

**PRODUCTION, OPTIMIZATION, PURIFICATION, CHARACTERIZATION AND
ANTI-CANCER APPLICATION OF EXTRACELLULAR L-GLUTAMINASE
PRODUCED FROM THE MARINE BACTERIAL ISOLATE**

*Submitted in partial fulfilment of the requirements for the award of Master of
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 **SANDHYA B (42101001)** who carried out Project entitled "**PRODUCTION, OPTIMIZATION, PURIFICATION, CHARACTERIZATION AND ANTI-CANCER APPLICATION OF EXTRACELLULAR L-GLUTAMINASE PRODUCED FROM THE MARINE BACTERIAL ISOLATE**" under the supervision from September 2023 to April 2024.

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DECLARATION

I, **SANDHYA B (42101001)** heraby declare that the Project Report entitled **"PRODUCTION, OPTIMIZATION, PURIFICATION, CHARACTERIZATION AND ANTI-CANCER APPLICATION OF EXTRACELLUAR L-GLUTAMINASE PRODUCED FROM THE MARINE BACTERIAL ISOLATE "** 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.

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Sandhya B

Abstract

There has been a lot of interest in L-glutaminase because of its prospective uses in medicine as an anticancer agent and as a taste enhancer in food products. In this study, the marine isolated microbial glutaminases were used to check for the anticancer activity. L-glutaminase is a significant anticancer agent that is widely utilized globally by depriving cancer cells of L-glutamine. Extracellular L-glutaminase-producing marine bacterial strains were screened using a dye-based technique using sediment and water samples that were obtained at several locations along the Bay of Bengal beach at Chennai. Marine microbial strains have been isolated from marine water samples and then studied for extracellular enzyme production. The optimum conditions were determined as temperature 37°C, pH- 8.2, Inoculum size- 5% and the incubation time 36h. L-glutaminase was purified in three simple steps and showed a partially purified band with a molecular weight of 40 kDa. Moreover, the purified L-glutaminase in the present study showed potent anti tumor activity against colorectal cancer cell lines (HCT116). This enzyme catalytic activity over a wide range of temperature and pH and its considerable stability makes it highly favourable for use as an effective anticancer agent.

Keywords: L-Glutaminase, Column Chromatography, Cytotoxicity, HCT116, Extracellular, Marine Bacteria.

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ABBREVIATIONS

DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
GAC	Kidney-type glutaminase
HIV	Human immunodeficiency virus
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
NACL	Sodium Chloride
PMSF	Phenylmethanesulfonyl fluoride
TLCK	N α -tosyl-L-lysine chloromethyl ketone hydrochloride Acute lymphoblastic leukemia
MTT	Dimethyl thiazolyl diphenyl tetrazolium salt
rRNA	Ribosomal ribonucleic acid
PCR	Polymerase chain reaction
EDTA	Ethylenediamine tetraacetic acid
DTT	Dithiothreitol
GAD	Glutamic acid decarboxylase
LAB	Lactic acid bacteria
MA	Marine Agar
TCA	Tricarboxylic acid cycle
CO ₂	Carbon dioxide
DMSO	Dimethyl sulfoxide
DEAE	Diethylaminoethyl
KDa	Kilo Dalton

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CHAPTER-1

INTRODUCTION

1.1 ENZYME

Enzymes are known as proteins catalyse chemical reactions in the body. For overall health, they are more significant than vitamins and minerals. An extensive class of enzymes found in everything from bacteria to higher eukaryotes are called hydrolases. (Council, G. M. (2000) All of the body's metabolic activities are governed and controlled by enzymes, which are extremely selective catalytic proteins. Living cells manufacture them to speed up metabolic operations and increase their selectivity. Because each enzyme is designed to do a single, unique job, the functions of enzymes are extremely specialized. Chemical reactions that take place in a living system are mediated by millions of enzymes (Sabu et al., 2000).

In the detection, treatment, biochemical analysis, and surveillance of numerous deadly illnesses, microbial enzymes are crucial. An outstanding example of microorganism biochemical diversity and genetic modification susceptibility make them a rich supply of therapeutic enzymes. According to (Saptarshi & Lele, 2011)), one of the key components of the pharmaceutical sector nowadays is the production of enzymes for use as medications. Enzymes are produced by all live cells. The industry uses very few, naturally occurring enzymes that are influenced by environmental conditions. These enzymes are extracted from plant and animal tissues for the synthesis of enzymes. Increased attempts are being made to convince enzyme synthesis using microbial sources.

1.2 MICROBIAL ENZYMES

The main source of enzymes is microorganisms because they can be grown in huge quantities quickly and because bacterial cells can be genetically modified to increase the production of enzymes. Enzymes from microbes have received greater attention than enzymes from plants and animals because of their active and stable nature. When compared to animal and plant sources, microbial enzymes are more in demand. This is due in large part to their high growth rate, low space needs, affordable media, ethical, and availability of enzymes with catalytic activity in many

different settings. Numerous biological effects, including those of antibacterial, antifungal, anticancer, antitumor, anti-inflammatory, antimalarial, antiviral, cytotoxic, and anti-angiogenesis medicines, are demonstrated by the abundance of such stable enzymes. A multitude of diverse environmental circumstances have shaped the evolution of the marine microbial community. Therefore, it's possible that certain organisms with potentially novel antimicrobial compounds exist in the marine ecosystem (Bülbül & Karakuş, 2013). Therefore, efforts must be made to utilize the rich biological diversity to its fullest.

There are two major categories into which enzymes fall. These are both external and intracellular enzymes. The cell produces the intracellular enzymes, which catalyze intracellular reactions. However, the extracellular enzymes are produced by cells and sent to various locations to initiate metabolic reactions.

1.2.1 ADVANTAGES OF MICROBIAL ENZYMES

- Easy and straightforward development processes
- Easy extraction and large-scale production.
- Compared to animal and plant sources, it requires less space and has a shorter generation time.
- Using genetic engineering to create strains with abnormally high amounts.
- Enzyme yields, activity, and lack of seasonal variation are predictable.
- Through a chemical process, the product's purity can reach 99.96% pure.

In marine environments, microbial communities are essential to the cycling of nutrients and organic carbon. One possible source of bioactive chemicals with significant commercial value is marine microorganisms. Of all the microbes, Because they are the most abundant source of bioactive secondary metabolites and antibiotics, bacteria have gained particular significance. The majority of bacterial research has been on antibiotic synthesis. Various enzymes are secreted by bacteria and fungi found in marine environments, depending on their habitat and ecological roles.

Many enzymes have caught the interest of microbial prospectors, and some have been isolated from marine sediments and saltwater. Marine microbial enzymes have

emerged as a focus point of research. The ability of enzymes to carrying out highly specialized molecular functions, such as restriction endonuclease, RNA, and DNA polymerase, which are typically connected to the modification of DNA or RNA of genetically modified organisms for diagnostic procedures. Due in part to their relative stability and activity over similar enzymes derived from plants and animals, marine microbes have become the focus of increasing amounts of research for novel enzyme discoveries(Krishnakumar, 2011)

The intricate nature of the marine environment, which is characterized by extreme salinity, high pressure, low temperature, and lightning conditions caused by microbes. These enzymes are utilized in food additives, medications, and fine compounds. A range of unique-activity enzymes have been identified by researchers from marine bacteria, actinomycetes, and fungi that yield certain compounds with industrial uses.

The enzymes that are now known to be produced by marine microbes include α -amylase, α -glucosidase, Agarase, α -galactosidase, chitinase, alkaline protease, collagenase, lipase, L-asparaginase, L-glutaminase, tyrosinase, cellulases, lignin peroxidase (LiP), hydrogenases, and other enzymes.

1.3 THERAPEUTIC ENZYMES

Therapeutic enzymes are those that are used in medical and therapeutic settings. Therapeutic enzymes are used as anticoagulants, thrombolytics, oncolytics, anti-inflammatory agents, and digestive aids, fibrinolytics, mucolytics, and antibiotics. The primary potential use of therapeutic enzymes is in the management of cancer. For the successful treatment of acute lymphoblastic leukemia (a condition in which blood cells turn malignant) and lymphosarcoma, the enzyme lipasaginase has been approved as a therapeutic anti-tumour medication. Relatively small doses of extremely pure and specialized enzymes are needed for therapeutic purposes. I.e. Table No. 1 contains the list of therapeutic enzymes. Therapeutic proteins, such as enzymes, have the potential to treat serious, uncommon, and horrifying diseases. For certain people, enzyme therapy is the sole option. Disorders.

Enzymes for therapeutic purposes are rarely used. Enzymes' potential usefulness as medicinal agents is diminished by the following factor:

- To be dispersed intracellularly within a bodily cell, they are too big. Four
- If used for an extended period, the antigenic foreign proteins trigger a strong and potentially fatal immune response.
- Their circulation-based effective lifespan is extremely short.

There is a high need for the use of enzymes as therapeutic agents to combat numerous terrible diseases. The oncolytic enzymes L-glutaminase and L-asparaginase are responsible for the deamination of L-glutamine and L-asparagine, respectively. It has been discovered that a variety of sources, including certain plant and animal species, fungus, and bacteria, are good makers of enzymes. When treating acute lymphoblastic leukemia and other cancers, L-asparaginase and L-glutaminase show great promise. While malignant and cancerous cells, in particular, require an external source of these amino acids for growth and multiplication, normal tissues are able to synthesis leucine and glutamine. L-asparaginase and L-glutaminase are strong antitumor or antileukemic medications that can be utilized as chemotherapy agents to treat, mostly in children. In addition to their duty in the care of 5 cancer, L-asparaginase, and L-glutaminase are also utilized in the food business; L-asparaginase lowers the production of acrylamide, a carcinogen present in starchy food goods, while L-glutaminase raises the food's glutamic acid level, which adds flavor.(Patel et al., n.d.)

1.4 L-GLUTAMINASE

The enzyme responsible for converting L-glutamine into L-glutamic acid and ammonia is called L-glutaminase (also known as L-glutamine amidohydrolase E.C 3.5.1.2) (Abell et al., 2012). An enzyme that is necessary for the manufacture of a range of nitrogenous metabolic intermediates. From its broad reporting as an antileukemic agent L-glutaminase has garnered a great deal of interest. The absence of L-glutamine synthetase in leukemic cells, in contrast to normal cells, means that they must obtain L-glutamine from outside sources in order to develop

and survive . Glutamate serves as a growth substrate that is absolutely necessary for tumor cells. In addition to serving as a respiratory substrate, glutamine is necessary as a precursor for the production of both proteins and DNA.

The use of microbial L-glutaminases in food flavoring, particularly in soy sauce and related sectors in the East, sparked a parallel interest in the enzyme and led to the search for industrial sources. With microbial L-glutaminases have more recent uses in clinical analysis and even metabolite production because to advancements in biotechnology. While modern biotechnological techniques suggest unique and alternative strategies for the treatment of cancer, amidohydrolases were once used. This led to the vast investigations on L-glutaminase.

The initial stage of the conversion of glutamine to glutamate is catalyzed by the amidohydrolase glutaminase. As liver and kidney glutaminases, it is found in two isoforms: glutaminase 1 (GLS1) and glutaminase 2 (GLS2). according to. The kidney-type glutaminase (KGA) isoform and the glutaminase C isoform (GAC), both of which are found in the mitochondria, are the two splice variants of GLS1, which is expressed more than GLS2. GLS1 is linked to the development of tumors and cancer in a variety of illnesses. Therefore, the inhibitor GLS1 may be helpful in treating various malignancies or in combination with other antitumor drugs.

1.5 MECHANISM OF L-GLUTAMINASE

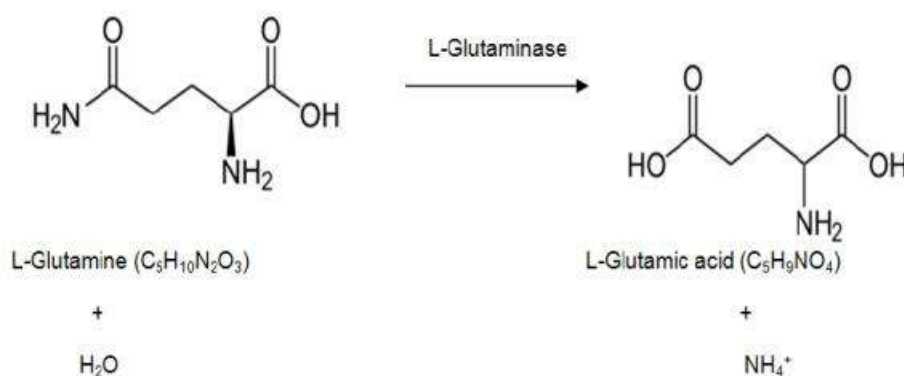


Fig 1.1 Mechanism of L-glutaminase

As a result of the enzyme glutamine synthetase (Fig. 1), which is present in high concentration in the skeletal muscle, liver, brain, and stomach tissue, human cells are able to rapidly manufacture glutamine, which is why it is not regarded as

indispensable. Considering that the body can produce this amino acid and that the body contains a rather large amount of glutamine.

1.6 L-GLUTAMINASE STRUCTURE

Using x-ray deflection, the structure of L-glutaminase was resolved to a maximum resolution of 1.73Å°. The dimeric protein is composed of two chains, each of 305 residues. 23% of the amino acid content on both strands, 71 Eight helices contain residues, and the 23 beta sheets strands seen in Figure 2 (Ramli et al., 2021)consist of 21%, or 95 residues.

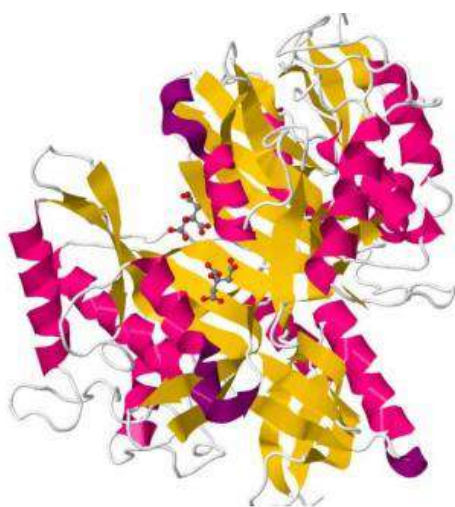


Fig 1.2 Structure of l-glutaminase

1.7 OCCURENCE AND DISTRIBUTION OF L-GLUTAMINASE

In both prokaryotes and eukaryotes, L-glutaminase is essential for nitrogen metabolism (Kumar Katikala et al., n.d.). It is discovered to be extensively dispersed in the tissues of plants, animals, and microbes including bacteria and yeast. plus, fungus. Although L-asparaginase has been linked to allergy reactions, L-glutaminase, which is produced by microorganisms from marine settings, may be more effective in treating leukemia. Boost their maritime-based commercial output Beyond their usage in the food business, microbes may enable its widespread use in cancer chemotherapy.

Therefore, in order to choose an appropriate fermentation method for these bacteria's commercial production of glutaminase, their production pattern in SSF and SmF was examined. Researchers have been isolating and screening organisms for L-glutaminase with unique properties like temperature (Roberts.J 2001, Sato.I 1999), pH (Kashyap et al., n.d.), and salt tolerance . These features increase the productivity of the enzyme in food industries.

1.8 PROPERTIES OF L-GLUTAMINASE

1.8.1 L-Glutaminase for therapeutic use

- 1.Glutaminase enzyme should ideally have a low K_M , or between 10^{-6} and 10^{-4} .
- Excellent stability at pH levels that are physiological.
- It should continue to have a high level of activity in human and animal serum and blood.
- It doesn't require prosthetic groups or cofactors that are difficult to separate from the enzyme.
- Its limited substrate specificity and poor immunogenicity

1.8.2 L-Glutaminase as chemotherapeutic agent

- In the past few years, L-glutaminases have been used extensively across a range of industrial domains. Despite being initially discovered as a strong anti-cancer medication with potential uses in enzyme therapy has been utilized to improve flavor in the food business.
- Nowadays, glutaminases are employed as biosensors to measure the amounts of glutamate and glutamine in fluids. They can be employed as free enzymes or immobilized on membrane forms.
- The use of L-glutaminase in clinical analysis has aided in the production of highly purified L-glutaminase and the hunt for L-glutaminases that are stable for extended periods of time for use in biosensors. The majority of the

therapeutically employed glutaminases, which are enzymes especially used for this purpose, are derived from mammals.

- The therapy of HIV is one of the most promising therapeutic uses for glutaminase that has ever been suggested. (Unissa et al., 2014) The administration of L-11 glutaminase from *Pseudomonas* sp. 7A inhibits HIV replication in infected cells. The enzyme inhibits DNA manufacture in impacted cells and causes tumors (melanoma).
- It is thought that microbial L-glutaminases are more stable than their equivalents in plants and animals (Review & Literature 2. Review and Literature, n.d.). However, as powerful anticancer drugs, microbial L-glutaminases are preferable. According to Roberts et al. (1979), glutaminase treatment has also been demonstrated to postpone the emergence of methotrixate resistance.
- If L-glutaminase enzyme is extremely stable at physiologic pH (pH 6.5–8.5), it is suitable for therapeutic use. It should have a long plasma half-life ($t_{1/2}$), minimal immunogenicity, a low K_m (ideally in the mM range), a high V_{max} , and a narrow substrate specificity.

CHAPTER-2

REVIEW OF LITERATURE

Microbial L-glutaminase synthesis can be achieved with the application of ideal 45 conditions, and the bacterial isolates have a great deal of promise for 46 producing glutaminase enzyme and its applications.

L-glutaminase is a significant anticancer agent that is widely utilized globally by depriving cancer cells of L-glutamine, according to (Mostafa et al., 2021) The Red Sea was the source of the marine bacterium *Halomonas meridian*, which was identified as the more active L-glutaminase-producing bacteria. Using 1.5% glucose and 2% soybean meal, the optimal conditions for L-glutaminase fermentation were 36 hours, pH 8.0, 37 °C, and 3.0% NaCl. The SDS-PAGE examination revealed that the molecular weight of the isolated enzyme was 57 kDa, and it exhibited a specific activity of 36.08 U/mg. At 37 °C and pH 8.0, the enzyme was very active. Higher affinity for its substrate is shown by the kinetics parameters K_m and V_{max} , which were 12.2×10^{-6} M and 121.95 $\mu\text{mol/mL/min}$, respectively. Early apoptosis was associated with a greater prevalence of cell death in HCT 116 compared to LS 174 T, but late apoptosis was more common in LS 174 T than in HCT 116. Furthermore, compared to LS 174T cells, HCT 116 had greater nuclear fragmentation after L-glutaminase induction. As an L-glutaminase producer utilized as an anti-colorectal cancer therapy, this is the first report on *Halomonas meridiana*. Keywords: purification, colorectal cancer, 16S rRNA gene, *Halomonas meridian*, L-glutaminase, production.

(Barzkar et al., n.d.) It is stated that the unique reaction known as "deamination of L-glutamine to glutamic acid with the concomitant release of ammonia by the activity of L-glutaminase (L-glutamine amidohydrolase EC 3.5.1.2)" has potential uses in a variety of fields, including medicine and the food industry. Microbial production of L-glutaminase is preferred over production from other sources due to its affordability, speed, and compatibility with downstream processes. Because of their extraordinary ability to manufacture L-glutaminase, marine microorganisms such as bacteria, yeasts, and molds are thought to be promising options for the large-scale synthesis of this enzyme. In order to comprehend the future possibilities, the primary goals of this article are to give a general overview of marine microorganisms that

produce L-glutaminase, to talk about the methods utilized to produce these enzymes on a large and in the lab, and to examine the uses of L-glutaminase derived from marine sources. L-glutaminase can be found here. creatures in the sea. The process of fermentation. use for therapeutic purposes. industry of food.

(Kashyap et al., n.d.) reported that two agro-industrial substrates, wheat bran and sesamum oil cake, were chosen after screening four substrates and two moistening media (10% NaCl in tap water and sea water). For the purpose of producing enzymes, adding different organic and inorganic nitrogen sources outside to the fermentation medium had no beneficial effect. Copyright 2002 by Elsevier Science Ltd. All rights reserved.

(El-Sayed, 2009) reported that solid state fermentation was carried out using various agro-industrial byproducts, such as wheat bran, groundnut residues, rice hulls, soy bean meal, corn steep, sesamum oil cake, cotton seed residues, and lentil industrial residues, in order to produce L-glutaminase by *Trichoderma koningii* Oud. aggr [pp4]

According to (Moriguchi et al., 1994), two salt-tolerant glutaminases, known as glutaminases I and II, were constitutively generated by Marine *Micrococcus luteus* K-3. Glutaminase I was a dimer with a molecular weight of roughly 86,000 that had been homogeneously purified about 1620 times with a 4% yield. With a yield of 0.04%, glutaminase II was partially purified about 190 times. Moreover, glutaminase II has a molecular weight of 86,000.

The amidohydrolase L-glutaminase is an essential chemotherapeutic drug against a variety of cancers, according to (Awad et al., n.d.). In their experiments, the assay parameters (temperature 60 °C, pH 7.0, glutamine substrate, substrate concentration 1.0%, and reaction time 60 min) were optimized to increase the enzyme activity. Due to total suppression by all serine protease inhibitors (PMSF, benzamidine, and TLCK), Faesay4 L-glutaminase was identified as a serine protease enzyme. The purified L-glutaminase that was isolated from *Aspergillus versicolor* Faesay4 demonstrated strong anticancer activities against human cell lines that represented the liver (HepG-2), colon (HCT-116), breast (MCF-7), lung (A-549), and cervical (Hela) cancers, with IC₅₀ values of 39.61, 12.8, 6.18, 11.48, and 7.25 µg/mL, respectively. L-glutaminase and *Aspergillus* sp. streamlining. clarification and description. antioxidants and cancer-preventive

According to (Kumar Katikala et al., n.d.), microbial glutaminases are used in a variety of industries. Recently, there has been a lot of interest in L-glutaminase because of its prospective uses in medicine as an anticancer agent and as a taste enhancer in food products. Extracellular L-glutaminase-producing marine bacterial strains were screened using a dye-based technique using sediment and water samples that were obtained at several locations along the Bay of Bengal beach at Visakhapatnam. Among the thirty isolates, LG24 exhibited the greatest activity of 22.68U/ml in 120 hours when the only carbon and nitrogen sources in the medium were supplemented with L-glutamine.

(Kiruthika & Saraswathy, 2013)) stated that a range of microorganisms, such as bacteria, yeast, and fungus, create the amidohydrolase L-glutaminase. At now, it is utilized for the management of HIV and acute lymphoblastic leukemia (ALL). L-glutaminase has the potential to be used as a flavor-enhancing agent in the fermentation of soy sauce. Marine bacteria produce extremely little L-glutaminase, despite the fact that L-glutaminase activity has been observed in a wide variety of microorganisms.

Actinomycetes from terrestrial and marine habitats have long been known to be either organisms of scientific interest or organisms that manufacture antibiotics, according to a 2010 study by (Balagurunathan & Radhakrishnan, 2010)). L-glutaminase is an enzyme that is produced by a variety of microorganisms that are being used as an enzyme sensor, flavor enhancer, and leukemia treatment. For therapeutic enzymes in general and L-glutaminase in particular, the selective isolation technique and semi-quantitative assay created in this work will be the most appropriate and effective approach.

Actinomycetes were identified for the synthesis of extracellular L-glutaminase from maritime sediments. *Streptomyces* sp.-SBU1 was the strain that exhibited the most promising L-glutaminase activity out of all 25 isolates evaluated. Thus far, the synthesis of L-glutaminase by a small number of marine actinomycetes has been investigated. Therefore, the goal of the current study is to improve the medium composition for the production of L-glutaminase.

According to (Iyer & Singhal, 2009)), the current work describes the screening of over 400 marine isolates from different marine environments; one isolate was ultimately chosen based on glutaminase productivity (71.23 U/l). According to response surface methods, a medium containing 10.0 g/l of glucose, 5.15 urea, 3.5 methionine, and succinic acid 6.0, ammonium sulfate 2.5, and yeast extract 6.0 are the ideal values for glutaminase synthesis. After 28 hours of incubation at 25°C, enzyme activity of 119 ± 0.12 U/l and specific activity of 0.63 U/mg protein were obtained, validating the employed methodology.

Microbial glutaminases have been used in a variety of disciplines, according to (Suneetha et al., 2015). Due to its possible uses in medicine as an anticancer agent and as a flavor enhancer in the food industry, L-glutaminase has attracted a lot of attention lately. Extracellular L-glutaminase-producing marine bacterial strains were screened using a dye-based technique using sediment and water samples that were obtained at several locations along the Bay of Bengal beach at Visakhapatnam. When L-glutamine was added to the media as the only source of carbon and nitrogen, LG24, out of the fifteen isolates, demonstrated the highest activity of 18.82 U/ml in 48 hours.

Extracellular L-glutaminase has been shown to be produced by mesophilic bacteria from soil habitats (Waykar et al., n.d.). The goal of the current investigation was to screen soil samples from cow feeding sites in Satara Parisar, Chhatrapati Sambhajnagar, for the L-glutaminase-producing bacteria ((Waykar et al., n.d.)), which is known to have anticancer properties. *Kurthia gibsonii* was isolated from three soil samples taken from cow feeding farms, according to the results. It had the maximum level of L-glutaminase activity. Additionally, the MTT assay was used to compare the in vitro cytotoxic activity of L-glutaminase against the epithelial human breast cancer cell line MDA-MB 231 and hepatocellular carcinoma cell lines HepG-2 at varying concentrations (0.47, 0.94, 1.88, 3.75, 7.50, 15.00, 30.00, and 60.00 µg/ml) and Lymph Node Carcinoma of the Prostate LNCaP. *Kurthia gibsonii*'s Lglutaminase demonstrated strong cytotoxic activity against a human liver cancer cell line in a dose-dependent manner, with an LC50 value of 4.1 µg/ml. This antitumor impact was shown against this particular cell line.

Using the MTT assay, the isolated enzyme's anticancer activity was assessed in vitro against five different types of human cancer cell lines. The evaluated enzyme was highly effective against Hep-G2 (IC₅₀: 6.8 µg/ml) and HeLa (IC₅₀: 8.3 µg/ml) cells, however it had no effect on MCF-7 cell proliferation. However, the evaluated enzyme had a mild cytotoxic effect on RAW 264.7 cells (IC₅₀, 59.3 µg/ml) and HCT-116 cells (IC₅₀, 64.7 µg/ml).

According to (Divya Teja et al., 2014), the enzyme L-glutaminase is produced by a variety of microorganisms and is being used as an enzyme sensor, flavor enhancer, and treatment for leukemia. Actinomycetes are known to be a rich source of the enzyme needed to produce glutaminase. There is a dearth of L-glutaminase from actinomycetes in general and marine actinomycetes in particular, despite reports of L-glutaminase activity in a variety of microorganisms. These days, attempts are being undertaken to substitute enzymes from microbes for those that were previously obtained from plant and animal tissues. There is extremely little L-glutaminase found in actinomycetes in general and marine actinomycetes in particular. Enzymes from microbes are currently being tried to replace those that have historically been extracted from plant and animal tissues. Economically, solid state fermentation is advantageous. For a considerable amount of time, actinomycetes from both marine and terrestrial ecosystems have been known to produce antibiotics, be intriguing to scientists, and be useful in the synthesis of enzymes. Wheat bran proved to be the most effective substrate among the others for L-glutaminase induction. Under ideal growth conditions, the Actinomycetes strain was employed to produce glutamic acid. A spectrophotometer was used to quantify L-glutaminase by direct Nesslerization at 480 nm. Glutamic acid was enhanced with the usage of actinomycetes. At pH 7, temperature 30°C, time 96 hours, and salinity 3.5%, the strain produced at its highest level.

In addition to being used in the food sector to break down acrylamide in fried foods, L-glutaminase is recognized as an anti-cancer medication for treating leukemic malignancy (Potla Durthi et al., 2019). A possible bacterial source for the production of L-glutaminase was identified through experiments using the newly obtained mutants of *Bacillus* spp. L-glutaminase Acrylamide are the keywords. anti-leukemia enzymatic The application of statistical optimization Methodology for response surfaces.

Microorganisms such as bacteria, yeast, and fungi are known to contain large amounts of L-glutaminase, according to a study by (kumarS et al., 2013). L-glutamine's γ -amido bond hydrolysis is mostly catalyzed by the enzyme. For *Streptomyces griseus*'s submerged production of L-glutaminase medium. L-glutamine production was found to be significantly impacted by the medium components (g/l) galactose 10.0, yeast extract 10.0, magnesium sulphate 0.5, KH_2PO_4 0.5, and NaCl 40. After 48 hours of incubation, an enzyme activity of 45 IU/ml was obtained, validating the employed technology.

A culture obtained from garden soil was discovered to be a promising generator of L-glutaminase (Iyer & Singhal, 2009). *Klebsiella oxytoca* was identified as the isolate by 16S rRNA sequencing and biochemical identification testing. Following that, response surface methodology and a one-factor-at-a-time strategy were used for media optimization. The synthesis of glutaminase was significantly impacted by the interactions of almost all parameters.

Microbial L-asparaginases and L-glutaminases have drawn a lot of attention lately because of their prospective uses, according to (Helmy El-Ghonemy et al., n.d.). L-asparaginase and L-glutaminase were shown to have the highest specific activity for both amidases, with 3.74 and 5.21 IU/mg protein, respectively.

The key enzyme that catalyzes the deamination of L-glutamine to L-glutamic acid and ammonium ions is called L-glutaminase (also known as L-glutamine amidohydrolase; EC 3.5.1.2), according to (Bülbül & Karakuş, 2013). Because of its potential uses in medicine and industry, L-glutaminase has drawn a lot of attention recently.

Using ammonium sulfate fractionation and gel filtering on Sephadex G-75, the glutaminase generated from *Aspergillus oryzae* NRRL 32567 was purified 10.2 times, according to (Bazaraa et al., 2016). The *Aspergillus oryzae* Features Improver of flavor L-glutaminase Cleaning up Capable of withstanding salt.

One of the top contenders for the therapy of crippling human tumors is a family of enzymes known as L-glutaminases, according to (Unissa et al., 2014). These enzymes have been shown to have antitumor action against Acute Lymphoblastic Leukemia (ALL), a pediatric malignancy that is frequently diagnosed. Furthermore,

it has been discovered to suppress four human tumor cell lines: A549 (human lung cancer), MCF-7 (breast cancer cell line), HCT-116 (colon cell line), and Hep-G2 (human hepatocellular carcinoma cell line). It is used in the food business, analysis, and the synthesis of fine compounds, among many other fields besides medicine. As a result, the primary focus of this review is on the biochemical elements of L-glutaminase production with the goal of understanding the physiochemical features, applications, and qualities of L-glutamine. Important biochemical processes are also covered, such as solid state and submerged fermentation of organisms that produce glutaminase. According to (Kannan et al., 2014), acute lymphoblastic leukemia can be successfully treated with L-glutaminase.

(Yulianti et al., n.d.) stated that because of its dual roles as an antileukemic drug and flavor enhancer, L-glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) is an extremely significant enzyme. Produced by marine microorganisms, salt-tolerant L-glutaminase has advantages in the food sector.

Rhizophora mucronata, a mangrove and a vital source of endophytic bacteria, was studied for its capacity to generate glutaminase-free L-asparaginase (Prihanto et al., 2019). In order to maximize enzyme synthesis, the study sought to extract L-asparaginase-producing endophytic bacteria from the mangrove that were free of glutaminase. M9 medium was adjusted for the purpose of screening for L-asparaginase-producing bacteria. Using 16S rRNA gene sequencing, the possible producer was examined in further detail with regard to species.

Streptomyces canarius FR (KC460654), which has an apparent molecular mass of 44 kDa, was the source of L-glutaminase, according to (Reda, 2015). It has a final specific activity of 132.2 U/mg proteins, a 17.9 purification fold, and a 28% yield recovery. When incubated at pH 8.0 for 30 minutes at 40°C, the isolated L-glutaminase exhibited its maximum activity against L-glutamine. It was thermally stable to 60 °C with a T_m value of 57.5 °C, and it remained stable over a broad pH range of 5.0–11.0.

According to (Jeon et al., 2009), we produced the glutaminase gene (EC 3.5.1.2) heterologously and examined the characteristics of *Lactobacillus reuteri* KCTC3594's glutaminase activity. By using PCR, the glutaminase gene was cloned from *Lactobacillus reuteri* KCTC3594 and subsequently inserted into two

Lactobacillus isolates from Korea. Every transformant carrying the *L. reuteri* KCTC3594 glutaminase gene was able to increase.

According to (Vineetha et al., 2024), L-glutaminase is a special enzyme having catalytic activity and the capacity to adjust glutamine levels, making it a useful enzyme with a wide range of possible uses. An approach that shows promise for the commercial synthesis of L-glutaminase is the selective isolation and optimization procedures outlined in this dissertation.

Actinomycetes are great microbiological sources for a variety of chemical structures, such as enzymes, the majority of which are used in industrial and pharmaceutical products (Wardah et al., 2023) stated. Using a combination of two solid substrates (wheat bran and sesame oil cake) incubated at pH 7.0 for six days at 33 °C, L-glutaminase activity increased to 1297.87U/gds, which is 3.21-fold higher than the unoptimized medium. Statistical approaches proved to be an effective method for increasing the yield of extracellular L-glutaminase from *S. pseudogriseolus* ZHG20.

L-glutaminase is a significant anticancer treatment that is widely utilized worldwide by depriving cancer cells of L-glutamine, according to (Mostafa et al., 2021). Isolated from the Red Sea, the marine bacterium *Halomonas meridian* was chosen as the more active L-glutaminase-producing bacterium. As an L-glutaminase producer and potential anti-colorectal cancer drug, this is the first report on *Halomonas meridiana*. Yeast, fungus, and bacteria are among the microorganisms that make L-glutaminase, an amidohydrolase, according to (Aly et al., 2017)). It has gained popularity recently as a flavor enhancer and anticancer medication. And Saline soil is a valuable source of bacteria, particularly those of the genus *Streptomyces*, which generate L-glutaminase, an anticancer drug with greater activity and lower toxicity that is salt tolerant.

In order to isolate a bacterial strain from maritime soil that might manufacture L-glutaminase, the current experiment was conducted, according to (More et al., 2018) pH 7.0, 37 C, and 0.3% L-glutamine were the ideal conditions for the isolated bacterium, *Bacillus cereus* strain LC13, to manufacture the enzyme, according to 16s rRNA analysis. Best carbon source addition was maltose (0.4 % w/v). Strong

radical scavenging activity has also been demonstrated by L-glutaminase, suggesting potential uses in medicine as an anti-tumor agent.

(Renu & Chandrasekaran, 1992) stated that glutaminase was discovered to be produced by four species of bacteria, including *Pseudomonas fluorescens*, *Vibrio cholerae*, and *Vibrio costicola*, in both external and intracellular fractions. In comparison, both fractions were greater in mineral media supplemented with 1% glutamine than in nutrient broth supplemented with or without glutamine. All of the investigated strains produced between 2.6 and 6.8 times as much glutaminase extracellularly as they did intracellularly.

(Lakshmi & Mari, 2020) stated that halophiles are a great source of enzymes that can endure and carry out reactions effectively in harsh environments in addition to being salt stable. 34 bacterial isolates exhibiting glutaminase and asparaginase activity were obtained through screening of halophiles from five distinct saline habitats. Out of the 34 isolates, the two most potent strains were chosen for additional research based on primary and secondary screening. Based on 16S rDNA sequencing, they were identified. Enzymes that are crucial to industry, including cellulase, phosphatase, amylase, and protease, were also searched for in these isolates. Along with these topics, the current study looks at the growth of two antineoplastic enzymes, glutaminase and asparaginase, as well as the optimization of their substrates and their enzyme activity throughout time.

Probiotic strains may be employed in the future as functional radical scavengers and bio-preservatives, according to (Hasson et al., 2021). Extracellular L-glutaminase isolated from *L. gasseri* BRLHM was evaluated for its antioxidant activities using several antioxidant tests, such as DPPH radical scavenging. This finding may hold promise for antioxidant prophylaxis using natural probiotics and treatments aimed at scavenging free radicals in the future.

Microbial glutaminases have been used in a variety of disciplines, according to (Kumar Katikala et al., n.d.). Due to its possible uses in medicine as an anticancer agent and as a flavor enhancer in the food industry, L-glutaminase has attracted a lot of attention lately.

(Yoshimune et al., 2004) and colleagues reported that protease produced from *Micrococcus luteus* K-3, a marine bacterium, was used to digest salt-tolerant glutaminase (*Micrococcus* glutaminase, with an apparent molecular mass of 48.3 kDa, intact glutaminase). A big fragment (the glutaminase fragment, with an apparent molecular mass of 38.5 kDa) and smaller fragments (with an apparent molecular mass of 8 kDa) were the breakdown products. Phenylmethanesulfonyl fluoride (PMSF) impeded the process of digestion. The lysyl endopeptidase, trypsin, elastase, and arginylendopeptidase are among the serine proteases that may digest intact glutaminase to create the glutaminase fragment. The glutaminase fragment's N-terminus matched that of the complete glutaminase. Two tiny fragments have the N-termini Ala394 and Ala396 respectively. With the exception of salt tolerance, the glutaminase fragment's enzymatic and kinetic characteristics were nearly identical to those of intact glutaminase. The glutaminase fragment had a greater level of salt tolerance compared to the complete glutaminase. This suggests that serine protease from *M. luteus* K-3 digests *Micrococcus* glutaminase in the C-terminal region, hence conferring salt tolerance on the enzyme. Words Glutaminase-producing marine bacteria The *Micrococcus luteus* Tolerance to Salt serine protease.

Microorganisms from harsh environments, like the Antarctic ecosystems, have a high potential to develop enzymes with novel features, according to a paper by (Sánchez-Moguel et al., 2023) In this regard, little research has been done on L-asparaginase (ASNase) derived from yeast species. In that work, the ability of yeasts isolated from materials gathered at Admiralty Bay (King George Island, Antarctica) to produce ASNase was examined. For the treatment of asparagine synthetase-negative cancer cells, this work is the first to report *L. scottii* as a source of glutaminase-activity free L-asparaginase, a medication feature appropriate for acute lymphoblastic leukemia.

Amidases (L-asparaginase and L-glutaminase) catalyze the deamination process of L-asparagine and L-glutamine to their corresponding acidic form with ammonia release, according to a report by (Orabi et al., 2019). In addition to their significant commercial contribution on a global scale, these enzymes are regarded as members of one of the most important groups of enzymes in the fields of

biotechnology and biomedicine. As antileukemic agents for the treatment of acute lymphoblastic leukemia (ALL) and other cancers, L-asparaginase and L-glutaminase have drawn increased attention. However, these enzymes are also employed in the production of food due to their hydrolysis effect, which may help reduce the amount of free L-asparagine in the raw materials and reduce the immediate risk of acrylamide compounds, which are carcinogenic and neurotoxic when food is heated above 120 °C. In addition to being essential amino acids for nutrition, glutamic and aspartic acid are used in food processing to produce a delightful, fine, sour, and umami taste. L-asparaginase and L-glutaminase's modes of action are covered in a recent review. This review also includes information on the origins of L-asparaginase and L-glutaminase, how to optimize enzyme production, and how to use the two enzymes for industrial and cancer treatment applications.

As an important protein, L-glutaminases find prospective uses in a range of sectors, from the food business to the medical and therapeutic fields (Ahmed, n.d.) Microbial L-glutaminase synthesis can be achieved with the application of ideal circumstances, and the bacterial isolates have a significant deal of promise for the manufacture of glutaminase enzyme and its uses.

Glutaminase was extracted from the cell-free extract of *Debaryomyces* spp. CECT 11815 using protamine sulphate treatment and a number of chromatographic techniques, such as anion exchange chromatography and gel filtration, according to (Durá et al., n.d.) The enzyme remained unaffected by EDTA, DTT, or PMSF. L-aspartate had no effect on the enzyme's activity, while sulfhydryl group reagents, DON, L-albizziin, L-asparagine, and large doses of L-glutamine and ammonium inhibited the enzyme. While lactate and borate marginally increased glutaminase activity, phosphate and acetate had no discernible effect. Reprinted by permission of (Gupta et al., 2003) Glutamic acid decarboxylase (GAD) and L-glutaminase catalyze the hydrolysis of glutamate and L-glutamine, respectively, according to (Woraharn et al., 2015). In addition to being a common ingredient in cancer treatments, L-glutaminase was also utilized as a key bioconversion catalyst in the food industry. In order to identify and filter lactic acid bacteria (LAB) that produce GAD and L-glutaminase, this study was conducted.

Purification and characterisation of halotolerant, thermostable alkaline L-glutaminase from *Bacillus* sp. LKG-01 (MTCC 10401), isolated from the Gangotri region of Uttarakhand Himalaya, is being reported in this research (Kumar et al., 2012). Compared to other soil microbe-derived L-glutaminase that has been reported thus far, this enzyme would be more appealing for food fermentation due to its higher specific activity, purification fold, improved halo-tolerance, and thermostability.

A thorough investigation of the production of L-glutaminase from marine bacteria has been conducted, according to (Ramli et al., 2021). The isolate with the greatest enzymatic activity among the tested isolates was discovered to be *Kosakonia radicincitans*, an L-glutaminase producer from a marine environment. For *K. radicincitans* to produce L-glutaminase, the ideal pH and temperature were 7.0 and 37 °C, respectively.

L-glutaminase is a significant enzyme because of its uses in the food and pharmaceutical industries, according to (Zeinat Kamel Mohamed Ibrahim et al., 2020). 142 L-glutaminase-producing bacterial species were identified in the current investigation from brackish water samples taken from Lake Mariout in northern Egypt. Findings showed that only eighteen of these strains produced extracellular enzyme, while the majority produced the enzyme intracellularly. Through the use of 16S rRNA gene analysis, the strains were identified as follows: GPBW1, *Legionella feeleeii*, GPBW2, *Enterobacter cloacae*, GPBW3, *Empedobacter brevis*, GPBW4, *Burkholderia plantarii*, GPBW5, *Lysobacter antibioticus*, GPBW6, *Vibrio parahaemolyticus*, GPBW7, *Serratia fonticola*, GPBW8, *Yokenella regensburgei*, GPBW9, and *Klebsiella oxytoca*, strain GPBW10. The research demonstrated the strain GPBW10 of *Klebsiella oxytoca*'s potential ability to produce L-glutaminase.

According to (Patel et al., n.d.) the enzyme L-glutaminase (EC 3.5.1.2) is receiving a lot of interest in the current situation. Because of its greater affinity to polymerize and alter peptidoglycan production, it is a member of the amidohydrolase class, which is attached to the family of serine-reliant β -lactamases and the penicillin-binding proteins. But because of its catalytic efficiency, L-glutaminase is classified as a proteolytic endopeptidase that releases ammonia in addition to glutamate by cleaving peptide bonds. According to reports, L-glutamine is a crucial amino acid

that is involved in several metabolic processes, including nitrogen metabolism. The current developments and factors related to the biotechnological applicability of L-glutaminase, mostly from marine habitats, are the main focus of this research. In addition, most L-glutaminases are used as therapeutic agents in cancer therapy, particularly in acute lymphocytic leukaemia. The in vitro investigations have demonstrated efficacy against diverse human cancer cell lines. Probiotic bacterial growth is aided by L-glutaminase. In addition to all of these uses, it can be effectively added to fermented foods to increase their flavour, particularly their umami content. The bio-catalytic potential of marine environments has been extensively utilized, yet medicinal enzymes have been found extremely rarely. There are a few papers in the domain about marine bacterial isolates from *Bacillus*, *Pseudomonas*, and *Vibrio* species, but none emphasize the therapeutic uses of these drugs, which are primarily anticancer and anti-proliferative. The term "L-glutaminase." oceanic enzymes. umami flavor. anti-tumor. capacity for biotechnology. lactic acid bacteria. Gujarat beaches

CHAPTER-3

AIM AND OBJECTIVE

3.1 AIM

To produce, optimize, purify, and characterize L-glutaminase isolated from marine microorganisms and its applications in cancer treatment.

3.2 OBJECTIVE

- To collect samples between the rocks on the seashore of Bay of Bengal.
- To isolate the bacterial culture from the sample by plating method.
- To screen L-Glutaminase producing marine organisms.
- To determine the L-Glutaminase enzyme activity.
- To produce L-Glutaminase under optimum conditions.
- To purify the enzyme.
- To characterize the produced enzyme.
- To determine its application in the field of cancer treatment.

CHAPTER- 4

MATERIALS AND METHODS

4.1 COLLECTION OF SAMPLES

The sample was collected the rocks on the seashore & transported carefully to the laboratory for the further processing.

4.2 ISOLATION OF L-GLUTAMINASE PRODUCING BACTERIA

Serial dilutions were prepared for 10^{-1} to 10^{-9} dilutions from the soil sample without clumps. The 0.1ml of 10^{-4} and 10^{-5} dilutions was cultivated on marine agar (MA) plates and incubated at 37°C for 24hrs to 48hrs for bacteria and the grown bacterial colonies were streaked individually and the pure culture was streaked in marine agar slants at 4°C for further analysis.

4.3 L-GLUTAMINASE SCREENING FOR BACTERIA

For bacteria, to marine agar which was autoclaved at 121°C, l-glutamine, D-glucose, and phenol red were added and the isolates have been grown on the phenol red agar base plates and incubated at 37°C for 24-48 hours. A colour change was observed from yellow to pink to identify the l-glutaminase production.

4.4 GRAM STAINING FOR BACTERIA

The purified isolated bacterial strain was smeared on the glass slide by heat fixing and then it was primary stained by crystal violet for 1 minute and washed with water and added Gram's iodine for 1minute then washed with water and it was treated by decolorizer with ethanol, acetone or by mixing both was washed with water and finally counterstained with safranin for 30 seconds and washed with water. After air drying, the slide was observed under the microscope to identify whether the bacteria were Gram positive or Gram negative.

4.5 MEDIA COMPOSITION

4.1 Table-1 Composition

Ingredients	g/L
Peptone	5.0

Sodium chloride	19.45
Yeast Extract	1.0
Magnesium Chloride	8.8
Ferric Citrate	0.1
Sodium Sulphate	3.24
Calcium Chloride	1.8
Potassium Chloride	0.55
Sodium Bicarbonate	0.16
Potassium Bromide	0.08
Strontium Chloride	0.034
Disodium Phosphate	0.0008
Boric Acid	0.022
Sodium Silicate	0.004
Sodium Fluorate	0.0024
Ammonium Nitrate	0.0016
D-Glucose	2
L-Glutaminase	10
Phenol red	0.009
Agar	15.0

Adjusted to suit performance parameters.

Final pH(at 25°C): 7.6±0.2

g/L: Suspend 55.25g of the powder in 1000mL purified /distilled water.

4.6 OPTIMIZATION FOR BACTERIA

For optimization, there are pH, Temperature, incubation time, carbon source, nitrogen source, agitation speed to identify the better l-glutaminase production from the bacteria in their favorable conditions.

4.6.1 pH

The optimum pH to produce enzyme from bacteria in marine broth was finalized by adjusting the pH from 2 to 11 with l-glutamine in the marine broth where the other

parameter range are unaltered. After incubation for 24hrs at 37°C, the enzyme activity was calculated to finalize the optimized pH.

4.6.2 Temperature

The optimum temperature was optimized for L-glutaminase production by ranging the temperature from 25 to 65°C in L-glutamine marine broth. After incubation for 24hrs at 37°C, the enzyme activity was calculated to finalize the optimized temperature.

4.6.3 Incubation time

The bacterial culture was inoculated in L-glutamine marine broth and incubated at the temperature of 37°C for time intervals of 24hrs to 72hrs and checked the enzyme activity by centrifuging at 4000rpm for 10mins for bacteria.

4.7 DETERMINATION OF L-GLUTAMINASE ACTIVITY BY SPECTROPHOTOMETER METHOD

For enzyme activity, the substrate solution was prepared as follows.

- 0.05M Tris HCl & 0.04M L-glutamine was mixed and made up to 100ml was named as solution A.
- 750 µl of solution A and 25 µl of enzyme extract was added and kept for dry bath at 37°C for 30 minutes.
- To stop reaction 0.25µl of 1.5M TCA was added and it was centrifuged at 10000rpm for 10 minutes and supernatant was separated.
- Now 50 µl of Nessler's reagent, 25 µl of supernatant and 925 µl of distilled water are added. The mixture was kept in room temperature for 20 minutes.
- And absorbance was measured using spectrophotometer at wavelength of 450nm to calibrate the release of ammonia.

4.8 PURIFICATION OF L-GLUTAMINASE

4.8.1 Ammonium sulphate precipitation

To the crude enzyme, ammonium sulphate (70 %) was added and was kept in stirring at the temperature of 4°C for 24hrs. The precipitation was seen, and it was centrifuged at 1000rpm for 5 minutes and the supernatant was discarded, and the pellet was mixed with the Tris HCl buffer for further use.

4.8.2 Dialysis

The precipitation mixed buffer was added to the membrane, and it was kept inside a flask filled with Tris Hcl buffer at 4°C for 24hrs. The dialysis membrane was prepared by boiling the membrane in 2 % sodium bicarbonate and 1mM EDTA for 10 mins. The membrane was then rinsed in distilled water. The membrane was stored in ethanol at 4°C.

4.9 COLUMN CHROMATOGRAPHY

Into the 400mg Sephadex, the sodium phosphate buffer was added and kept at 4°C for 4hrs. Then the Sephadex with the Tris Hcl buffer was added slowly in the column chromatography to this the dialyzed L-glutaminase was added. Then the enzyme was eluted with the same Tris Hcl buffer and collected 1ml into 20 tubes as elution fractions. The enzyme activity was carried out for all the fractions.

4.10 SDS-PAGE

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was carried out to measure the molecular weight of the purified lipase from GFC. The concentrations of the separating and stacking gel were 12.5% and 4.5%, respectively. The L-glutaminase sample and sample buffer were mixed in a volume ratio of 3:1 and were kept in a boiling water bath for 5 min. The supernatant was injected into the gel lane after the mixture was centrifuged for 5 minutes a speed of 1000×g. Electrophoresis was performed at a constant voltage of 80 V for approximately 1–2 h. The gel was stained with a Coomassie Brilliant Blue R250 of 0.25% (w/v), 50% (v/v) methanol and 10% (v/v) acetic acid. The gel was destained using the buffer with 25%(v/v) methanol and 7% (v/v) acetic acid.

4.11 CHARACTERIZATION OF L-GLUTAMINASE

The purified enzyme was characterized by metal ions and detergent to determine the stability and relative activity of the enzyme.

4.11.1 Metal ions

0.5ml of crude enzyme, 0.5ml L-glutamine and 0.5ml metal ions which are Magnesium chloride, manganese was added to each tube and kept in dry bath at 37°C for 30 minutes and the enzyme activity and the relative activity was calculated.

4.11.2 Detergent

0.1 ml of SDS, Tween (80), Triton x-100 are taken into each tube along with 0.5ml of crude enzyme, 0.5ml of L-glutamine. The mixture was kept in dry bath at 38°C for 45 minutes. The enzyme activity and the relative activity was calculated.

4.12 MTT ASSAY

Colorectal carcinoma cell lines (HCT1166) were used. These cells were cultured in DMEM media containing 10 % fetal bovine serum at 37 °C, in a CO₂ incubator in the presence or absence of test compounds. Cytotoxicity activity was measured using the MTT assay, according to the method of Mosmann. Cancer cells (5×10^4) were seeded in each well containing 0.1 ml of RPMI medium in 96-well plates, and then L-glutaminase of different concentrations (10–100 µg/ml) was added after 24 h. MTT of 10 µl (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, 5 mg ml⁻¹, stock solution; Sigma) was then added to each well, and cell viability was assessed after 48 h. The medium from the well was discarded, and formazan blue crystals, which formed in the cells, were dissolved with 100 µl DMSO. The rate of color production was measured at 570 nm in a spectrophotometer (Scigenics, India). All experiments were conducted under standard laboratory illumination. The relation between the viability of cell and L-glutaminase concentration was plotted in order to get the viability curve of each HCT116 cell line for the specified enzyme. The effective dose required to inhibit cell growth by 50 % (IC₅₀ in micrograms per milliliter) was determined. (Mitra et al., 2016).

CHAPTER-5

RESULT & DISCUSSION:

5.1 MICROBIAL ISOLATION AND IDENTIFICATION

About 3 samples taken from marine sources were serially diluted and plated from 10^{-1} to 10^{-7} dilution, 23 strains were isolated by plating serially to get the pure cultures as shown in Fig 1. The bacterial colonies were isolated using manual into separate plates. Based on the colony morphology, the samples with good enzymatic activity were taken forward for the production.

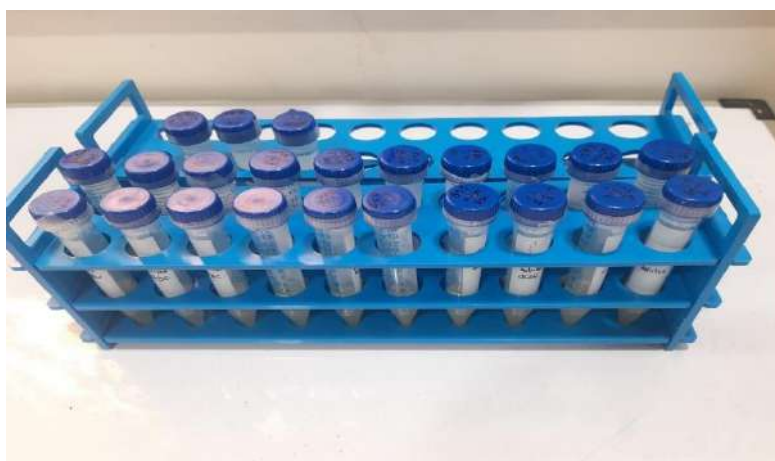


Fig 5.1 Isolation of marine isolates

5.2 SCREENING

Based on the isolates tested for L-glutaminase activity by Plate assay method, 23 isolates were showed the colonies. The samples 23 isolates labelled S6C, S5A, S8A, S1C, S3C, S8B, S4A1, S1A, S8C, S4B, S3C, S7A1, S1D, S1A, S8D, S6A, S4A, S1B, SBD, S4A2, S6B, S7B, control, that showed respectively (figure 2,3).

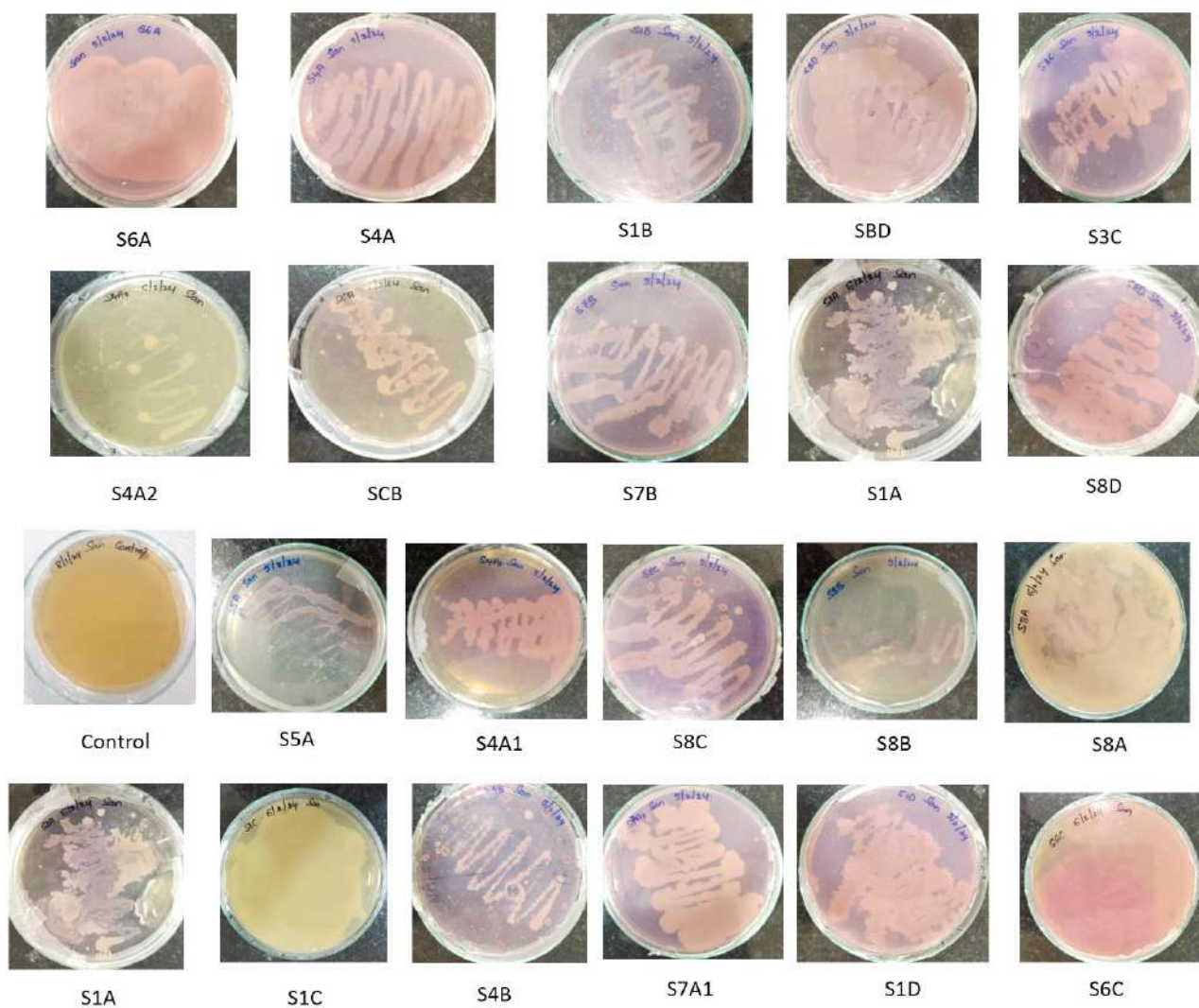


Fig 5.2 Screening of marine isolates for the presence of L-glutaminase

Table 5.1 Screening of marine isolates for the presence of L-glutaminase

Sl.no	Bacteria	24 hrs	48hrs
1	S6C	+ve	+ve
2	S5A	-ve	+ve
3	S8A	-ve	+ve
4	S1C	-ve	-ve
5	S3C	-ve	+ve
6	S8B	+ve	+ve
7	S4A1	+ve	+ve
8	S1A	-ve	+ve
9	S8C	-ve	+ve
10	S4B	-ve	+ve
11	S7B	-ve	+ve
12	S7A1	+ve	+ve
13	S1D	+ve	+ve
14	S1A	-ve	+ve
15	S8D	+ve	+ve
16	S6A	+ve	+ve
17	S4A	-ve	+ve
18	S1B	-ve	+ve
19	SBD	+ve	+ve
20	S4A2	-ve	+ve
21	S6B	-ve	+ve
22	S8D	+ve	+ve
23	Control		

5.3 BEST SELECTED BACTERIA FOR FURTHER OPTIMISATION STUDIES:



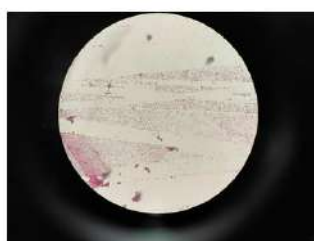
Fig5.3. Best marine isolates showing better L-glutaminase

The sample labelled S6C, S7A1 and S4A1 was selected for the further studies as it had the best outcome of extracellular I-ase production among all the strains (fig 4)

5.4 GRAM STAINING OF L-GLUTAMINASE PRODUCING BACTERIA



S4A1-Gram
Negative, Cocci



S7A1-Gram
Negative, Short rod



S6C-Gram Negative,
short rod

Fig 5.4. Gram Staining results of I-Glutaminase producing bacteria.

From 23 bacteria, 3 positive samples showing better zone were proceeded for gram staining to study the cellular morphology. In S4A1 sample got, Gram negative, Cocci. And in S7A1 sample got Gram negative, Short rod. And in S6C sample got, Gram negative, Short rod.

Table 5.2 Gram staining of l-glutaminase producing bacteria

S.no	Sample	Morphology	Gram staining
1	S4A1	Cocci	Gram negative
2	S7A1	Short rod	Gram negative
3	S6C	Short rod	Gram negative

Table 5.3 L-Glutaminase Activity

S.NO	Sample	Enzyme Activity(U/ml)	Protein (mg/ml)	Specific Activity(U/ml)
1	S4A1	293.35	6.2	47.95
2	S7A1	232.70	7.8	29.83
3	S6C	273.27	5.2	49.68

In l-glutaminase activity, the sample S4A1 enzyme activity was 293.35 (U/ml) and the protein was 6.2(mg/ml) and the specific activity was 47.95 (U/mg). The sample S7A1 the enzyme activity was 232.70(U/ml) and the protein was 7.8 (mg/ml) and the specific activity was 29.83(U/mg). The sample S6C the enzyme activity was

273.27(U/mg), and the protein was 5.2(mg/ml),and the specific activity was 49.68(U/mg).

5.5 OPTIMUM CONDITIONS FOR ENZYME PRODUCTION:

5.5.1 Temperature:

The enzyme activity observed for the temperatures 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C and 65°C are 220 U/ml, 250U/ml, 290 U/ml, 297 U/ml, 240 U/ml, 200 U/ml, 110 U/ml, 50 U/ml and 40 U/ml respectively. The highest l-glutaminase production was observed at 35°C. While in another study, the enzyme production was decreased when the bacterial culture was subjected to growing at temperatures other than the optimum value. The enzyme productivity was decreased by 35.57% when the incubation temperature increased from 37 °C (1.1470 U/mL/h) to 46 °C (0.408 U/mL/h).

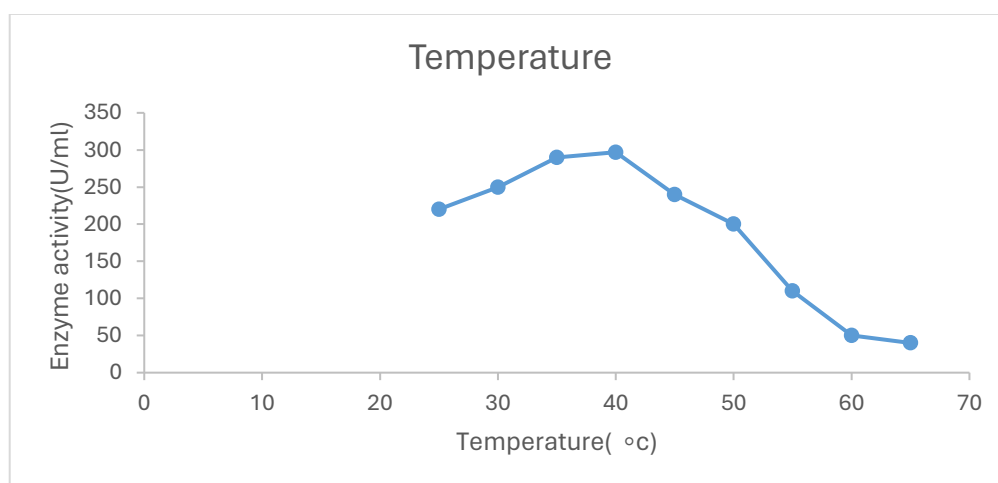


Fig 5.5 Effect of temperature

5.5.2 pH:

The influence of different pH conditions on the enzyme activity such as 4, 5, 6, 7, 8, 9 and 10 were found to be 80U/ml, 90 U/ml, 95 U/ml, 210 U/ml, 293 U/ml, 230 U/ml and 110 U/ml. In the previous studies conducted by (Moriguchi et al., 1994), the highest activity at pH under the standard assay conditions was observed using l-glutaminase as the substrate. Since the optimal pH for the tested strain in the

present study was around the neutral range, no additional acid or alkaline was needed.

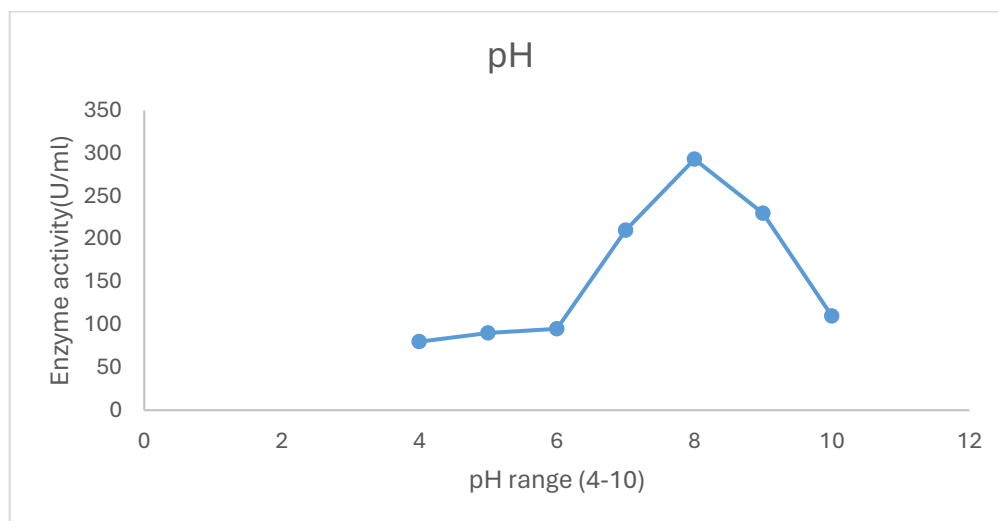


Fig 5.6. Effect of pH

5.5.3 Incubation time

The influence of different incubation times in the range 12, 18, 24, 36, 48, 72, and 96 h was observed as 210U/ml, 240 U/ml, 260U/ml, 350 U/ml, 335 U/ml, 320 U/ml and 322U/ml of enzyme activity respectively. The highest l-glutaminase production was observed around the incubation time of 36 h. Further increase in the incubation time decreased the production and activity of the enzyme. The observed is in accordance with literature reports(Kiruthika & Saraswathy, 2013), where it is noticed that the optimum incubation period for were within 24 h with the maximal enzyme production of 246.86 U/ml.

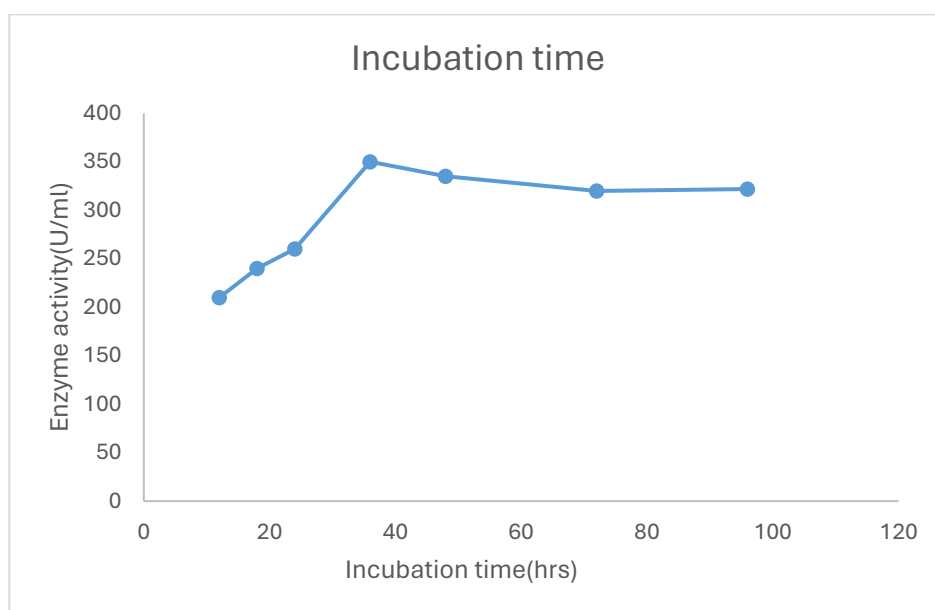


Fig 5.7. Effect of incubation time

5.5.4 Inoculum size:

By varying the inoculum size for the enzyme production, the results observed were 20 U/ml, 80 U/ml, 350 U/ml, 345 U/ml and 320 for the respective concentrations 0.5%, 1%, 5%, 10% and 20%. The highest production was observed around 5%. The initial inoculum size controls the kinetics of growth and several metabolic functions leading to overall biomass and extracellular product formation (Kiruthika & Saraswathy, 2013). To evaluate same, experiments were planned with increasing inoculums concentration from 1 to 5%. The results indicated that the kinetics of L-glutaminase production varied with variation in inoculums concentration. The maximal enzyme production (246 U/ml) was observed at 1% initial inoculums supplementation.

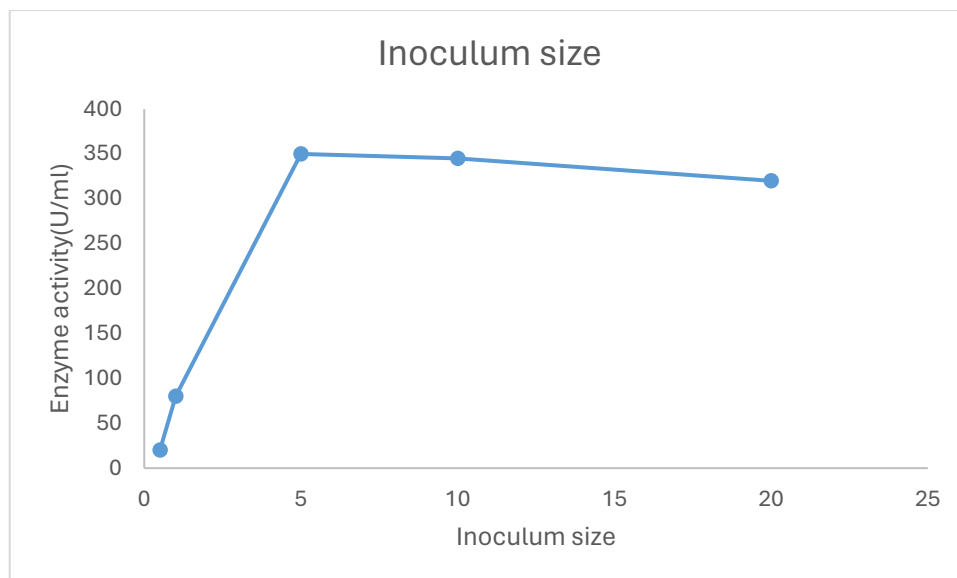


Fig 5.8. Effect of inoculum size

5.6 PURIFICATION OF L-GLUTAMINASE:

5.6.1 Ammonium sulphate precipitation & Dialysis: After the precipitation of crude enzyme with 70% Ammonium sulfate solution, concentrated pellet with a protein was obtained. After the partial purification of the enzyme with dialysis, the activity was found to be effective.



Ammonium sulfate + Enzyme



DIALYSIS



Dialysis sample

Fig 5.9.

Fig 5.10

Fig 5.11

5.6.2 COLUMN CHROMATOGRAPHY:

Then to purified further by column chromatography using Sephadex g-300 was performed. While the elution fraction labelled 6,7 and 8, had the highest enzyme activity of 0.288U/ml, 0.296 U/ml and 0.231 U/ml respectively (fig9).



Fig 5.12 COLUMNCHROMATOGRAPHY



Fig 5.13 ELUSIONS

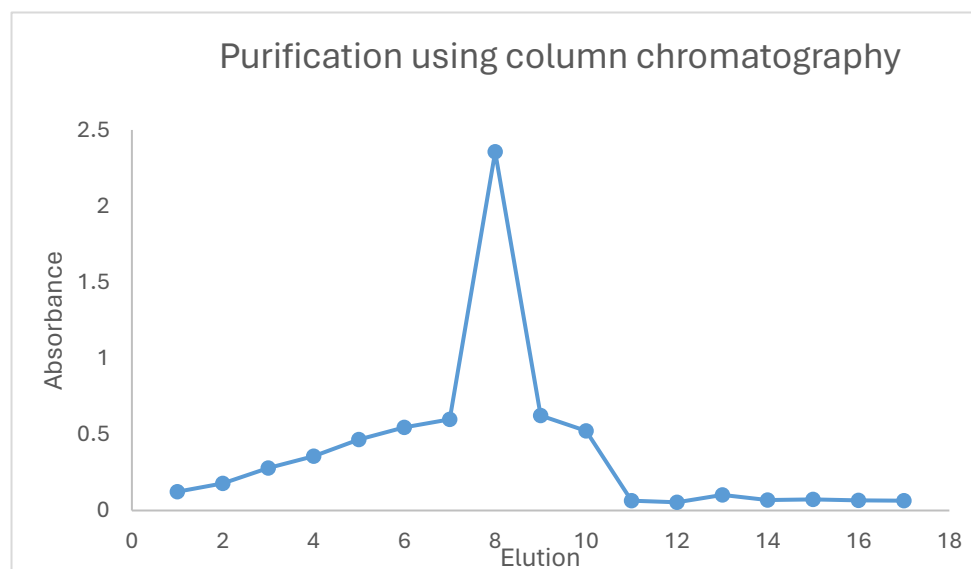


Fig 5.14. Absorbance of the elution fractions of the dialysed L-glutaminase

These three fractions were mixed to obtain maximum., Compared to the another. Further dialyzed sample was subjected to anion exchange chromatography on a Diethylamino ethyl (Desai et al., 2016)(DEAE)-cellulose column, pre-equilibrated same buffer. The column was washed thoroughly with the same buffer, and the bound proteins were eluted with a linear salt gradient of NaCl (0.1–0.25).

5.6.3 Enzyme activity

Table 5. 4 Enzyme activity of purified L-Glutaminase enzyme

S4A1	TOTAL VOLUME	ENZYME ACTIVITY (u/ml)	TOTAL PROTEIN (mg/ml)	SPECIFIC ACTIVITY (u/mg)
Crude enzyme	100	293.9	5.678	51.579
Dialysis	5	240.10	4.23	56.761
Column	2	230.59	0.121	1905.70

In this for S4A1,the crude enzyme total volume is 100ml,and enzyme activity is 293.9(U/ml) and total protein is 5.678(mg/ml)and the specific activity is 51.579(U/mg).Then dialysis total volume is 5ml,enzyme activity is 240.10(U/ml),total protein is 4.23(mg/ml) and specific activity is 56.761(U/mg). Finally the partially purified column chromatography has the total volume is 2ml,and enzyme activity is 230.59(U/ml),and total protein is 0.121(mg/ml), and specific activity is 1905.70(U/mg).

5.6.4 SDS-PAGE:

SDS PAGE was used to determine the molecular weight of L-glutaminase enzyme produced. The figure shows that the molecular weight of L-glutaminase enzyme to be 40 kDa with the help of Bio-helix protein marker (broad range 3 kDa to 260 kDa). As resulted in the other article (Moriguchi et al., 1994) it was found that the molecular weight of L- glutaminase was to be 85 kDa produced by the organism. The result of SDS-PAGE after the final step of purification revealed a single major band corresponding to molecular mass of approximately 50 kDa(Desai et al., 2016)

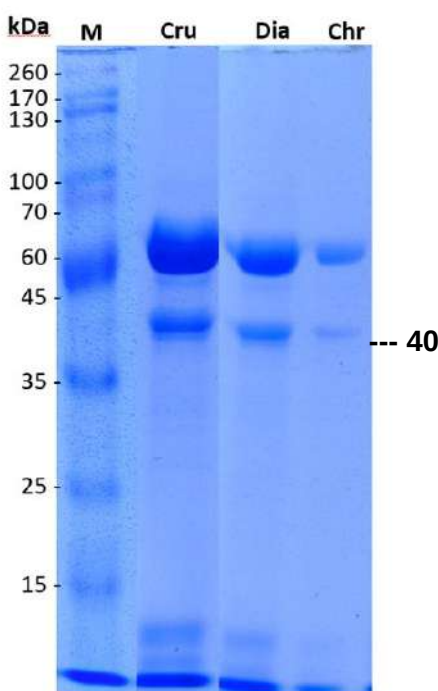


Fig 5.15. SDS PAGE

5.7 CHARACTERIZATION OF L-GLUTAMINASE

5.7.1 Temperature

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 L-glutaminase 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 L-glutaminase 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. compared to others The best temperature for the production of L-glutaminase from *K. radicincitans* LG8 were established, and the outcomes are portrayed in Fig. 5. Difference in temperature (31–43 °C) had led the purified enzyme to become active between of 4 and 8, with the maximal L-glutaminase production was observed at 37 °C. There was a gradual increase in activity of the L-glutaminase enzyme until temperature 37 °C and a before the activity decreased briskly(Ramli et al., 2021)

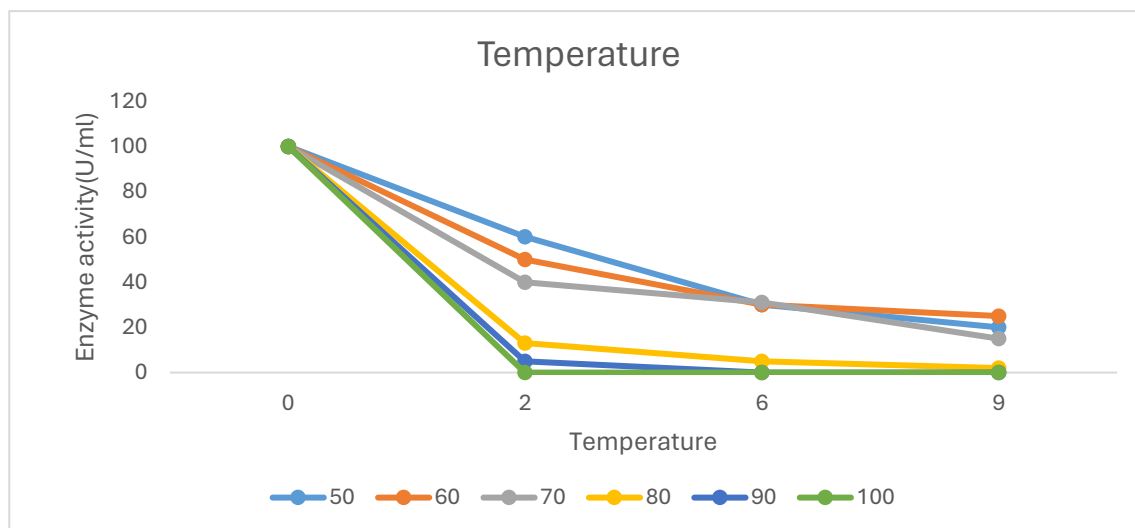


Fig 5.16 Effect of temperature on purified enzyme

5.7.2 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 8.2, 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. The best pH and for the production of L-glutaminase from *K. radicincitans* LG8 were established and the outcomes are portrayed in Fig. 5. Difference in had led the purified enzyme to become active between a pH of 4 and 8, with the maximal L-glutaminase production was observed at pH 7. There was a gradual increase in activity of the L-glutaminase enzyme until a pH value of 7 before the activity decreased briskly(Ramli et al., 2021)

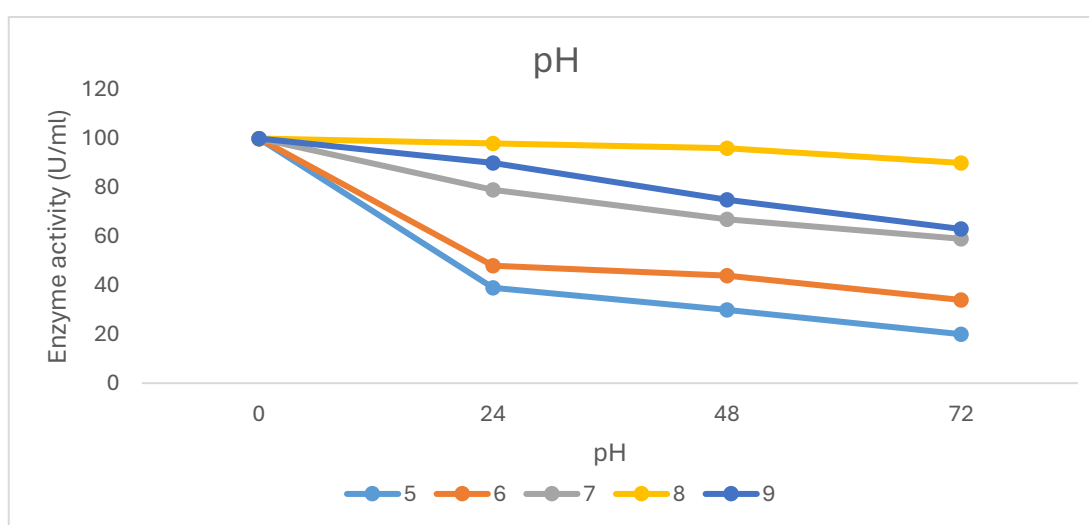


Fig 5.17. Effect of pH on the purified enzyme

5.7.3 Ions

Two different concentrations of each metal ions i.e. 1 mM and 5 mM was used determine their effect on α -amylase activity at pH 7.2 and 60°C. The enzymatic activity was inhibited by Mn^{2+} . However, no significant difference was observed in I-glutaminase activity of enzyme in the presence of Ca^{2+} ions. In the presence of Mg^{2+} , the enzyme retained 69% activity but the high concentration of Mg^{2+} ions reduced the I-glutaminase activity. These results showed that no ion was able to enhance the I-glutaminase activity. In the present study, Mn^{2+} was found to inhibit the I-glutaminase activity which was also reported with another I-glutaminase enzyme. Enhancement effect on I-glutaminase activity by Ca^{2+} is well known (Dheeran et al., 2010) but a few Ca^{2+} independent I-glutaminase also known (Asoodeh et al., 2010; Hmidet et al., 2008). The I-glutaminase reported in this study was also characterized to be Ca^{2+} independent. The I-glutaminase 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.

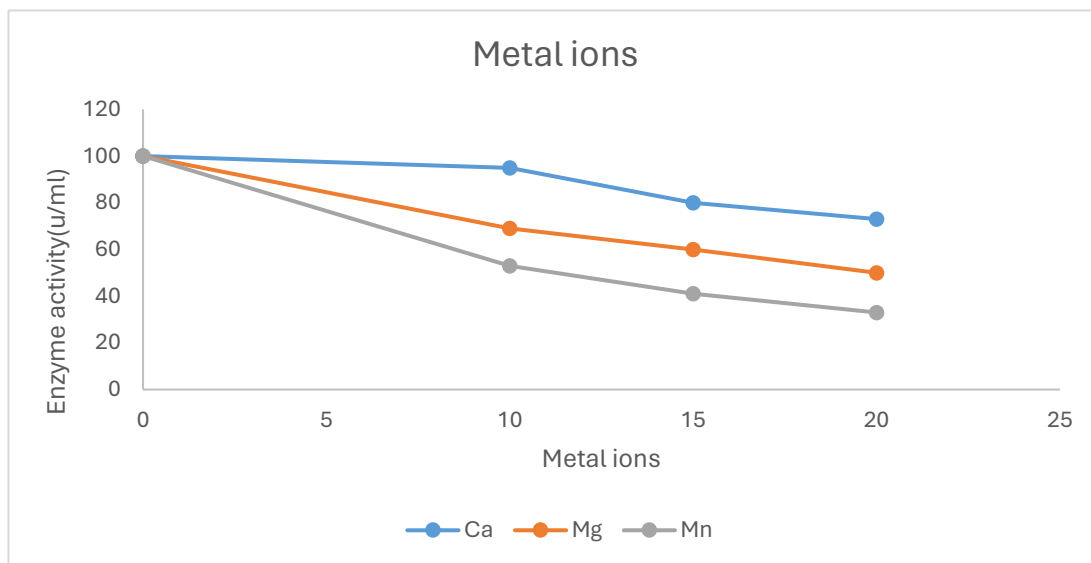


Fig 5.18. Effect of metal ions on the purified enzyme

5.7.4 Detergents

Apparent differences were found in the l-glutaminase activity with addition of different detergents to the reaction mixture (Fig 7c). In the presence of Triton X-100, Tween 20 and Tween 80, about 80% enzyme activity was retained. In case of its treatment with SDS, 60% activity was retained.

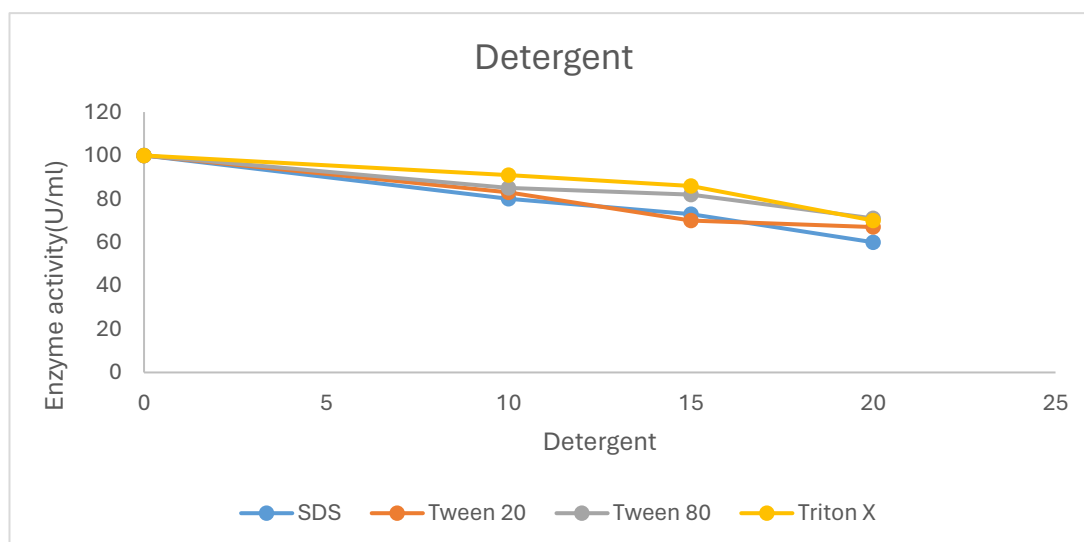


Fig 5.19. Effect of detergent on purified enzyme

5.7.5 Inhibitors:

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

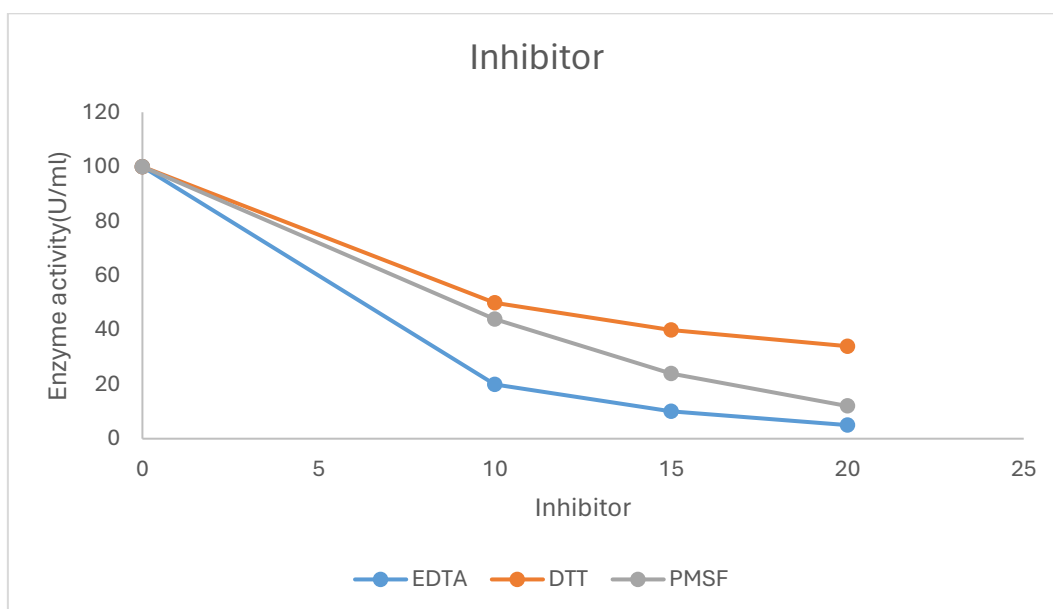


Fig 5.20. Effect of inhibitor on purified enzyme

5.8 CYTOTOXICITY ASSAY:

The effect of L-glutaminase against cancer cells was analysed by the MTT assay. The enzyme was able to reduce the viability of HCT116 cells in a dose-dependent manner, as shown in Fig 5.



Fig 5.21. MTT assay

The IC_{50} value of the enzyme was found to be $6.12 \mu\text{g}/\mu\text{L}$ when the cells were treated with the enzyme for 24 h. This result proved the cytotoxicity of L-glutaminase against HCT116 cells and indicated that the toxic effect was dose and time-dependent. The IC_{50} concentration mentioned above was adopted in further experiments.

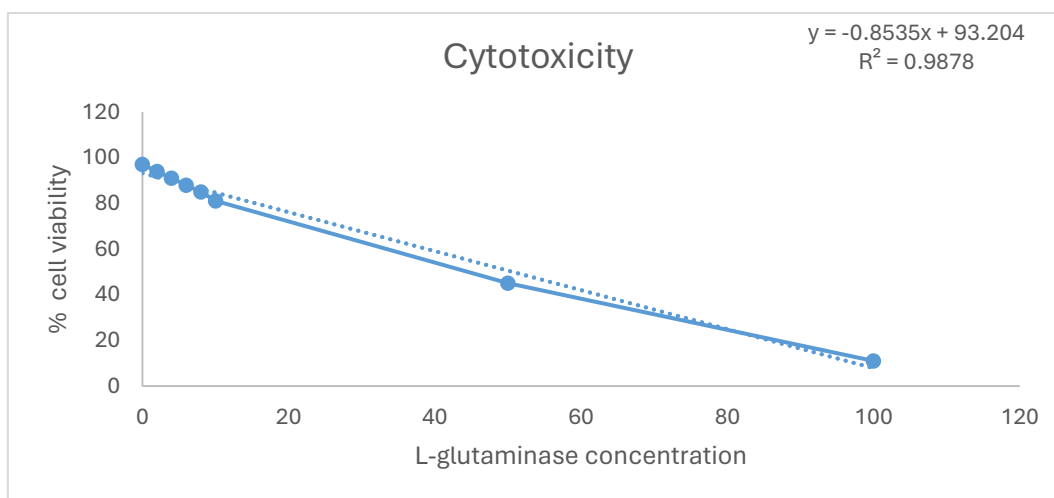


Fig 5.22. Cytotoxicity assay for L-Glutaminase

CHAPTER-6

SUMMARY AND CONCLUSION

Recently, there has been a lot of interest in L-glutaminase because of its prospective uses in medicine as an anticancer agent and as a taste enhancer in food products. Extracellular L-glutaminase-producing marine bacterial strains were screened using a dye-based technique using sediment and water samples that were obtained at several locations along the Bay of Bengal beach at Chennai. In this study, the marine isolated microbial glutaminases were used to check for the anticancer activity. L-glutaminase is a significant anticancer agent that is widely utilized globally by depriving cancer cells of L-glutamine. Marine microbial strains has been isolated from marine water samples and then studied for extracellular enzyme production. A great promise of the observations made in this work; from optimization parameters, various purification steps, molecular weight determination by using SDS-PAGE, enzyme kinetic parameters and substrate specificity were also determined. L-glutaminase was purified in three simple steps and showed a highly purified band with a molecular weight of 40 kDa. Moreover, the purified L-glutaminase in the present study showed potent antitumor activity against colorectal cancer cell lines (HCT1166). This enzyme catalytic activity over a wide range of temperature and pH and its considerable stability makes it highly favourable for use as an effective anticancer agent.

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