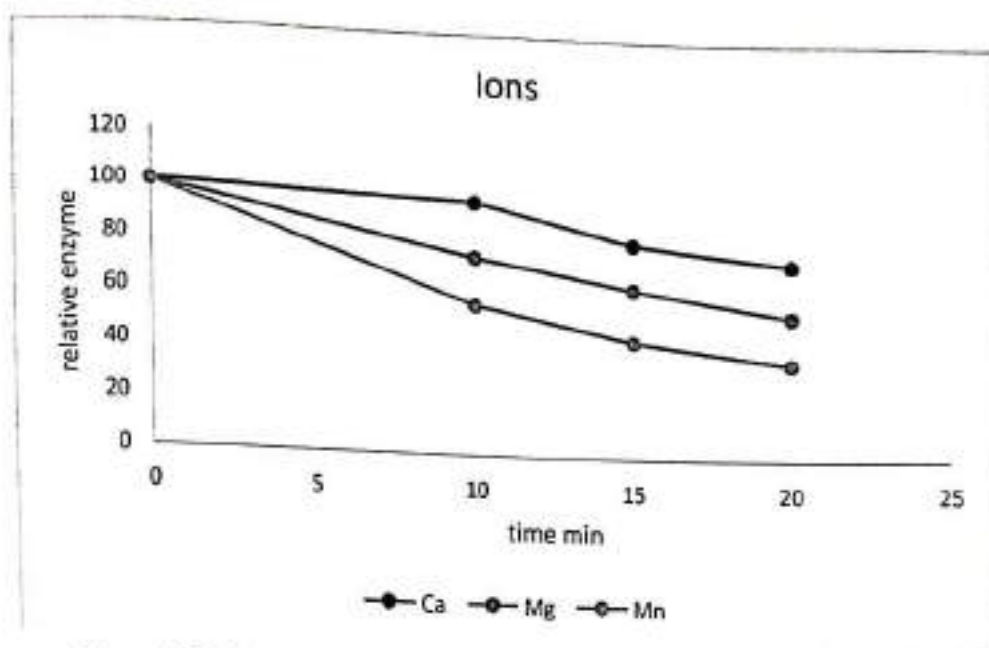
The activity of the enzyme was also maintained even after its pH found to be optimal for the enzyme by determining the enzyme activity. The purified enzyme was stable at pH ranging from 6.0 to 8.5, with an optimum at pH 7.2, which indicates that this could be potentially to be used for starch hydrolysis process. As well as, the enzyme retained more than 80% activity at different pH 6.0, pH 7.0 and pH 8.0. The enzyme was also found to be stable at different pH and retained more than 70% of the activity even after 48 hr incubation at pH 6.0, 7.0 and 8.0. The excellent stability over pH range would enable the enzyme to work efficiently during starch hydrolysis that reduces the need for pH adjustment. Consequently, the potential of this enzyme in the starch hydrolysis industry was exhibited.



Fig;- 5.6.2: Effect of pH on the purified L-asparaginase

5.6.3. Ions

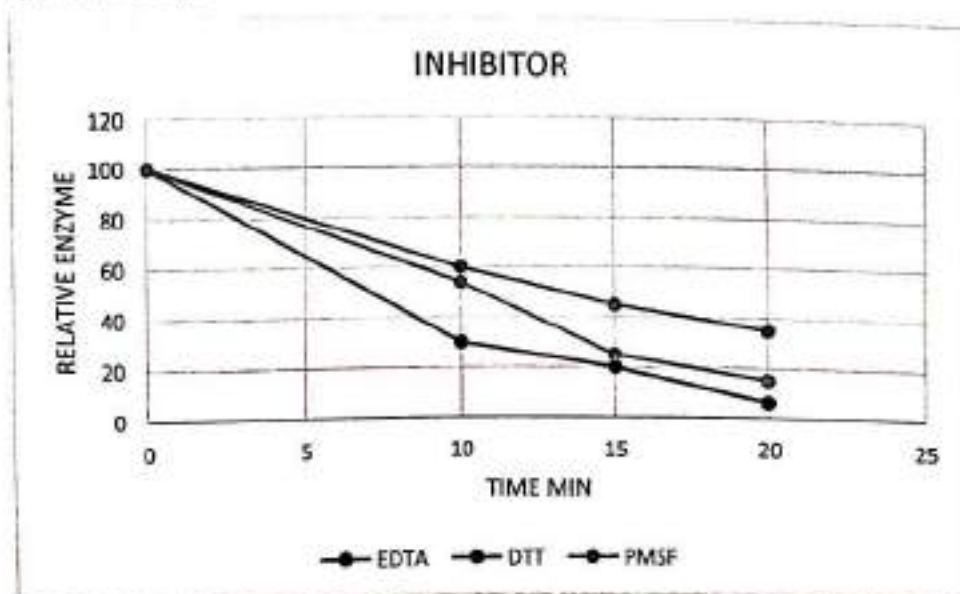
Two different concentrations of each metal ions i.e. 1 mM and 5 mM was used to determine their effect on α -l-asparaginase activity at pH 7.2 and 60°C. The enzymatic activity was inhibited by Mn^{2+} . However, no significant difference was observed in l-asparaginase activity of enzyme in the presence of Ca^{2+} ions. In the presence of Mg^{2+} , the enzyme retained 70% activity but the high concentration of Mg^{2+} ions reduced the α -l-asparaginase activity (Fig. 5.6.2). These results showed that no ion was able to enhance the α -l-asparaginase activity. In the present study, Mn^{2+} was found to inhibit the α -l-asparaginase activity which was also reported with another α -l-asparaginase enzyme. Enhancement effect on α -l-asparaginase activity by Ca^{2+} is well known but a few Ca^{2+} independent l-asparaginases are also known. The l-asparaginase reported in this study was also characterized to be Ca^{2+} independent. The α -l-asparaginase we have isolated and characterized was found to be metal ion independent so, no extra ion needs to be added to increase the enzyme activity during saccharification processes.



Fig;- 5.6.3: Effect of ions on the purified L-asparaginase

5.6.4. Inhibitors

The effect of inhibitors such as EDTA, DTT and PMSF was observed for the enzyme. Among the inhibitors, EDTA induced significant reduction in α -l-asparaginase activity whereas in case of DTT, about 80% activity was observed in α -l-asparaginase (Fig 7c). At higher concentration of PMSF (5mM) only 15% activity was retained (Fig 5e). The PMSF inhibition of the α -l-asparaginase of *Bacillus* sp. indicates the involvement of the seryl hydroxyl group in the enzyme catalysis. Similar results were found for other thermostable L-asparaginase from *Geobacillus* sp. (Uma Maheswar Rao and Satyanarayana, 2007).



Fig;- 5.6.4 Effect of inhibitor on the purified L-asparaginase

5.7. Antimicrobial activity

To check the antimicrobial activity the purified samples were used. And the antimicrobial activity of the enzyme was checked against clinical pathogens. The cultures *E. coli* and *Streptococcus* were cultured from clinical samples. The resistance and susceptibility of the clinical pathogens *Streptococcus*, *E. coli* strain was tested by adding 10 μ l of the purified enzyme in MH agar. And the zone on inhibition for the *Streptococcus* and *E. coli* strain observed was about 12mm and 13mm respectively. Hence, enzyme from the isolated organism was found to be an effective antimicrobial agent.



A



B

Fig 5.7 Effect of ions on the purified L-asparaginase(A, B)

5.RESULTS AND DISCUSSION

5.1. SCREENING

Based on the isolates tested for L-asparaginase activity by Plate assay method, 8 isolates showed more than 3mm zone of clearance from the colony. The samples 8 isolates labelled pr1, pr2, pr3, pr4, pr5, pr6, pr7, pr8, that showed 4 mm, 3 mm, 3.5 mm, 3.5 mm, 3 mm, 3 mm, 4.5 mm, 3 mm and 3 mm respectively (Table 1). The sample labelled S24A was selected for the further studies as it had the best outcome of extracellular L-asparaginase production among all the strains.

A



FIG 1 (PR3)

B



FIG 2(PR5)

Fig:- 5.1: Screening plates showing extracellular production of L-asparaginase(A, B)

5.2. MORPHOLOGY OF L-ASPARGINASE PRODUCTION BACTERIAL STRAIN

Size and shape of the organism was determined using a light microscope with the use of a stage and ocular micrometer. Simple staining method using crystal violet as a single stain was used for the purpose.

CHAPTER 6

6. SUMMARY AND CONCLUSION

In conclusion, the antimicrobial activity of L-Asparaginase represents a promising avenue in the fight against infectious diseases. Through mechanisms involving the depletion of extracellular asparagine and disruption of microbial metabolism, L-asparaginase demonstrates broad-spectrum activity against various bacterial, fungal, and parasitic pathogens. The versatility of its antimicrobial effects underscores the potential for L-Asparaginase to serve as a valuable therapeutic agent in combating microbial infections. While further research is warranted to elucidate the full extent of L-asparaginase's antimicrobial properties and optimize its therapeutic efficacy, the existing evidence highlights its potential as a novel approach to antimicrobial therapy. Continued exploration of L-asparaginase-based interventions may lead to the development of new treatment strategies that complement existing antimicrobial agents and address the challenges posed by antimicrobial resistance.

In summary, L-Asparaginase holds promise as an antimicrobial agent, offering a multifaceted approach to combating infectious diseases and improving patient outcomes. Future research endeavours in this field have the potential to yield valuable insights and contribute to the development of innovative therapeutic interventions targeting microbial pathogens.

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