PRODUCTION, PURIFICATION, AND CHARACTERIZATION OF MICROBIAL L-GLUTAMINASE

By

Rabia Saleem

Department of Microbiology
Faculty of Biological Sciences
Quaid-i-Azam University
Islamabad Pakistan

2021

PRODUCTION, PURIFICATION, AND CHARACTERIZATION OF MICROBIAL L-GLUTAMINASE

A thesis submitted in partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

In

MICROBIOLOGY

By

Rabia Saleem

Department of Microbiology
Faculty of Biological Sciences
Quaid-i-Azam University,
Islamabad Pakistan
2021

Author's Declaration

I **Ms. Rabia Saleem** hereby state that my Ph.D. thesis titled **"Production, Purification and Characterization of Microbial L-Glutaminase"** is my own work and has not been submitted previously by me for taking any degree from Quaid-i-Azam University, Islamabad, Pakistan.

At any time if my statement is found to be incorrect even after I Graduate, the University has the right to withdraw my Ph.D. degree.

Rabia Saleem

Ms. Rabia Saleem
Date:_____

Plagiarism Undertaking

"Production, Purification and Characterization of Microbial L-Glutaminase" is solely my research work with no significant contribution from any other person. Small contribution / help wherever taken has been duly acknowledged and that complete thesis has been written by me.

I understand the zero tolerance policy of the HEC and Quaid-i-Azam University towards plagiarism. Therefore I as an Author of the above titled thesis declare that no portion of my thesis has been plagiarized and any material used as reference is properly referred/cited.

I undertake that if I am found guilty of any formal plagiarism in the above titled thesis even after award of Ph.D degree and that HEC and the University has the right to publish my name on the HEC/University Website on which names of students are placed who submitted plagiarized thesis.

Student / Author Signature: Rabia Saleem

Name: **Ms. Rabia Saleem**

Certificate of Approval

This is to certify that the research work presented in this thesis, entitled titled **"Production, Purification and Characterization of Microbial L-Glutaminase"** was conducted by **Ms. Rabia Saleem** under the supervision of **Prof. Dr. Safia Ahmed.** No part of this thesis has been submitted anywhere else for any other degree. This thesis is submitted to the Department of Microbiology, Quaid-i-Azam University, Islamabad in partial fulfillment of the requirements for the degree of Doctor of Philosophy in field of **Microbiology**.

Student Name: **Ms. Rabia Saleem** Signature: Rabia Saleem

Examination Committee:

a) External Examiner 1: Signature:

Dr. Hussnain A Janjua
Professor and Principal
ASAB, National University of Sciences and Technology
(NUST), Campus H-12, Islamabad

b) External Examiner 2: Signature:

Dr. Tayyaba Yasmin
Associate Professor
Department of Biosciences,
COMSATS University Park Road,
Chak Shahzad, Islamabad.

Supervisor Name: **Prof. Dr. Safia Ahmed** Signature: Safia Ahmed

Name of HOD: **Prof. Dr. Aamer Ali Shah** Signature:

DEDICATION

"Dedicated to my beloved ***PARENTS & TEACHERS*** because whatever I am today could never be possible without their continuous effort, guidance, sincerity and prayers. I am heartily grateful to them."

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

rpm	Revolutions per minute
HIF	hypoxia-inducible factor
GCL	glutamate-cysteine ligase
BAX	Bcl-2 related X protein
PDG	phosphate-dependent glutamine
PTEN	phosphatase and tensin homolog
ROS	Reactive oxygen species
PI3K	phosphatidylinositol-3 kinase
AKT	protein kinase B
mTOR	Mammalian target of rapamycin
PDK1	pyruvate dehydrogenase kinase 1
ASCT2	Alanine, Serine, Cysteine Transporter
SLC7A5	solute carrier family 7 member 5
GSH	Glutathione
GLS1	glutaminase1
F-type	F1Fo ATPase proton translocating ATPase
α-KGDH	ketoglutarate dehydrogenase
CNS	Central Nervous System
CPS-II	Carbamoyl phosphate synthetase II
CSF	cerebrospinal fluid
GABA	γ-amino butyric acid
LKB1	liver kinase B 1
Vmax	Maximum Velocity
Km	Michaelis and Menten constant
U/mg	Units per milligram
TEMED	Tetramethylethylenediamine
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
KDa	Killodaltons
PGRs	Plant growth regulators
SSF	Solid-state fermentation
SMF	Submerged fermentation
V. costicola	*Vibrio costicola*
C. welchii	*Clostridium welchii*
GLUT1	Glucose transporters
TCA	Tricarboxylic acid cycle
A. nidulans	*Aspergillus nidulans*
SLC7A11	Solute Carrier Family 7 Member 11
PCR	Polymerase chain reaction
E. coli	*Escherichia coli*
B. subtilis	*Bacillus subtilis*

B. amyloliquefaciens	*Bacillus amyloliquefaciens*
S. griseus	*Streptomyces griseus*
B. cereus	*Bacillus cereus*
M. luteus	*Micrococcus luteus*
B. endophyticus	*Bacillus endophyticus*
A. faecalis	*Alcaligenes faecalis*
S. cerevisiae	*Saccharomyces cerevisiae*
P. aeruginosa	*Pseudomonas aeruginosa*
S. avermitilis	*Streptomyces avermitilis*
C. welchii	*Clostridium welchii*
H. jecorina	*Hypocria jecorina*
B. amyloliquefaciens	*Bacillus amyloliquefaciens*
S. maltophilia	*Stenotrophomonas maltophilia*
P. brevicompactum	*Penicillium brevicompactum*

ACKNOWLEDGMENT

This proposition shows up in its current content as my greatest accomplishment in life and pride for my loved ones. This proposal isn't exclusively my own achievement, however the resultant contribution of many people to whom I might want to offer my gratitude. I would like to acknowledge to:

I find tremendous delight in communicating my sincere respect and appreciation to my Supervisor Prof. Dr. Safia Ahmed for her supervision, brilliant help, support, important remarks, and suggestions until end result of my work. She has consistently given vital assistance and co-activity all through the time of my review. She stayed a ceaseless wellspring of support for me.

I recognize her arrangement for drafting my thesis. Her knowledge of microbiological sciences demonstrated productivity in understanding the process.

I'm grateful to Prof. Dr. Aamir Ali Shah (Chairman Dept Microbiology, QAU); Prof. Dr. Rani Faryal, Prof. Dr. Fariha Hasan, Dr. Samiullah Khan, Dr. Malik Badshah and all the regarded employees and supporting staff of Department of Microbiology, Quaid-I-Azam University Islamabad for their unlimited help and direction all through the period.

I am highly grateful to Dean Basic science, Prof. Uzma Ghazanfar, Chairperson Biosciences Dr. Waqas and Co director UWLC Dr. Shumaila Naz Department of Biosciences, University of Wah, Pakistan for helping me to complete my PhD research work.

I am also thankful to my lab fellows at Quaid-i-Azam University and University of Wah. Equally, I greatly appreciate friends Ms. Naheed Malik, Ms. Sania Rauf, Yasmeen Saif for their encouragement and affection on many occasions. Thanks to my students Ms. Madiha Rashid and Ms. Iqra Tariq for their support.

Over this proposition is devoted to my consistently loving father, mother, sisters whose constant supplications, backing and motivation gave me the mental fortitude to dream high and open the roads for me to investigate my capacities. I wish to communicate my unique gratitude to my sweet and cherishing mother whose benevolent love will consistently be valued however can never be reimbursed. I can always remember the penances of my family to satisfy my fantasies, particularly forfeits of my better half who is consistently there for my assistance.

I can never forget the sacrifices of my family to fulfill my dreams, especially sacrifices of my husband who is always there for my help. So I am heartedly thankful to my beloved Husband for his extreme support, caring attitude and always presence whenever I needed his help.

Rabia Saleem

Abstract

A significant protein L-glutaminases discovers potential applications in various divisions running from the nourishment industry to remedy and cure. L-glutaminase is the principal enzyme that changes L-glutamine to L-glutamate and is generally distributed in bacteria, actinomycetes, fungi, and higher organisms. The present study was focused on the isolation and characterization of glutaminase from microbial sources. Different bacterial and fungal strains were isolated. One bacterial strain RSHG1 was isolated from an expired glutamine sample, while 4bacterial strains were isolated from different soil samples. One endophytic fungal strain *Epicocum* sp. NFW1 previously isolated was also included in the study. Selected bacterial strains were identified as *Achromobacter xylosoxidans* RSHG1, *Bacillus subtilis* RSHU1, *Bacillus halotolerance* RSHQ1, *Stenotrophomonas maltophilia* RSHU3, and *Alcaligenes* sp. RSHS3 by 16S rDNA sequence analyses. All the bacterial strains were processed for the production of glutaminase, and different parameters were optimized. Results indicated that *A. xylosoxidans* RSHG1 showed maximum production of L-glutaminase at pH 9, 30˚C, sorbitol, and glutamine as carbon source and nitrogen source, respectively. The optimum pH for glutaminase production from other bacterial strains was between 6-8. *Alcaligenes* sp. RSHS3 achieved the highest L-glutaminase production at 25˚C, *A xylosoxidans* RSHG1, and *S. maltophilia* RSHU3, at 30˚C while for *B. subtilis* RSHU1 and *B. halotolerans* RSHQ1 optimum temperature was 37˚C. Sorbitol was the best carbon source for L-glutaminase production for *S. maltophilia* RSHU3 while *B. subtilis* RSHU1, and *Alcaligenes* sp. RSHS3 and *B. halotolerans* RSHQ1 showed maximum production with glucose sucrose as a carbon source, respectively. L-Glutamine was the best nitrogen source for Lglutaminase production by *A. xylosoxidans* RSHG1 and *B. subtilis* RSHU1, and 1.5 % glutamine was inducer for glutaminase production by all the strains. Optimization of enzyme production for fungal strain *Epicoccum* sp. NFW1 showed the best production at pH 7, 30˚C, sucrose as carbon source and glutamine as nitrogen source and inducer.

Enzymes from all the bacterial strains and fungal isolate were purified using ammonium sulfate precipitation and size exclusion column chromatography. Further purified enzyme (*B. subtilis* RSHU1, *A xylosoxidans* RSHG1, and *Epicoccum* sp. NFW1) and partially purified enzyme (*B. halotolerans* RSHQ1, *S. maltophilia* RSHU3, and *Alcaligenes* sp. RSHS3) were characterized for their kinetic behavior. L-Glutaminase

from *B. subtilis* RSHU1 showed the highest Vmax 522U/mg among all the selected strains and best affinity for its substrate with Km of 0.0591mM. While Vmax of other enzymes from *A. xylosoxidans* RSHG1, *B. halotolerans* RSHQ1, *S. maltophilia* RSHU3, *Alcaligenes* sp. RSHS3, and *Epicoccum* sp. NFW1 was 443U/mg, 343.6 U/mg, 351 U/mg, 232 U/mg, and 450 U/mg, respectively. The L-glutaminases from *A xylosoxidans* RSHG1, *B. halotolerans* RSHQ1, *S. maltophilia* RSHU3, *Alcaligenes* sp. RSHS3, and *Epicoccum* sp. NFW1 showed Km values of 0.235mM, 0.37mM, 0.166mM, 0.186mM, and 0.424mM, respectively.

Optimum pH L-glutaminase produced by *A. xylosoxidans* RSHG1, *Alcaligenes* sp. RSHS3, and *Epicoccum* sp. NFW1 was 7 while the enzyme from *B. subtilis* RSHU1 and *B. halotolerans* RSHQ1 were active at pH 6-6.5, and optimum pH8 was observed for the enzyme from *S. maltophilia*. At temperature 40˚C, the best activities were recorded for L-glutaminase produced by all the strains except for *B. halotolerans* RSHQ1, which showed the best activity at 50˚C. All the L-glutaminases were inhibited by EDTA, $MnSO_4$, $FeSO_4$ while *A. xylosoxidans* RSHG1, *B. subtilis* RSHU1, and *Epicoccum* sp. NFW1 were also inhibited by HgCl and activated by 20 mM $CoCl_2$, $CaCl_2$, $BaCl_2$, $ZnSO_4$, KCl, $MgSO_4$, and NaCl. *B. halotolerans* RSHQ1 L-glutaminase was also inhibited by $CaCl_2$, and increased activities were observed in the presence of $CoCl_2$, $BaCl_2$, $ZnSO_4$, KCl, $MgSO_4$, and NaCl. *S. maltophilia* RSHU3 L-glutaminase was additionally inhibited by $MgSO_4$, and raised activities were observed by $CoCl_2$, $BaCl_2$, KCl, $ZnSO_4$, and NaCl. *Alcaligenes* sp. RSHS3 L-glutaminase activity was additionally decreased by $ZnSO_4$ and improved by $CoCl_2$, $BaCl_2$, KCl, $MgSO_4$, and NaCl is shown in Figures 4.54, 4.69, 5.8. *A. xylosoxidans* RSHG1 L-glutaminase activity was enhanced 64.4% by 8% NaCl, *S. maltophilia* RSHU3 and *Alcaligenes* sp. RSHS3 using 12% NaCl with a rise of 32 % and 44 %, respectively, and *B. subtilis* RSHU1, *B. halotolerans* RSHQ1, and *Epicoccum* sp. NFW1 L-glutaminase activity enhanced by 16 % NaCl 65%, 53%, and 64.9% (Figure 5.9).

The molecular weight of *A. xylosoxidans* RSHG1, *B. subtilis* RSHU1, and *Epicoccum* sp. NFW1 L-glutaminases were 40, 60, and 38KDa approximately (Figure 4.71 & 4.72). Partially purified L-glutaminase produced by *A. xylosoxidans* RSHG1, *B. subtilis* RSHU1, and *Epicoccum* sp. NFW1 L-glutaminases were immobilized on 3.6% agar. The immobilized L-glutaminase showed stability up to 3 weeks. L-Glutaminase, produced by local indigenous bacterial strain *A. xylosoxidans* RSHG1, was stable at

wide pH and temperature conditions, with a high substrate affinity. Salt-tolerant bacteria may produce halophilic L-glutaminases, which can be used in the food industry and tolerate high salt concentrations. The bacterial and fungal strains produced relatively stable L-glutaminase, which have great potential to be used in the food and pharmaceutical industry, particularly as an antileukemic agent and as biosensors.

Keywords: *Achromobacter xylosoxidans* RSHG1; *B. subtilis* RSHU1; Characterization; *Epicoccum* sp NFW1; L-Glutaminase production; Molecular identification; Optimization; Purification.

1. INTRODUCTION

Microbial enzymes play a significant role in different industries that replace toxic chemicals in various industrial processes. Microbial enzymes are significantly required due to their economic production in huge quantities in a lesser time for use in many industrial processes, such as the food and beverages, pharmaceutical products, textile, and leather industry, and biodegradation and waste management (Anbu *et al.,*2015; Chapman *et al.,* 2018; Raveendran *et al.,* 2018). There is a high need for enzyme utilization produced by microorganisms. For instance, α-amylase in the baking industry, brewing and clarifying juices, and protease make meat tender (Raveendran *et al.,* 2018). β-galactosidase as a prebiotic component of food (Clark *et al.,* 2000). L-Glutaminase enhances food taste and treats certain cancers (Amobonye *et al.,* 2019; Mostafa *et al.,* 2021).

L-Glutaminase (L-glutaminase amidohydrolase EC 3.5.1.2), a hydrolase class of enzyme, removes an amino group of the substrate L-glutamine and produces L-glutamic acid and ammonia. L-Glutaminases, are widely distributed in living organisms (Iyer and Singhal, 2010; Kiruthika. 2013; Amobonye *et al.,* 2019). Present in all organisms, animals, plants, and microbes, ranging from prokaryotic bacteria to eukaryotic cells and higher animals like human beings. But microbial L-glutaminases are primarily used due to ease of processing and economic feasibility. Glutamine, nonessential amino acid, and the substrate for L-glutaminase are present in high quantities in the blood and fuels cell growth and nucleic acid synthesis (Wade 1980; Rao *et al.,* 2012). Mammalian cells also synthesize this enzyme which is involved in energy production by using L-glutamine as the significant respiratory fuel. So, many cancerous cells and efficiently generating normal cells show high L-glutamine consumption levels (Iyer and Singhal, 2009; Yoo *et al.,* 2020).

Currently, L-glutaminase has gained more attention concerning its application as an anti-cancerous agent in the pharmaceutical industry (Kumar and Chandrasekaran, 2003, Abdallah *et al.,* 2012; Jesuraj *et al.,* 2017) and for the enhancement of the taste of foods by the production of L-theanine (γ-glutamyl ethyl amide) and monosodium L-glutamate in the food industry (Amobonye *et al.,* 2019; Ito and Matsuyama, 2021; Ferreira *et al.,* 2021). L-Glutaminase, along with or as a replacement of L-asparaginase,

could be significant in the therapy for cancer treatment, mainly for acute leukemia (Rashmi *et al.,* 2012; Orabi *et al.,* 2021). Commercially, it is essential as an anti-leukemic and flavor-enhancing component that requires discovering good producers of L-glutaminase viable microbial strains (Nandakumar *et al.,* 2003; Orabi *et al.,* 2021) and economically viable bioprocesses for its large-scale production. An additional vital application of L-glutaminase is developing an L-glutamine biosensor made by immobilizing L-glutaminase to measure L-glutamine quantities monitoring in animals and hybridoma cells (Kashyap *et al.,* 2002; Bülbül and Karakuş, 2013; Albayrak and Karakuş, 2016). The immobilized L-glutaminase was used in the development of a novel biosensor for estimating L-glutamine for the pharmaceutical purpose on zinc oxide was produced by *Hypocria jecorina* (Albayrak and Karakuş, 2016).

At present, microbial L-glutaminase has found various medical and food industry applications. For industrial applications L-glutaminase is preferentially produced by microorganisms like bacteria, actinomycetes, fungi, and yeast than animal glutaminases due to increased stability and affordability (Amobonye *et al.,* 2019). Numerous bacterial species synthesize extracellularly and intracellularly. Important bacterial sources are *Bacillus sp*., *Pseudomonas*, *Actinobacterium* sp., and *E. coli* (Yano *et al.,*1988; Amobonye *et al.,* 2019). *Aspergillus, Penicillium,* and *Trichoderma* species are commonly used as fungal sources (Singh *et al.,* 2013; Ali *et al.,* 2009). Different species of actinomycetes also produce L-glutaminase (Teja *et al.,*2014). Plant tissues are also used to extract L-glutaminases, but the evidence of isolation from plants is unclear because of complex approaches (Yang *et al.,* 2017). Animals also have a complex organization; that's why not preferred for the isolation of L-glutaminases from animal tissues (Maharem *et al.,* 2020). Therefore, microorganisms are the primary sources of L-glutaminases. Two types of fermentation techniques are primarily used solid-state fermentation (SSF) process and submerged fermentation (SMF) process (Sabu *et al.,* 2000; Orabi *et al.,* 2020).

The role of production kinetics has been involved mainly in better-controlled fermentation on a large scale, such as industries. Different types of dynamics restrict fermentation processes and influence the growth or metabolism of microbes. That directly or indirectly may include environmental and self-parameters, physiological and psychological behavioral issues, intensity and rate of aeration, speed of agitation, expanse, and amount of dissolved oxygen (Kumar *et al.,* 2021; Orabi *et al.,* 2020), among which aeration and agitation considered as the furthermost grave parameters for

achieving best yield in fermentation (Iyer and Singhal, 2009). There may be three modes of achieving fermentation: batch fermentation, fed-batch, and continuous fermentation. Although large-scale construction and continuous application fermentation are used because of their higher throughput but due to many compensations such as stable genetics and less chance of adulteration, batch fermentation is also considered (Shuler *et al.,*1992). The production of L-glutaminase by solid-state fermentation increased manifolds (Iyer and Singhal, 2008).

The physical and chemical composition of the medium plays an essential role in the production of L-glutaminases. The chemical parameters effecting enzyme production are carbon sources like glucose, fructose, and lactose, and nitrogen sources such as casein, yeast extract, sodium nitrate, and other sources (Keerthi *et al.,* 1999; Jesuraj *et al.,* 2017). While the physical parameters, including the effect of pH, temperature, and incubation time, are optimized for maximum L-glutaminase production (Mostafa *et al.,* 2021). L-glutamine is used for L-glutaminase production as a nitrogen source or carbon/nitrogen source (Amobonye *et al.,* 2019). Soil is one of the most important reservoirs for L-glutaminase-producing bacteria under the submerged fermentation method (Aly *et al.,* 2017; Sinha and Nigam, 2016). Optimization of fermentation conditions and nutritional factors for L-glutaminase production under submerged fermentation enhances enzyme production (Nagaraju and Ram 2019; Aly *et al.,* 2017; Orabi *et al.,* 2020; El-Sousy *et al.,* 2021). L-Glutaminase was partially purified using ammonium sulfate and further purified on gel filtration column chromatography on Sephadex G-100 (Awad *et al.,* 2019; Aly *et al.,* 2017). L-Glutaminase was characterized for the kinetic parameters, i.e., incubation time, pH, temperature, and metal ions effect, to achieve the maximum activity (Sinha and Nigam 2016; Awad *et al.,* 2019; El-Sousy *et al.,* 2021). Immobilization studies were carried out to increase the stability and reuse of the L-glutaminase (Mahmod, 2016; Karahan *et al.,* 2014; Pandian *et al.,* 2015).

L-Glutaminase is involved in L-glutamine breakdown in microorganisms. Higher animals like human cells synthesize L-glutamine required for energy production as the major respiratory fuel (Mazat and Ransac, 2019). Therefore, various cancerous cells and efficiently reproducing cells show high L-glutamine consumptions (Cluntun *et al.,* 2010). Tumor cells, like leukemic cells, require high levels of L-glutamine. These cells cannot synthesize L-glutamine, so they need extracellular L-glutamine to keep them

alive and actively divide. Hence, the use of L-glutaminases degrades L-glutamine and kills the L-glutamine-dependent cancer cells (Fung and Chan, 2017).

L–Glutaminase has been used to treat leukemia, and one restriction in glutaminase therapy is the immune response generated against the L-glutaminase. Spiers and Wade 1976 used L-glutaminase for leukemia treatment. The human pharmacology and toxicology of L-glutaminase were described (Holcenberg *et al.,*1979). Although the enzyme is injected intravenously at the tumor site, it elutes from the kidneys after some time. When covalently linked with polyethylene glycol, the enzyme was active and very stable in the blood circulation and non–immunogenic compared with the native enzyme. Further, the enzyme can penetrate the peritoneal cavity. Extracorporeal injection of L–Glutaminase for treating acute lymphoblastic leukemia can be used (Giordano *et al.,* 1981).

Immobilization nanoparticles are a practicable and encouraging approach for the delivery of drugs (Hassan *et al.,* 2019). The availability of the enzyme in the blood is improved by using polymeric nanoparticles for immobilization (Jobmann *et al.,* 2002; Patra *et al.,* 2018). The human capillaries have a minimum diameter of 5-6 μm; thus, nanoparticle polymer does not cause clumping while circulating in the blood (Fullstone *et al.,* 2015; Chenthamara *et al.,* 2019). Polyhydroxybutyrate (PHB) is a polymer used to encapsulate various pharmaceutical drugs and enzymes. Therefore, it is preferentially used for immobilization (Chapman *et al.,* 2018). Another material used for immobilization is polyethylene glycol—a precisely used in medicine that enhances the shelf-life of proteins and enzymes. Immobilized L-glutaminase on polyethylene glycol reduces the toxicity, antigenicity, and immunogenicity during circulation in the blood (Shiraishi and Yokoyama, 2019). L-Glutaminase produced by marine *Halomonas meridiana* showed higher anticancer activity towards colorectal cancer cell lines (Mostafa *et al.,* 2021). The immobilization of enzymes by the entrapment method increases the thermostability of the enzymes. Natural polysaccharides like agar and agarose are used for the entrapment method. Agar is a natural polysaccharide that enhances the stability of the immobilized serine protease on 3% agar (Sattar *et al.,* 2018). L-Glutaminase was immobilized on the gelatin-agar matrix to increase the stability of the enzyme for colon cancer treatment (Jesuraj et al., 2015).

L–Glutaminase in the treatment of HIV is one of the most promising therapeutic applications (Kumar and Chandrasekaran, 2003). L-Glutamine depletion potentially inhibits the replication of Rauscher murine leukaemia retrovirus (RLV) in vitro. L–

Glutaminase from *Pseudomonas* sp. 7A effectively removes L-glutamine mice infected with RLV and was patented for HIV therapy and administered HIV replication in infected cells (Roberts and McGregor, 1991). L–Glutaminase in the treatment of HIV is a standout amongst the most encouraging remedial applications (Desai *et al.,* 2016; Klimberg *et al.,*1996). In other findings in HIV infection, GLS1-overexpressing as a result, increased extracellular vesicles (EVs) are observed, which was enhanced during neuroinflammation. Using specific GLS inhibitors effectively decreases EV release and shows a crucial role of GLS1 in EV release (Wu *et al.,* 2018).

L-Glutamine is generally produced by adding ammonium ions to the L-glutamic acid. Somewhat, from the transamination and subsequent amidation of α-ketoglutarate (Cruzat *et al.,* 2018). L-Glutamine is sequentially degraded into α-ketoglutarate by glutamate dehydrogenase. And α-ketoglutarate enters the tricarboxylic acid cycle (TCA) to provide energy. And the other molecular sources by transferring the carbon for oxaloacetic acid, citrate production, acetyl-CoA, and nitrogen for the synthesis of purine and pyrimidine, ultimately used for the DNA synthesis (Wang *et al.,* 2019), and reducing component NADPH to help the proliferative cells (DeBerardinis *et al.,* 2010). Immune cells need large quantities of exogenous supply of L-glutamine for their activation and functioning. It is observed that leukocytes utilize L-glutamine comparable to glucose consumption and higher levels in some cases (Shah *et al.,* 2020). The biosensor was prepared by immobilizing L-glutaminase on polyvinyl chloride (PVC) ammonium membrane electrode. An ammonium-selective glutamine biosensor. These biosensors detect L-glutamine in the biological samples such as healthy human serum, cerebrospinal fluid (CSF), and commercial glutamine capsules (Kumar and Chandrasekaran 2003). A biosensor was prepared by using L-glutaminase produced by *Hypocria jecorina*, to check the reproducibility and accuracy by immobilizing the enzyme on zinc oxide (ZnO) nanorod and chitosan (Amobonye *et al.,*2019).

L-Glutaminase is one of the most important enzymes used to enhance fermented foods. L-glutaminase imparts taste flavor to the foods (Dube *et al.,*2007; Viniegra-Gonzalez *et al.,* 2003; Vo *et al.,* 2020). L-Glutaminase is one of the essential enzymes used to enhance foods' taste. Soy sauce, Miso, and Sufu are considered delightful and palatable foods because of the presence of L-glutamic acid in their content (Orabi *et al.,* 2020; Ito *et al.,* 2013). L-Glutaminase is an essential ingredient of many fermented products of the food industry for enhancing the taste and aroma by hydrolysis of L-glutamine into glutamic acid that gives umami flavor (Vo *et al.,* 2020; Awad *et al.,* 2019). Salt

tolerant L-glutaminases were patented for use in industrial processes (Sabu, 2000). Salt-tolerant bacteria may produce halophilic L-glutaminases, which can be used in the food industry and tolerate high salt concentrations.

Due to the importance of L-glutaminase, the current research study was conducted to isolate new indigenous bacterial strains. With high L-glutaminase production, high pH stability, and salt tolerance, which can be used in food, and the development of biosensors in the pharmaceutical industry. For this comparative study, different microbial strains were be screened and isolated. Isolated bacterial strains were biochemically characterized, and 16S rDNA-based identification of the isolated bacterial strains was performed. Fermentation conditions were optimized, i.e., pH, temperature, carbon, nitrogen, and inducer sources for the maximum production of L-glutaminase. L-Glutaminases by selected strains were purified by using ammonium sulfate and column chromatography. Then partially purified and purified enzyme was characterized for the kinetic parameters, i.e., incubation time, pH, temperature, substrate, and metal ions effect on the enzyme activity. And immobilization of glutaminase to increase the stability and reuse of enzyme.

AIM AND OBJECTIVES

The aim of the following study is the production, purification, and characterization of microbial L-glutaminase.

The objectives of the study include:

1. Isolation and identification of L-glutaminase producing bacteria and fungi.
2. Optimization of cultural conditions for the production of L-glutaminase by selected bacterial and fungal strains.
3. Purification of L-glutaminases from selected strains.
4. Characterization of partially purified L-glutaminase of bacterial and fungal source.
5. Immobilization of L-glutaminase.
6. Comparative study of Microbial L-glutaminases.

2. REVIEW OF LITERATURE

Biocatalysts are mostly the protein in nature that work underneath gentle conditions because they require premium conditions for their capacity and safeguard the usefulness of natural associations. Catalysts are likewise considered natural synergist substance specialists framed in living elements, whereas catalysis is the organic response inside or outside to the body by specific catalysts. Comprising all cellular processes and accelerating the chemical reaction rates without connecting themselves to the final product. Compounds are positive for a substrate, implying they catalyze explicit response (Illanes *et al.,* 2008; Chapman *et al.,* 2018; Nikulin and Švedas 2021).

2.1. L-Glutaminase Enzyme

In humans, two tissue-specific isozymes of L-glutaminase are encoded by two genes present on chromosome 2 and 12. Kidney-specific located in chromosome 2, and liver-specific located on chromosome 12. Expression of Kidney-specific L-glutaminase is widely distributed in tissues such as kidney, brain and lymphocytes, resulting in ammonia release without generation of urea. While liver-specific occurs only in the periportal cells of the postnatal liver, it takes part in hepatic ureagenesis. Both vary in structure and kinetics from each other (Liang et al., 2021).

In *Escherichia coli*, there are 2 genes for L-glutaminase yneH (308 aa) and ybaS (310 aa). The ybaS gene encodes an acidic L-glutaminase that is not active in the physiological pH. On the other hand, the yneH gene encodes an enzyme which is active on the physiological pH (Irajie et al., 2A16).

A recombinant L-glutaminase from *Geobacillus thermodenitrificans* DS expressed in *E. coli* was compared with the human kidney-specific GLS-K by ConSurf and TM-align servers for evolutionarily conserved residues and structural domains. Results showed less than 40% of the amino acid were identical, but the superimposed monomers of both enzymes exhibited ~94% structural identity. On the active site, the positional differences occur at residues Ser65, Asn117, Glu162, Asn169, Tyr193, Tyr245, and Val263 found in the bacterial enzyme were also conserved in the human GLS-K (Shah et al., 2019).

L-Glutaminase assumes a commanding role inside the mammalian body where it is segregated into kidney type (GLS 1) and liver type (GLS 2) (Heine *et al.,*1987). L-Glutamine is a principal substrate for the amalgamation of protein and nucleic acid. The tumor cells deprived of glutamine requires a combative supply of glutamine for its

development. The utilization of L-glutaminase eliminates the glutamine levels and causes the selective cell death of the tumor cell. Because of this glutamine's hostility to tumor properties, endeavors have been made to separate bacterial Glutaminase for treatment of tumors. L-Glutamine metabolic processes play a crucial role in energy production. It acts as a precursor of purine and other important cellular products required for DNA and protein synthesis. It maintains the cell's redox potential, which is needed for actively dividing or cancerous cells. (Guzman *et al.,*1996; Orabi *et al.,* 2020; Matés *et al.,* 2020).

As of late, L-glutaminase has pulled in much consideration for proposed applications in pharmaceuticals as hostile to the leukemic operator (Kumar and Chandrasekaran, 2003, Abdallah *et al.,* 2012; Amobonye *et al.,* 2019) and sustenance industry flavor upgrading specialist (Loeliger 2000; Mosallatpour *et al.,* 2019). L-glutaminase in blend with or as an option in contrast to asparaginase could be centrality in catalyst treatment for malignant growth, particularly intense lymphocytic leukemia. It's business significance as anticancer and flavor upgrading specialist requests, not just the scan for better yielding suitable strains (Nandakumar *et al.,* 2003), yet additionally monetarily feasible bioprocesses for its huge scale creation. Another imperative utilization of L-glutaminase is developing biosensors to estimate glutamine quantities in mammalian and hybridoma cell societies without independently estimating glutamic acid (Kashyap *et al.,* 2002; Albayrak and Karakuş 2016).

2.2.History of L-Glutaminase

Research takes a shot at the enzyme L-glutaminase, which was begun in 1956 A.D. Gutman and Yue coincidentally found the significance of this enzyme in 1963 amid their work on the estimation of all-out uric acid-N15 improvement their abundance in every one of the four uric acid nitrogen. These researchers found that essential gout is because of powerlessness or less immunity of the body to finish glutamine digestion. Because of fragmented glutamine digestion, they are not used by the liver for the generation of alkali; hence additional glutamine begins to shape uric acid lastly convert into uric acid stone. They also recommend that variation from the norm in glutamine digestion is critical in the pathogenesis of essential gout. Researchers look into quarrelsome to find the protein further.

Right off the bat, the counter malignant growth movement of the enzyme was proposed by Greenberg in 1964 (Greenberg *et al.,* 1964). In 1966 just after two years, El-Asmar

and Greenberg purified the L-glutaminase enzyme from microorganisms *Pseudomonas*. They found that compounds captured the development of murine carcinomas and impacted creatures' survival (El-Asmar *et al.,* 1966). In 1970 Robert and his associate discovered that L-glutaminase detached from Gram-negative *Bacilli* rods captured the development rate of Ehrlich ascites carcinoma (kind of breast cancer) (Roberts et al.1970). Two years after it was reported, L-glutaminase isolated from microscopic soil organisms has a place with *Achromobactereace* that specifically murdered human leukemia cells in tissue culture (Roberts *et al.,* 1972). Imada *et al.,* 1973 contemplate the action, nearness, and circulation of L-glutaminase in numerous microorganisms. Their examination looked at 464 bacterial isolates, 1326 yeast isolates, and 4185 fungal isolates. (Imada *et al.,* 1973).

In Japan, soya sauce is conventional sustenance to expand the flavor of sauce microorganisms like yeast or a fungus mold. Yamamoto and Hirooka disengage two strains of *Aspergillus sojae* and recommend that chemicals assume an essential job in improving nourishment taste (Yamamoto and Hirooka, 1974).

2.3. Glutamine, the Substrate of L-Glutaminase

Glucose, an imperative metabolite required by all body cells to complete the ordinary working, goes about as a fuel for cells. A steady motion of glucose is kept up to counteract the unfavorable impact on the body. Like glucose, glutamine is also the basic for assortments of cells (Cruzat *et al.,* 2018).

The glutamine is most copiously present as a free amino acid in our body. L-Glutamine is abundantly present in our diet. It is also synthesized by the action of enzyme L-glutamine synthetase from L-glutamate and ammonium ions, mainly in skeletal muscle, hepatocytes, and adipocytes. The essential wellspring of glutamine in skeletal muscle had presented 10-100folds abundance when contrasted with some other amino acids (Watford, 2015). The physiology and significance of L-glutamine in cell expansion and cell working are presently generally acknowledged. The significance of glutamine to cell survival and expansion *in-vitro* was first announced by(Ehrensvard *et al.,* 1949).

2.4.Pathways of glutamine in major organs

Glutamine is one of the non-essential amino acids and probably the most studied one because of its sweeping scientific attention and reputation among cultures of either cell, tissue, plant, or animal. This amino acid is sole in providing fuel for rapid and instant growth and development. Some studies suggested that glutamine can also be under conditionally essential amino acids in some critical conditions. Oral or non-oral

feedings supplement the L-glutamine to boost recovery in ill situations (Meynial-Denis 2016; Cruzat *et al.,* 2018).

The glutamine trail all over the human body is similar in one way, such as its descendant or successor is glutamate in most cases. Then glutamate can be transformed into either glucose or glutathione and maybe into GABA (gamma-aminobutyric acid) (Albrecht *et al.,* 2010; Schousboe *et al.,* 2014).

2.4.1. Glutamine in the Central Nervous System

The manifestation of glutamine has been definite by many scientists (Castegna *et al.,* 2018). In the astrocytes of the central nervous system, the originator of glutamine in CNS is glutamate, devouring energy in the form of adenosine triphosphate, abbreviated as ATP. It must be converted back to glutamate again by the enzyme glutaminase (Shulman *et al.,* 2001; Schousboe *et al.,* 2014). In this process, glutamate escapes from neurons and enters astrocytes, where it forms glutamine by adding ammonia. The hydrolysis of glutamine causes the formation of glutamate in neurons; thus, one cycle completes in this way. According to nuclear magnetic resonance spectroscopic studies, there is an association between breakdown and overall metabolic rate of glutamate and glucose in the cortex of cerebral fluid (Sibson *et al.,* 2001; Schousboe *et al.,* 2014).

2.4.2. Gonads (Ovary and Testis)

Like most other body cells, ovaries and testis also revenue energy from glucose and glutamine sources; in the normalized states, they harvest energy from them and maintain normoxic circumstances in the gonads. Studies have been done, and experiments were primarily conducted on rats' gonads to check the effect of glutamine metabolism on the normal and cancerous cells to diagnose, treat and testify many diseases. Ovarian cancerous cells acquire energy by increasing glucose and amino acid metabolism, specifically glucose and glutamine. In the treatment, these pathways usually are rehabilitated, so there is an elevation of glutamine in serum, tumor tissues, and the patients' urine (Zhou *et al.,* 2010).

L-Glutamine was taken by Sertoli cells of testis cross the plasma membrane by ASCT2 (Alanine, Serine, Cysteine Transporter) amino acid-specific transporter and converted to glutamate in the mitochondria of Sertoli cells. This glutamate enters the TCA cycle ultimately in the mitochondria by aiding pyruvate from glucose metabolism in the cells, thus, ATP is engendered (Mateus *et al.,* 2018).

2.4.3. Heart

Being the most abundant amino acid in the circulatory system, L-glutamine has considered its defensive properties in the heart. According to some studies, in the heart tissue the main effect of L-glutamine is normally supposed to be facilitated through its anaplerotic metabolism to citric acid cycle (CAC) intermediates. It also replenishes the citric acid cycle's intermediates and affects the hexosamine biosynthetic pathway (Lauzier *et al.,* 2013; Durante, 2019). The major energy releasing pathways may be one among them. L-glutamine reduces the available pyruvate, constraining the anaplerotic carboxylation of pyruvate, which manipulates the TCA cycle. This indicates the process of anaplerosis done by L-glutamine specifically affecting the TCA cycle in the heart's cells (Fulop *et al.,* 2007; Lauzier *et al.,* 2013; Durante, 2019).

2.4.4. Immune System

The immunogenic cells such as neutrophils, macrophages, and lymphocytes inside the immune system consume glutamine at a higher rate for the sake of energy (Rogeri *et al.,* 2020; Shah *et al.,* 2020). These cells possess a high amount of phosphate-dependent L-glutaminase to halt glutamine. In the immune system, L-glutamine performs many functions such as multiplication of diversification of T and B cells, respectively, phagocytosis of macrophages (Newsholme et al., 2001; Shah et al., 2020), marking of antigenic cells, and synthesis of cytokines, synthesis, and apoptosis of neutrophils (Garcia et al.,1999). The glutamine may be converted to CO2, lactate, aspartate, and glutamate. In this case, CO2 requires special conditions, and aspartate may be produced by the TCA cycle bustle (Hertz et al., 2017; Cruzat et al., 2018). If glutamine does not perform its function, rather converted to glutamate, glutamate will perform many important functions, including transamination of neutrophils, lymphocytes, macrophages, etc. (Newsholme *et al.,* 2003).

2.4.5. Intestine

Glutamine is converted to glutamate in the human intestine by releasing ammonium ions. L-glutamate participates in the formation of 2-oxoglutarate, and as a result, alanine becomes a terminal product that is transported to the liver by the hepatic portal vein. L-alanine is formed from pyruvate coming from malate, which is produced in TCA and hence glutamate-pyruvate interference produced oxoglutarate and alanine, among which 2-oxoglutarate becomes part of TCA again, and pyruvate forms alanine with the aid of an enzyme known as alanine aminotransferase (Windmueller *et al.,*1978; Curi *et al.,* 2007). In this way, electrons are transported to mitochondria, incorporating with

electron transport chain for ATP production from $FADH_2$ and NADH (Kimura *et al.,*1998; Curi *et al.,* 2007).

2.4.6. Kidney

The chief contributor of ammonia inside the kidney is glutamine. The presence of phosphoenolpyruvate carboxykinase (PEPCK) inside the nephron indicates that there must be linkages and associations of gluconeogenic and ammoniagenic routes inside the kidney (Schoolwerth et al., 1991; Tannen et al., 1990). The main track of glutamine breakdown or metabolism inside the kidney is the deamination of glutamine inside the cell's mitochondria, initiated by glutaminase and glutamate dehydrogenase. In contrast, glutamate is converted to α-ketoglutarate by alanine aminotransferase. For balancing the acid-base concentration inside the kidney, the glutamine produces NH_3 with the help of the enzyme glutaminase, which is phosphate-dependent (PDG) (Gstraunthaler *et al.,* 2000; Lister *et al.,* 2018). Ammonia is disseminated toward collecting tubule and finally excreted out along with urine out of the body in the form of ammonium ion (NH_4^+), which is formed by ammonia reduction. This trail produces only one ammonium ion. For this purpose, H^+ arises from the disassociation of carbonic acid (H_2CO_3), the remaining part that is HCO_3^- maintain the body's pH where required (Curthoys *et al.,* 2001; Lister *et al.,* 2018).

2.4.7. Liver

Being the dominant site of nitrogen metabolism in L-glutamine and ammonia, the liver plays a crucial role in glutamine transformation from and to all over the body (Häussinger, 1990; Shi *et al.,* 2018). L-glutamine acts as a precursor of glutamate, and NH_3 is produced. With the utilization of ATP, this ammonia interacts with HCO_3^- to form Carbamoyl phosphate with the help of carbamoyl phosphate synthetase (CPS I). This enzyme also maintains glutamate concentration because it requires Nacetylglutamate for its activation (Häussinger, 1990; Shi *et al.,* 2018).

Carbamoyl phosphate synthetase II (CPS-II) is the isoform of Carbamoyl phosphate synthetase I, forming carbamoyl phosphate. Still, the reactants and products are different as in the case CPS I in such a way that in this case, glutamine is combined with HCO_3^- and water and utilize ATP again to form carbamoyl phosphate and glutamate as a primary product and inorganic phosphate and ATP for the sake of fuel as by-products. Glutamine metabolism occurs in periportal cells because of more enzyme concentration at this site, and ultimately glutamine forms carbamoyl phosphate.

Glutamate is also produced at the periportal site of the liver, but it may take part in the formation of amino acid or may form phosphoenolpyruvate, or it may enter to TCA cycle (Curthoys *et al.,* 1995; Shah *et al.,* 2020). In the presence of glutamate-derived carbon, glutamine is formed into the liver, the formation achieved by glutamine synthetase, glutamine can release from the liver. According to some studies, arginine catabolism causes the formation of glutamate, and ultimately glutamine is formed along with ADP and Pi production. (De-Souza *et al.,* 2005; Cruzat *et al.,* 2018).

2.4.8. Muscles

High levels of L-glutamine are present in the blood plasma and skeletal muscles. Muscle cells synthesize L-glutamine from α-ketoglutarate, intermediate TCA cycle (Raizel and Tirapegui, 2018). Like hepatocytes, muscles also showed an adaptive regulation of amino acids such as glutamine (Gulve *et al.,* 1991). The low amount of glutamine in muscle cells enhances glutamate entry, ultimately acting as an L-glutamine precursor (Low *et al.,* 1994). Reduction in glutamine levels mediated by vigorous heavy exercise is related to lowering the number of immune cells in the blood circulation (Raizel and Tirapegui, 2018).

2.4.9. Pancreas

The β-cells of the pancreas significantly impact the regulation of carbohydrates, lipids, and proteins due to insulin production at this site. Glucose does not involve in the release of insulin-stimulated glucose. Instead, it causes oxidation of insulin catalytically by forming 2-oxoglutarate and glutamate. This glutamate transformed into γ-amino butyric acid (GABA), an essential signaler with glutamic acid decarboxylase. (Rubi *et al.,* 2001). The insulin emission is also dependent on GABA (Winnock *et al.,* 2002; Jenstad and Chaudhry, 2013; Choat *et al.,* 2019).

2.5. Glutamine and Cancer Cell Metabolism

Glucose and glutamine metabolism have been integrated by a molecule synthesis known as hexosamine. The primary mechanism behind this metabolism involved transferring the amino group from glutamine to six carbon sugar with amidotransferase. By this mechanism, it can be clarified that normal or cancerous cells both require an uptake of carbon and nitrogen from glucose and glutamine sources, respectively. Carbon is most important concerning normal cell metabolism, while with those of cancerous tumorous cells, nitrogen is an essential element in normal and cancerous cells. It is also a major component of glutamine (Gaglio *et al.,* 2009; Shariatpanahi *et al.,* 2019). Most cell cycles either involved adding or removing nitrogen possessing

amination and deamination, respectively. It can be practically observable that if a cell is deprived of nitrogen, there will be an effect on the greater extent of the growth of a cell that will defiantly decline (Berardinis *et al.,* 2007). For example, some hexosamines like uridine diphosphate linked with acetylglucosamine do not grow or proliferate in the absence of nitrogen (Lagranha *et al.,* 2008). If L-glutamine introduction occurs experimentally or by nature, the cell can resume normal or uncontrolled growth in normal and tumor conditions. In the cancerous cell, glutamine insertion is assisted by some transporters such as SN_2 and ASC. After the entrance, this nitrogen assembled to form a macromolecule. The enzyme glutaminase inside the cell will act upon this macromolecule, so glutamate formation occurs.

The forerunner for glutathione synthesis is glutamate, which must be moved towards the mitochondria for further processing. Inside mitochondria, a-ketoglutarate bioproduction occurs from glutathione abetted by biocatalyst known as glutamate dehydrogenase. It can be supported by aminotransferases depending upon the source and supply of cells and products of its pathways. The overall regulatory mechanism of the enzyme glutaminase and glutaminase-2 may be backed by c-Myc and p53, respectively. Glutamine synthetase (GS) expression flourished by β-catenin (β-cat). So, the regulation of enzymes is critical for overall pathways. The regulation and activation of enzymes also require a specific site inside the cell (Hensley *et al.,* 2013; Chen *et al.,*2018; Li *et al.,* 2021) (Figure 2.1).

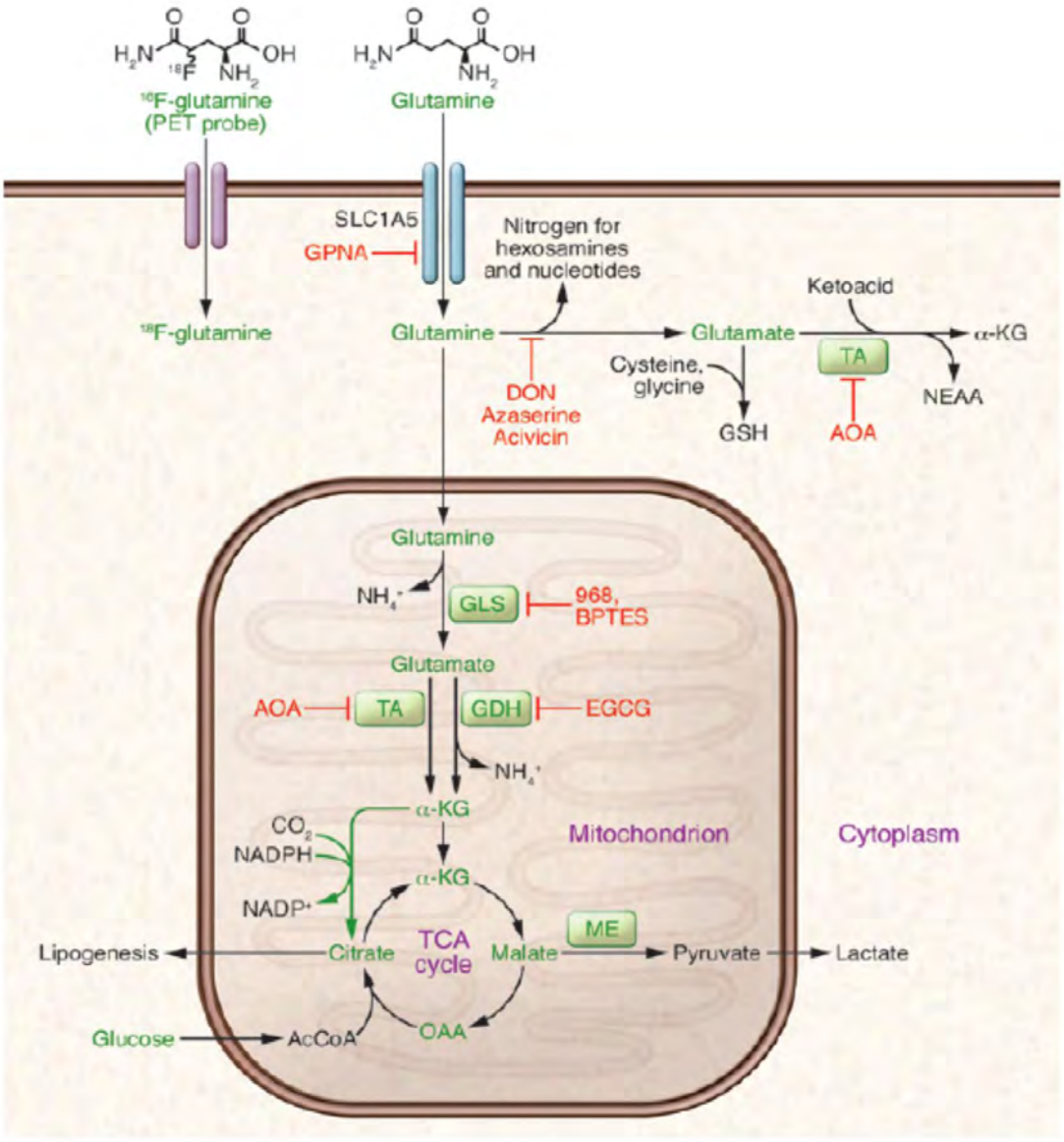

Figure 2.1 Glutamine metabolic pathway used for clinical imagery and cancer treatment. Glutamine enters the cells by SLC1A5 transporter and other transporters. Later by the number of processes, carbon and nitrogen of glutamine are provided to metabolic pathways required for cell growth and division (Hensley *et al.*, 2013; Li *et al.*, 2021).

2.5.1. Regulatory Factors in L-Glutamine Metabolism

During cancer, glutamine digestion is exceptionally managed and regulated by a few factors: MYC, p53, Ras, and HIF (hypoxia-inducible factor). This regulation keeps up malignancy development and is one reason for carcinogenesis (Wang *et al.,* 2021).

2.5.1.1. MYC as a Regulator

The proto-oncogene MYC directs about 15% of qualities in genomes from flies to people (Dang *et al.,* 2006). It incorporates N-MYC, C-MYC, and L-MYC, deregulated, transformed, or intensified in most human tumors (Vita *et al.,* 2006; Madden *et al.,* 2021). They can be actuated by the mitogenic flag and drive cell expansion. C-MYC is extensively deregulated in numerous human malignant growths, N-MYC articulation is increasingly confined to neural tumors, and L-MYC is prevalent in little cell lung disease. In certain malignant growths, MYC intensification is engaged with glutamine fixation (Wise *et al.,* 2008; Wise and Thompson, 2010). Glutamine enslavement is related to MYC-instigated redirection of glucose carbon far from mitochondria that are a consequence of LDHA (lactate dehydrogenase) initiation. All the more explicitly, MYC-acceptance prompts the preoccupation of glucose-got pyruvate far from mitochondria and is changed over to lactate (Wise *et al.,* 2008). Accordingly, MYC-changed cells become subject to glutamine anaplerosis for keeping up the mitochondrial respectability and TCA cycle work. Likewise, MYC will probably build exercises of glutaminase1 (GLS1) and glutamine synthetase. Also, MYC likely ties to the advertiser components of glutamine transporters, which is related to upgraded dimensions of glutamine transporters, e.g., SLC7A5 and ASCT2 (Gao *et al.,*2009). N-MYC overexpression invigorates mRNA and protein articulation of the synergist subunit of GCL (glutamate-cysteine ligase). It causes rate-restricting advances in Glutathione (GSH) biosynthesis, which builds GSH levels and protects against oxidative harm (Gao *et al.,* 2009). In this way, focusing on MYC can give a restorative window to malignant growths that have MYC enhancement. Down-guideline of N-MYC articulation has been demonstrated to actuate apoptosis and diminish expansion and neuronal separation in neuroblastoma cells in vitro (Westermark *et al.,* 2011). Comparative outcomes are likewise seen in leukemia, osteosarcoma, squamous carcinoma, hepatocellular carcinoma, and pancreatic cancer (Felsher *et al.,* 2010).

2.5.1.2. p53 as Regulator

one predetermined cell outcomes, p53 quality is observed to be changed or disassembled in most human tumors. It is generally acknowledged that p53 is a tumor silencer quality, inciting cell cycle capture and apoptosis under DNA harm, hypoxia, or oncogene enactment conditions (Sherr *et al.,* 2000; Zhao *et al.,* 2009). Typically, p53 quality is situated in the atomic (Nieminen *et al.,* 2013). It translocates to cytosol and ties to its cytosolic MDM2 after interpretation, and this coupling hinders p53

enactment. Upon stress signal, p53 is phosphorylated at serine. It discharges from MDM2 (twofold mouse moment 2 homolog), and after that actuates its downstream factors, for example, p21, BAX (Bcl-2 related X protein), PUMA (p53 upregulated modulator of apoptosis), NOXA (phorbol-12-myristate-13-acetic acid derivation instigated protein 1) or PTEN (phosphatase and tensin homolog) to play out its capacity. GLS2 has been demonstrated to be an objective of p53 (Lane and Levine, 2010; Suzuki *et al.,*2010). By up-directing GLS2 articulation, p53 expands GSH levels and decreases ROS levels, which hinders tumorigenesis. Sadly, p53 is changed in numerous diseases, which demonstrates loss of capacities. Aside from taking a shot at GLS2, p53 is, as of late, answered to quell articulation of SLC7A11, a key part of the cysteine/glutamate antiporter. SLC7A11 intercedes the trade of extracellular cysteine to intracellular glutamate and is overexpressed in a few human tumors (Jiang *et al.,*2015). Likewise, p53 can stifle GLUT1 (glucose transporters) and GLUT4 and hinder PI3K (phosphatidylinositol-3 kinase)–AKT (protein kinase B) and mTOR pathways. These impacts of p53 result in cell development suppression and afterward invert the malignant growth (Lane and Levine, 2010). In light of its significantly inhibitive job in malignancies, it is incredibly fascinating in attempting to reestablish or increment p53 action in p53 changes or loss of capacity diseases.

2.5.1.3. Ras as a Regulator

Oncogenic Ras proteins are recognized in 25% of human malignant growths and are associated with metabolic changes. Ras builds usage of the carbon spine and amino-nitrogen moieties of L-glutamine and advances glucose utilization (Gaglio *et al.,* 2011). Ras-driven diseases can fulfill their dietary needs by enacting liquid stage endocytic supplement take-up and elevating angiogenesis to build the tumor blood supply (White *et al.,* 2013). It has been uncovered that glutamine is the real carbon hotspot for the TCA cycle when Ras is enacted (Ying *et al.,* 2012). The reconstructing of glutamine digestion is interceded by oncogenic K-Ras in human pancreatic ductal adenocarcinoma. K-Ras can quell glutamate dehydrogenase articulation and increment aspartate transaminase articulation (Son *et al.,* 2013). In the meantime, glutamine consumption can actuate expansion capture of K-Ras-changed cells (Gaglio *et al.,* 2009).

2.5.1.4. HIF as Regulator

HIF-1, a heterodimer, is made out of HIF-1α and HIF-1β subunits. The articulation and action of the HIF-1α subunit are firmly directed by cell oxygen focus. The articulation

dimensions of the α subunit are expanded amid hypoxia while the β subunit is constitutively communicated (Chen *et al.,* 2012).

HIF-1 assumes a key job in reconstructing malignant growth digestion by initiating translation of numerous qualities that encode glucose transporters just as glycolytic proteins, and it additionally advances angiogenesis (Chen *et al.,* 2012). HIF-1 is vital and adequate for lessening mitochondrial oxygen utilization in hypoxia by initiating PDK1 (pyruvate dehydrogenase kinase 1), tumor cells can survive under hypoxia (Papandreou *et al.,* 2006). In any case, if PDK1 movement is repressed, proceeded mitochondrial breath and coming about oxidative pressure will initiate cell demise. Certain tumors are regularly ineffectively vascularized and contain district hypoxia. This extraordinary tumor microenvironment upgrades HIF-1α over-articulation in most human malignant growths and their metastases, where HIF-1α actuates quality articulation to advance survival. Under hypoxia, diminished F1Fo ATPase movement can build phosphate fixation in mitochondria (Chiu *et al.,* 2019). Expanded phosphate advances GAC (extended kidney glutaminase variation)- based glutaminase action, which improves glutaminolysis to give expanded metabolic and biosynthetic requirements for presenting specific preferred standpoints to harmful cells. The previously mentioned metabolic guideline makes dangerous cells progressively helpless to GLS inhibitor and, consequently, focuses on malignancy treatment. Amid hypoxia, there is a decline in glucose-determined citrate because of diminished pyruvate dehydrogenase action and an expansion in α-KG levels brought about by decreased α-KGDH (ketoglutarate dehydrogenase) movement. These progressions drive the switch response at IDH (isocitrate dehydrogenase) and afterward increment citrate generation, lastly influence cells to depend on reductive glutamine digestion in the lipid combination. LKB1 (liver kinase B 1) is a serine/threonine kinase frequently inactivated in human disease. It has been shown that loss of LKB1 influences malignancy cells to depend on HIF-1α in the ATP supply, which incites an expansion in glycolysis and glutamine utilization. Sensibly, knockdown of HIF-1α in cells without LKB1 advances a lessening in glutamine utilization by these cells (Katt *et al.,* 2017; Li *et al.,* 2017).

2.6. Sources of Glutaminase

Enzymes are the biocatalysts that have gained chief importance inside and outside the body, causing multiple chemical reactions. They play a crucial role and are chiefly important industrially, economically, socially, and commercially. The enzyme got

much attentiveness after an investigation in the therapeutic arena. Major sources involve bacteria, actinomycetes, yeast, and fungi and animals, plants. The most authentic and feasible approaches are microbes like yeast, fungi, and bacteria (Binod *et al.,* 2017).

2.6.1. Bacterial Sources For L-Glutaminase Production

Numerous bacteria synthesize extracellular and intracellular glutaminases, such as *Bacillus*, *Actinobacterium*, *E. coli,* and *Pseudomonas*. These organisms can be unglued from many sources such as soil (which serves as an essential and significant source for microbes by acting as a medium for nutrients), stagnant or motile water bodies such as marine environment, rivers, ponds, lakes, etc. The air may also become a microbial source, but it depends on the location and flow of air at a particular site or environment (Sabu, 2003; Priyanka *et al.,* 2019). Marine bacteria are more exceptional in their natural potential and character because they are more actively involved in therapeutic tasks than terrestrial bacteria. The bacteria, in this case, maybe *Micrococcus*, *Beauveria bassiana*, *Vibrio*, and *Pseudomonas fluorescent* (Chandrasekaran *et al.,* 1997). Many marine bacteria like *Pseudomonas fluorescens*, *Vibrio cholerae*, *V. costicola*, *Micrococcus luteus K-3,* and *S. maltophilia* NYW-81L-glutaminase can tolerate salt at a very high concentration. Salt tolerant L-glutaminases are preferably used in the food industry (Amobonye *et al.,* 2019; Patel *et al.,* 2021).

The basic protocol for isolation of this enzyme includes isolation and screening of glutaminase-producing bacteria, fermentation, centrifugation, chromatography or ion exchange, gel filtration, and characterization of the enzyme (Gerber *et al.,* 1994), (Ando *et al.,* 1989).

2.6.2. Fungal Sources for L-Glutaminase Production

The primary sources of fungal glutaminases are *Aspergillus* and *Trichoderma* species (El-Sayed *et al.,* 2009). The approaches towards enzymatic production involved fermentation techniques, mainly of two types, i.e., solid-state fermentation (SSF) and submerged fermentation (SMF). In most cases, submerged fermentation is practiced for commercial production and industrial use. Submerged fermentation has been used to produce L-glutaminases by *Tilachlidium humicola* and *Penicillium notatum* (Shindia *et al.,* 2007), *A. nidulans*, *Aspergillus oryzae* (Yano *et al.,*1988; Thammarongtham *et al.,* 2001), *Aspergillus sojae* (Hirooka *et al.,* 1974; Ito *et al.,* 2013), *Verticillium malthousei* (Imada *et al.,*1973). The nutrition source for submerged fermentation can be taken from wastes such as agro-waste. In this way, L-glutaminase production may become eco-

friendly (Soren *et al.,* 2020). The basics of the procedure are the same as in bacteria. Fungal L-glutaminase production involves the fermentation of fungal strains. Then centrifugation, filtration, and chromatographic techniques such as ion exchange. This approach is to isolate trans glutaminases (Khalil *et al.,* 2020).

2.6.3. Actinomycetes

Actinomycetes have the sole cultural characters, physiology, morphology, and biochemical properties; actinomycetes also act as a source of enzymes. Actinomycetes, a nutrition mode, are heterotrophic filamentous in their cellular organization. Containing higher guanine and cytosine in its DNA made up of gram-positive bacteria. Actinomycetes from marine and terrestrial the basic steps for isolation are the same as fungi and bacteria. The glutaminase-producing actinomycetes include *Streptomyces avermitilis*, *Streptomyces canarius, Micromonospora* (Abdallah *et al.,* 2013; Teja *et al.,* 2014; Reda *et al.,* 2015; Jagannathan *et al.,* 2021). Actinomycetes are well known for producing multiple chemical compounds, which are of great importance medically and therapeutically (Reda *et al.,* 2015).

2.6.4. Floral source

Plant tissues also extract L-glutaminases involving steps slightly different from a microbial source. For example, plant tissue extraction, followed by filtration, leads to an extract free from impurities or extra materials. the filtrate is then subjected to centrifugation to separate the protein part containing the enzyme. Chromatography can be performed for screening and testification and to get extra pour enzymes (Falcone *et al.,* 1993). The shreds of evidence of extraction glutaminases from plants are frequently used due to less practicable approaches.

2.6.5. Faunal Source

Animals have complex organization; animals are not well known to put enzymatic isolation from their tissues in practice. Animal L-glutaminase extraction is similar to that of plants in which the first step is the homogenization of tissue. Then after filtration, dialysis, and chromatography is done multiple times depending upon the separation of different animals to attain transglutaminases (Botman *et al.,* 2014).

2.7. Production of L-Glutaminase from Microbial sources

Kikkoman Corporation, the Japan researcher, performed remarkable work on the L-glutaminase enzyme. Many microbial source glutaminases are commercially available (Table 2.1).

Table 2.1 L-Glutaminase commercially available

Sr.	Manufacturer	Brand Name	Species Name
1.	Ajinoto Co Inc, Japan	Glutaminase	*Bacillus sp.*
2.	Amano enzymes inc, Japan	Glutaminase F "Amano 100"	*Bacillus subtilis*
3.	Biocatalysts, UK	Flavorpro B73P	*Bacillus sp.*
4.	Enzyme Development Corporation, Japan	Enzeco	*Bacillus subtilis*
5.	Kikkoman Corporation, Japan	GLN	*Bacillus sp.*

2.7.1. Microbial Sources of L-Glutaminases

Microorganisms are widely used in the detachment of remedial protein because of their assorted biochemical variety. After discovering L-glutaminase and its restorative movement, researchers begin looking through the microorganisms that discharge this protein. This compound likewise assumes a significant job in nitrogen digestion in the prokaryotes and eukaryotes (Katikala *et al.,* 2009). L-Glutaminase is available in plants tissues creatures and broadly appropriated in organisms, such as microorganisms, parasites, actinomycetes, yeasts, fungi, and humans. L-Glutaminase can be confined from plants and animal tissue; however, microbial glutaminase takes much consideration. Microbial sources are generally utilized in enterprises since they can undoubtedly be used in biotechnology for their extensive scale creation, effectively balanced, with little size and consistency. Microbial sources have an advantage over different sources in that it requires a brief period and little space for huge scale creation (Binod *et al.,* 2017). Many organisms were screened and testified for L-glutaminase action by (Imada *et al.,* 1973). In the wake of discovering helpful movement of chemicals, they were confined and refined from microbial sources (Eremenko *et al.,*1975). Their structure was likewise contemplated (Ammon *et al.,* 1988; Tanaka *et al.,* 1988; Wlodawer *et al.,* 1977).

Chemicals additionally have application in sustenance ventures, so salt and thermo-tolerant glutaminase were required. The screening was disagreeable, which discovered that the perfect hotspot for salt and thermo-tolerant microorganisms normally happened in marine conditions (Sato *et al.,* 1999). A few endeavors were made to segregate corona-tolerant protein (Moriguchi *et al.,* 1994; Sabu *et al.,* 2000). Because of the

extracellular nature of this catalyst, they are all the more productively assault the crude substrate and effectively sanitized. A few microorganisms screened for L-glutaminase are recorded (Table 2.2, 2.3, and 2.4).

Table 2.2 Lists of microorganisms screened for L-glutaminase activity.

Bacteria		
Sr. No.	Name of organisms	Reference
1	*Bacillus licheniformis*	(Cook *et al.,* 1981)
2	*Bacillus subtilis*	(Sathish & Prakasham, 2010)
3	*Stenotrophomonas maltophilia*	(Wakayama *et al.,* 2005)
4	*Lactobacillus brevis*	(Woraharn *et al.,* 2014)
5	*Lactobacillus fermentum*	(Woraharn *et al.,* 2014)
6	*Meyerozyma (Pichia) guilliermondii*	(Aryuman *et al.,* 2015)
7	*Micrococcus luteus*	(Moriguchi *et al.,* 1994)
8	*Providencia sp*	(Iyer and Singhal, 2009)
9	*Pseudomonas aurantiaca*	(Lebedeva *et al.,* 1981)
10	*Pseudomonas fluorescens*	(Eremenko *et al.,* 1975)
11	*Pseudomonas nitroreducens*	(Tachiki *et al.,* 1998)
12	*Penicillium politans*	(Ali *et al.,* 2009)
13	*Pseudomonas sp*	(Kumar and Chandrasekaran, 2003)
14	*Pseudomonas stutzeri*	(Athira *et al.,* 2014)
15	*Streptomyces griseus*	(Muthuvelayudham et al. 2013)
16	*Vibrio costicola*	(Prabhu and Chandrasekaran 1999)

Table 2.3 Yeast screened for L-glutaminase activity.

Yeasts		
Sr. No.	**Name of organisms**	**Reference**
1	*Cryptococcus albidus*	(Fukushima & Motai, 1990)
2	*Cryptococcus nodaensis*	(Sato et al. 1999)
3	*Saccharomyces cerevisiae*	(Penninckx *et al.,* 1985)
4	*Zygosaccharomyces rouxii*	(Kashyap *et al.,* 2002)

Table 2.4 List of fungi screened for L-Glutaminase activity.

Fungi		
Sr. No.	**Name of organisms**	**Reference**
1	*Actinomucor elegans*	(Chou et al. 1993)
2	*Aspergillus oryzae*	(Yano *et al.,* 1988)
3	*Aspergillus flavus*	(Nathiya *et al.,* 2011)
4	*Aspergillus sojae*	(Thammarongtham *et al.,* 2001)
5	*Beauveria* sp.	(Sabu *et al.,* 2000)
6	*Beauveria bassiana*	(Keerthi *et al.,*1999)
7	*Penicillium citrinum*	(Sajitha *et al.,* 2014)
8	*Penicillium politans*	(Ali *et al.,* 2009)
9	*Streptomyces olivochromogenes*	(Balagurunathan *et al.,* 2010)
10	*Trichoderma koningii*	(El-Sayed, 2009)

2.8. Microbial Production

Microbes are a diversified group of creatures responsible for formulating multiple life accessories by providing a biological tool for multitasking in medical and health sciences. One of the main biological tools in this era are enzymes which can also be extracted from plants and animals but are the most feasible, and microbes are the primary source of enzymes. Microbial formulation of enzymes such as glutaminases is done with the help of a process termed fermentation. There are two types of fermentation usually:

2.8.1. Solid-state Fermentation (SSF)

As the name indicates, solid-state fermentation is accomplished by providing a solid medium along with a liquid and gaseous phase. It is mainly practiced and is more auspicious because it increases the production of enzymes up to 25 to 30 times more than that of conformist submerged fermentation (Kiruthika *et al.,*2018). Most researchers and scientists practice solid-state fermentation mainly consisting of a solid substrate with a minute quantity of water or no free moisture content, less sterilizing, and mixing cost (Kiruthika *et al.,*2018). An orthodox case is of "Koji", which contains many enzymes, including L-glutaminase, whose dense sustenance is rice (Orabi *et al.,*2020).

The solid support may be any carbon source such as starch, cellulose, chitin hemicellulose, lignin, waste from agro-industries such as bagasse, wheat, and rice bran or straws, and polystyrene (Doelle *et al.,*1992; Bajar *et al.,*2020). A medium, suitable temperature, pH moisture content, and supplements such as glucose and maltose are pre-requisite for biomolecule production. The usual range of temperature and acid-base balance for L-glutaminases are similar to those of the human body's optimum requirements to achieve high yield (ElSayed *et al.,*2009).

2.8.1.1.Enyme Production by Solid-State Fermentation

With the development and aid of biotechnological tools and techniques, solid-state fermentation can harvest multiple fruitages at the laboratory as well as at an industrial scale to relieve us scientifically, socially, commercially, industrially, and economically important enzymes such as Glutaminase (Bajar *et al.,* 2020), Amylases (Souza *et al.,* 2010), Glucoamylase (Pavezzi *et al.,* 2008), Lipase (Chandra *et al.,* 2020), Acid protease (Gomri *et al.,* 2018), Cellulase (Ejaz *et al.,* 2021), Glucosidase (Zang *et al.,* 2018), Xylanase (Bhardwaj *et al.,* 2019), Hemicellulase (Houfani *et al.,* 2019), Phytase (Priyodip *et al.,* 2017), Mannanase (Liu *et al.,* 2020), Pectinase (El Enshasy *et*

al., 2018), Pectin lyase (Hugouvieux-Cotte-Pattat *et al.,*2014), Chitinase (Le and Yang, 2019) and Tannase (Dhiman *et al.,* 2018) are produced by solid-state fermentation.

Other fallouts from solid-state fermentation may also include;

- Organic acids (Miura *et al.,* 2004)
- Secondary metabolites such as antibiotics (Charlton *et al.,* 1965)
- Biofuels such as ethanol
- Aromatic compounds (Adegboye *et al.,* 2021)
- Bioactive compounds include hormones, PGRs (Plant growth regulators), and mycotoxins (Bartholomew *et al.,* 2021).

2.8.2. Submerged Fermentation (SMF)

A type of basal synthetic media, also known as liquid fermentation, contains some nutritional components such as L-glutamic acid, sorbitol, glucose, sodium chloride, trace minerals, methionine, yeast extract, and ammonium sulfate, etc. in a liquid media requiring a high amount of oxygen intake (Kumar *et al.,* 2003; Orabi *et al.,* 2020). Submerged fermentation is used chiefly to produce L-glutaminases (Sinha and Nigam, 2016). The amount of carbon and nitrogen source may also significantly affect the productivity of L-glutaminase species to species (Orabi *et al.,* 2020; Amobonye *et al.,* 2019).

2.8.3. Basics of Microbial L-Glutaminases

The first stage in any protocol is optimizing the parameters and conditions leading toward productivity, such as the medial composition orientation, temperature pH optimization, air content and duration of incubation, source allocation, bioreactor statement, estimation, and characterization output.

2.8.3.1.Medial Components

The media must contain the essential macro and microelements such as carbon source (glucose, sucrose, starch, maltose, fructose, lactose, sorbitol, etc.) (Keerthi *et al.,* 1999), glutamine, glutamic acid. And nitrogen sources (Tullimilli *et al.,* 2014) such as nitric acid or sodium nitrate and ammonium acetate some time also supplemented with malt or yeast extract and a solidifying agent such as agar (Sivakumar *et al.,* 2006; Orabi *et al.,* 2020; Amobonye *et al.,* 2019). Other chemicals such as HCl, NaOH, K_2HPO_4, KH_2PO_4, CH_3COOH, NH_4SO_4, and H_3BO_4 can also stabilize. Along with all these, some buffers may also be used to stabilize the pH of media.

2.8.3.2. pH Stability

The acid-base balance in the media is a critical issue because it depends on the source from where it has been extracted. So, pH ranges from 6 to 9, indicating that glutaminases from different origins can work under different conditions. (Ali Mohamed *et al.,* 2014; Athira *et al.,* 2014). L-glutaminase isolated from marine isolated *Halomonas meridiana* with the molecular weight of 57KDa was maximally active at pH8 (Mostafa *et al.,* 2021). Recombinant L-glutaminase isolated from *Geobacillus thermodenitrificans* DSM-465 was best at pH9 (Shah *et al.,* 2019). A novel halophilic and thermophilic L-glutaminase produced by *Cohnella* sp. A01 has a wide pH stability range of 4-11 and was most active at pH8 (Mosallatpour *et al.,* 2019). Soil isolated *Streptomyces rochei* SAH2_CWMSG L-glutaminase showed maximum activity at pH7.5 (Awad *et al.,*2019). L-glutaminase produced by marine isolated *Bacillus subtilis* OHEM11 having a molecular weight of 54.8KDa was maximally active at pH8.2 (Orabi *et al.,* 2020). Soil isolated *Bacillus* B12 was used to produce extracellular L-glutaminase with a wide range of pH stability 6-9.5 and monitored to be best at pH8(Abdelhameed *et al.,* 2020).

2.8.3.3.Temperature Stability

The enzyme glutaminases also disclosed an eclectic discrepancy by acting on different temperatures. Most L-glutaminases are mesophilic (Amobonye *et al.,* 2019), fluctuating from room temperature up to 70 ^{o}C. This variable range indicates that the cradle of glutaminases must activate at a specific temperature, showing the specificity of different sources (Ali *et al.,* 2014; Bülbül and Karakuş, 2013; Prusiner *et al.,* 1976). Mostafa *et al.,* 2021 isolated L-glutaminase from Red sea samples, *Halomonas meridian* with anticancer activity against colorectal oncogenic cell lines, most active at 37^{o}C. Recombinant L-glutaminase by *Geobacillus thermodenitrificans* DSM-465, noted maximally active at 70^{o}C (Shah *et al.,* 2019). L-glutaminase produced by extremophilic bacteria *Cohnella sp.* A01 achieved the best activity at 50^{o}C (Mosallatpour *et al.,* 2019). L-glutaminase produced by soil isolated *Streptomyces rochei* SAH2_CWMSG showed maximum activity at 40 ^{o}C (Awad *et al.,* 2019). The L-glutaminase produced by Bacillus subtilis OHEM1 from the marine environment was best at 40 ^{o}C (Orabi *et al.,* 2020). L-glutaminase, produced by a novel soil isolated strain, *Bacillus* B12, achieved maximum activity at 40^{o}C (Abdelhameed *et al.,* 2020).

2.8.3.4.Effect of Other Substances

Glutaminases ought to be deactivated by contamination of heavy metals in the media. Still, some sources accelerated by incorporating heavy metals such as *E. coli* and *Clostridium welchii,* but this activation occurs in crude form (Hughes *et al.,* 1952). Some are activated or inhibited depending on the source and state. They are extracted by certain chemicals such as ethylenediaminetetraacetic acid (EDTA), glutamate, ammonia, certain divalent anion, and cation. (Robert *et al.,* 1972). Some other chemicals such as mercury, iron, and chromium also inhibit in one way and activate in others depending upon the origin of L-glutamines (Kumar *et al.,* 2003). Hence a wide range of other substances has a variety of activities on the production and stability of glutaminase enzymes. Such as Na^{+}, Co^{+2}, and Mn^{+2}, increased the activity while Ca^{+2}, Cu^{+}, Hg^{+2}, Zn^{+2}, Ba^{+2}, Cd^{+2}, and Fe^{+3} inhibited the L-glutaminase activity (Reda *et al.,* 2015). Extremophilic bacterial *Cohnella* sp. A01, L-glutaminase, inhibited by iodoacetate and iodoacetamide and most resistant towards sodium chloride, dithiothreitol, magnesium ions, and glycerol (Mosallatpour *et al.,* 2019). Novel *Bacillus* B12 L-glutaminase activity, increased by using Mn^{2+}, Mg^{2+}, Ca^{+2}, and Na^{+}, and activity reduction, monitored with Hg^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+}(Abdelhameed *et al.,* 2020).

2.9. Immobilization

Immobilization of any enzymes leads to efficient and practical activity, which is cost-effective as well. Usually, inactive constituents are inert, which means more stable and do not react with protein or enzyme overall. The immobilization makes the enzyme more stable, effective, catalytically stable, efficient, resistant to drastic changes in pH and temperature. And give ionic strength and reduce the need for time and space, such as large volumes of bioreactors, etc. (Zaak *et al.,* 2017; Karakus et al. 2013). The inert stuff that will immobilize an enzyme may be organic or inorganic. The material may be synthetic, innovative, or bio-polymer and hydrogels essential for better performance, retention, and stability of enzymes (Zhang *et al.,* 2009). There may be many approaches leading toward immobilization, among which three are practicable (Karakuş and Pekyardimci, 2012; Kujawa *et al.,* 2021).

- Adsorption method
- Entrapment method
- Covalent binding method

Above all methods, the covalent binding method is most effective because it binds the enzyme with surface covalently, neglecting the chance of leaking and destroying by environmental instability. Many attempts have been made for immobilizing glutaminase enzymes and determining the stable and optimal temperature and pH, some of which are as follows, which can be made practically feasible and more accurate.

Immobilizing L-glutaminases with polyacrylic acid in the presence of Cu^{2+} makes the enzyme more stable at pH8 (Karahan *et al.,* 2014). Immobilization on silica gel and alginate silica gel makes the enzyme more active and yields more product than without immobilization (Fukushima *et al.,* 1990). For the production of L-glutaminase, immobilization is also done by entrapment method facilitated by Ca-alginate gel using packed bed reactor by continuous and batch fermentation. This showed the best productivity for marine fungal species. (Kumar *et al.,* 2003)

2.10. Therapeutic Application of L-Glutaminase

This enzyme glutaminase is engaged with glutamine catabolism in small-scale creatures. Mammalian cells likewise combine this chemical associated with the age of vitality, utilizing glutamine as the major respiratory fuel. In this manner, numerous kinds of tumor cells just as effectively isolating ordinary cells display high rates of glutamine use. Diseased cells, particularly Lymphatic tumor cells, can't integrate L-glutamine and subsequently rely upon the expansive measure of L-glutamine for their fast development. Subsequently, these cells rely upon the exogenous supply of L-glutamine for their survival and quick cell division. Subsequently, the utilization of amidases denies the tumor cells from L-glutamine. It causes specific passing of L-glutamine subordinate tumor cells by depriving the tumor cell of L-Glutamine, vitality wellspring of malignancy cell for cell multiplication and protein amalgamation (Sarada 2013; Wang *et al.,* 2020).

L–Glutaminase has been utilized to treat leukemia, and one disadvantage in glutaminase treatment is that the body begins creating a resistant reaction against the compound. Although the catalyst is infused intravenously at the tumor site, it appears that it stays available for use for a brief timeframe and is observed to be cleaned from the kidney. A few examinations were made to dodge this issue. Spiers *et al.,* 1976 tried L-glutaminase as a medication in leukemia treatment. The human pharmacology and toxicology of L-glutaminase, portrayed by Holcenberg (Holcenberg et al. 1979). Extracorporeal infusion of L-glutaminase to treat intense lymphoblastic leukemia, proposed by Giordano *et al.,* 1981. Be that as it may, elective strategy is additionally

seen that utilized for the treatment of malignancy, for example, utilization of phenylacetic acid derivation for bringing down the dimension of L-glutamine. Afterward, antisense mRNA, used for phosphate actuated L– glutaminase in Ehrlich ascites tumor (sort of bosom carcinoma).

2.10.1. HIV Treatment

L–Glutaminase in the treatment of HIV is a standout amongst the most encouraging remedial applications (Kumar and Chandrasekaran, 2003). They go about as an imperative enemy of a retroviral specialist in treating HIV. Protein decreases the plasma L-glutamine level for a significant lot of time, bringing about lessening the action of serum invert transcriptase (RTA) of Human Immunodeficiency Infection (HIV) (Sarada, 2013). L–Glutaminase from *Pseudomonas* sp. 7A was protected for HIV treatment and regulated for HIV replication in tainted cells (Robert *et al.,* 2001).

2.10.2. Role of L-Glutaminase in Industries

Compounds accepted more noteworthy hugeness step by step because of their biocatalyst movement and their significance for life maintenance. They can catalyze response, so they are being produced substantially for utilization in businesses. Increment examines or progresses in chemical microbiology, sub-atomic, and biotechnology increment the utilization of catalysts on the modern scale and at the industrial level (Binod *et al.,* 2017).

Catalysts represent 80% of the entire mechanical market (Van Oort, 2010). Requests for chemicals utilized in ventures are expanding step by step to improve the procedures. Proteins have various applications in material, nourishment, cowhide, and restorative businesses. Compounds are utilized to empower the usage of crude material. In biotechnology, they increment the generation of metabolites, also utilized for waste treatment, and different proteins are used to control contamination by corrupting the poison from the condition (Binod *et al.,* 2017). Over 500 ventures utilized chemicals to make items (Kumar and Singh, 2013). The primary or helpful hotspot for modern proteins is microorganisms (Adrio and Demain, 2014).

Steady innovative biotechnology work and recombinant procedures improve the compound yield and soundness amid maturation forms (Shah *et al.,* 2019). Further research or redesigning in protein biotechnology fringe and broaden applications in sustenance, restorative and different enterprises (Petersen, 2005).

2.10.3. Food

L-Glutaminase is considered the most important enzyme in food industries to enhance the taste and aroma of fermented food such as soya sauce and miso (Fukushima and Motai, 1990; Nandakumar *et al.,* 2003). This enzyme is very popular in Asian countries because it imparts a pleasant and palatable taste to food. The presence of proteolytic enzymes such as L-glutaminase, protease, and peptidases hydrolysis the protein content and increases the L-glutamic ratio in fermented food, which improves the food taste (Fukushima & Motai, 1990). Low L-glutaminase levels and activity reduce the L-Glutamic acid contents, and L-glutamine is irreversibly converted into pyroglutamic acid, a flavorless compound (Unissa *et al.,* 2014).

L-Glutaminase is viewed as the most imperative catalyst in sustenance ventures, for upgrading the taste and fragrance of matured nourishment, for example, soya sauce and in miso (Fukushima and Motai, 1990; Nandakumar *et al.,* 2003). Unissa and Iwasa also used L-Glutaminase for the same purpose (Iwasa *et al.,* 1987; Unissa *et al.,* 2014).

This protein is well known in Asian nations since it grants excellent and attractive taste to sustenance. The nearness of proteolytic chemicals, for example, L-glutaminase, protease, and peptidases, hydrolysis the protein substance and expands L-Glutamic proportion in matured sustenance, which improves the nourishment taste (Fukushima and Motai, 1990). Hydrolysis of L-Glutamine by L-Glutaminase is viewed as an imperative wellspring of expanding L-Glutamic content (Nakadai and Nasuno, 1989; Sugiyama, 1984). Low L-glutaminase level and action lessen the L-Glutamic corrosive substance, and L-glutamine is artificially and irreversibly changed over into pyroglutamic corrosive, a flavorless compound (Unissa, 2014). L-Glutamic corrosive and L-aspartic corrosive not just build the flavor, taste, and sharp preference for the sustenance things yet additionally the wholesome nature of nourishment.

L-Glutaminase detached from microbial source is used to build taste. Unissa and Iwasa utilized the Koji form to expand the L-glutaminase proportion in soya sauce (Iwasa *et al.,* 1987; Unissa *et al.,* 2014). In the food industry, L-glutaminase is also used in the synthesis of γ-glutamylethylamide (L-Theanine), a non-standard amino acid, which imparts umami flavor (Mu *et al.,* 2015; Ferreira *et al.,* 2021). γ-glutamyl-ethyl amide is an ingredient in food and beverages, and it is naturally present in green tea. It can also be produced by L-glutaminase (Ferreira *et al.,* 2021). L-Glutaminase extensively used proteins degradation in the food industry. More particularly, soy sauce, produced by koji molds produced by the fermentation of soybeans and wheat. L-Glutaminase

converts L-glutamine into L-glutamic acid (monosodium l-glutamate), giving the soya sauce an umami flavor (Ito and Matsuyama, 2021). Usually, high NaCl concentrations are used in fermented foods. That needs L-glutaminases can tolerate and remain functionally active at high salt concentrations (Zhang *et al.,* 2019). *S. maltophilia* is one of the bacterial strains that produce highly salt-tolerant L-glutaminase can be used in the food industry. *B. amyloliquefaciens* Y-9 L-glutaminase showed a 68% increase with 20% sodium chloride (Ye *et al.,* 2013).

2.10.4. Biosensor

Biosensors are measurement tools that convert the living signal into an electronic response (Mehrotra, 2016). Previously glucose oxidase biosensors were used to determine human serum samples (Karakuş *et al.,* 2013). Now L-glutaminase biosensors are also used in health. It is broadly utilized as a biosensor to assure glutamine fixation in mammalian cell culture or hybridoma cell culture media by stream infusion technique (Sarada, 2013). Observing glutamine or glutamate level in body liquid is also critical for clinical examination and well-being checking. A free and immobilized protein on the layer is utilized as a biosensor for deciding glutamine and glutamate. L-Glutaminase as a biosensor, utilized by Kikkoman Company, Japan, stands out among the essential makers of L-glutaminase (Sabu *et al.,* 2000). Enzymatic assurance of glutamine and glutamate level is increasingly exact and exact when contrasted with another strategy, for example, the Nesslerization technique pursued by the assurance of freed smelling salts. Lunda depicts the effective spectrophotometric strategy for assurance of L-Glutamine or L-Glutamate level by utilizing L-Glutaminase. The need for authenticity for the continuous quantification of compounds in medicine and research unfolds the requirement to develop economic instruments for their detection. A new biosensor was created by using L-glutaminase produced by *Hypocria jecorina* immobilized on zinc oxide nanorod. This biosensor determines L-glutamine levels and is essential in medicine (Bülbül and Karakuş, 2013).

3. MATERIALS AND METHODS

In the present research, Bacterial and fungal strains are used to produce industrially important enzyme L-glutaminase. Microbial strains mainly screened from the soils of Pakistan. Screening of bacteria that produce L-glutaminase followed by biochemical tests and 16S rRNA-based identification. Optimization of media composition and fermentation conditions for the production of L-glutaminase by isolated and selected bacterial and fungal strains. L-Glutaminases produced by selected bacterial and fungal strains were partially purified, characterized by various kinetic parameters, and immobilized to increase the stability and reuse of enzymes.

3.1. Microbial Strains

Soil samples, collected from various areas such as Taxila graveyard, Wah University, Wah Cantt, Quetta, and Quaid-i-Azam University Islamabad, Pakistan, stored in an aseptic condition and maintained at 4°C. A fungal strain *Epicoccum* sp. NFW1 (Jx402049.1), an endophyte isolated from the *Taxus fauna* plant (Fatima *et al.,* 2016), was taken from the Microbiology Laboratory (Department of Microbiology) Quaid-i-Azam University, Islamabad. One bacterial strain was also isolated from an expired glutamine sample taken from Microbiology Lab, Quaid-i-Azam University.

3.2. Isolation and Screening of Microorganisms

3.2.1. Bacterial Primary Screening

Glutamine Salt agar was used for bacterial isolation from soil samples (Ahmed *et al.,* 2016). The plate media prepared (Table 3.1) has phenol red in this medium as an indicator that detects pH change in a medium. The pH of the medium was adjusted to 6.6 using 0.1N HCl and 0.1N NaOH. After sterilizing the medium in an autoclave at 121°C for 15 min at 15psi pressure, L-glutamine was added after filter sterilization by syringe filter.

Collected soil samples, inoculated on the Glutamine salt agar plates by sprinkling the soil on the dishes, and incubated at 37°C in a thermal incubator for 24-48 hours. Bacteria that showed changes in color yellow to red around the colony due to basic pH (by ammonia release from L-glutamine) were considered as L-glutaminase producers and were used later for secondary screening.

The fungal strain grew on Glutamine salt medium and Czapek dox media (Ali *et al.,* 2009), Potato dextrose agar, and Sabouraud dextrose agar containing composition as follows (Table 3.1-3.4);

Table. 3.1. Composition Glutamine salt agar with Phenol pH 6.6

Sr.No	Chemicals	Composition g/L
1	KCl	0.5
2	$MgSO_4.7H_2O$	0.5
3	K_2HPO_4	1
4	NaCl	0.5
5	$FeSO_4.7H_2O$	0.01
6	$ZnSO_4.7H_2O$	0.1
7	L-Glutamine	05
8	Phenol red	0.02
9	Agar	16

Table 3.2. Composition of Czapek dox agar pH6.8.

Sr No	Chemicals	Composition g/L
1	Sucrose	30
2	Sodium nitrate	2
3	Dipotassium phosphate	1
4	Magnesium sulphate	0.5
5	Potassium chloride	0.5
6	Ferrous sulfate	0.01
7	Agar	16

Table 3.3. Medium Composition of Potato dextrose agar pH5.6.

Sr No	Chemicals	Composition g/L
1	Dextrose	20
2	Potato extract	4
3	Agar	15

Table 3.4. Medium Composition of Sabouraud dextrose agar pH5.6.

Sr No	Chemicals	Composition g/L
1	Dextrose	40
2	Peptone	10
3	Agar	15

3.2.2. Screening in the Absence and Presence of L-Glutamine

Mineral salt media (50 ml, Table 3.5) of pH 6.6 was prepared with phenol red as an indicator in a 100 mL flask with glutamine as a substrate and without adding glutamine. Then L-glutamine (5g/L) was added after sterilization by syringe filter, and media suspended with phenol red incubated at 37°C. Strains that impart pink color were selected.

The bacterial screening was performed in the presence and absence of L-glutamine on Mineral salt medium. In the existence of glutamine (hydrolysis of glutamine), turning color from yellow to red. In contrast, the media did not change its color without glutamine.

Table 3.5. Composition of Mineral salt media with L-Glutamine pH 6.8

Sr No	Chemicals	Composition g/L
1	Glucose	10
2	Glutamine	5
3	$Na_2HPO_4.2H_2O$	6
4	KH_2PO_4	3
5	$MgSO_4$	0.49
6	$CaCl_2$	0.002
7	Agar	16

3.2.3. Secondary Screening

Glutamine salt agar medium (with phenol red as an indicator) of pH 6.6 was prepared in 300 mL distilled water. The medium was poured into Petri plates, and selected bacterial strains were point inoculated. Each bacterial strain was inoculated in three Petri dishes and incubated at 37°C. Zone of hydrolysis, measured after 24 hours of incubation.

3.2.4. Extracellular L-Glutaminase Screening by Well Diffusion Method

The selected bacterial strains were testified for L-glutaminase activity by well diffusion assay. The bacterial strains were cultivated on Mineral salt broth (50 mL) of pH 7 with L-glutamine at 37°C for 24 hours. Liquid broth after 24hrs, centrifuged at 6000-10000 rpm at 4°C for 15-20 minutes, the supernatant was used as a source of L-glutaminase. The wells were prepared on Glutamine salt agar plates, and 100 µl of the supernatant or extracellular enzymes were then poured into the well and incubated at 37°C for 24

hours. The next day, the medium's color change was observed and recorded; the crude enzyme from fungus *Epicoccum* sp. NFW1 was separated with the help of the filtration process and dispensed into the well, and incubated for 24 hours.

3. 3 Production of L-Glutaminase in Fermentation Medium

Selected bacterial strains (5 bacteria) and one fungal strain produced L-glutaminase in a Glutamine salt medium. The selected bacterial and fungal strains were inoculated in 100 mL flasks containing 50 mL of Glutamine Salt media at 37ºC for bacteria and 30ºC for fungal at 120rpm. The sample was collected at a regular 24 hours and centrifuged at 6000-10000 rpm for 15-20 min at 4ºC. The liquid supernatant containing the L-glutaminase was used for enzyme assay and stored at 4ºC.

3.3.1. Enzyme assay

The presence of an enzyme in the supernatant, checked by the Nesslerization method (Imada *et al.,* 1973). The enzyme assay reaction mixture of L-glutaminase assay contains:

- 0.5 ml of Phosphate buffer of pH 7.0
- 0.5 ml of 40 mM L-Glutamine substrate
- 0.5 ml of supernatant containing L-glutaminase

The enzyme assay reaction mixture containing test tubes was kept in a shaking water bath at the temperature 37$\pm$2ºC for 30 min of incubation. After incubation, Trichloroacetic acid (TCA) was added 0.5 ml to the assay mixture as a stopping reagent. 3.7 ml of distilled water, taken in the separate test tubes, and 0.1ml, of the incubated reaction mixture, added in the end, 0.2 ml of Nessler reagent, added to each test tube to estimate ammonia produced in the assay. Absorbance, measured at 450 nm. By comparing with the standard curve, L-glutaminase units, calculated. One unit of the enzyme is the quantity of enzyme that liberated 1µmol of ammonia per milliliter per minute.

3.3.2 Ammonia Standard Curve

The quantification of L-glutaminase was performed by ammonium sulfate standard curve using the concentration (60-360 µmol/mL). Different dilutions of ammonium sulfate were prepared. 0.5 mL of ammonium sulfate taken from each dilution and added to 0.5 mL phosphate buffer of pH 7 and 0.5 mL 1.5 M Trichloroacetic acid (TCA). From each test tube, 0.1mL of the reaction mixture, added in 3.7 mL of distilled water in a new test tube, and finally, 0.2 mL of Nessler reagent, added to each tube.

Absorbance, measured at 450 nm. One unit of enzyme is the enzyme that liberates 1µmol of ammonia per milliliter per minute (Figure 3.1).

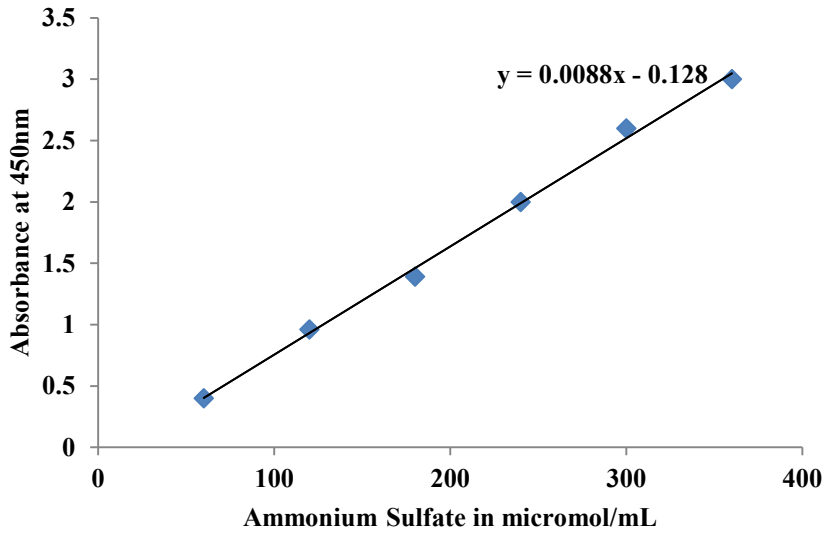

Figure 3.1. Standard curve of ammonium sulfate concentration.

3. 3. 3. Protein Estimation

The Lowry method estimated the extracellular protein existence in the supernatant (Lowry *et al.,* 1951). Reagents of the lowery method are in Table 3.6.

Bovine serum albumin (BSA) protein was used for the standard curve formation. 0.1% BSA stock solution was used for making different dilutions such as 20, 40, 60, to 200 µl/100 ml. in each dilution concentration of BSA was about 20, 40, 60, 80, 100, 120, 140, 160, 180, 200 µg/ml.

In each test tube, 1mL of diluted BSA sample and 1ml freshly prepared solution D was added and incubated at room temperature for 10 min. After 10 min, 0.1ml of folin phenol was added and incubated for 30minutes. Change in the color of the solution into blue indicated the presence of protein in the solution. O.D, taken at 650 nm with the help of a spectrophotometer. The standard curve was used to calculate the amount of protein in µg/mL.

Table 3.6. Reagents used in protein estimation by Lowery method.

Solution	Chemicals	Quantity
A	Sodium bicarbonate	4.0 g/100 ml
	Sodium hydroxide	0.4 g/100 ml
B	Copper sulfate	0.5 g/100 ml
C	Sodium potassium tartrate	1 g/100 ml
D	Soln. A	48 mL
	Soln. B	1 mL
	Soln. C	1mL
E	Folin phenol	1:1 with distilled H_2O

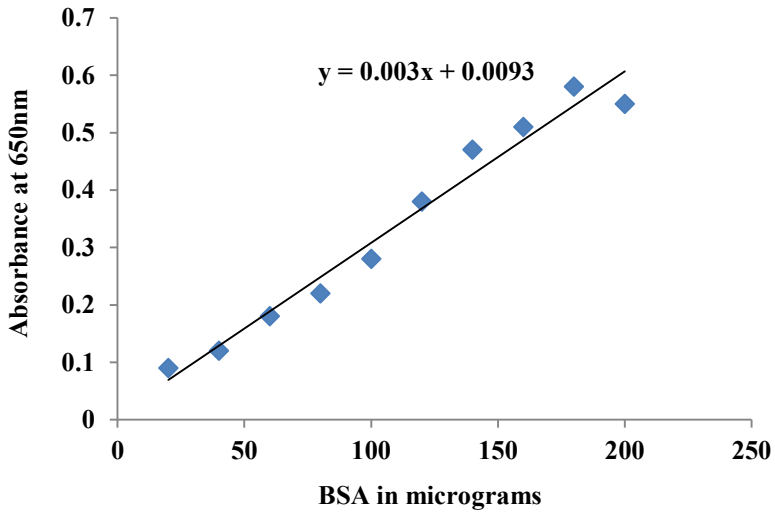

Figure 3.2. Standard curve of protein estimation by Lowry method.

3.4. Maintenance of Isolates

The bacterial strains were maintained by making 80% glycerol stock solution, while the fungal strain was kept in 20% glycerol stock. Glycerol stocks were preserved at -40ºC.

3.5. Bacterial Identification

To identify and morphological characterize bacterial isolates, different biochemical tests, performed.

3.5.1. Gram Staining

All the selected strains were purified on the Nutrient agar plates. Morphology of colonies studied on Nutrient agar plates. Singles colonies, used for the gram staining

procedure. It is a technique used to identify gram-positive or gram-negative bacteria. It is the most commonly used technique of differential staining in microbiology named after the scientist Dr. Christian Gram. The four reagents used during gram staining are shown in Table 3.7. All reagents, given a reaction time of 60 seconds. In contrast, ethanol was used for 5 seconds on each slide. The gram staining results, monitored under a light microscope and the other features of the selected strains.

Table 3.7. Reagents and Chemicals used in the performance of Gram staining.

Chemicals	Chemical's property	Reaction time	Mode of action
Crystal violet	Primary stain	60 sec	Give purple color to each cell.
Gram iodine	Mordant	60 sec	Bind with primary stain form insoluble complex and make stain more intensity.
Ethanol	Decolorizing agent	5sec	During the action of 95%, ethanol gram-positive bacteria retain purple stain. In contrast, gram-negative bacteria become colorless.
Safranin	Counter-stain	60 sec	stains were red to those bacteria, which decolorized with ethanol.

3.5.2. Morphological Characterization

The isolated bacterial strains' morphology, observed on the nutrient agar plates—selected isolates, streaked in various ways to study the colonies morphologies and incubated overnight. The colony morphology was observed according to as performed by "Bergey's Manual of Systematic Bacteriology" (Krieg *et al.,*1984); the sizes of the colonies were either small, moderate, or large. Their color (pigmentation) colonies shape circular, irregular, and rhizoidal. Colonies margins like entire, lobed, and undulate, colonies elevations, either domed shaped, flat, and raised, and opacity of the colonies like opaque, transparent, and semi-transparent, noted.

3.5.3. Biochemical test

A total of 24 biochemical tests, performed to check bacteria's biochemical nature, their enzymes, and their byproduct Bergey's Manual (Krieg *et al.,*1984; MacFaddin, 2000). And for the additional biochemical testing, API kit 20 Ne was used (Figure 3.4). These biochemical tests are listed **below:**

3.5.3.1. MacCkonkey Agar Test

MacConkey agar (HiMedia Laboratories used) is a selective medium. Containing bile salts and dyes are used to isolate gram-ve bacteria by suppressing the growth of gram+ve bacteria. The composition of the medium is given in Table 3.8. MacConkey agar was prepared in 300 mL of distilling water, autoclaved for 15min at 15lbs pressure, and poured into Petri plates. And all 5selected bacterial isolates were inoculated on plates and incubated at 37^{o}C for 24 hours.

Table 3.8 MacConkey agar Medium composition pH7.

Sr. No	Chemicals	Composition g/L
1	Peptones	3
2	Pancreatic digest of gelatin	17
3	Lactose	10
4	Bile salts	1.5
5	Sodium chloride	5
6	Crystal violet	0.001
7	Neutral red	0.030
8	Agar	13.5

3.5.3.2. Indole Test

Enzyme tryptophanase is produced by certain microorganisms that hydrolyze amino acid tryptophan into ammonia, pyruvic acid, and indole. Kovac's reagent addition observed indole production.

Tryptophan (1%) containing medium was inoculated with the bacterial cultures and kept in the incubator for 1-2days (24-48 hrs) at 37^{o}C. Kovac's reagent (2-5drops) was added after incubation of the bacterial isolates and left for a few minutes. The appearance of the red ring indicated indole production.

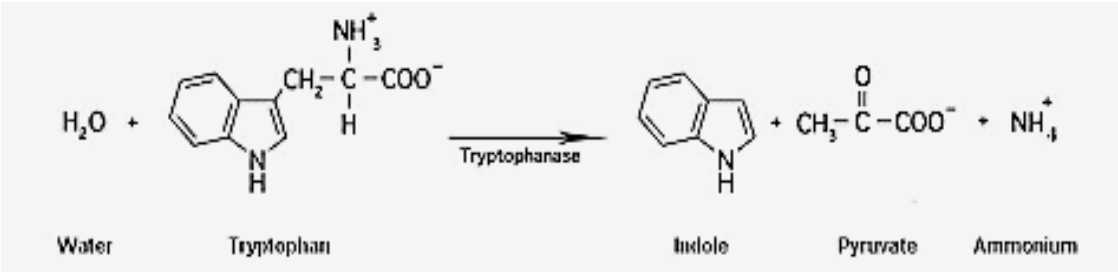

Figure 3.3. Tryptophanase activity on amino acid tryptophan

3.5.3.3. Urease Test

Urea, hydrolyzed by the urease enzyme, is produced by some bacterial strains. The urease production can be estimated by inoculating bacteria on the urease broth medium. The composition of Urea broth is as follows Table 3.9:

Table 3.9 Urea broth composition

Sr. No.	Chemicals	Composition (g/L)
1	Urea	20
2	NaCl	5
3	KH2PO4	2
4	Phenol red	0.012
5	Dextrose	1
6	Peptone	1

Urease hydrolyzes urea by attacking its amide linkage, resulting in ammonia releasing byproducts such as water and carbon dioxide. The following reaction takes place in media during incubation;

$$(NH_2)_2CO + 2H_2O \xrightarrow{\text{yields}} 2H_2O+2NH_3$$

All the medial chemicals except urea, autoclaved, and urea was then added through the syringe filter for sterilization. Phenol red was used as a pH indicator. The urease enzyme breaks down urea and releases ammonia which causes media to decline its pH and form an alkaline media, observed as a change in color of phenol red. As a positive result, the media change its color from red to the original medial color again.

3.5.3.4. Catalase Test

Some microorganism degrades hydrogen peroxide by producing catalase enzyme. The enzyme catalase also has detoxifying effects in eukaryotic cells, which detoxify H_2O_2 formulated from alcohol as the precursor.

The test was performed by adding culture on the glass slide and then adding a few drops of 30% H_2O_2. Bubbles formed within a few seconds due to oxygen release indicate a positive result.

3.5.3.5. Oxidase Test

The test indicated the enzyme cytochrome c oxidase used in the electron transport chain. The enzyme oxidizes the reagent tetramethyl-p-phenylenediamine dihydrochloride and produces indophenol that is purple. The bacteria with negative results also have an aerobic mode of respiration, but instead of using cytochrome c oxidase, they use another oxidase enzyme for respiration.

A bacterial colony was put on filter paper, and added few drops of reagent were on the bacterial colony. Positive results were observed as bacteria turned their color into purple.

3.5.3.6. Carbohydrate Fermentation Test

Most bacteria can ferment carbohydrates and use them as a source of energy. The carbohydrate fermentation media contain:

- **Nutrient broth**: used for the growth of bacteria. The composition of Nutrient broth in table 3.10.

Table 3.10. Composition of Nutrient Broth pH 7.2.

Sr No	Chemicals	Composition g/L
1	beef extract	1
2	Yeast extract	2
3	Peptone	5
4	Sodium chloride (NaCl)	5

- **Specific carbohydrate**: different carbohydrates act as a substrate to determine the fermentation ability of bacteria.
- **Phenol red**: pH indicator with red color at neutral pH, and changes into yellow at slightly alkaline pH.

Fresh culture of bacteria was inoculated in a fermentation medium and incubated overnight at 37 °C. The next day the color change in a media, recorded (Hemraj *et al.,* 2013).

3.5.3.7. Mannitol Salt Agar

The ability of fermentation of mannitol, bacteria were inoculated on an MSA medium. The medium has high quantities of salt (7.5% NaCl) and mannitol as a carbon source. If bacteria ferment mannitol, they show a yellow zone around the colony. In contrast, if they cannot ferment mannitol but can withstand high salt concentration, they grow on medium without forming yellow zones (Hemraj *et al.,* 2013).

3.5.3.8. Simmon Citrate Agar Test

This test contains citrate as a carbon source, and the nitrogen source is ammonium salt. The media also include pH indicator bromothymol blue. The pH of media was changed into alkaline if bacteria utilized citrate and ammonium salt as a carbon and nitrogen source. This alkaline condition will change media color from green to Royal blue; otherwise, media retain color.

The pure culture was inoculated on plates and incubated for 24-48 hours in a thermal incubator. Change in color from green to royal blue indicates positive results.

3.5.4. API Kit 20Ne

For API kit 20Ne, initially, 4-5 colonies were added in a sterile ampule of 7 mL mixed, and 200 µL, added into each well of API stripe containing a dried form of medium. Then for each API stripe, some water was put into the given tray and put Api stripe that tray covered it and incubated for 24hrs at 37^{o}C. After 24hrs, the change in coloration was monitored, as shown in Figure 3.4.

Figure 3.4 API kit results of Selected Bacterial strains

3. 5. 5. Identification at Molecular level (16S rDNA Sequencing)

3.5.5.1. DNA Isolation

The genomic DNA of all selected bacterial (5bacteria) strains was isolated using the "Solarbio bacterial DNA extraction kit" using the standard protocol given in the kit. For this sake, microorganisms inoculated on liquid media such as nutrient broth and kept in the incubator for 24hours at 37°C. The grown culture was centrifuge at 11000 rpm for 5 minutes. The extracted DNA samples were added in 30µl of Tris-EDTA buffer and mixed the DNA samples, maintained at -20°C.

3.5.5.2. Agarose Gel Electrophoresis (AGE)

The purity of extracted DNA was confirmed on the agarose gel electrophoresis. The DNA, resolved using 0.8% (0.32g) agarose, was added in 40mL of 1X TAE buffer and heated until wholly dissolved, cooled the mixture up to 50°C and added 3µL of ethidium bromide and poured into the gel casting apparatus. The gel, stained by using 1µg/ml of ethidium bromide. 0.5µl of DNA was loaded in a gel stained with a DNA dye and observed using a gel doc or transilluminator.

3.5.5.3. Quantification of DNA

Extracted DNA samples were quantified using a Naono drop and spectrophotometry with the ratio of absorbance A260/280, confirming the purity of DNA. Hence, spectrophotometry was performed to quantify and qualify DNA.

3.5.5.4. PCR Amplification of 16s rDNA

Genomic DNA extracted from 5 bacterial strains, amplified using PCR (Labnet International, Model: MultiGene OptiMax, USA). The reaction mixture, prepared in the 25µl final volume, contains 1x PCR buffer of 2.5µl, 5µl of dNTPs, 3µl of 1mM $MgSO_4$, 0.75µl Taq DNA Polymerase, Forward primer 1 µl of 10 µM, Reverse Primer 1µl of 10 µM, extracted bacterial DNATemplate DNA (2 µl) and double-distilled H_2O (10.75µl) (Table 3.10).

Table 3.11. Reagents for PCR for Amplification of 16S rDNA.

	Ingredients	Stock solutions		Concentrations for PCR
1	PCR buffer	10X	1X	2.5µl
2	dNTPs	200mM	10mM	5µl
3	Forward primer	100µM	10µM	1µl
4	Reverse primer	100µM	10µM	1µl
5	Taq DNA polymerase	5U	0.625	0.125µl
6	$MgSO_4$	25mM	1.5mM	3µl
7	Template DNA	-	-	2µl
8	Nuclease free water	-	-	14.625µl

PCR optimization of 16S rRNA gene using 27F(5′-AGAGTTTGATCMTGGCTCAG-3′) forward primer and 1492 R (5′-TACGGYTACCTTGTTACGACTT-3′) reverse primers. The PCR mixture was placed into the thermocycler for amplification purposes. PCR cycles are optimized by checking the cycle times monitoring melting and annealing temperatures. DNA amplification was carried out as follows: 10 seconds at 98^{o}C, 5 minutes at 94^{o}C, 50 seconds at 52^{o}C, 1 minute at 72^{o}C, and finished with 10 minutes at 72^{o}C for the final extension. DNA, amplified on the PCR for 30 cycles, took the total time 1hour and 40 minutes. PCR products, monitored on 0.8% agarose gel. The amplified PCR products were purified using the gel extraction kit (Geneaid cat. No. DFG100).

3.5.5.5. 16S rDNA Sequencing and Identification

All the polymerase chain reaction products were sequenced commercially for all bacteria samples. All the commercially sequenced 16S rDNA, submitted online on the NCBI (National Centre for Biotechnology Information) site to get accession numbers.

3.5.5.6. Phylogenetic Analysis

For the phylogenetic analysis**,** nucleotide BLAST (BLASTn) was used to get the identified Fasta aligned sequences. These sequences are used in the Mega X software. The sequences are aligned by Clustal Won Mega X software. The final phylogenetic trees, obtained by the neighbor-joining method (using 1000 bootstrap replicates), cleared the evolutionary basis of strains (Awad *et al.,* 2019).

3. 6. Optimization for the maximum Production of L-glutaminase by Selected Bacterial Strains

All selected bacterial strains were optimized for maximum L-glutaminase production for various fermentation conditions. Bacterial isolates were optimized for incubation time, pH effect, temperature effect, carbon and nitrogen sources effect, and inducers effect on the L-glutaminase production (Sathish *et al.,* 2018).

3.6.1. Effect of Incubation Time on L-Glutaminase Production

Enzyme production, optimized in 100 mL Erlenmeyer flasks containing 50 mL Glutamine salt media of pH7. Selected bacterial strain inoculated (0.5mL inoculum) (RSHG1, RSHQ1, RSHU1, RSHU3, and RSHS3) and incubated in a shaking incubator at 37°C. L-Glutaminase activity, monitored after 24hours of incubation.

3.6.2. Effect of pH on L-Glutaminase Production

The isolated bacterial strains, optimized for the effect of pH. For this purpose, 50 mL of Glutamine salt media, prepared in 100 mL Erlenmeyer flasks, then pH, adjusted from pH6 to 9. Media was inoculated with 1% inoculum and incubated at 37°C. Enzyme activity, measured after every 24hrs of incubation.

3.6.3. Effect of Temperature on L-Glutaminase Production

Selected bacterial isolates, optimized for the effect of temperature. Glutamine salt medium (50 mL), prepared in a 100 mL flask. The pH of the medium adjusted to the optimum. And inoculated with 1% inoculum for all bacterial strains and incubated at 25°C, 30°C, and 37°C. The enzyme activity is observed after every 24hrs of incubation.

3.6.4. Effect of carbon sources on L-glutaminase Production

The selected strains were observed for the effect of carbon sources on L-glutaminase production. 50 mL of Glutamine salt medium, prepared in 100 mL flasks with different 0.5gm (1%) carbon sources such as glucose, sucrose, lactose, maltose, xylose, and sorbitol. pH was adjusted to optimum and incubated at optimum temperature.

3.6.5. Effect of nitrogen source on L-Glutaminase Production

Different nitrogen sources of 0.1% were added to 50 mL of Glutamine salt media into a 100 mL flask with an optimum carbon source, pH, and temperature of respective strains. The nitrogen sources used were Tryptone, Yeast extract, Ammonium chloride, and Sodium nitrate. Samples, collected after 24 hours, and L-glutaminase activities were recorded.

3.6.6. Effect of Inducers on L-Glutaminase Production

The L-glutaminase-producing selected strains optimized for the inducer effects in Glutamine salt media. Medium (50 mL), prepared in a 100 mL flask with 1% inoculum. Three inducers (1.5%) glycine, glutamine, and lysine, added by syringe filter method for L-glutaminase production, and enzyme activity by all the selected bacteria, measured every 24hrs.

3.7. Optimization for L-Glutaminase Production by Fungal Strain *Epicoccum* sp. NFW1

Fungal culture was grown in a controlled environment to check the effect of different conditions regarding its activity of glutaminase production to achieve the maximum yield of the enzyme.

3.7.1. Effect of Incubation Time on L-Glutaminase Production by *Epicoccum* sp. NFW1

L-Glutaminase, produced by *Epicoccum* sp. NFW1 on Glutamine salt media and observed the effect of incubation time. 50mL medium, prepared in the 100mL flask. And incubated at 30ºC for 5days, and activity was measured after every 24hrs of incubation.

3.7.2. Effect of Media on L-Glutaminase Production by *Epicoccum* sp. NFW1

The effect of the 3media monitored for L-glutaminase production by *Epicoccum* sp. NFW1 on Glutamine salt media, Czapek dox, Sabouraud dextrose, and potato dextrose broth. All media, prepared in a 100 mL flask containing 50mL of media, incubated at 30ºC. The activity was checked for five days of incubation, after every 24hours.

3.7.3. Effect of inducers on L-Glutaminase Production by *Epicoccum* sp. NFW1

The strain *Epicoccum* sp. NFW1 checked for glycine, glutamine, and Lysine effects on L-glutaminase production. 50 mL Czapek dox and potato dextrose broth, prepared in a 100 mL flask incubated at 30°C, L-glutaminase activity, measured every 24 hrs.

3.7.4. Effect of Salt and Sucrose on L-Glutaminase Production by *Epicoccum* sp. NFW1

Effect of 0.3% NaCl and 1% sucrose on *Epicoccum* sp. NFW1 for maximum enzyme production was studied. For this purpose, 50 mL of Potato dextrose broth and Sabouraud dextrose broth, prepared in 100 mL flasks inoculated with selected fungal strain and incubated at 30ºC. Enzyme activity was monitored every 24hrs, for 5days of incubation.

3.7.5. Effect of Substrate on L-Glutaminase Production by *Epicoccum* sp. NFW1

The effect of different carbon sources checked out for *Epicoccum* sp. NFW1 in 100 mL flask containing 50mL of Glutamine salt media for L-glutaminase production of pH7, incubated at 30°C. The carbon sources used; as glucose, sucrose, lactose, maltose, xylose, and sorbitol.

3.7.6. Effect of pH on L-Glutaminase Production by *Epicoccum* sp. NFW1

The effect of pH on L-glutaminase production by *Epicoccum* sp. NFW1 in 50 mL (Glutamine salt media) in 100 mL flasks. The pH values ranged from pH 3 to 9 at 30°C with glutamine 1% as substrate. The effect of pH checked out for 5days, and activity was determined every 24hours.

3.7.7. Effect of nitrogen source on L-Glutaminase Production by *Epicoccum* sp. NFW1

The effect of 1% nitrogen source monitored in 50 mL of Glutamine salt media in 100 mL flasks for L-glutaminase production by *Epicoccum* sp. NFW1. The potent nitrogen sources were Tryptone, Yeast extract, Ammonium chloride, and Sodium nitrate.

3.8. Production of L-glutaminase by selected bacterial strains under optimized conditions.

L-Glutaminase production by strain RSHG1was carried out in 600 mL Glutamine salt media in a 1Litre flask at pH9 at 30°C with 1% sorbitol as a carbon source, 1.5% L-glutamine as a nitrogen source, and as an inducer at incubated for 3days. Production of RSHU1 L-glutaminase, conducted in 1litre flasks, containing 600 mL in Glutamine salt media with 1% glucose, 1.5% L-glutamine 600 mL in each flask at pH6 and incubated on 37°C at 120 rpm. The culture was harvested after 3days. The strain RSHQ1 conditions for Lglutaminase production were, Glutamine salt media with sucrose as carbon and L-glutamine as a nitrogen and inducer source, at pH7 on 37°C incubated till the 4^{th} day of incubation. L-Glutaminase production by RSHU3 was carried out on glutamine salt media with sorbitol as carbon and L-glutamine as a nitrogen source, respectively incubated at 30°C at pH8 for 3days. For RSHS3, L-glutaminase production glutamine salt media, used with sucrose as carbon source, L-glutamine as nitrogen and inducer at pH8 on 25°C incubated for 3days. For harvesting centrifuged at 10,000 rpm for 10-12minutes at 4°C, the supernatant, used for further purification. Pellet, discarded. NFW1 was grown for glutaminase production on glutamine salt media with sucrose as carbon source L-glutamine

as inducer at pH7 on 30°C for 4days of incubation. The culture, harvested by filtration by cellulose filter, mycelial mass, removed and the crude extract, used for partial purification.

3.9. L-Glutaminase Purification

L-Glutaminase produced by *A. xylosoxidans* RSHG1, *B. subtilis* RSHU1, *B. halotolerans* RSHQ1, *S. maltophilia* RSHU3, and *Alcaligenes* sp. RSHS3 and *Epicoccum* sp. NFW1 was partially purified, and RSHG1, RSHU1, and *Epicoccum* sp. NFW1 purified on gel filtration column chromatography (More *et al.,* 2018; Bazaraa *et al.,*2016).

3.9.1. L-Glutaminase Partial Purification

Partial purification, performed with the help of ammonium sulfate precipitation, was initially optimized at 10, 20, 30, 40, 50, 60, 70, 80, and 90% fractional saturation of ammonium sulfate. Partial purification with 70% ammonium sulfate was for all bacterial and fungal strains.

3.9.2. Ammonium sulfate precipitation

After centrifugation, supernatants, taken in 1L flask by taking 600ml crude enzyme in each flask, and 70% of ammonium sulfate, slowly added with stirring and kept at a low temperature (4°C). The mixture was put into the refrigerator overnight, then centrifuged the next day at 10,000 rpm for 12 min at 4°C in 50ml falcon tubes. The pellet, dissolved in 15ml of 0.1M phosphate buffer of pH7, discarded the supernatant.

3.9.3. Dialysis

All the precipitated protein samples were dialyzed against a low ionic strength buffer to remove excess amounts of salt. The pellet dissolved in buffer and dialyzed against 0.01M phosphate buffer of pH7. The dialysis process is done by placing a small piece of dialysis tube in a beaker containing buffer. The enzyme was added to the dialysis tube and clamped from both sides. The beaker containing buffer, and dialysis tube, kept in the fridge at 4^{o}C. The buffer changed after every 3 hours to remove salts. Salt removal was confirmed by the Nesslerization of the buffer surrounding the dialysis beaker. After dialyzing the enzyme, it was subjected to column chromatographic technique.

3.10. Characterization of L-Glutaminase

The partially purified and purified enzyme, optimized for kinetic parameters such as incubation time, substrate concentration, pH, temperature, metal ions, and sodium chloride concentration (Bülbül and Karakuş 2013).

3.10.1 Effect of Incubation time on L-Glutaminase Activity

The effect of incubation time, observed after 15, 30, 45, and 60 minutes of incubation at pH7 using 0.5M phosphate buffer at 37°C.

3.10.2. Effect of Substrate Concentration on L-Glutaminase Activity

The effect of the substrate was monitored by adding the substrate concentrations ranges 0.04-0.56 mM at pH 7 (0.5M phosphate buffer) at 40°C for *A. xylosoxidans* RSHG1, *B. subtilis* RSHU1, *S. maltophilia* RSHU3, and *Alcaligenes* sp. RSHS3, *Epicoccum* sp. NFW1 L-glutaminase and 50°C for *B. halotolerans* RSHQ1 L-glutaminase.

3.10.3. Effect of pH on L-Glutaminase Activity

The effect of 0.5M buffer ranges pH4-9.5 was checked out on the partially purified and purified L-glutaminase from different strains *A. xylosoxidans* RSHG1, *B. Subtilis* RSHU1, *B. halotolerans* RSHQ1, *S. maltophilia* RSHU3, and *Alcaligenes* sp. RSHS3, and *Epicoccum* sp. NFW1.

3.10.4. Effect of Temperature on L-Glutaminase Activity

The effect of temperature 20°C to 70°C, tested on L-glutaminase of selected bacterial and fungal strains *A. xylosoxidans* RSHG1, *Alcaligenes* sp. RSHS3, and *Epicoccum* sp. NFW1 at pH7 of 0.5M phosphate buffer, *B. subtilis* RSHU1 at pH 6.5 of 0.5M phosphate buffer, *B. halotolerans* RSHQ1 at pH 6 of 0.5M phosphate buffer, and *S. maltophilia* RSHU3 at pH 8 of 0.5M phosphate buffer.

3.10.5. Effect of Metal ions on L-Glutaminase Activity

The effect of different inhibitors was measured, like EDTA, cobalt chloride, calcium chloride, barium chloride, mercuric chloride, zinc sulfate, manganese sulfate, potassium chloride, magnesium sulfate, sodium chloride. A control without inhibitor was included in the study. L-Glutaminase, incubated with 20 mmol of different metal ions, then used for enzyme assay at their optimum conditions.

3.10.6. Effect of NaCl (sodium chloride) on L-Glutaminase Activity.

L-glutaminase from all the selected strains was incubated at different concentrations of NaCl (sodium chloride) (4-20%) to check out the effect of increasing concentration of sodium chloride on L-glutaminase activity.

3.11 Purification of L-Glutaminase on Gel Filtration Column Chromatography

The partially purified L-glutaminase was further purified on the column chromatographic technique using Sephadex G75 for *A. xylosoxidans* RSHG1 and *Epicoccum* sp. NFW1 and

Sephadex G 100 for *B. subtilis* RSHU1. A column of 27cm length and 1.5cm in diameter, containing 21cm gel. The column was packed with gel, prepared in sterilized buffer, and left to settle overnight. The samples were concentrated after dialysis by lyophilization. 2.5 ml of the samples, run after passing through a syringe filter of 0.45 micron. After filtration, the samples were run in a column and later eluted by phosphate buffer. Then collected in fractions of 3ml, and the total number of aliquots was 25—all the fractions, estimated for both protein and enzyme activity.

3.12. Molecular Weight Determination of L-Glutaminases

The molecular weights were determined by SDS gel electrophoresis of the partially purified and purified L-glutaminase (Laemmli *et al.,* 1970). The reagents are given in Table 3.10.

Table 3.12 Reagent of Gel electrophoresis

Sr. No	Reagents required	Components of reagents
1	Solution A	3M Tris base (36.34g), SDS 0.4% dissolved in 100 mL (pH 9).
2	Solution B	0.439 MTrisbase of 5.98g, 0.4% SDS in100 mL with distilled water, and pH adjusted to 7.
3	Solution C	Acrylamide 29g and bisacrylamide 1g dissolved and Make up the final volume using distilled water to 100 mL.
4	1 x TGS Buffer	Tris base 3g, glycine 14.4g, and SDS1.25g was dissolved in 1000mL of distilled water.
5	2x Laemmli loading buffer	0.5MTris-HCl of pH 6.8, 1.25 g, SDS 0.2 g, glycerol 2 mL, β-mercaptoethanol 0.5 mL, Bromophenol blue 0.02 g dissolved in 7.5 mL of distilled water.
6	Staining solution	Methanol 220mL, acetic acid 30mL, Coomassie brilliant blue G-250 made up to 250 mL

3.12.1. SDS Gel Electrophoresis Gel Preparation for the Separation of Proteins

For the molecular weight determination, 7.5% resolving gel and 4.5% stacking gel were prepared composition given in Table 3.12. Ammonium persulfate solution is freshly

prepared every time. Then comb was placed into the stacking gel and allowed to polymerize. After the polymerization of the resolving gel, then stacking gel was poured on the resolving gel.

Table 3.13. Composition of Resolving and Stacking Gel

Gel type	Gel %age	Solution A	Solution B	Solution C	distilled water	Ammonium persulphate	Tetramethylethylenediamine (TEMED)
Resolving gel	7.5%	10 mL	-	10 mL	10 mL	10% (400µl)	15 µl
Stacking gel	4.5%	-	2.5 mL	1.5 mL	6.0 mL	10%APS (70µl)	17 µl

3.12.2. Samples Preparation and Gel Running

The samples (20µl, 10µl protein in 10µl 2x loading buffer) were prepared by heating in the water bath at 90°C for 5-8min. The gel plates were placed in an electrophoresis tank and submerged with 1X TGS buffer. 4µl of BenchMark" (Cat No. 10747-012) and "ThermoFisher scientific" (Cat No. 00819260) protein ladder, used and 20µl of samples, added in the wells. The gel was placed for 1hr in the staining solution and then destained overnight.

3.13. Immobilization of L-Glutaminase

3.13.1. Immobilization on Agar

The partially purified L-glutaminase from RSHG1, RSHU1, and *Epicoccum* sp. NFW1, subjected to immobilization on agar. 3.6% agar in 15ml of buffer, autoclaved. After cooling up to 60°C, 5ml of the partially purified L-glutaminase was added to autoclaved agar, poured in Petri dishes, and kept for solidification. The plates were put into the refrigerator at 4°C for 2 hours. The solidified agar with immobilized enzyme was cut into small pieces, washed with 0.5M phosphate buffer of pH 7, and then assayed for enzyme activity. The immobilized enzyme, stored at 4°C in a 0.2M phosphate buffer of pH 7. Immobilized L-glutaminase stability, checked out for 3weeks.

3.13.2. Enzyme Assay After Immobilization

0.5gram of immobilized cubes and 0.5 ml of 80 mmol substrates (glutamine), added to the tube containing 0.5mL of 0.5M buffer and 0.5 mL distilled water. The enzyme assay

reaction mixture was incubated in a shaking water bath at 37±2ºC for 30 min. After incubation, 0.5ml of Trichloroacetic acid (TCA), added as a stopping reagent. 3.7ml of distilled water was added to new test tubes, then 0.1mL of the incubated reaction mixture was added. Finally, 0.2ml of Nessler reagent was added to estimate ammonia produced in the mixture.

3.14 Statistical Analysis

All experiments were conducted multiple times. L-Glutaminase activities were monitored in triplicate (n = 3); all graphs were plotted with their mean values with error bars of the standard deviation of the means. Kinetic parameters, analyzed by using non-linear regression and correlation.

4. RESULTS

The present study showed remarkable results ranging from screening, isolation of bacterial strains, cultural characters up to biochemical testing, molecular identification, and optimization of conditions regarding maximum L-glutaminases from microbial sources, specifically bacteria and fungi. Then purification, characterization, immobilization, and comparative study of L-glutaminases produced by selected strains.

4.1. Isolation of L-glutaminase Producing Microorganisms and Initial Screening

The soils samples of Taxila, Wah Cantt, Quaid-i-Azam University Islamabad, Quetta, were sprinkled on glutamine salt agar plates. An expired glutamine sample, inoculated by the spread plate method used for initial screening at 37°C for 2days pH of the medium was pH 6.6 (Figure 4.1). The glutamine salt medium, along with phenol red as an indicator, was used to grow bacteria. This media grew specified bacteria and screened that bacteria depending upon phenol red color change yellow to red upon hydrolysis of glutamine into glutamate, and ammonium ions released on hydrolysis were converted color of media from yellow to red. Phenol red is an indicator that changes its color from yellow to red or the original color of the medium at pH7 or higher than 7. Bacterial colonies were randomly selected and purified on nutrient agar plates, then screened on glutamine salt agar plates, for fungal screening soil sample taken from Quaid-i-Azam University Islamabad and sprinkled on glutamine salt agar of pH6.6, incubated at 30°C for 7days. One fungal strain, *Epicoccum* sp. NFW1, taken from the Microbiology lab, Quaid-i-Azam University, was also screened on glutamine salt agar.

4.2. Primary Screening of L-Glutaminase Producing Bacteria

Initially, ten strains were isolated from each sample inoculated plate based on change of coloration from yellow to red from various soils and expired glutamine samples. The strains that showed no difference in coloration of media were negative for glutaminase production. All randomly selected strains were further screened on a glutamine salt agar medium of pH 6.6 incubated for 24hours at 37°C red colorations around the colony, indicating L-glutaminase production (Figure 4.2 & 4.3). Four strains were selected as RSHU1, isolated from Wah Cantt, RSHU3 from Quid-i-Azam University Islamabad soil, RSHQ1 bacterial strain isolated from Quetta soil, and RSHS3 isolated from Taxila soil. While one isolate was selected from an expired glutamine sample named RSHG1.

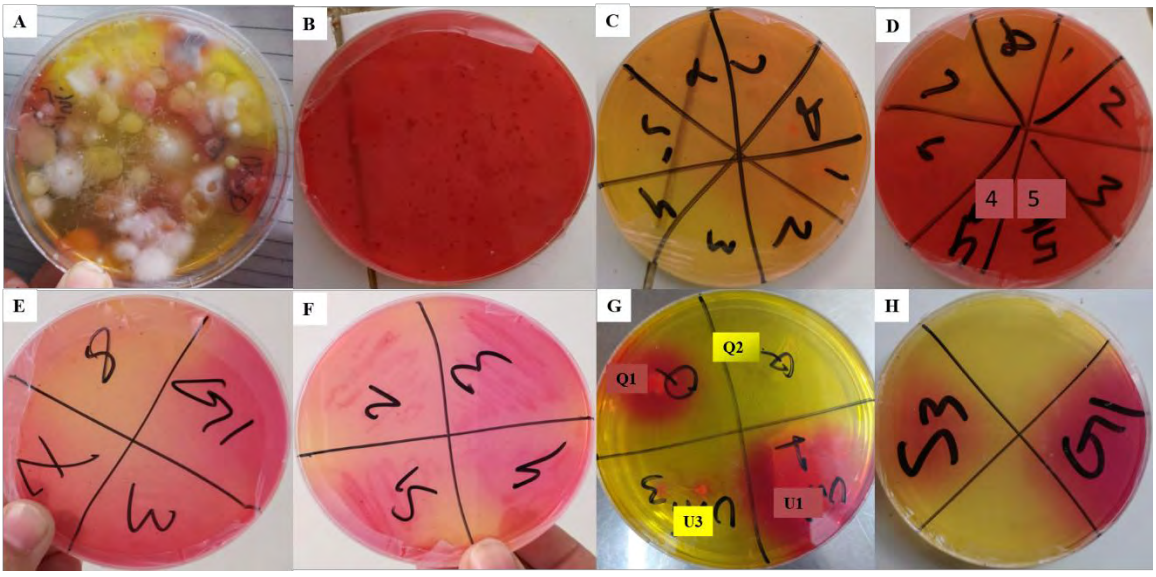

Figure 4.1 A, Isolation of Fungi from soil, B Isolation of bacteria from soil sample by sprinkling method on Glutamine Salt media with phenol red. C, D, E stages of expired glutamine isolated stains, F, Taxila graveyard isolated strains screening. G, Quetta soil, Quaid-i-Azam University and University of wah soil isolated strains. H, Taxila soil isolates stain S3 and expired Glutamine isolated strain G1.

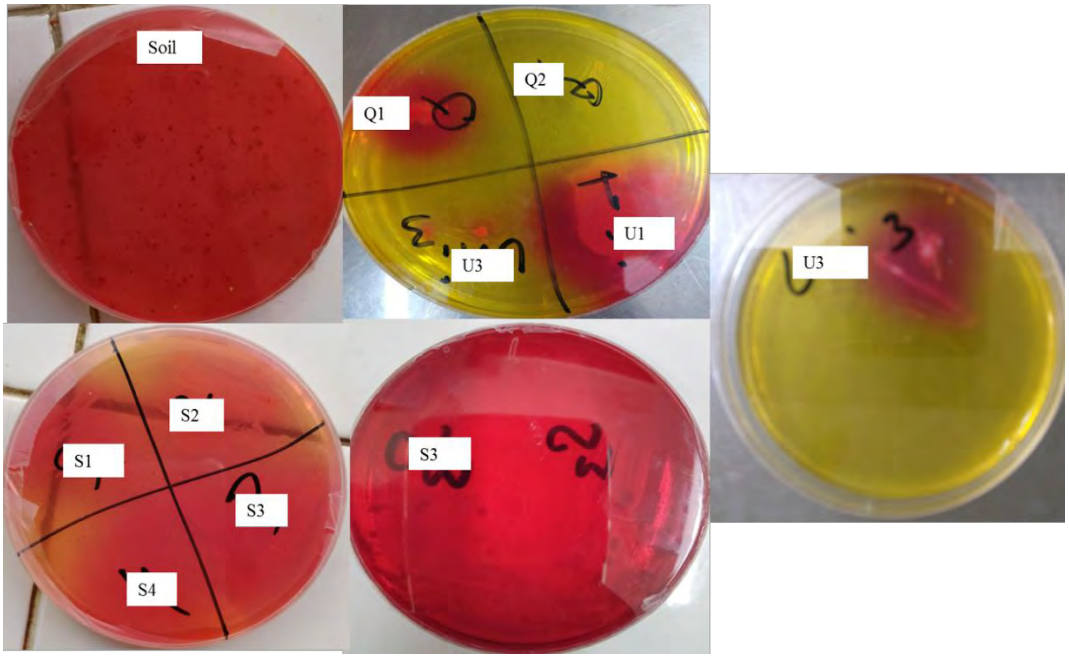

Figure 4.2 Screening of bacterial isolates from different soil samples indicated as S1-S4, Q1, Q2, and U1 and U3 on glutamine salt media showing positive result with red coloration and negative with the yellow color of media. RSHU1, isolated from Wah Cantt, RSHU3 from Quid-i-Azam University Islamabad soil, RSHQ1 bacterial strain isolated from Quetta soil, and RSHS3 isolated from Taxila soil.

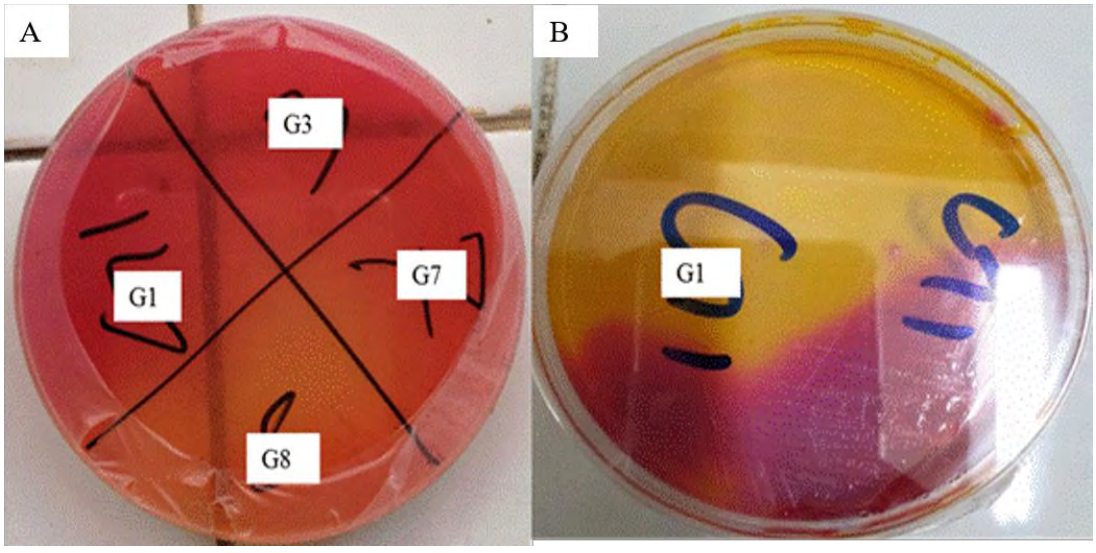

Figure 4.3. A Expired glutamine isolated strains on glutamine salt agar. B G1 isolated from Expired glutamine sample grown on Glutamine Salt medium.

4.2.1. Screening of Fungal Isolates

Different fungal strains FS1, FS2, FS3, and FS4, were isolated from the soil and screened for glutaminase production on glutamine salt media with phenol red as an indicator. Fungi isolated from samples FS1 and FS3 showed positive results while FS4 showed a slight change of color yellow to pink while FS2 didn't change the color and showed negative results as shown in Figure 4.4.

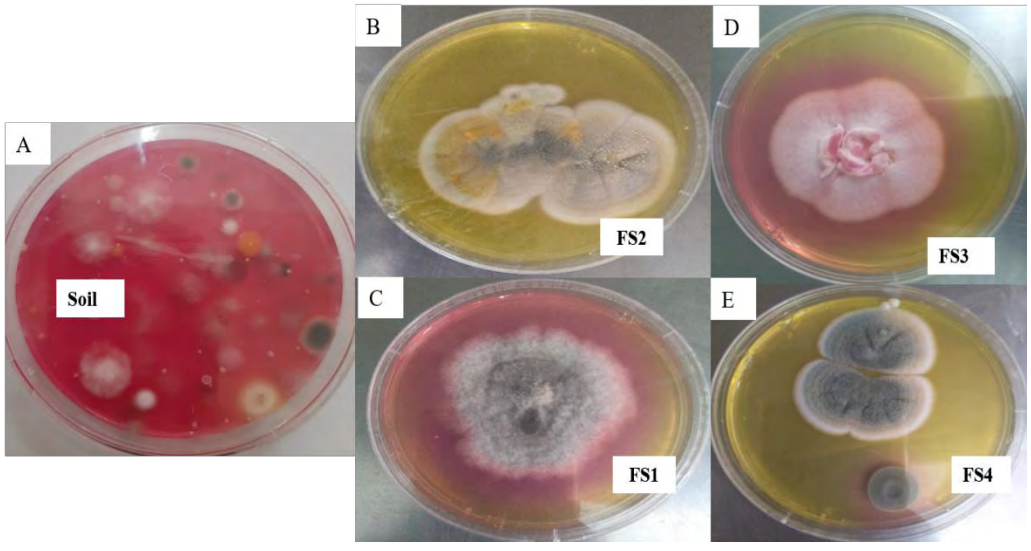

Figure 4.4. A Primary screening of fungal isolates from the soil on glutamine salt medium with phenol red as an indicator. B, C, D and E isolated bacteria screened on Glutamine Salt agar.

4.2.2. Screening of *Epicoccum* sp. NFW1

Fungal strain, *Epicoccum* sp. NFW1, taken from the Microbiology laboratory Quaid-i-Azam University Islamabad. Initially, *Epicoccum* sp. NFW1 screened on glutamine salt agar of pH 6. NFW1 exhibited the highest activity and changed color from yellow to red in glutamine salt and Sabouraud dextrose agar. *Epicoccum* sp. NFW1, used for further studies (Figure 4.5).

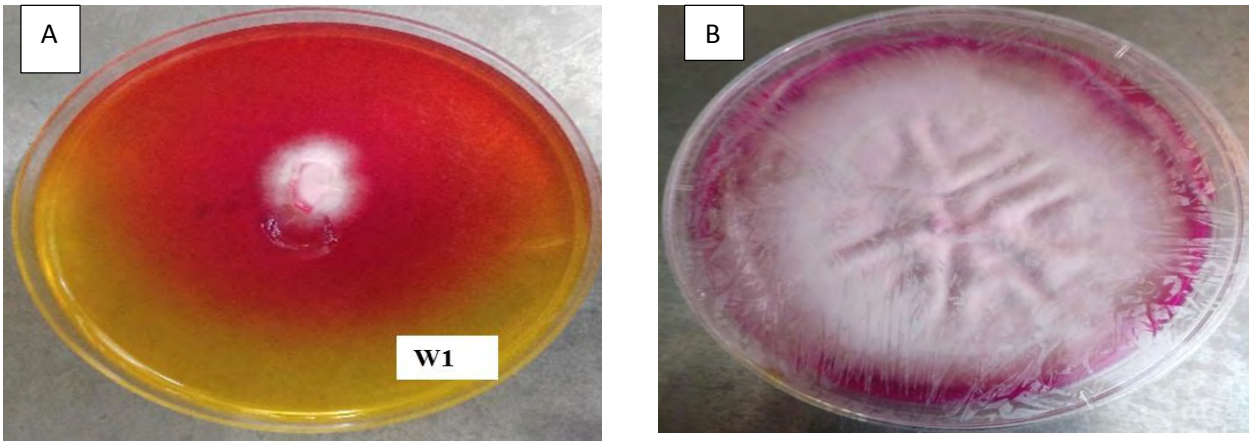

Figure 4.5. *Epicoccum* sp. NFW1, on Glutamine salt media, with phenol red as an indicator (A) and Sabouraud dextrose agar with Phenol red (B)

4.3. Secondary Screening

Selected bacterial strains RSHG1, RSHU1, RSHQ1, RSHU3, and RSHS3, further screened by measuring the zone of hydrolysis, well diffusion assay of liquid enzyme filtrate, and with and between without substrate screening of selected strains.

4.3.1 Zone of Hydrolysis

Selected bacterial strains were point inoculated on Glutamine Salt Medium incubated for 24hours at 37°C, and the zone of hydrolysis was measured in millimeters. The highest hydrolysis zone was shown by RSHU3 of 33 mm, and the smallest zone was shown by RSHU1 of 14 mm, while RSHG1, RSHQ1, and RSHS3 showed 26 mm, 20 mm, and 32 mm zones of hydrolysis, respectively Table 4.1 and Figure 4.6.

Table 4.1. Screening of bacterial isolates for L-glutaminase production by Zone of hydrolysis

Bacterial isolates	**Zone of hydrolysis (mm)** Secondary screening
RSHG1	26
RSHQ1	20
RSHS3	32
RSHU1	14
RSHU3	33

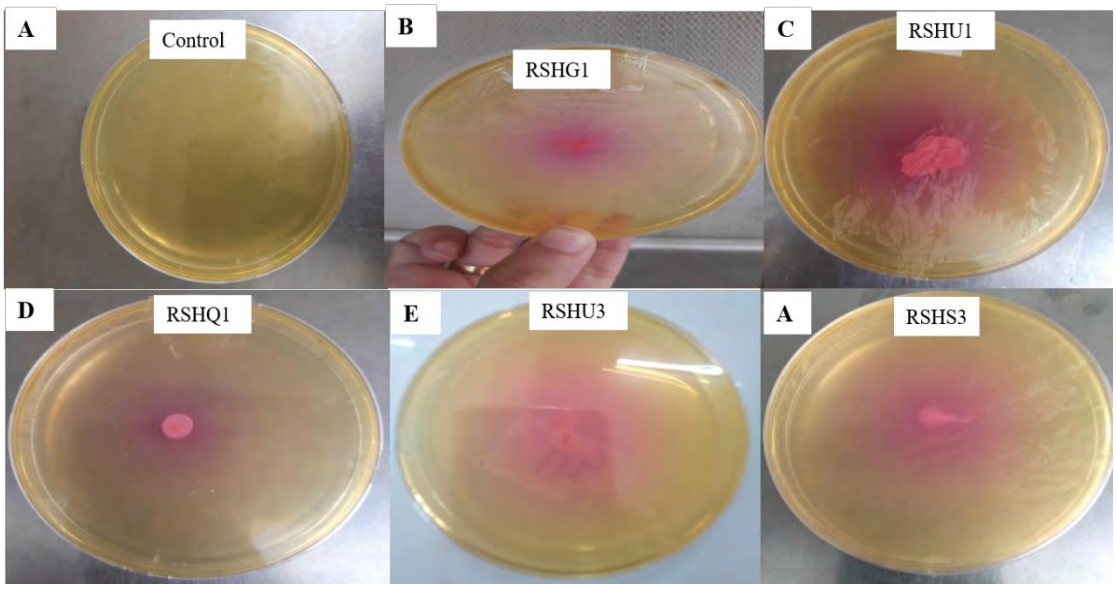

Figure 4.6. Zone of hydrolysis formed by selected bacterial strains A, Control without bacterial inoculation, B, C, D, E, and F showed the zone of hydrolysis by RSHG1 RSHU1, RSHQ1, RSHU3, and RSHS3, respectively.

Change of coloration yellow to red, not observed in the absence of glutamine Figure 4.7. So all of these strains showed positive results only in the presence of L-glutamine in a medium.

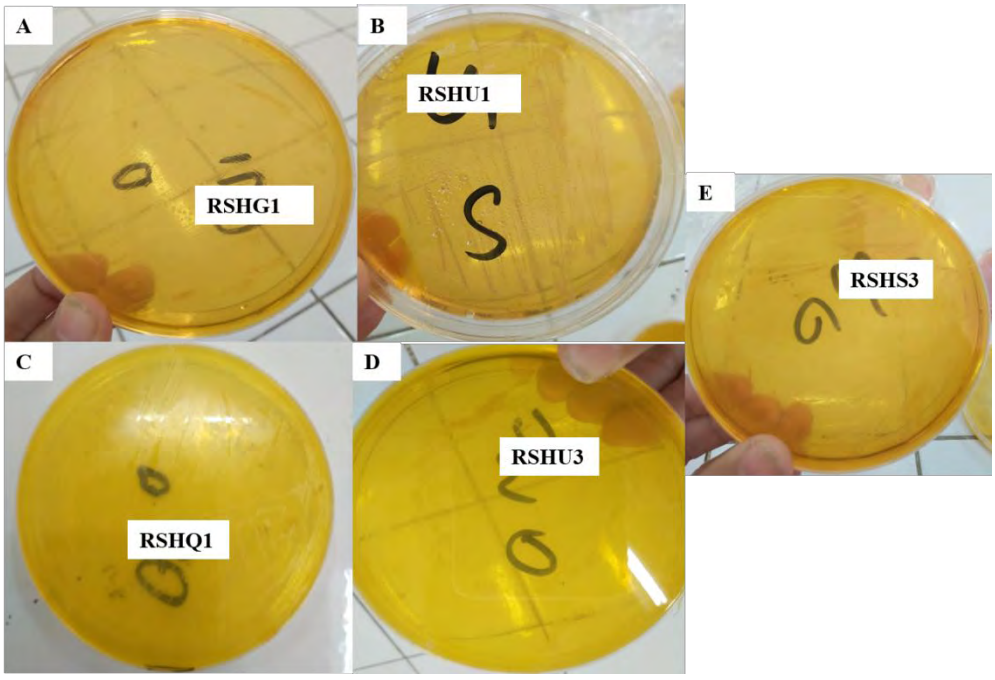

Figure 4.7. Bacterial Screening without Substrate showing no color production. A, B,C,D, and E are the negative results for RSHG1, RSHU1, RSHQ1, RSHU3, and RSHS3, respectively.

4.3.2. Extracellular Enzyme Activity

Extracellular enzyme activity, detected by well diffusion method. The highest activity in terms of zones of hydrolysis was shown by RSHU1 of 34 mm while RSHQ1 showed the lowest activity (zone of 16mm), and other strains RSHG1, RSHS3, and RSHU3 showed 30 mm, 30 mm, and 23mm zones, respectively Table 4.2 and Figure 4.8.

Table 4.2. Secondary Screening of bacterial isolates for glutaminase by well diffusion assay

Bacterial isolates	**Well Diffusion Assay** **Zone of hydrolysis (mm)**
RSHG1	30
RSHQ1	16
RSHS3	30
RSHU1	34
RSHU3	23

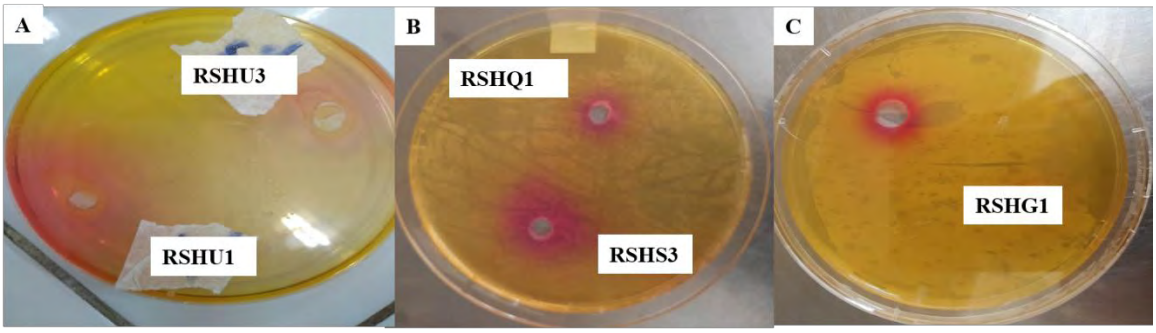

Figure 4.8. Extracellular enzyme activity by well diffusion assay, A showed RSHU1 and RSHU3, B, RSHS3 and RSHQ1, and C for RSHG1.

4.3.3. Secondary Screening (Well Diffusion Assay for Fungi)

Well diffusion assay test was also performed for endophytic fungi *Epicoccum* sp. NFW1 showed measurable values in Figure 4.9. The trial, conducted by pouring 60µl; the zone size was 1.7 cm and for of 50µl was 1.65cm. The value in mm was 17 mm.

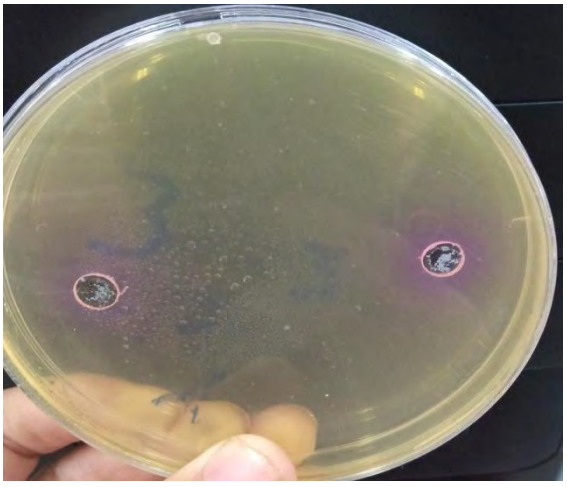

Figure 4.9. Secondary screening of *Epicoccum* sp. NFW1 by well diffusion assay.

4.3.4. Bacterial Screening With and Without Substrate in Liquid Medium

After screening with and without glutamine in solid media, the same performance was tested for the activity of the L-glutaminase enzyme in liquid media. All the selected bacterial strains were grown on mineral salt media containing phenol red with and without substrate and with phenol red as an indicator on liquid media. All bacterial isolates RSHG1, RSHU1, RSHQ1, RSHU3, and RSHS3 showed positive results by changing the color of media yellow to red Figure 4.10. While in the case of without substrate, none of them changed the color of media. An enzyme assay for L-glutaminase activity was performed in liquid culture media after 24 hours of incubation. RSHG1 showed 0.64, RSHU1 showed 0.88, RSHQ1 showed 1.79, RSHU3 showed 1.8, and RSHS3 showed 0.51U/mg.

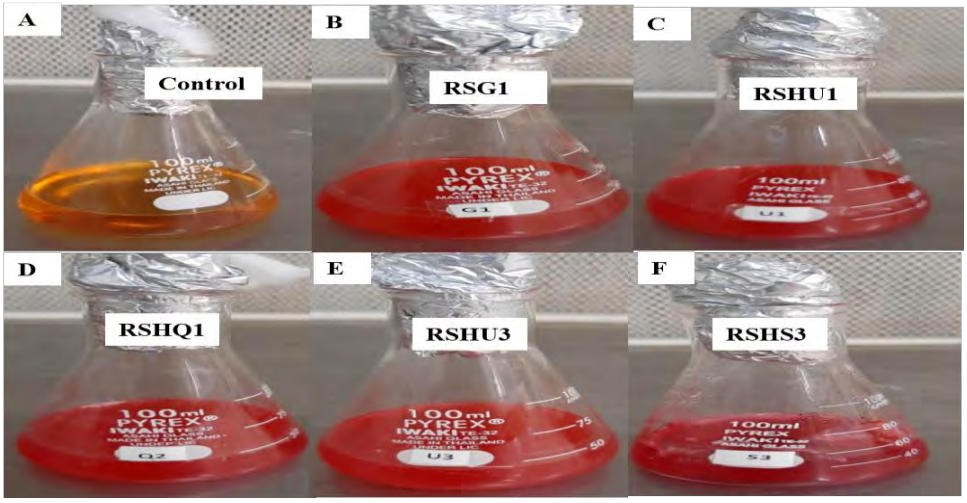

Figure 4.10. Screening of L-Glutaminase producing bacteria on Glutamine Salt medium with subtrte addition at 37℃. A is the control sample without microbial inoculation, B is the screening of RSHG1, C screening of RSHU1, D screening of RSHQ1, E screening of RSHQ1, F is the screening of RSHQ1.

All the strains in liquid mineral salt media without glutamine addition did not change their colors from yellow to red in the absence of glutamine Figure 4.11.

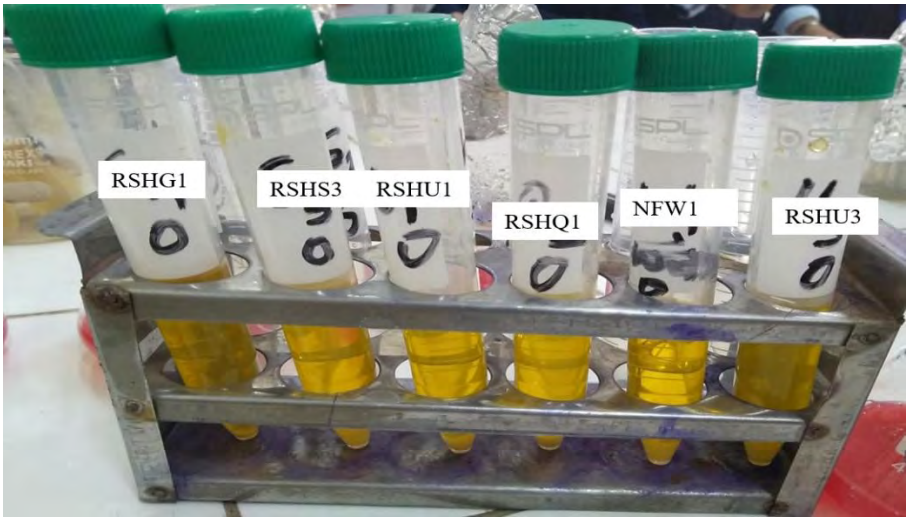

Figure 4.11. Selected bacterial strains on Glutamine Salt medium without substrate.

The screening in liquid broth was observed till 9days of incubation, the color of medium inoculated with RSHQ1 and RSHU3 flasks again changed color to yellow from red, indicating utilization of all the substrate (Figure 4.12).

Figure 4.12. Screening of bacterial strains in glutamine slat media after 9days of incubation.

4.4. Identification of Bacterial Strains

The features like size, shape, margins, elevations, consistency, opacity, and pigmentations showed slight differences in all the strains.

4.4.1. Colony Morphology

All the bacterial colonies were streaked on nutrient agar and broth media to get pure bacterial strains Figure 4.13. Strain RSHS3 had smaller circular colonies, RSHG1 medium size rhizoidal in shape, while RSHU3 colonies were medium and filamentous, and RSHQ1 and RSHU1 had large filamentous colonies.

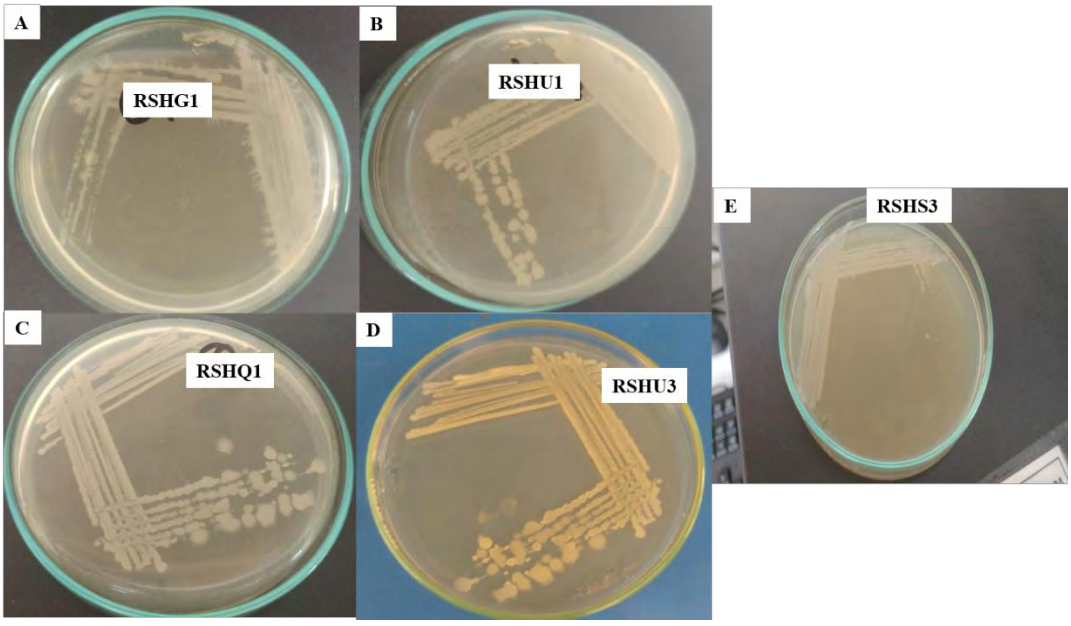

Figure 4.13. Purified bacterial strains on Nutrient agar. A, B, C, D, and E purification streaking results for RSHG1 reults, RSHU1, RSHQ1, RSHU3, and RSHS3, respectively.

There were three features named consistency, opacity, and pigmentation, which were 100% the same for all strains; 80% of the elevations were dome-shaped while 20% were flat. All the strains were dome-shaped except RSHU1, which was of flat elevation. All the strains were opaque and showed no pigmentation at all. The consistency of all samples was moist. Among all strains, RSHS3 and RSHQ1 showed 94% similarity in all morphological characters and 14% dissimilarity in their size. RSHU1 and RSHU3 are 78% similar, and their sizes and elevations contributed towards their 28% dissimilarity (Table 4.3).

4.4.2. Microscopic Characters of Strains

The microscopic observations such as shape, size, and arrangement of cells were of different phases, and features and the gram staining revealed that RSHG1, RSHU3, and RSHS3 strains were gram-negative while RSHU1 and RSHQ1 were gram-positive Table 4.4. All the inoculants showed single in arrangements, and the majority of them were of rod shapes;

Table 4.3. Cultural characteristics of Selected bacterial isolates

Strains	Cultural characteristics						
	Size	**Shape**	**Margins**	**Elevations**	**Consistency**	**Opacity**	**Pigmentation**
RSHG1	Medium	Rhizoids	Lobate	Dome	Moist	Opaque	Nil
RSHS3	Small	Circular	Entire	Dome	Moist	Opaque	Nil
RSHQ1	Large	Circular	Entire	Dome	Moist	Opaque	Nil
RSHU1	Large	Filamentous	Filiform	Flat	Moist	Opaque	Nil
RSHU3	Medium	Filamentous	Filiform	Dome	Moist	Opaque	Nil

Table 4.4. The Gram staining and microscopic features.

Strains	**Cultural Characteristics**		
	Shape	**Arrangement**	**Gram reaction**
RSHG1	Cocci	Single	Negative
RSHS3	Rod	Single	Negative
RSHQ1	Rod	Single	Positive
RSHU1	Rod	Single	Positive
RSHU3	Rod	Single	Negative

4.4.3. Biochemical Tests of Strains

The biochemical tests were performed for all five strains using different media and API 20Ne kit. Maximum tests were positive for RSHU1, and maximum tests were negative for RSHU3, Table 4.5. API kit 20Ne, used for additional biochemical testing for the selected bacterial strains.

Table 4.5. Biochemical Characterization of Bacterial Isolates.

Bacterial strains	Biochemical Testing																					
	Oxidase	Catalase	Mannitol Salt agar test	Simmon Citrate	Indole Production	MacCkonkey Agar	Glucose fermentation	Arginine Hydrolase	Urease	Esculin ferric citrate	Gelatin hydrolysis	β-Galactosidase	D-Glucose assimilation	L-Arabinose assimilation	D-Mannose assimilation	D-Mannitol assimilation	N-acetyl-glucosamine	D-Maltose assimilation	Potassium gluconate	Capric acid assimilation	Adipic acid assimilation	Malic Acid assimilation	Trisodium Citrate	Phenylacetic acid assimilation
RSHG1	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
RSHU1	+	-	+	-	+	-	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-
RSHQ1	+	+	+	+	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
RSHU3	-	-	-	-	-	+	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-
RSHS3	+	+	-	+	-	+	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-

The biochemical tests were performed for all 5selected bacterial strains. RSHG1 showed positive results for oxidase, catalase, Simmon citrate, arginine hydrolase, urease, and esculin ferric citrate and was negative for other tests. RSHU1 was observed negative for catalase, Simmon citrate, D-mannose assimilation, capric acid assimilation, adipic acid assimilation, malic acid assimilation, trisodium citrate assimilation, and phenylacetic acid assimilation and positive for other tests. Biochemical testing for RSHQ1 gave positive results for oxidase, catalase, Simmon citrate, glucose fermentation, arginine hydrolase, urease, gelatin hydrolase, β-Galactosidase. RSHU3 showed positive results only for urease, gelatin hydrolysis, and arginine hydrolase and negative for all the other biochemical testing. RSHS3 monitored positive results for oxidase, catalase, Simmon citrate, arginine hydrolase, urease, and gelatin hydrolase and negative results for other biochemical tests (Table 4.5).

4.5. Molecular Analysis

4.5.1. Gel Electrophoresis After DNA Isolation

All the strains showed almost equal amounts and sizes of DNA isolated from bacterial culture Figure 4.14.

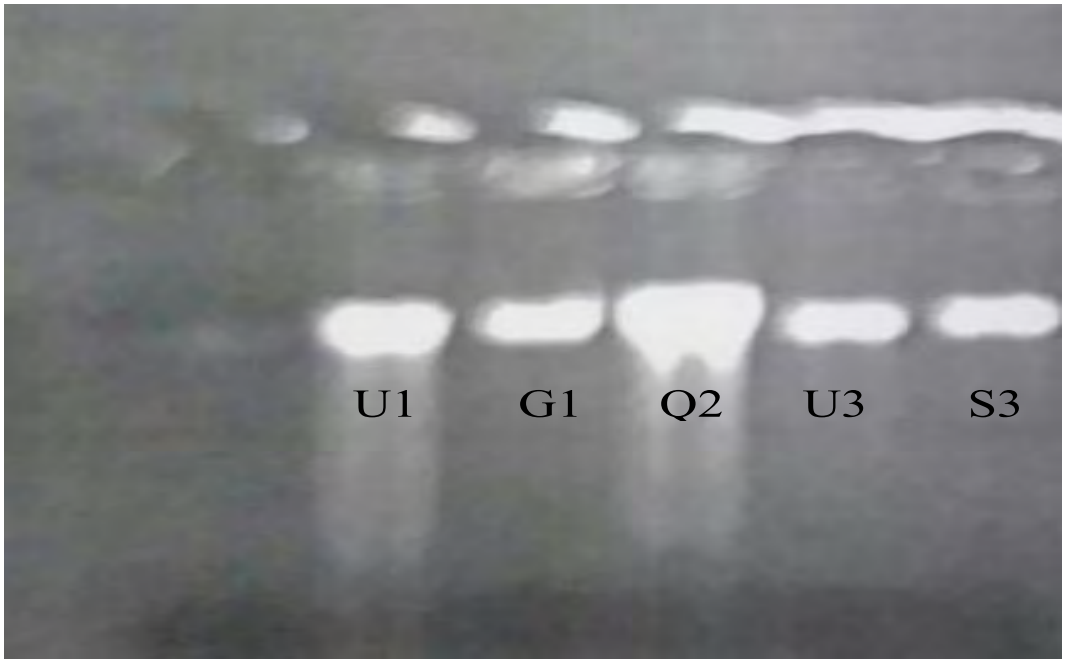

Figure 4.14. Agarose gel electrophoresis showing DNA

4.5.2. 16S rRNA Amplification

The agarose gel electrophoresis showed amplified sequences that were almost equal to the size of 1500bp Figure 4.15. The bands showed size similarity after amplification.

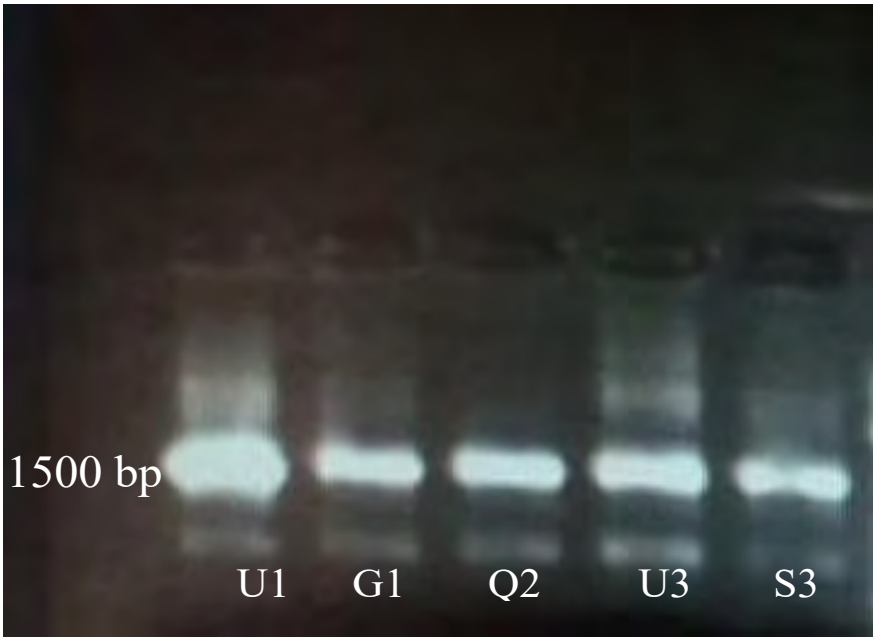

Figure 4.15. Gel electroporation of PCR amplified sequence.

4.5.3. Sequencing Results

4.5.3.1. Blast for RSHU1 Sequence

The evolutionary history, deduced using the Neighbor-Joining method (Saitou and Nei 1987). The bootstrap consensus tree inferred from 1000 replicates (Felsenstein 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein 1985). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein, 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.,*2004) and are in the units of the number of base substitutions per site. This analysis involved 8nucleotide sequences, all vague positions, removed for each pair (by pairwise deletion option). There were 1010 positions in the final dataset, and the final phylogenetic tree was constructed on MEGA X software (Kumar *et al.,*2018; Figure 4.16-4.20).

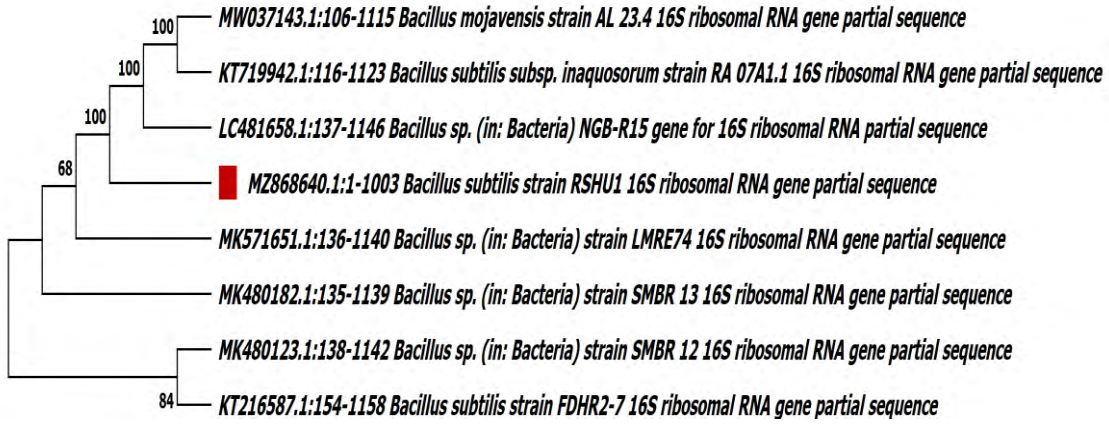

Figure 4.16. Phylogenetic tree of RSHU1

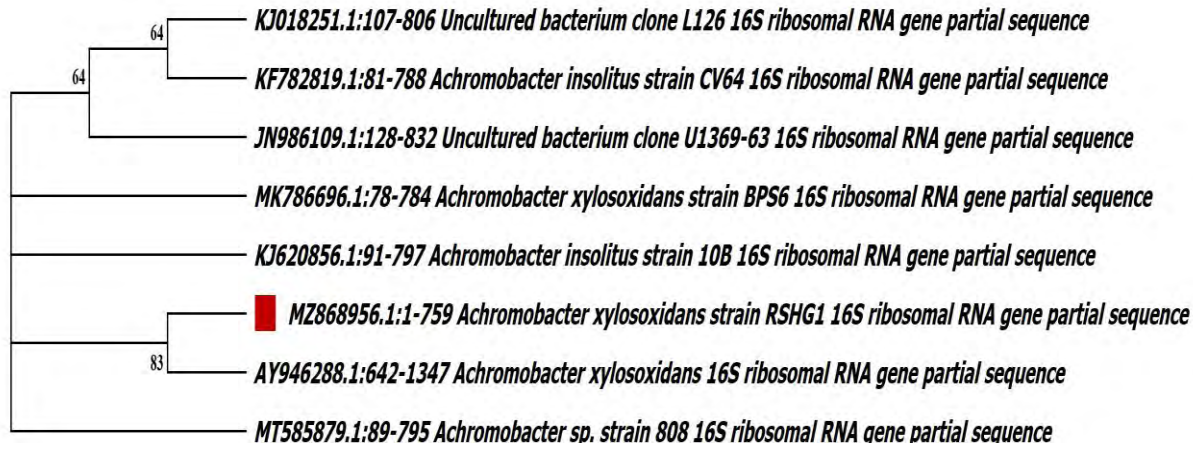

Figure 4.17. Phylogenetic tree of RSHG1

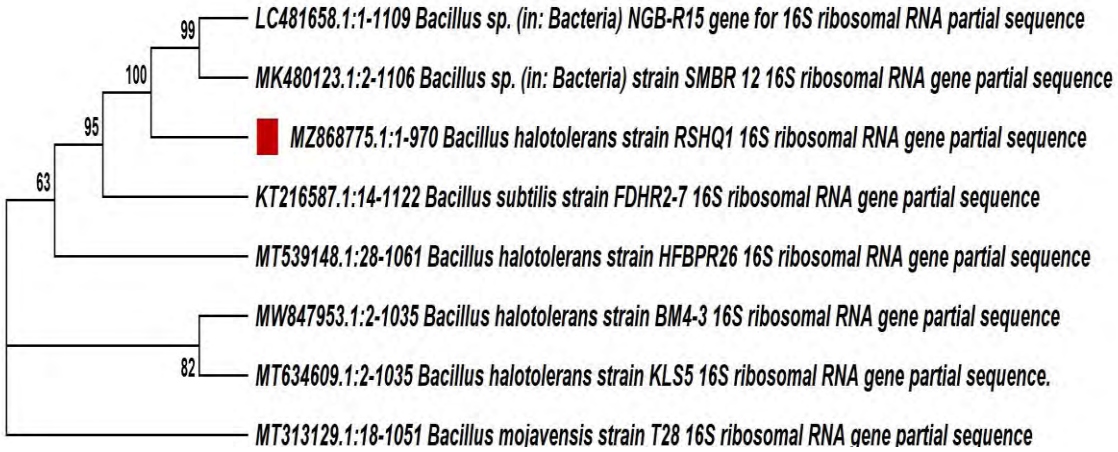

Figure 4.18. Phylogenetic tree of RSHQ1.

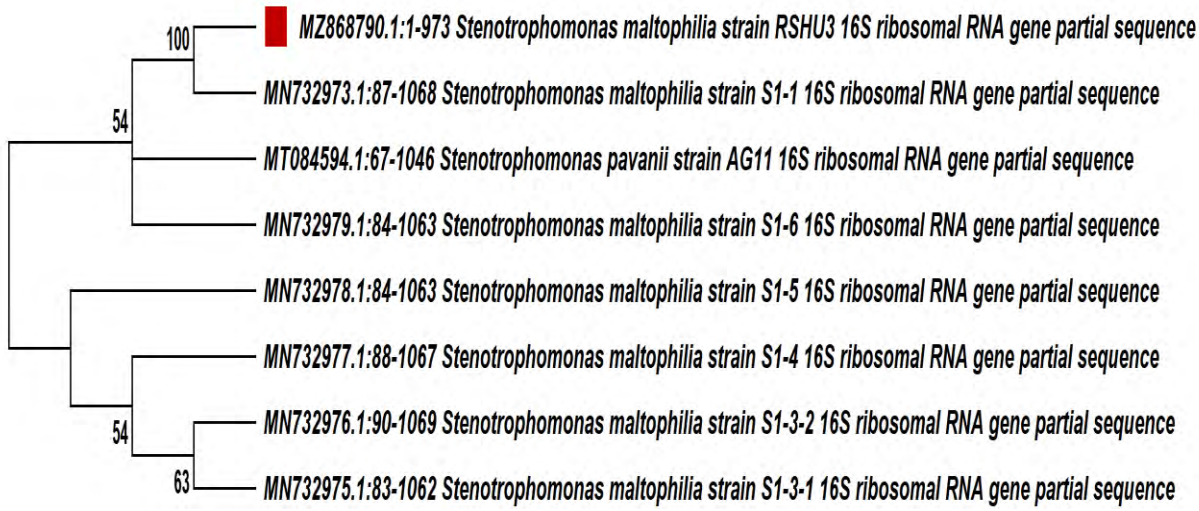

Figure 4.19. Phylogenetic tree of RSHU3

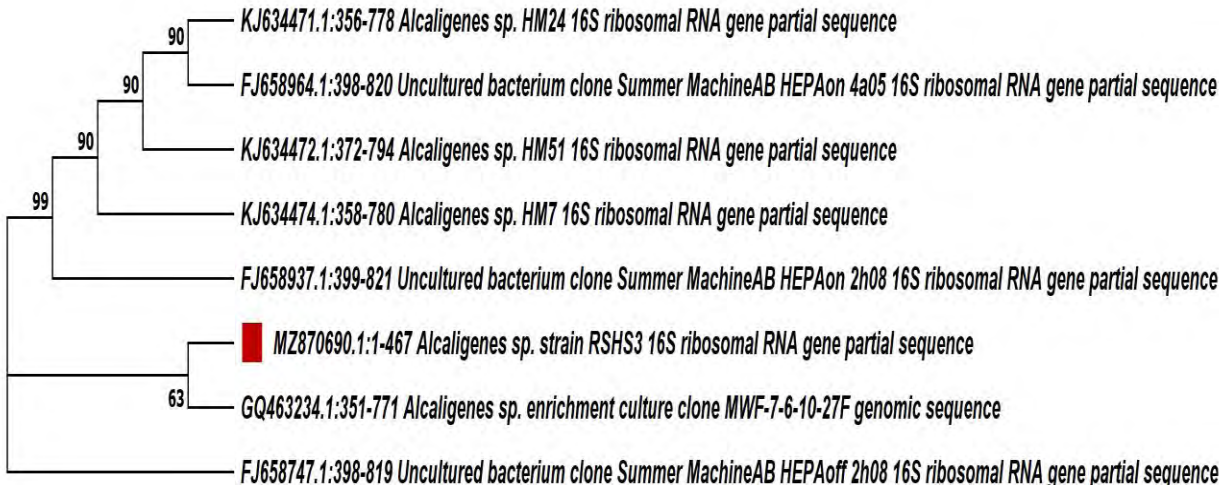

Figure 4.20. Phylogenetic tree of RSHS3

4.5.4. Identification of Bacterial Isolates

The sequences of five all the selected bacterial isolates registered on the NCBI. RSHU1, RSHS3, RSHG1, RSHQ1, and RSHU3, identified as *Bacillus subtilis* RSHU1with the accession number MZ868640.1, *Alcaligenes* sp. RSHS3 with MZ870690.1, *Achromobacter xylosoxidans* RSHG1 with accession number MZ868956, *Bacillus halotolerance* RSHQ1 accession number MZ868775, and *Stenotrophomona maltophilia* RSHU3 accession number MZ868790, respectively Table 4.6.

Table 4.6. Bacterial strains 16S rDNA sequence Homology and Identification

Strains	**Sequence homology with**	**Accession number**	**Identified as**
RSHU1	*Bacillus subtilis* ribosomal RNA gene, partial sequence.	MZ868640.1	*Bacillus subtilis* RSHU1
RSHS3	*Alcaligenes* sp. *ribosomal RNA gene, partial sequence*	MZ870690.1	*Alcaligenes* sp. RSHS3
RSHG1	*Achromobacter xylosoxidans* 16S ribosomal RNA gene, partial sequence	MZ868956	*Achromobacter xylosoxidans* RSHG1
RSHQ1	*Bacillus halotolerance*	MZ868775	*Bacillus halotolerance* RSHQ1
RSHU3	*Stenotrophomonas maltophilia*	MZ868790	*Stenotrophomonas maltophilia* RSHU3

4.6. Optimization of Selected Bacterial Strains for L-Glutaminase Production

L-Glutaminase production media, optimized for different effectors like temperature, pH, inducers, nitrogen source, carbon source, and incubation time.

4.6.1. Effect of Incubation Time on the Production of L-Glutaminase

L-Glutaminase production done by all the strains on Glutamine Salt Medium of pH6.6 37°C. The figure illustrates the amount of enzyme produced from *A. xylosoxidans* RSHG1, the highest amount of enzyme produced 1.39U/mg on the 3rd day of incubation. Maximum L-glutaminase production by *B. subtilis* RSHU1on glutamine salt broth medium was 3.6U/mg on the 3rd day of incubation, as shown in Figure 4.21. *B. halotolerans* Q1 L-glutaminase gained the best activity 2.6U/mg on the 3rd day of incubation on glutamine salt broth. *S. maltophilia* RSHU3 L-glutaminase on glutamine salt broth achieved maximum activity of 3U/mg of protein on the 2nd day of incubation. L-glutaminase produced by *Alcaligene* sp. RSHS3 on glutamine

salt broth medium showed the best activity of 1.3U/mg shown in Figure 4.21 on the 3rd day of incubation.

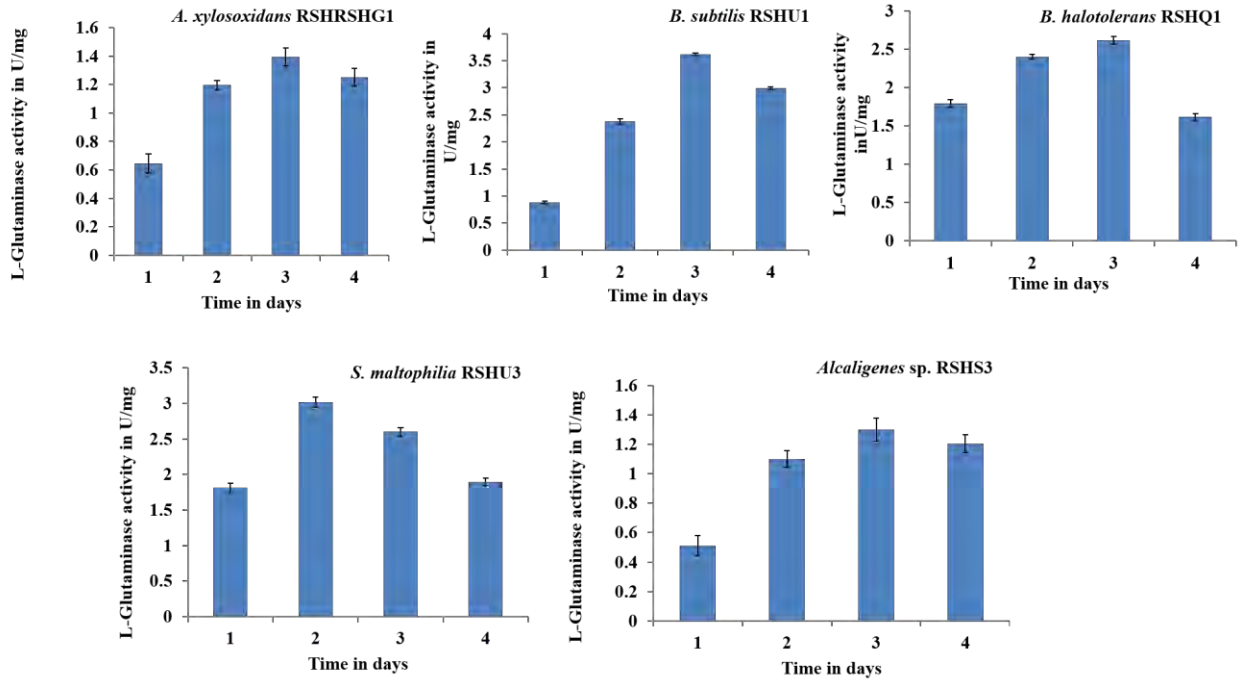

Figure 4.21. Effect of incubation time on L-glutaminase production by *A. xylosoxidans* RSHG1, *B. subtilis* RSHU1, *B. halotolerans* RSHQ1, *S. maltophilia* RSHU3, and *Alcaligenes* sp. RSHS3 on glutamine salt broth at 37°C.

4.6.2. Effect of pH on the L-Glutaminase Production

The pH of media greatly influenced the growth, time, and overall activity of bacteria and bacterial enzymes. The optimum pH was supposed to be in the range of pH6 to 9. Effect of pH on L-glutaminase production by *A. xylosoxidans* RSHG1 on Glutamine salt media at 37°C showed that optimum pH in the range of 8 to 9. In this case, the maximum activity was 3.26 U/mg on the 3rd day of incubation at pH 9. The activity of strains *B. subtilis* RSHU1 was the best activity, 4.2U/mg, at pH6 on the 3rd day of incubation, but on the 4th-day decline in the enzymatic activity was observed, as shown in Figure 4.22. *B. halotolerans* RSHQ1 showed a maximum of 3.9U/mg activity at pH7 on the 3rd day of incubation. L-glutaminase activity of *S. maltophilia* RSHU3 was highest at pH8 on the 3rd day of incubation, and activity was 4.3U/mg. Concerning the *Alcaligenes* sp. RSHS3 gained maximum activity of 1.85 U/mg at pH8 on the 3rd day of incubation (Figure 4.22).

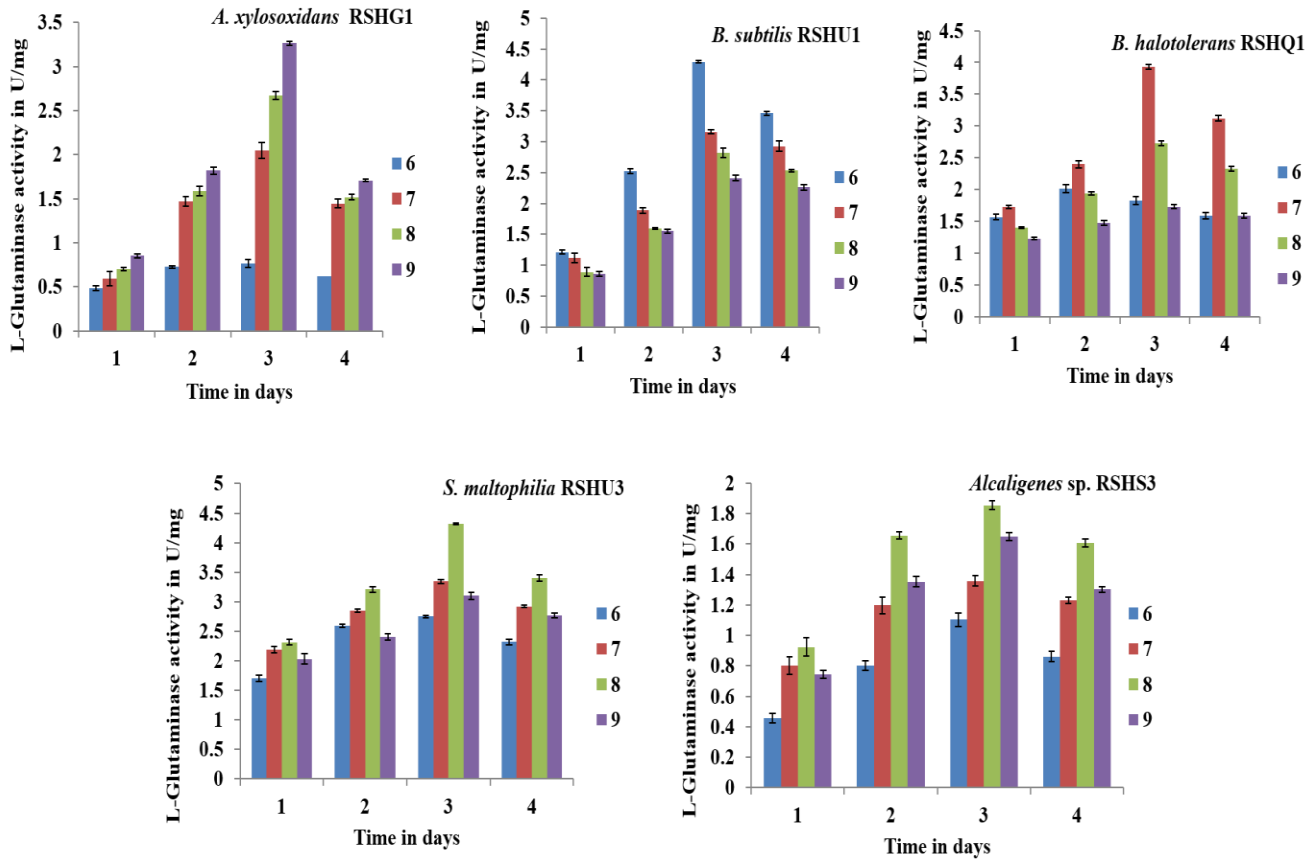

Figure 4.22. Effect of pH on L-glutaminase production by *A. xylosoxidans* RSHG1, *B. subtilis* RSHU1, *B. halotolerans* RSHQ1, *S. maltophilia* RSHU3, and *Alcaligenes* sp. RSHS3 strain on Glutamine Salt Medium at 37°C.

4.6.3. Effect of Temperature

Glutaminase production at 25°C, 30°C, and 37°C by all the selected bacterial strains was analyzed. The strains *A. xylosoxidans* RSHG1 showed maximum enzyme production on the 3rd day of incubation 3.2U/mg at 30°C and pH9, as shown in Figure 4.23. The optimum temperature for *B. subtilis* RSHU1and *B. halotolerans* RSHQ1 for glutaminase production was 37°C, *S. maltophilia* RSHU3 showed 30°C as optimum temperature for L-glutaminase production. The highest Glutaminase activity was shown at 37°C by *B. subtilis* RSHU1 achieved on the 3rd day was 4.62U/mg. *B. halotolerans* RSHQ1 gained maximum activity of 3.89U/mg at 37°C on the 3rd day of incubation. L-glutaminase by *S. maltophilia* RSHU3, maximally achieved at 30°C on the 3rd day of incubation with the activity of 4.62U/mg. While *Alcaligenes* sp. RSHS3 best activity of 2.7U/mg, as shown in Figure 4.23, was observed at 25°C on the 3rd day of incubation. While *Alcaligenes* sp. RSHS3 best activity of 2.7U/mg, observed at 25°C on the 3rd day of incubation.

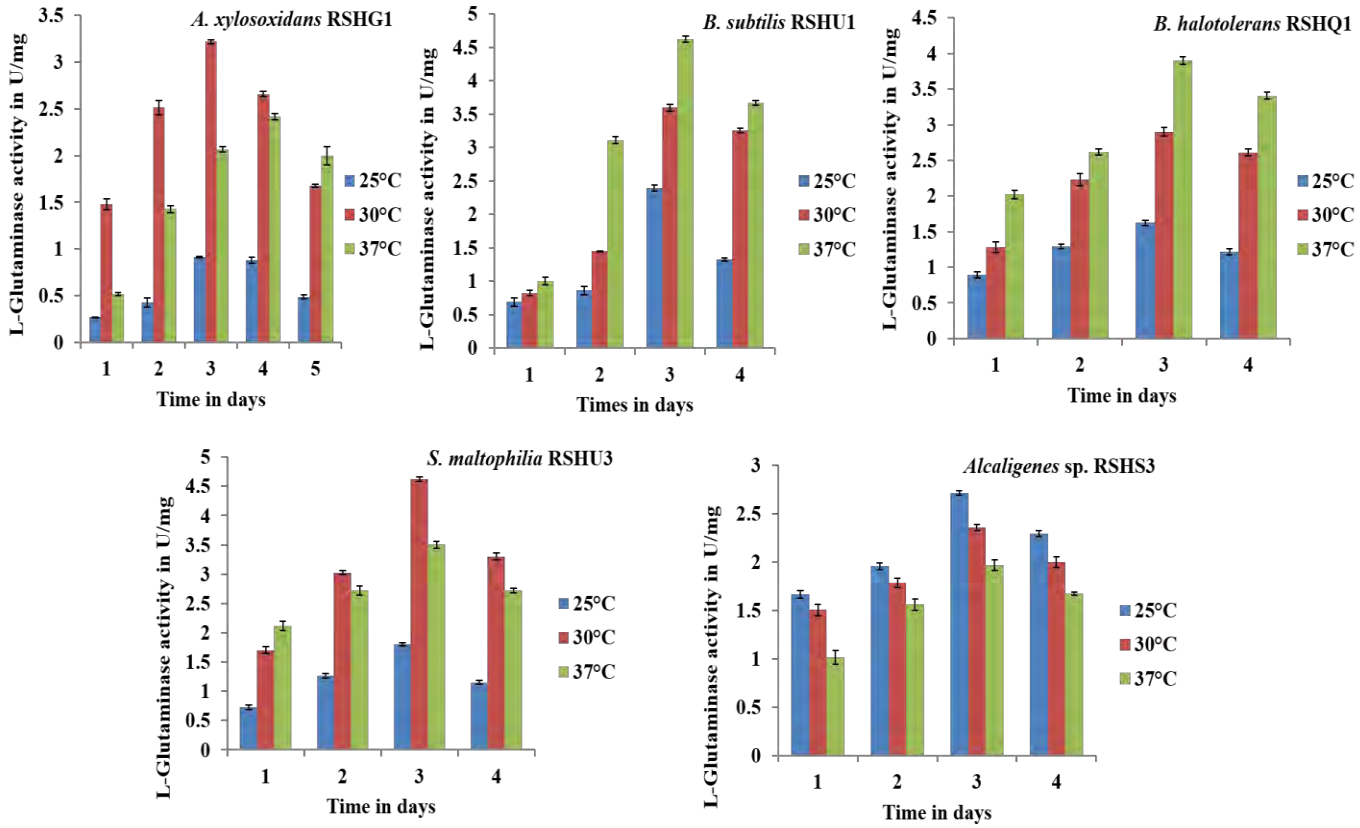

Figure 4.23. Effect of Temperature on Glutaminase production on Glutamine Salt Medium by *A. xylosoxidans* RSHG1 at pH9, by *B. subtilis* RSHU1 at pH6, *B. halotolerans* RSHQ1 at pH7, by *S. maltophilia* RSHU3 at pH8, by *Alcaligenes* sp. RSHS3 at pH8.

4.6.4. Effect of Carbon Sources

Different carbon sources optimized for glutaminase production on glutamine salt media. *A. xylosoxidans* RSHG1 showed the highest activity, 3.18U/mg with sorbitol, on the 3rd day of incubation, as shown in Figure 4.24. The minimum activity was observed with glucose. Other carbon sources showed the highest activities of 2.1U/mg with sucrose and 2.2U/mg with lactose, both observed on the 3rd day of incubation. The nutrient broth was also tested for glutamine production without adding any carbon source, but the maximum activity of 1.8U/mg was achieved on the 2nd day of incubation.

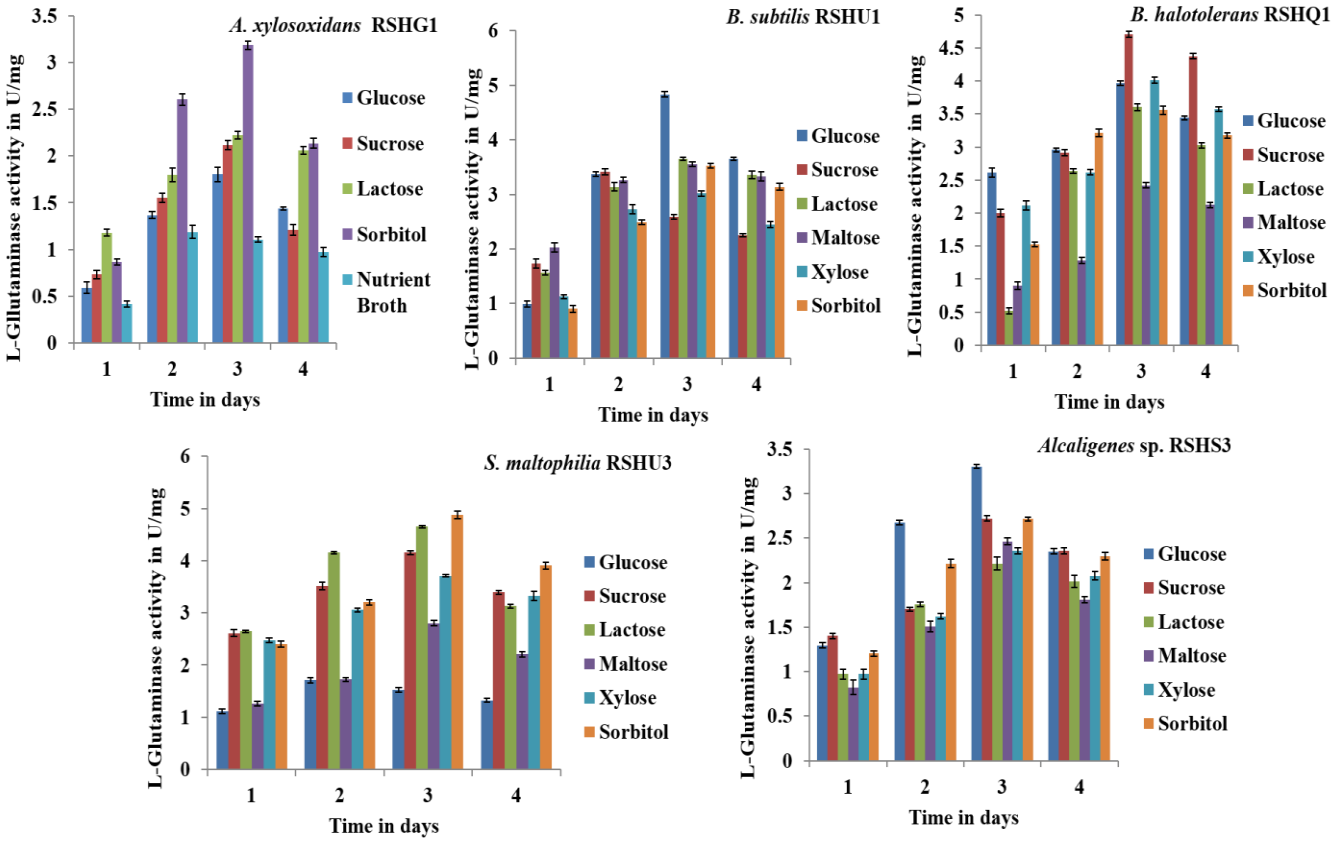

Figure 4.24. Effect of carbon source on Glutaminase production by *A. xylosoxidans* RSHG1 on Glutamine Salt Medium 30°C at pH9, by *B. Subtilis* RSHU1 on Glutamine Salt Medium 37°C at pH6, *B. halotolerans* RSHQ1, *S. maltophilia* RSHU3 on Glutamine Salt Medium 30°C at pH8, and *Alcaligenes* sp. RSHS3 on Glutamine Salt Medium at 25°C on pH8.

L-Glutaminase production by *B. subtilis* RSHU1 influenced by different carbon sources. The highest L-glutaminase activity, 4.8 U/mg, was gained on the 3rd day of incubation with glucose and the lowest activity with sucrose. The maximum activity with sucrose was 2.8U/mg on the 3rd day of incubation. While maximum activities on other carbon sources were 3.65 U/mg on the 3rd day with lactose, 3.55 U/mg on the 3rd day with maltose, and 3U/mg on the 3rd day with xylose 3.52U/mg on the 3rd day with sorbitol (Figure 4.24).

L-Glutaminase production by *B. halotolerans* RSHQ1 was optimized using six carbon sources with the best activity, 4.7 U/mg gained with sucrose on the 3rd-day of incubation, and the lowest activity 2.4 U/mg with maltose on the 3rd-day of incubation. While on glucose, lactose, xylose, and sorbitol activities were 3.96 U/mg on the 3rd day, 3.6 U/mg on the 3rd day, 4U/mg on the 3rd day, and 3.55U/mg on the 3rd day of incubation time, respectively (Figure 4.24).

The strain *S. maltophilia* RSHU3 got the highest activity, 4.9U/mg, on the 3rd day of incubation with sorbitol and showed the minimum activity of 1.7U/mg on the 2nd day of incubation with glucose. The rest of the carbon sources, sucrose, lactose, maltose, and xylose, showed 4.1U/mg

on the 3rd day, 4.65U/mg on the 4th day, 2.8U/mg on the 3rd day, and 3.8U/mg on the 3rd day of incubation, respectively.

Alcaligenes sp. RSHS3 is also optimized for carbon sources for L-glutaminase production. *Alcaligenes* sp. RSHS3 showed the best activity of 3.3U/mg on the 3rd day of incubation with glucose and minimum activity of 2.2 U/mg with lactose on the 3rd day of incubation. While activities with other carbon sources sucrose, maltose, xylose, and sorbitol were 2.7U/mg on the 3rd day, 2.46U/mg on the 3rd day, 2.35U/mg on the 3rd day, and 2.71U/mg on the 3rd day of incubation, respectively (Figure 4.24).

4.6.5. Nitrogen Sources

Effect of various nitrogen sources monitored on the L-glutaminase production. Maximum glutaminase activity by *A. xylosoxidans* RSHG1 achieved 3.1U/mg on the 3rd day of incubation with L-glutamine (Figure 4.25), while the lowest activity, 0.6 U/mg, showed with Tryptone on the 3rd day of incubation. The effect of other nitrogen sources yeast extract, ammonium sulfate, and sodium nitrate was 2.6 U/mg on the 3rd day, 1.18 U/mg on the 3rd day, and 1.16 U/mg on the 3rd-day incubation, respectively.

B. subtilis RSHU1, optimized against various nitrogen sources. Maximum activity 4.5U/mg gained with L-glutamine on the 3rd day of incubation, and minimum activity was monitored 2.3U/mg with ammonium sulfate on the 3rd day of incubation. While other sources, yeast extract, Tryptone, and sodium nitrate, showed 3.68U/mg on the 3rd day, 3.66U/mg on the 3rd day, and 3.2U/mg on the 3rd day of incubation (Figure 4.25).

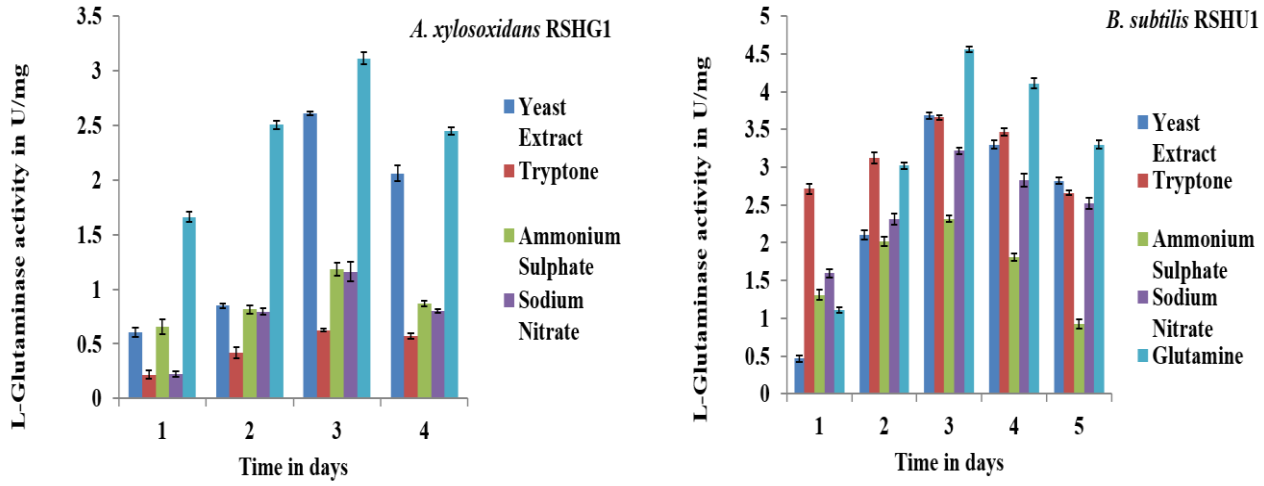

Figure 4.25. Effect of Nitrogen source Glutaminase production by *A. xylosoxidans* RSHG1 on Glutamine salt Medium on 30°C at pH9 and production by *B. subtilis* RSHU1 on at 37°C on pH 6.

4.6.6. Effect of Different Inducers on L-Glutaminase Production.

Glycine, glutamine, and lysine effects were observed on all selected strains as an inducer for 4days of incubation. *A. xylosoxidans* RSHG1 achieved the best activity 6.87U/mg (Figure 4.26) with L-glutamine as an inducer on the 3rd day of incubation. Glutaminase activity with glycine and lysine was 6.13U/mg and 5.3U/mg on the 3rd day of incubation, respectively.
For glutaminase production, *B. subtilis* RSHU1 was optimized against glycine, glutamine, and lysine. The highest activity, 5.3U/mg achieved with L-glutamine on the 3rd day of incubation, as shown in Figure 4.44. In contrast, activities with glycine and lysine were 3.5U/mg on the 2nd day and 2.9U/mg on the 3rd day of incubation, respectively.

L-Glutaminase activity by *B. halotolerans* RSHQ1 was optimized using three inhibitors glycine, glutamine, and lysine. Maximum activity 5.6U/mg shown in Figure 4.26 on the 3rd day achieved with glutamine, and activities with other sources glycine and lysine were 2.38 on the 3rd day and 2.88 U/mg on the 3rd day of incubation, respectively.

S. maltophilia RSHU3 maximum production of 5.46 U/mg of L-glutaminase was achieved with L-glutamine as an inducer on the 3rd day of incubation. Activities with glycine and lysine were 2.66 U/mg on the 3rd day and 3.06 U/mg on the 3rd day of incubation, respectively.

Effect of induces on L-glutaminase production by *Alcaligenes* sp. RSHS3 showed the best activity of 5.65U/mg on the 3rd day with inducer L-glutamine, while glycine and lysine showed 3.6U/mg on the 3rd day and 4.2U/mg on the 3rd-day incubation, respectively.

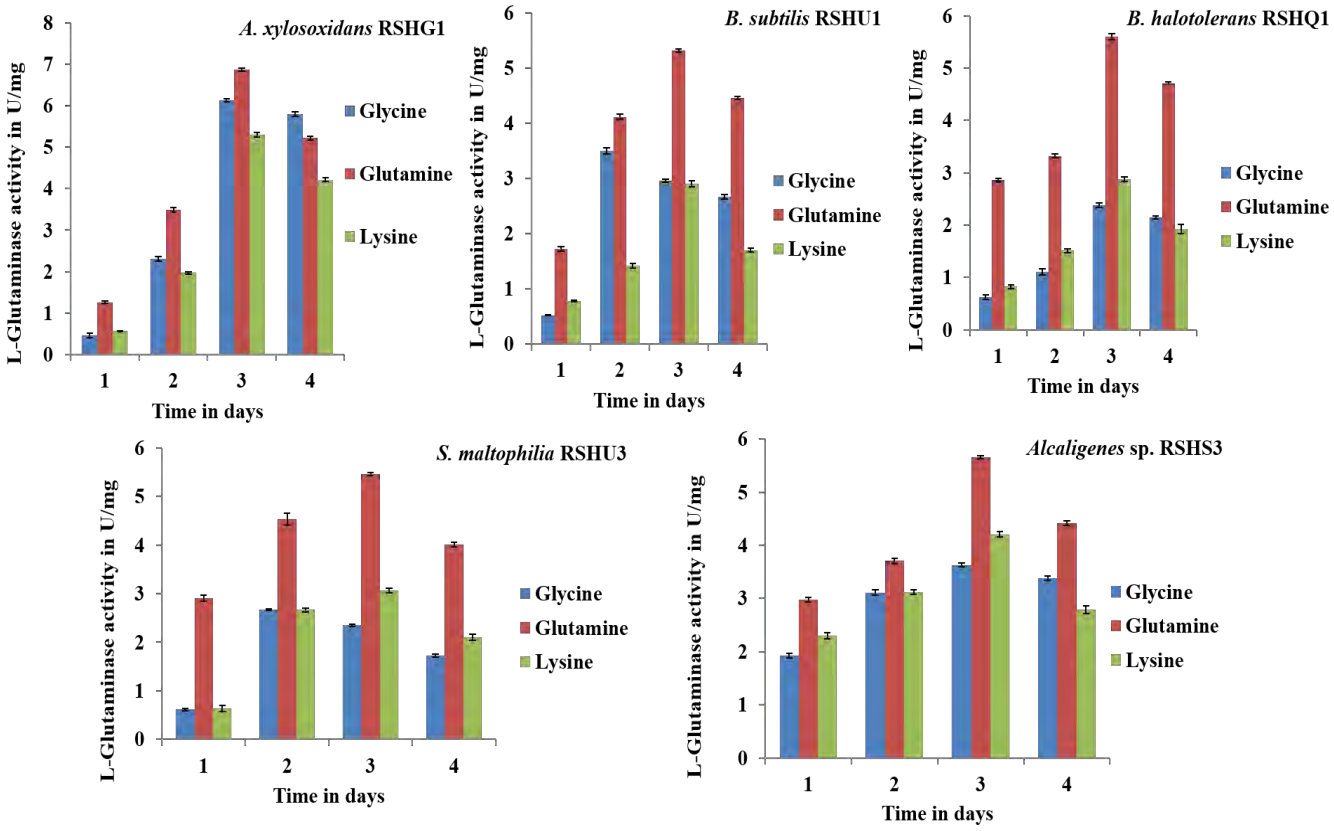

Figure 4.26. Inducers Effect on Glutaminase production on Glutamine salt medium by *A. xylosoxidans* RSHG1 at 30°C on pH 9, by *B. Subtilis* RSHU1on 37°C at pH 6, by *B. halotolerans* RSHQ1 at 37°C at pH7, by *S. maltophilia* RSHU3 on 30°C at pH 8, and by *Alcaligenes* sp. RSHS3 on 25°C at pH 8.

4.7. L-Glutaminase Production by Selected Bacterial Strains

L-glutaminase produced under optimized fermentation conditions by five selected bacterial strains *A. xylosoxidans* RSHG1, *B. subtilis* RSHU1, *B. halotolerans* RSHQ1, *S. maltophilia* RSHU3, and *Alcaligenes* sp. RSHS3 were partially purified using 70% fractional saturation of ammonium sulfate. Good enzyme activities were observed for all the strains except *B. subtilis* RSHU1. Maximum specific activity (6.7 U/mg of protein) was for *Alcaligenes* sp. RSHS3 (Table 4.7).

Partially purified L-glutaminase was dialyzed against a low ionic strength buffer to remove an excessive amount of ammonium sulfate. L-Glutaminase from *A. xylosoxidans* RSHG1 and *B. subtilis* RSHU1 has been purified on gel filtration chromatographic column with G75 and G-100.

Table 4.7. Glutaminase activities produced by selected strains before and after dialysis

Bacterial strains	Total Enzyme Units (IU)	Total protein (mg)	Specific Activity (U/mg) Before Ammonium sulfate precipitation	Total units after dialysis (mg)	Total protein after dialysis	Specific Activity (U/mg) After Dialysis
***A. xylosoxidans* RSHG1**	2340.0	750	3.12	1500	35	42
***B. subtilis* RSHU1**	2820.0	720	3.9	760	30	25
***B. halotolerans* RSHQ1**	1134.0	520.5	2.1	817.8	15.38	54
***S maltophilia* RSHU3**	1800.0	450.48	3.9	1120	13.6	82
***Alcaligenes* sp. RSHS3**	2025.6	380.48	3.3	1620	12.02	135

4.8. Bacterial L-Glutaminase Purification

Two partially purified bacterial L-glutaminases, further purified on gel filtration column chromatography. One L-glutaminase was produced by an expired L-glutamine sample *A. xylosoxidans* RSHG1, second L-glutaminase was isolated from isolated soil strain *B. subtilis* RSHU1.

4.8.1. Purification L-Glutaminase Produced by *B. subtilis* RSHU1

After ammonium sulfate precipitation and dialysis, L-Glutaminase produced by *B. subtilis* RSHU1, purified on gel filtration column chromatography, with Sephadex G-100. The sample volume of 2.5mL, loaded on the gel filtration column. Later, the sample was collected in 25fractions at the flow rate of 0.5 mL/min; 3ml fractions were collected. The fractions estimated for protein and glutaminase activity; 6-12 fractions were pooled based on their activity. The enzyme was 12.5 fold purified after gel filtration giving a 14.5 percent yield (Table 4.8 & Figure 4.27).

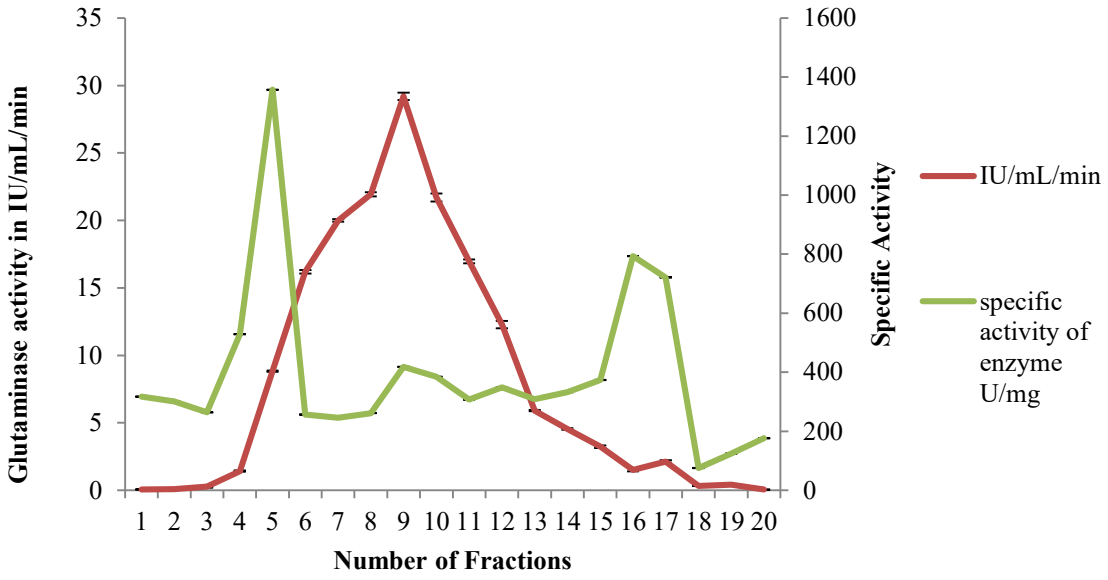

Figure 4.27. Gel filtration column chromatography results for *B. subtilis* RSHU1 L-glutaminase on Sephadex G-100.

Table 4.8. Sequential purification stages *B. subtilis* RSHU1

Method	Total Protein (mg)	Specific Activity (U/mg)	Purification (fold)	Total Activity (IU)	Yield (%)
Crude Extract	720	3.9	1	2820.0	100
Ammonium sulphate (70%) and dialysis	30	25	6.4	760	26.9
Gel filtration chromatography	1.3	314	12.5	409.42	14.5

A. xylosoxidans RSHG1 L-glutaminase, after ammonium sulfate precipitation and dialysis purified on Sephadex G-75 Table 4.9 & Figure 4.49. 2.5mL sample was loaded on column and collected with the flow rate of 0.5 mL/min in 25 fractions, 3 mL collected in each fraction. 6-14 fractions were pooled based on their activities. Collected fractions checked out for protein and then for glutaminase activity. There were 19.7 folds of purification after gel chromatography.

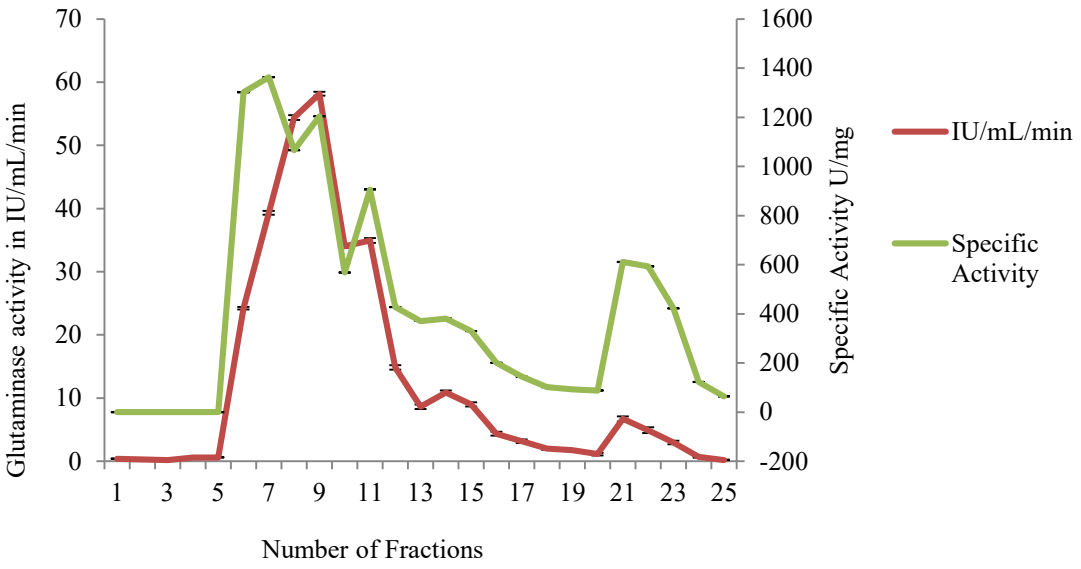

Figure 4.28. Gel filtration column chromatography results for *A. xylosoxidans* RSHG1 L-glutaminase on Sephadex G-75.

Table 4.9. Sequential purification stages *A. xylosoxidans* RSHG1

Method	**Total Protein (mg)**	**Specific Activity (U/mg)**	**Purification (fold)**	**Total Activity (IU)**	**Yield (%)**
Crude Extract	750	3.12	1	2340.0	100
Ammonium sulphate (75%) and dialysis	35	42.8	13.7	1500	64
Gel filtration chromatography	1.04	840	19.7	874	37.3

4.9. Characterization of Bacterial L-Glutaminase

After production, a crude enzyme, isolated from all selected strains, partially purified, using ammonium sulfate, and a run on a manual column filled with G-100, characterized for incubation time, temperature, pH, sodium chloride concentration, and metal ion effects.

4.9.1. Effect of Incubation Time on Bacterial L-Glutaminase

Purified L-glutaminase produced by *A. xylosoxidans* RSHG1 showed maximum activity of 511U/mg after 30 minutes of incubation at pH7 and temperature 37°C while after that activity, reduced. L-Glutaminase produced by *B. subtilis* RSHU1 was partially purified and observed the highest activity, 445 U/mg, after 30 minutes of incubation at pH7 and temperature 37°C. *B.*

halotolerans RSHQ1 L-glutaminase showed the best activity after 30mintues of incubation (203.6 U/mg). L-Glutaminase produced by *S. maltophilia* RSHU3 was checked out for the incubation time effects on L-glutaminase activity. Best activity 248.8U/mg was observed after 30 minutes of incubation; afterward, activity decreased. L-Glutaminase produced by *Alcaligenes* sp. RSHS3, partially purified and observed the incubation time effect, maximum activity 211.2U/mg achieved after 30minutes of incubation (Figure 4.29).

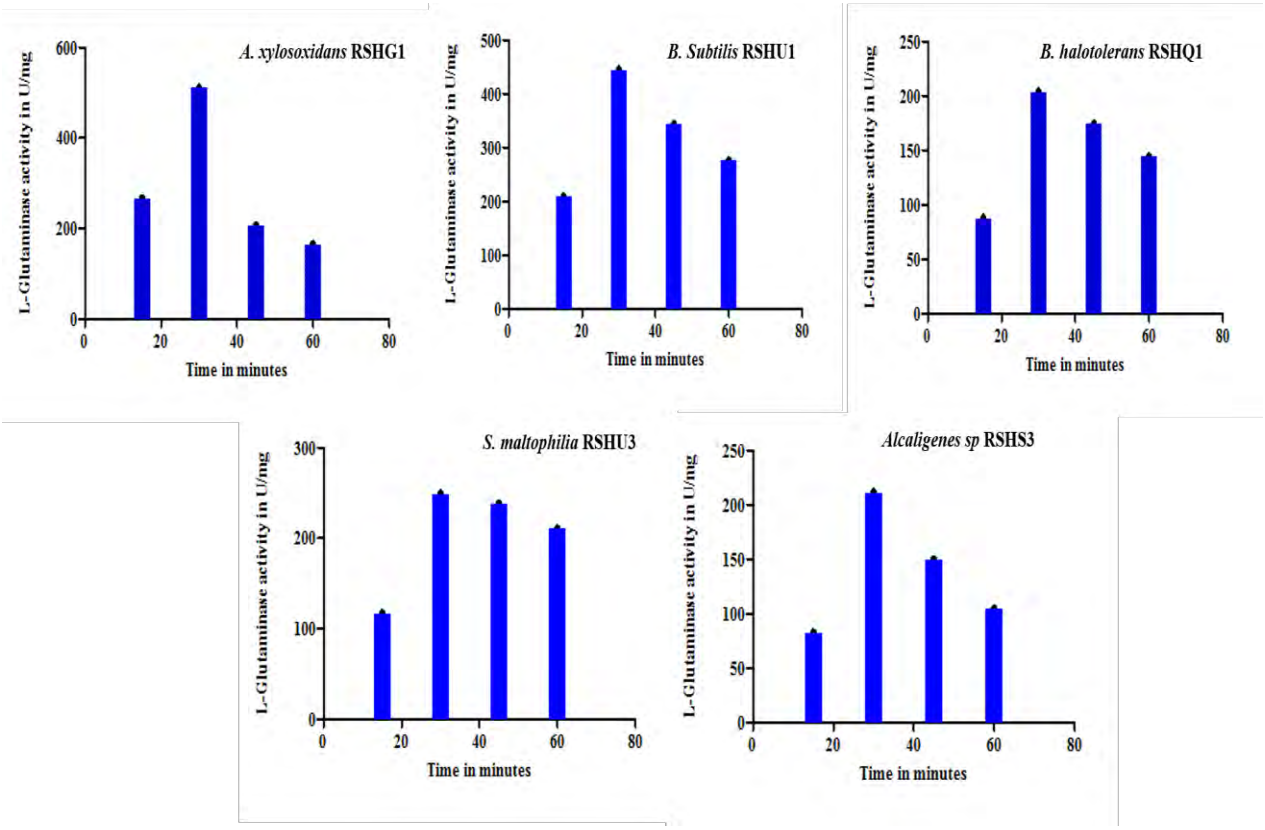

Figure 4.29. Effect of Incubation Time on L-Glutaminase produced by Selected Bacterial Strains at 37°C.

4.9.2. Effect of Substrate Concentration

The effect of various substrate concentrations on the purified and partially purified L-glutaminase from five bacterial strains was monitored.

The value of Vmax for *A. xylosoxidans* RSHG1 was 443.8U/mg, then the value of Km was calculated by Michaelis and Menten plot. The value of Km was 0.235 mM, Vmax of Glutaminase produced by *B. subtilis* RSHU1 was 522 U/mg, and KM was 0.059 mM. L-Glutaminase produced by *B. halotolerans* RSHQ1 showed Vmax 343.6U/mg, and KM is 0.37 mM while *S. maltophilia* RSHU3 glutaminase observed Vmax was 351.6 U/mg, and KM was 0.166 mM. Vmax of L-glutaminase, produced by *Alcaligenes* sp. RSHS3 achieved Vmax 232.9 U/mg, and KM was 0.186mM, as shown in Figure 4.30 by Michaelis and Menten plot.

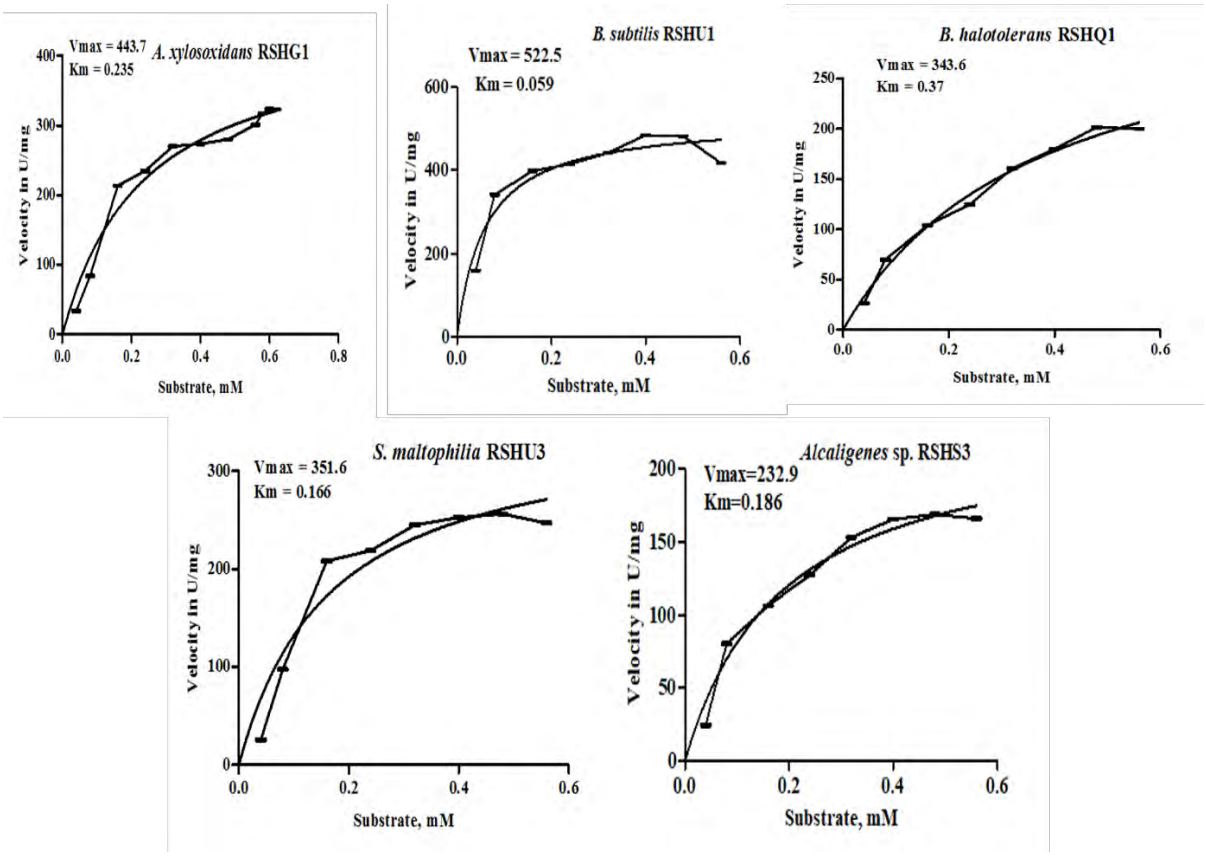

Figure 4.30. Effect of Substrate Concentration on L-Glutaminase activity produced by *A. xylosoxidans* RSHG1 incubated at 40°C on pH7, *B. subtilis* RSHU1 at 40°C on pH6.5, *B. halotolerans* RSHQ1 at 50°C on pH6, *S. maltophilia* RSHU3 at 40°C on pH8, *Alcaligenes* sp. RSHS3 at 40°C on pH7.

4.9.3. Effect of pH on Bacterial L-Glutaminase Activity

Partially purified L-glutaminase produced by *A. xylosoxidans* RSHG1 showed maximum activity of 505 U/mg at pH 7. The enzyme showed good activity within pH 6-7.5. Glutaminase produced by *B. subtilis* RSHU1 gained the highest activity, 516U/mg at pH 6.5. The enzyme was stable over the range of pH range 6-7. *B. halotolerans* RSHQ1 L-glutaminase was stable between pH 5.5-7 and maximum activity of 268 U/mg at pH 6. Optimum pH for L-glutaminase produced by *S. maltophilia* RSHU3 was 8 with activity 303U/mg, and the enzyme was relatively stable over pH range 7.5-8.5 Figure 4.78. *Alcaligenes* sp. RSHS3 glutaminase gained optimum activity 219 U/mg at pH 7, and the enzyme was stable between pH range 6.5-7.5 (Figure 4.31).

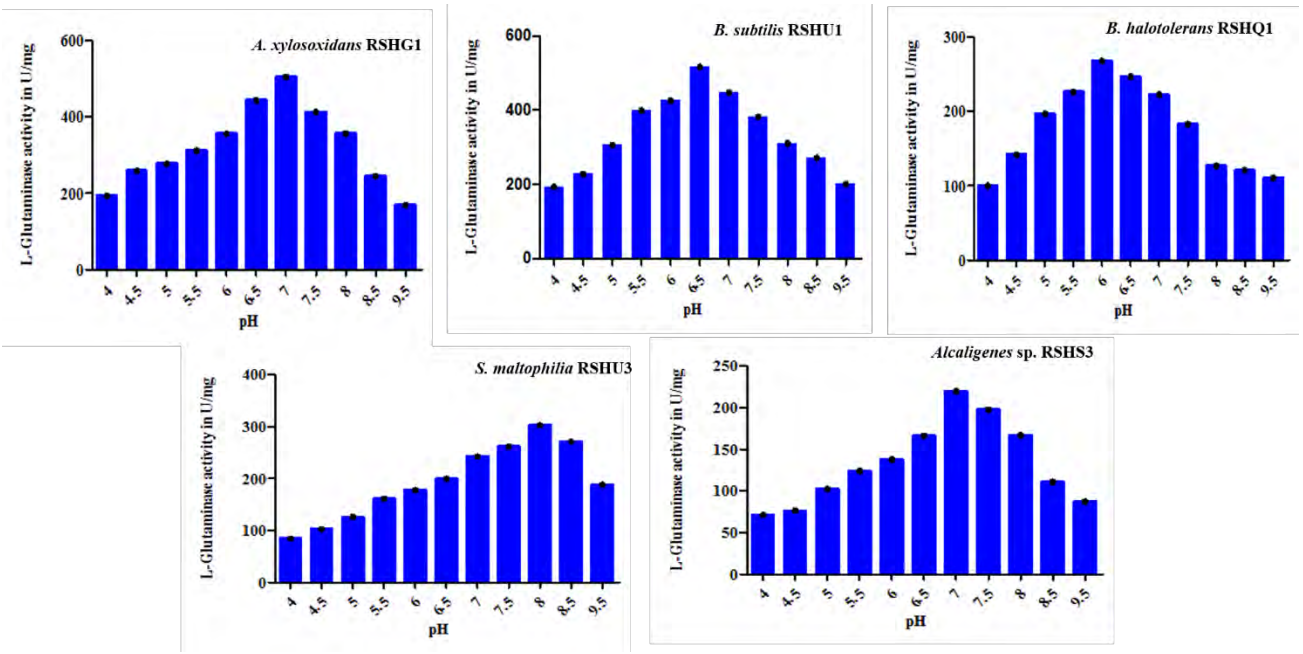

Figure 4.31. Effect of pH on L-Glutaminase produced by *A. xylosoxidans* RSHG1, *B. subtilis* RSHU1, *B. halotolerans* RSHQ1, *S. maltophilia* RSHU3, and *Alcaligenes* sp. RSHS3 at 37°C.

4.9.4. Effect of Temperature on Bacterial L-Glutaminase Activity

L-Glutaminase produced by *A. xylosoxidans* RSHG1 showed the best activity 520 U/mg at 40°C. The enzyme was stable at 40-50°C. After 50°C sharp decrease in activity was observed. L-glutaminase by *B. subtilis* RSHU1 showed the highest activity, 512U/mg at 40°C; the enzyme was stable at the range of 40-50°C. Reduction in enzyme activity, observed after 40°C temperature. *B. halotolerans* RSHQ1 L-glutaminase was gained maximum activity of 291U/mg at 50°C, and activity decreased after 50°C. L-glutaminase isolated from *S. maltophilia* RSHU3 achieved the highest activity of 270.9U/mg at 40°C and decreased activity, observed after 40°C. L-Glutaminase produced by *Alcaligenes* sp. RSHS3 showed the best activity 225U/mg at 40°C; activity decreased after 40°C (Figure 4.32).

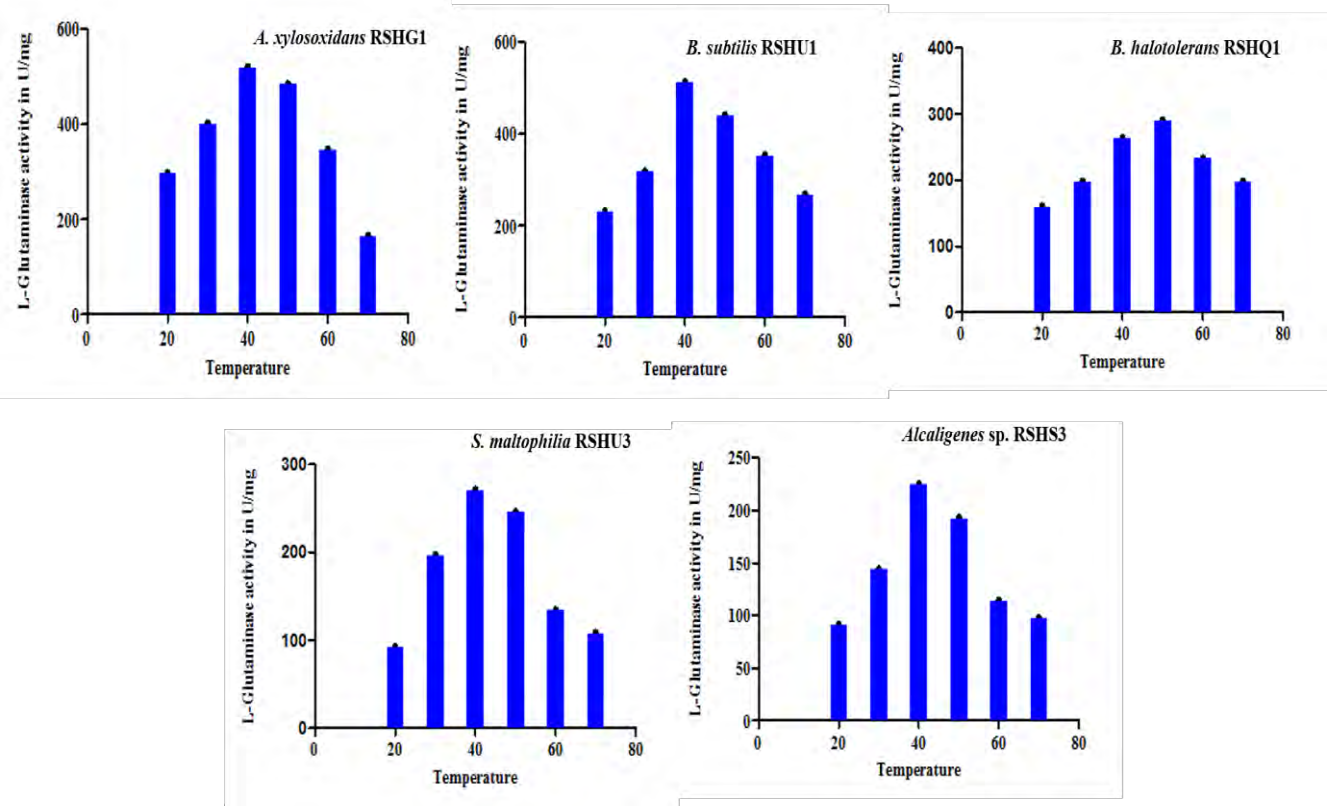

Figure 4.32. Effect of Temperature on L-glutaminase produced by *A. xylosoxidans* RSHG1 at pH7, *B. subtilis* RSHU1 at pH6.5, *B. halotolerans* RSHQ1 at pH6, *S. maltophilia* RSHU3 at pH8, and *Alcaligenes* sp RSHS3 at pH7.

4.9.5. Effect of metal ion on Bacterial L-Glutaminase Activity

The effect of inhibitors checked out on partially purified glutaminases produced by all the selected bacterial strains. L-Glutaminase produced by *A. xylosoxidans* RSHG1 showed activity 506.8U/mg without the use of metal ions. An increase in activity observed with cobalt chloride, calcium chloride, barium chloride, zinc sulfate, potassium chloride, and magnesium sulfate and sodium chloride activities were 596, 832, 844, 684, 559, 850, and 558U/mg, respectively (Figure 4.87). While decreased activity with EDTA, mercuric chloride, manganese sulfate, and ferrous sulfate with activities 224, 374, 396, and 414U/mg, respectively.

B. subtilis RSHU1 L-glutaminase activity without metal ions addition was 511U/mg. Enzyme activity increased with cobalt chloride, calcium chloride, barium chloride, zinc sulfate, potassium chloride, magnesium sulfate, and sodium chloride with activities 836, 793, 733, 755, 601, 647, and 528, respectively. L-Glutaminase activity decreased by adding EDTA, mercuric chloride, manganese sulfate, ferrous sulfate, and activities monitored were 97, 289, 206, and 289, respectively. L-glutaminase produced by *B. halotolerans* RSHQ1 showed activity 296 U/mg without the addition of inhibitor. The rise in activity monitored with cobalt chloride,

barium chloride, mercuric chloride, zinc sulfate, potassium chloride, magnesium sulfate, and sodium chloride; activities 451, 468, 302, 385, 304, 453, and 306 U/mg, respectively. Activity reduction with EDTA, calcium chloride, manganese sulfate, and ferrous sulfate with activities 47, 150, 162, and 226 U/mg, respectively. L-glutaminase produced by *S. maltophilia* RSHU3 activity without metal 295U/mg. Enzyme gained activity with cobalt chloride, calcium chloride, barium chloride, mercuric chloride, zinc sulfate, potassium chloride, and sodium chloride; 477, 460, 360, 413, 47, 372, and 319U/mg, respectively. While the metal ions lowered the activity of enzymes were EDTA, manganese sulfate, magnesium sulfate, and ferrous sulfate, activities were 53.8, 177, 168, and 62 U/mg, respectively.

L-glutaminase produced by *Alcaligenes* sp. RSHS3 activity without metal ions was 213U/mg. The addition of metal ions gave higher activities: cobalt chloride, calcium chloride, barium chloride, potassium chloride, magnesium sulfate, and sodium chloride with activities 336, 328, 229, 231, 305, and 261, respectively. L-Glutaminase activity, reduced by adding EDTA, mercuric chloride, zinc sulfate, manganese sulfate, and ferrous sulfate with activities 57, 202, 196, and 133, respectively (Figure 4.33).

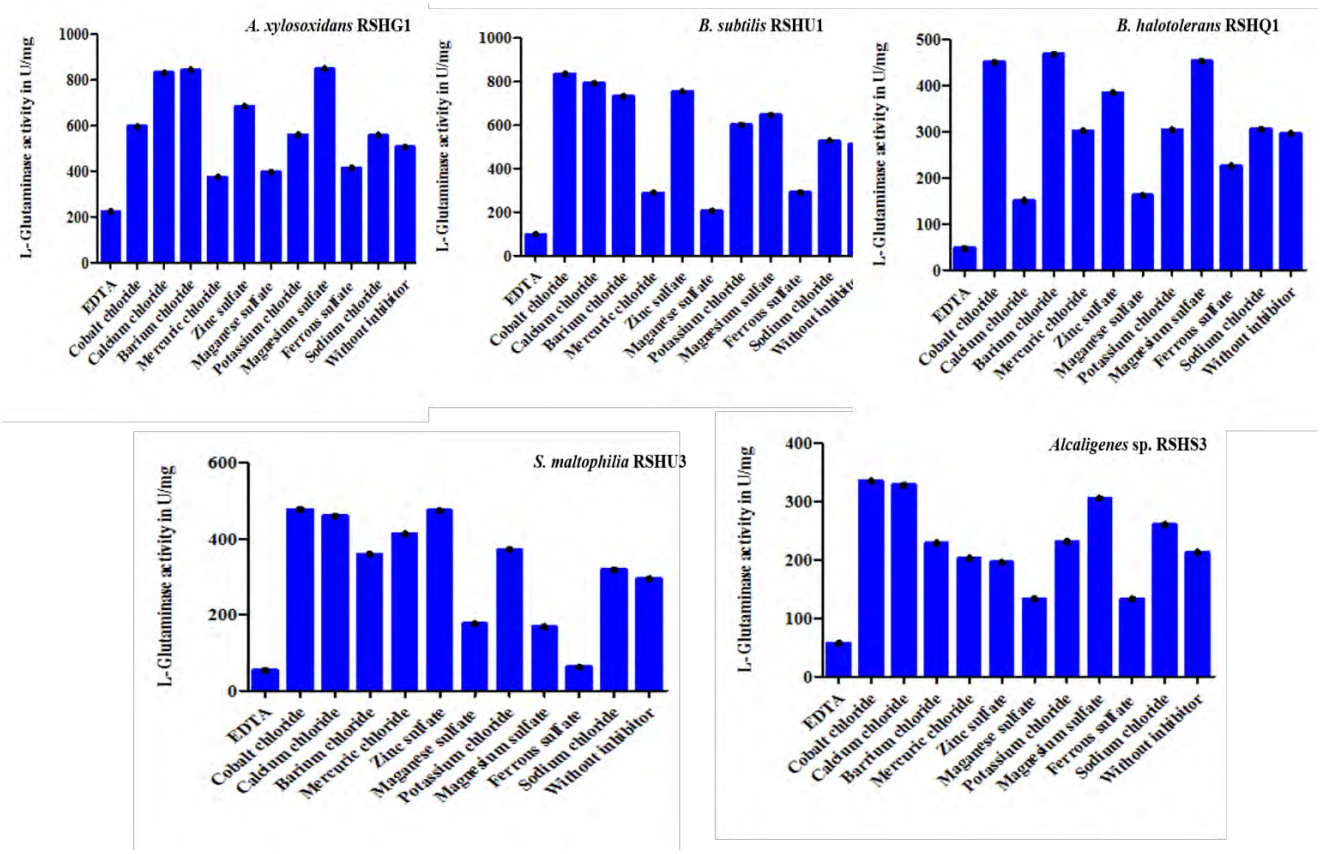

Figure 4.33. Effect of Metal ions on L-glutaminase activity produced by *A. xylosoxidans* RSHG1 at pH7 at 40°C, *B. subtilis* RSHU1 on 40°C at pH6.5, *B. halotolerans* RSHQ1 on 50°C at pH6, *S. maltophilia RSHU3* at pH8 on 40°C, and *Alcaligenes* sp. RSHS3 on 40°Cat pH7.

4.9.6. Effect of Sodium Chloride on Bacterial L-Glutaminase Activity

Partially purified L-glutaminase produced by selected bacterial strains and fungal strain optimized for the effect of sodium chloride (4-20%) concentration on glutaminase activity.

A. xylosoxidans RSHG1 partially purified glutaminase activity was 500U/mg without sodium chloride. The addition of 8% NaCl observed an increase of 64% in enzyme activity, and the best activity was 822 U/mg. L-glutaminase produced *B. subtilis* RSHU1 showed 517 U/mg without sodium chloride, and sodium chloride gave a 65% increase in enzyme activity and highest activity 855 U/mg on 16% NaCl concentration. L-Glutaminase produced by *B. halotolerans* RSHQ1 monitored for the effect of sodium chloride on enzyme activity. Without sodium chloride, activity was 295U/mg, 53% activity increased by adding 16% NaCl; the best activity was 452 U/mg. L-glutaminase produced by *S. maltophilia* RSHU3 activity was 305U/mg without sodium chloride. Sodium chloride increased the activity by 32% with 12% NaCl, and the maximum enzyme activity was 403U/mg. L-Glutaminase produced by *Alcaligenes* sp. RSHS3 showed activity of 212 U/mg without sodium chloride. Figure 4.34 illustrates a 44 % increase in activity observed with 12 % sodium NaCl on glutaminase highest activity was 306 U/mg.

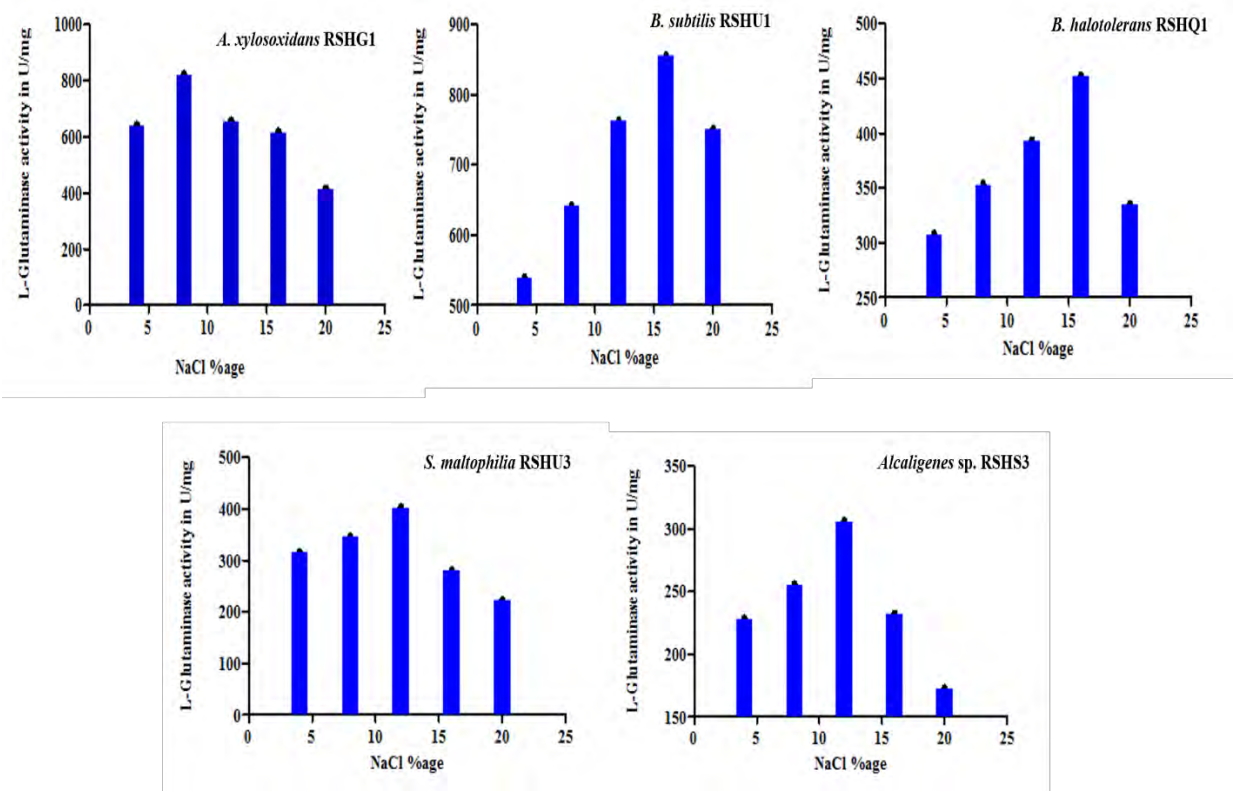

Figure 4.34. Effect of NaCl, on L-Glutaminase activity by *A. xylosoxidans* RSHG1 on 40°C at pH7, *B. subtilis* RSHU1 on 40°C at pH6.5, *B. halotolerans* RSHQ1 at 50°C on pH6, *S. maltophilia* RSHU3 at 40°C on pH8, and *Alcaligenes* sp. RSHS3 at 40°C on pH7.

4.10. Cultural Optimization of L-Glutaminase Production by Fungal strain *Epicoccum* sp. NFW1

4.10.1. Effect of Incubation Time on L-Glutaminase Production

The *Epicoccum* sp. NFW1 was grown on Glutamine salt media with phenol red as an indicator that showed the best activity of 1.75U/mg on the 4th day at 30°C (Figure 4.35).

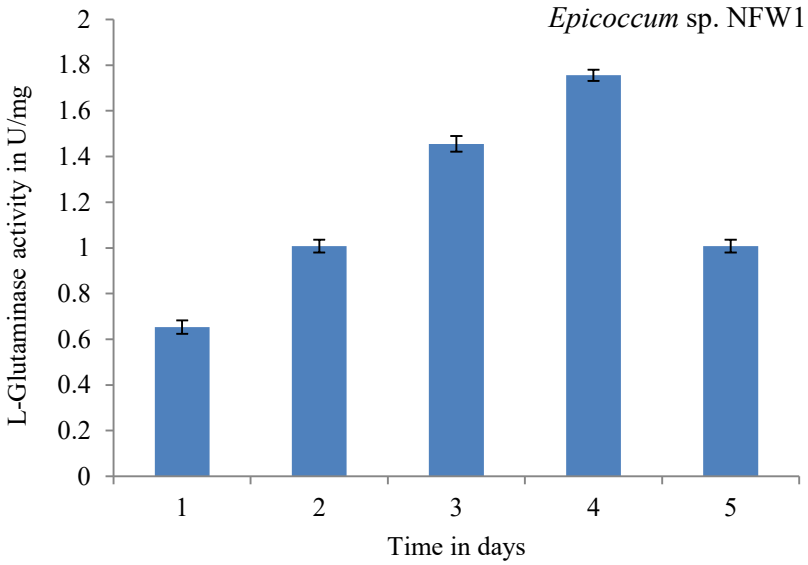

Figure 4.35. L-Glutaminase production by *Epicoccum* sp. NFW1 on Glutamine salt media at 30°C at pH6.6.

4.10.2. Effect of Media on L-Glutaminase Production

Potato dextrose broth, Sabouruad dextrose broth, and Czapek dox broth media, used for the maximum production of L-glutaminase. *Epicoccum* sp. NFW1 showed the best glutaminase activity 2.75 U/mg on Czapek dox broth on the 4th day of incubation, as shown in Figure 4.36. while the strain was grown on Potato dextrose broth and Sabouruad dextrose broth gave activities of 1.36 U/mg on the 4th day of incubation and 2.15U/mg on the 4th day of incubation, respectively.

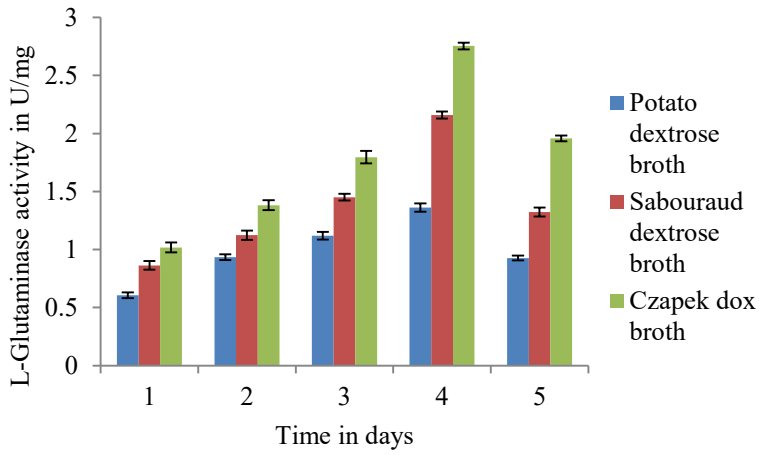

Figure 4.36. Effect of Media on L-Glutaminase production by *Epicoccum* sp. NFW1 at 30°C.

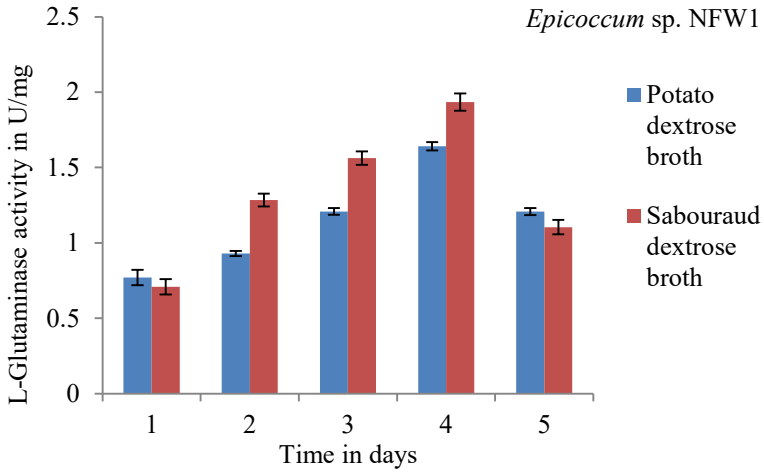

Figure 4.37. Effect of 1% sucrose and 0.3% NaCl on Glutaminase production by *Epicoccum* sp. NFW1 on Potato dextrose broth and Sabouraud dextrose broth incubated at 30°C.

Epicoccum sp. NFW1 optimized using Potato dextrose broth and Sabouraud dextrose broth with 1% sucrose and 0.3% sodium chloride. Maximum activities were 1.64U/mg on the 4th day and 2.27U/mg (Figure 4.37) on the 4th day of incubation.

4.10.3. Effect of Inducers on L-Glutaminase Production

Epicoccum sp. NFW1on Czapek dox media showed maximum activity of 4.4U/mg with L-glutamine as an inducer on the 4th day of incubation (Figure 4.38). Glycine and lysine also showed a significant 1.58 and 2U/mg activity on the 4th day of incubation, respectively.

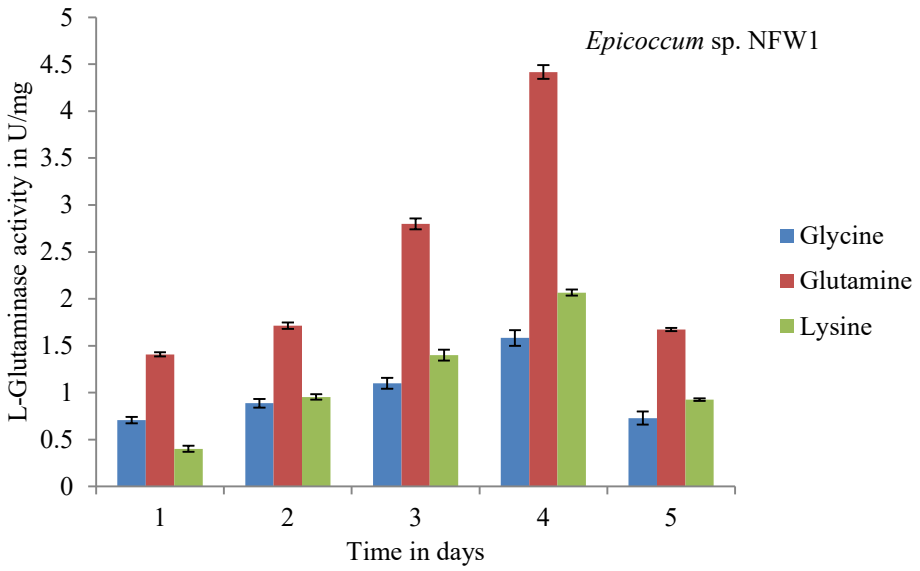

Figure 4.38. Effect of Inducers on L-Glutaminase production by *Epicoccum* sp. NFW1 on Czapek dox medium on 30°C.

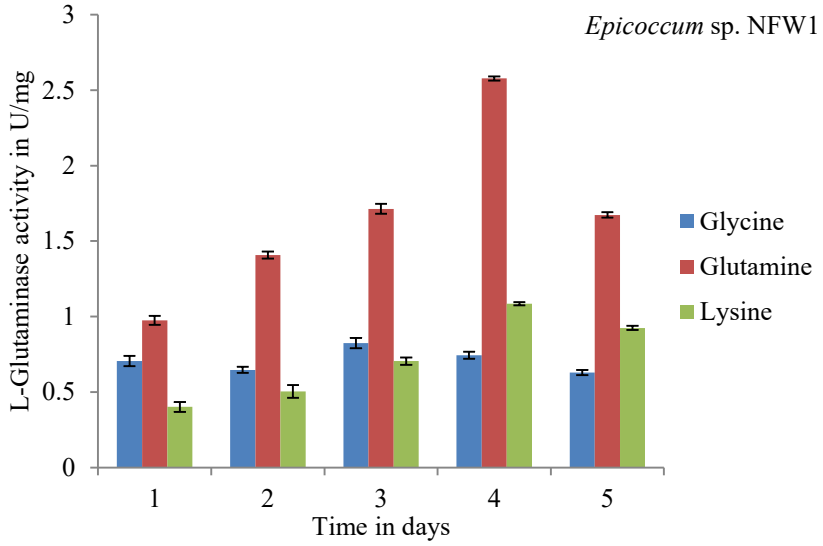

Figure 4.39. Inducers Effect with 1% sucrose and 0.3% NaCl on Potato Dextrose broth for L-Glutaminase production by *Epicoccum* sp. NFW1 at 30°C.

L-glutaminase produced by *Epicoccum* sp. NFW1on potato dextrose broth with inducers showed a maximum of 2.7U/mg activity on the 4th day of incubation with glutamine. While with glycine and lysine highest activities were 1.32 and 1.54 U/mg on the 4th day of incubation, respectively (Figure 4.39).

4.10.4. Effect of Carbon Source on L-Glutaminase Production

The effect of six-carbon sources, optimized for glutaminase production by *Epicoccum* sp. NFW1 on fermentation media at 30°C. Maximum activity of 2.3U/mg, achieved with sucrose on the 4th day of incubation, and lowest activity of 1U/mg with sorbitol on the 4th day of

incubation. Maximum activities with other carbon sources lactose, maltose, glucose, and xylose, were 1.36U/mg on the 3rd day of incubation, 1.12U/mg on the 3rd day of incubation, 1.38U/mg on the 3rd-day, 1.78 U/mg on the 4th day of incubation, respectively as shown in Figure 4.40.

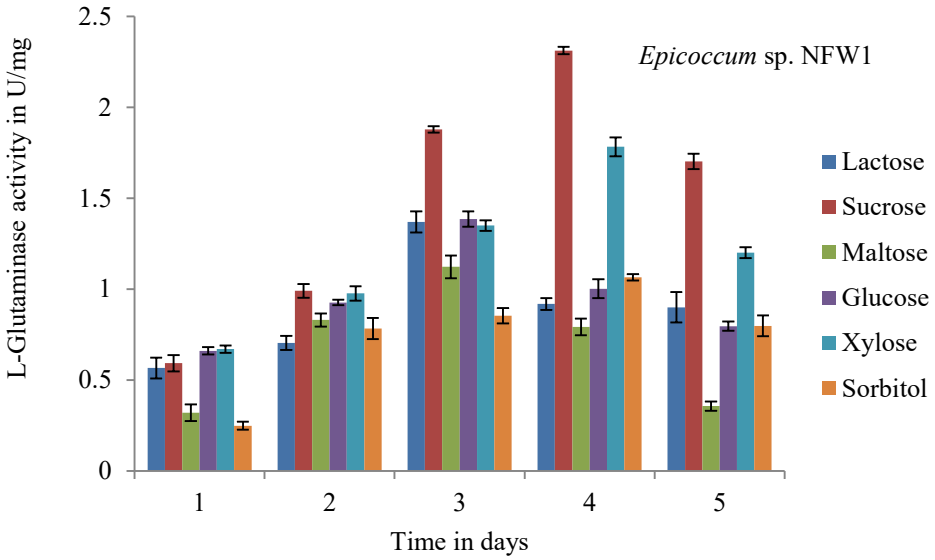

Figure 4.40. Effect of Substrate on Glutaminase production by *Epicoccum* sp. NFW1 on Glutamine Salt Medium at 30°C.

4.10.5. Effect of pH on L-Glutaminase Production

Effect of 3-9 pH was observed on glutaminase production by *Epicoccum* sp. NFW1 as shown in Figure 4.41. At pH 4, 5, 6, 8, and 9, activities were 1.38U/mg on the 4th day of incubation, 1.54U/mg, 1.97U/mg, 1.97U/mg, 2.3U/mg on the 4th day, and 1.1 U/mg on the 3rd day of incubation respectively The highest activity, 2.67U/mg achieved on the 4th day of incubation at pH7, and the lowest activity, 1U/mg, was at pH3 on the 4th day of incubation.

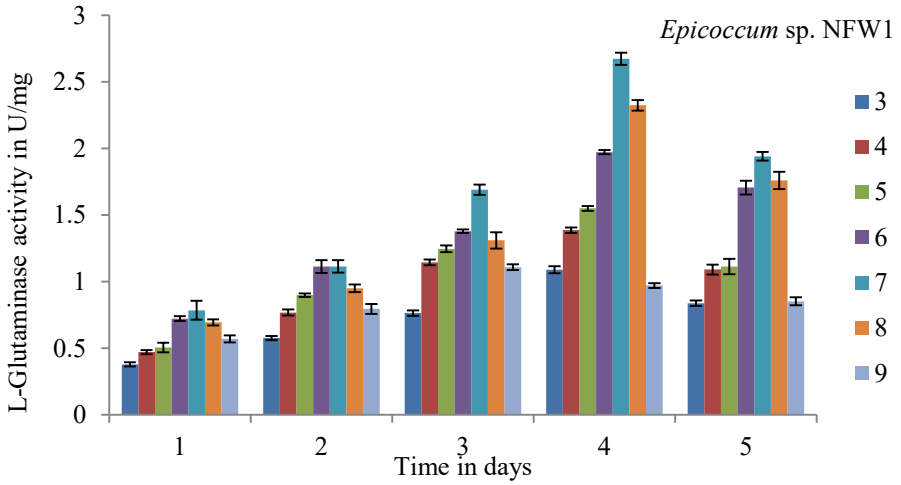

Figure 4.41. Effect of pH on Glutaminase production by *Epicoccum* sp. NFW1 on Glutamine Salt Medium with 1% sucrose as a carbon source on 30°C.

4.10.6. Effect of Nitrogen Source

Epicoccum sp. NFW1 was optimized for nitrogen sources for glutaminase production on fermentation media with 1% sucrose as a carbon source at 30°C, at pH 7 in a shaking incubator. The highest activity, 3U/mg, was achieved on the 4th day of incubation with glutamine and minimum activity 0.85U/mg on the 4th day with ammonium chloride. While activities with other nitrogen sources yeast extract, Tryptone, ammonium sulfate, and sodium nitrate activities were 1.7 U/mg on the 3rd day, 1.9U/mg on the 3rd day, 0.95 U/mg on the 4th day, and 2.53 U/mg on the 4th day (Figure 4.42) of incubation respectively.

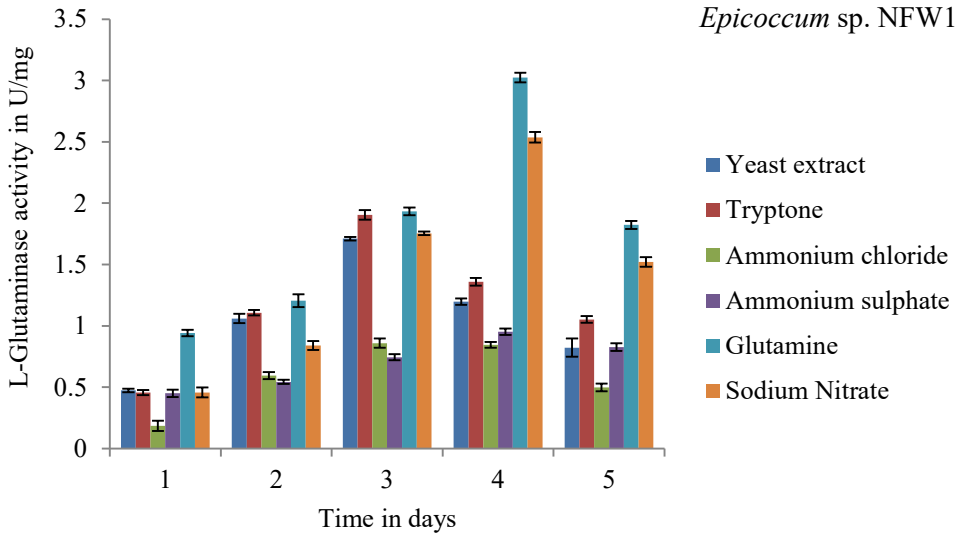

Figure 4.42. Effect of 1% Nitrogen source on Glutaminase production by *Epicoccum* sp. NFW1 on fermentation media with 1% sucrose at pH7 on 30°C.

4.11. L-Glutaminase production by *Epicoccum* sp. NFW1

L-glutaminase also produced one fungal strain, *Epicoccum* sp. NFW1. Then partially purified with 70% of ammonium sulfate fractional saturation Table 4.10.

Later ammonium sulfate precipitated L-glutaminase was dialyzed against a 10mM phosphate buffer to remove an excessive amount of ammonium sulfate. *Epicoccum* sp. NFW1, L-glutaminase purified on gel filtration chromatographic column with G75.

Table 4.10. Glutaminase activities produced by selected strains before and after dialysis

Fungal strain	Total Enzyme Units (IU)	Total protein (mg)	Specific Activity (U/mg) Before Ammonium sulfate precipitation	Total units after dialysis	Total protein after dialysis (mg)	Specific Activity (U/mg) After Dialysis
***Epicoccum* sp. NFW1**	2520.0	660.6	3.8	1160	24	48

4.12. *Epicoccum* sp. NFW1 L-Glutaminase Purification

Partially purified L-glutaminase produced *Epicoccum* sp. NFW1was further purified on a gel filtration column chromatography.

Sephadex G-75 gel filtration column chromatography was used to purify L-glutaminase produced by *Epicoccum* sp. NFW1after ammonium sulfate precipitation and dialysis. 2.5mL of the concentrated sample, added in a column, and collected in 25 of 3 mL fractions with the flow rate of 0.5mL/min. Fractions were assayed for protein and glutaminase activity; 6-13 fractions were pooled based on their activities (Table 4.11 & Figure 4.43).

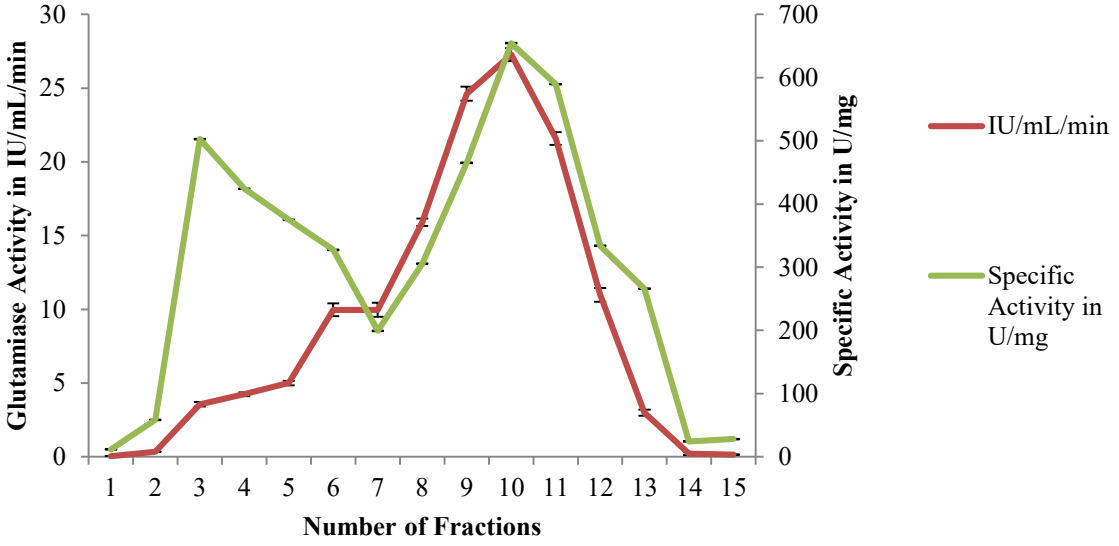

Figure 4.43. Gel filtration column chromatography results for *Epicoccum* sp. NFW1 L-glutaminase on Sephadex G-75.

Table 411. Sequential purification stages *Epicoccum* sp. NFW1

Method	Total Protein (mg)	Specific Activity (U/mg)	Purification (fold)	Total Activity (IU)	Yield (%)
Crude Extract	660.6	3.8	1	2520.0	100
Ammonium sulphate (70%) and dialysis	24	48.3	12.7	1160	46
Gel filtration chromatography	0.88	409	8.47	360	12.4

4.13. Characterization of Fungal L-Glutaminase

The crude enzyme from *Epicoccum* sp. NFW1, purified using a Sephadex G-75 gel filtration column, characterized for various kinetic parameters like incubation time, temperature, pH, sodium chloride concentration, and metal ion effects.

4.13.1. Effect of Incubation Time on *Epicoccum* sp. NFW1 L-Glutaminase

L-glutaminase produced by *Epicoccum* sp. NFW1 optimized for incubation time effect, highest activity 252.7U/mg shown in Figure 4.44, decreased activity was observed after 30 min of incubation.

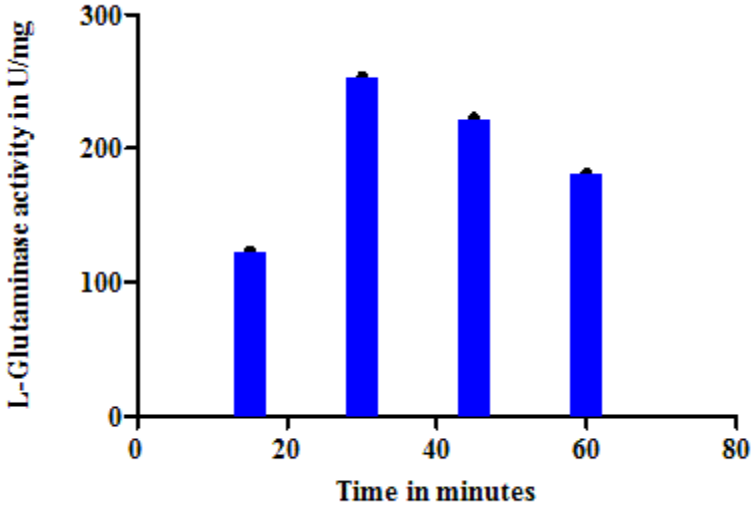

Figure 4.44. Effect of Incubation time on L-Glutaminase produced by *Epicoccum* sp. NFW1 at 37°C.

4.13.2. Effect of Substrate on *Epicoccum* sp. NFW1 L-Glutaminase Activity

The value of Vmax produced by endophytic fungi *Epicoccum* sp. NFW1 was 450.4U/mg, and the Km 0.424mM, calculated by Michaelis and Menten plot shown in Figure 4.45.

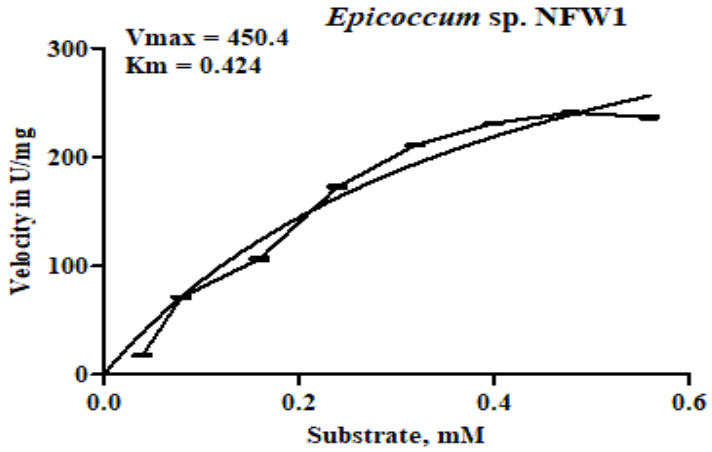

Figure 4.45. Effect of substrate concentration on the activity of enzyme isolated from *Epicoccum* sp. NFW1 at 40°C on pH7.

4.13.3. Effect of pH on *Epicoccum* sp. NFW1 L-Glutaminase Activity

The effect of pH on enzyme produced by fungal strain *Epicoccum* sp. NFW1 showed maximum activity of 253U/mg at pH 7. The enzyme also showed good activities between pH range 6-7.6 (Figure 4.46).

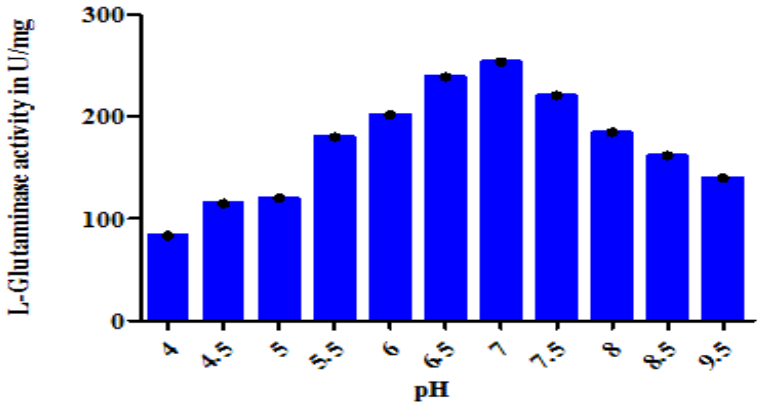

Figure 4.46. Effect of pH on L-Glutaminase produced by *Epicoccum* sp. NFW1 at 37°C**.**

4.13.4. Effect of Temperature on *Epicoccum* sp. NFW1 L-Glutaminase Activity

L-glutaminase produced by *Epicoccum* sp. NFW1 achieved a maximum activity of 268U/mg at 40°C; a decrease, observed after 40°C Figure 4.47.

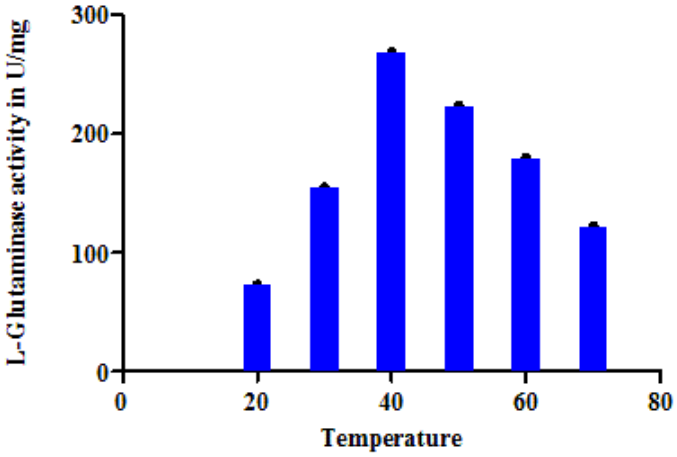

Figure 4.47. Effect of Temperature on L-glutaminase produced by *Epicoccum* sp. NFW1 at pH7.

4.13.5. Effect of Inhibitors on *Epicoccum* sp. NFW1 L-Glutaminase Activity

L-Glutaminase produced by endophytic fungi *Epicoccum* sp. NFW1 showed activity 257U/mg without metal ion addition. An increase in enzyme activity was monitored, with cobalt chloride, calcium chloride, barium chloride, zinc sulfate, potassium chloride, magnesium sulfate, and sodium chloride with activities 413, 414, 419, 368, 319, 311, and 320U/mg, respectively. Reduced enzyme activity was noticed with EDTA, mercuric chloride, magnesium sulfate, and ferrous sulfate with 106, 222, 223, and 213U/mg activities, respectively, as shown in Figure 4.48.

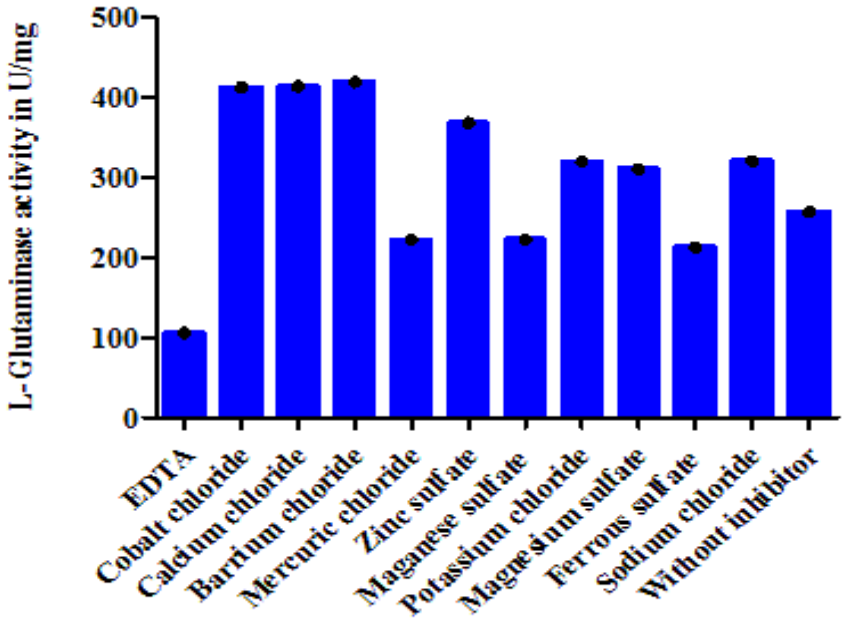

Figure 4.48. Effect of inhibitors on the L-glutaminase from *Epicoccum* sp. NFW1 on 40°C at pH7.

4.13.6. Effect of Sodium Chloride on *Epicoccum* sp. NFW1 L-Glutaminase Activity

Epicoccum sp. NFW1 L-glutaminase activity was 285U/mg without sodium chloride, 64.8% increase in activity with 16% NaCl, and the best activity achieved was 469U/mg (Figure 4.49).

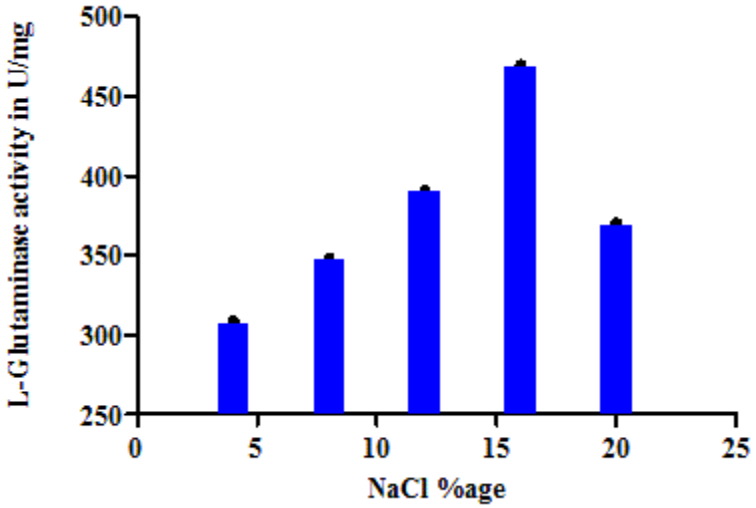

Figure 4.49. Effect of NaCl on L-Glutaminase from *Epicoccum* sp. NFW1 at 40°C on pH 7.

4.14. SDS Gel Electrophoresis

SDS gel electrophoresis was performed to check out the molecular weights of purified L-glutaminases by using "BenchMark" (Cat No. 10747-012) and "ThermoFisher scientific" (Cat No. 00819260) protein ladder. *A. xylosoxidans* RSHG1 L-glutaminase molecular weight was 40KDa, *B. subtilis* RSHU1 L-glutaminase showed a molecular weight of 60KDa and *Epicoccum* sp. NFW1 showed a molecular weight of 38KDa, approximately Figure 4.50 & 451.

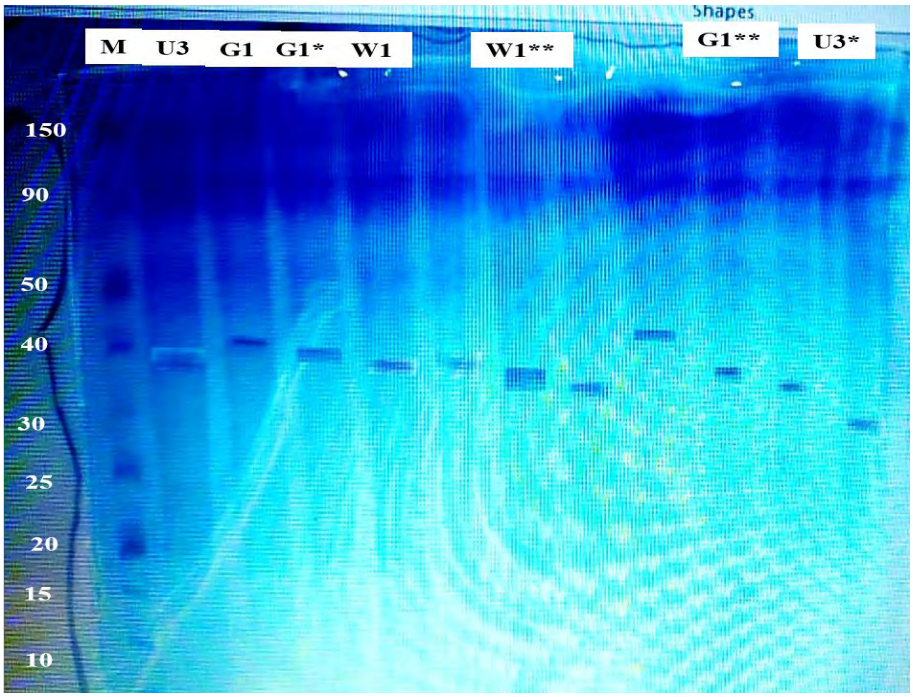

Figure 4.50. SDS gel labelled from left to right M is the marker band, U3 (RSHU3) is after dialysis band, G1(RSHG1) crude band G1*(RSHG1) after dialysis, W1 (NFW1) after dialysis, W1 (NFW1) after column chromatography, and G1**(RSHG1) after column chromatography, U3*(RSHU3) after column chromatography.**

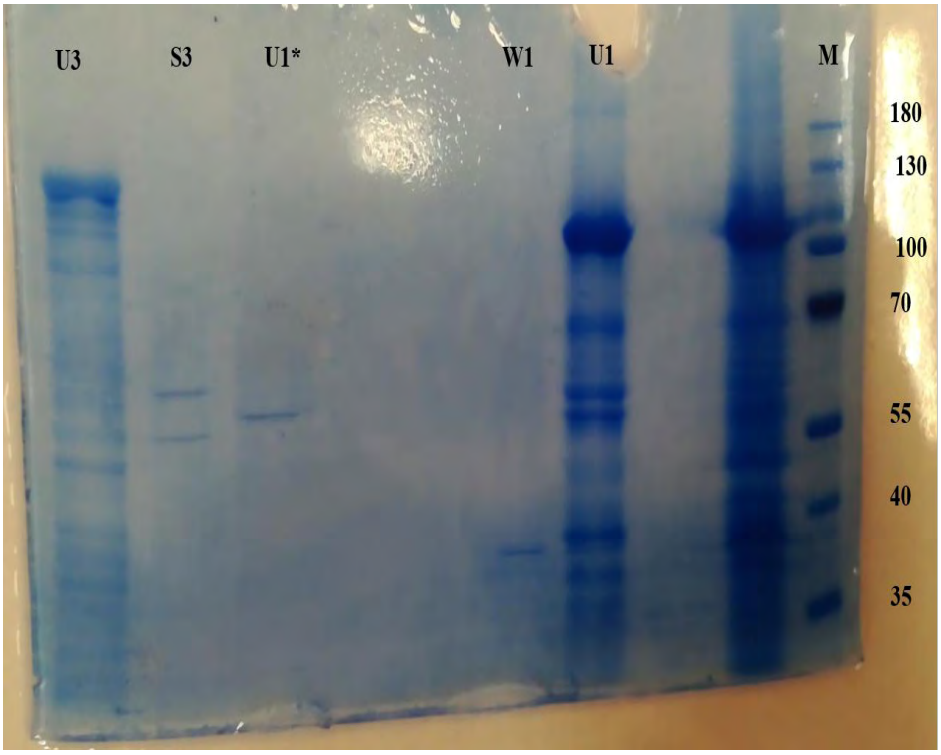

Figure 4.451. SDS gel labelled from right to left M is the marker band, U1(RSHU1) after dialysis, NFW1 after column chromatography U1*(RSHU1) after column chromatography, S3(RSHS3) after partial purification, U3(RSHU3) is the crude band.

2.15. Enzyme Assay After Immobilization

After immobilization, the enzyme was stable for three weeks. Its activity was almost similar up to 21days. The L-glutaminase units, from RSHG1, 23U/mg, RSHU1, 18U/mg, and *Epicoccum* sp. NFW1, 16U/mg.

4.16. Comparative Study of all the L-Glutaminases Produced By Microbial Sources.

The L-glutaminases produced by all selected strains were compared for the enzyme production and kinetic parameters.

4.16.1. Fermentation Conditions for L-Glutaminase Production

A. xylosoxidans RSHG1 showed the best production at pH9, *B. subtilis* RSHU1 at pH6, *B. halotolerans* RSHQ1, and *Epicoccum* sp. NFW1at pH7, *S. maltophilia* RSHU3, and *Alcaligenes* sp. RSHS3 at pH 8. *A. xylosoxidans* RSHG1, *Epicoccum* sp. NFW1and *S. maltophilia* RSHU3 L-glutaminase showed maximum production at 30°C, *B. subtilis* RSHU1 and *B. halotolerans* RSHQ1 at 37°C, and *Alcaligenes* sp. RSHS3 at 25°C. *A. xylosoxidans* RSHG1 and *S. maltophilia* RSHU3 L-glutaminase were highest with sorbitol as a carbon source, *B. subtilis* RSHU1, and *Alcaligenes* sp. RSHS3 with glucose, *B. halotolerans* RSHQ1, and *Epicocum* sp. NFW1 with sucrose as a carbon source. All strains showed maximum L-glutaminase production with L-glutamine as an inducer Table 4.12.

Table 4.12. Comparative study of Different L-glutaminase by selected strains

Strains	**Culture conditions**
A. xylosoxidans RSHG1	pH9 at 30°C with sorbitol as a carbon source and L-glutamine as a nitrogen source and inducer.
B. subtilis RSHU1	pH6 at 37°C, glucose as carbon source, L-glutamine as nitrogen, and inducer source.
B. halotolerans RSHQ1	pH7 at 37°C with sucrose as a carbon source and L-glutamine as a nitrogen and inducer source.
S. maltophilia RSHU3	pH8 on 30°C with sorbitol as carbon source and L-glutamine as nitrogen and inducer source.
Alcaligenes sp. RSHS3	pH8 on 25°C with glucose as a carbon source and L-glutamine as an inducer and nitrogen source
Epicoccum sp. NFW1	pH7 on 30°C with sucrose as a carbon source and L-glutamine as a nitrogen and inducer source.

4.16.2. Kinetic Parameters

A. xylosoxidans RSHG1, *B. subtilis* RSHU1, *B. halotolerans* RSHQ1, *S maltophilia* RSHU3, *Alcaligenes* sp RSHS3, and *Epicocum* sp NFW1L-glutaminases Vmax and Km 443U/mg & 0.235mM, 522U/mg & 0.0591mM, 343.6U/mg &0.37mM, 351U/mg & 0.166mM, 232U/mg & 0.186mM, and 450U/mg & 0.424mM respectively (Table 4.12). *A. xylosoxidans* RSHG1, *Alcaligenes* sp RSHS3, and *Epicocum* sp NFW1L-glutaminases showed maximum activity at pH7, while *B. subtilis* RSHU1, *B. halotolerans* RSHQ1, and *S. maltophilia* RSHU3 L-glutaminase showed maximum activity at pH6.5, 6, and 8, respectively. All L-glutaminases showed maximum activity at 40°C except *B. halotolerans* RSHQ1 L-glutaminase showed maximum activity at 50°C temperature. EDTA, HgCl, $MnSO_{4,}$ and $FeSO_4$ inhibited all the glutaminases *A. xylosoxidans* RSHG1, *B. subtilis* RSHU1, *S. maltophilia* RSHU3, *Alcaligenes* sp. RSHS3, and *Epicocum* sp. NFW1, L-glutaminases are additionally inhibited by HgCl. *B. halotolerans* RSHQ1 L-glutaminase, additionally inhibited by $CaCl_2$. *S maltophilia* RSHU3, *Alcaligenes* sp. RSHS3 glutaminases also inhibited $MgSO_4$ and $ZnSO_4$, respectively.

A. xylosoxidans RSHG1, L-glutaminase showed the best activity with 8% NaCl, *S maltophilia* RSHU3, *Alcaligenes* sp RSHS3 with 12% NaCl and *B. subtilis* RSHU1, *B. halotolerans* RSHQ1, and *Epiccocum* sp. NFW1with 16% NaCl Table 4.12.

Table 4.13. Kinetic Parameters of L-glutaminases produced by selected strains

Strains	Vmax U/mg	KM mM	pH Effect	Temperature Effect	Inhibitors	Activators	Effect of NaCl
A. xylosoxidans **RSHG1**	443	0.235	7	40°C	EDTA, HgCl, $MnSO_4$, and $FeSO_4$	$CoCl_2$, $CaCl_2$, $BaCl_2$, $ZnSO_4$, KCl, $MgSO_4$ and NaCl	8%
B. subtilis **RSHU1**	522	0.0591	6.5	40°C	EDTA, HgCl, $MnSO_4$, and $FeSO_4$	$CoCl_2$, $CaCl_2$, $BaCl_2$, $MgSO_4$, KCl, $ZnSO_4$, and NaCl	16%
B. halotolerans **RSHQ1**	343.6	0.37	6	50°C	EDTA, $CaCl_2$, $MnSO_4$, and $FeSO_4$	$CoCl_2$, $BaCl_2$, HgCl, $MgSO_4$, KCl, $ZnSO_4$, and NaCl	16%
S. maltophilia **RSHU3**	351	0.166	8	40°C	EDTA, $MnSO_4$, $MgSO_4$, and $FeSO_4$	$CoCl_2$, $CaCl_2$, $BaCl_2$, HgCl, KCl, $ZnSO_4$, and NaCl	12%
Alcaligenes **sp. RSHS3**	232	0.186	7	40°C	EDTA, HgCl, $ZnSO_4$, $MnSO_4$, and $FeSO_4$	$CoCl_2$, $CaCl_2$, $BaCl_2$, $MgSO_4$, KCl, and NaCl	12%
Epicoccum **sp. NFW1**	450	0.424	7	40°C	EDTA, HgCl, $MnSO_4$, and $FeSO_4$	$CoCl_2$, $CaCl_2$, $BaCl_2$, KCl, $ZnSO_4$, $MgSO_4$ and NaCl	16%

5. DISCUSSION

The insignificant amino acid L-glutamine has been the focal point of broad logical intrigue due to its significance in cell and tissue societies and its physiologic job in creatures and people. L-Glutamine accounts to be an exceptional amino acid, filling in as a favored respiratory fuel for quickly multiplying cells, for example, enterocytes also, lymphocytes; a controller of acid-base parity through the generation of urinary smelling salts; a bearer of nitrogen among tissues; and a significant precursor of nucleic acids, nucleotides, amino sugars, and proteins. L-Glutamine may turn into an essential amino acid in the sick. During stress, the body's necessities for glutamine seem to exceed the person's capacity to create adequate measures of this amino acid. Arrangement of supplemental glutamine in specific enteral or parenteral encouraging may upgrade nourishing the board and increase recovery of the genuinely sick while limiting clinical remains.

Microbial L-glutaminases have acquired a more distinguished reflection for their potential applications in biotechnology and efficacy in a broad range. Microorganisms are generally an excellent reservoir of enzymes such as L-glutaminase. Since the declaration of L-glutaminase for its valuable effects like antitumor activity, various microbial glutaminases are the focus of attention of eagerness to isolate microbial glutaminases. L-Glutaminase action is monitored in various living organisms like animals, plants, and microorganisms, including bacteria, actinomycetes, yeast, and fungi. L-Glutaminases are produced mainly by *Bacillus* and *Pseudomonas* species. In general, L-glutaminases are produced from *E. coli*, *Bacillus* spp (Irajie *et al.,*2016), *Pseudomonas* spp., *Citrobacter*, and *Staphylococcus* have been well-studied (Karim and Thalij 2016). L-Glutaminase can also be extracted from animal and plant sources. The microbial sources usually are preferred for mechanical application considering their financial creation, consistency, office of balance, and streamlining ways.

The present study was focused on the isolation, screening, and characterization of L-glutaminase-producing bacteria and fungi. Bacteria were isolated from soil samples, and one sample for the isolation was an expired glutaminase in the lab. One fungal strain previously isolated and identified as *Epicoccum* sp. NFW1, was isolated from *Taxus fauna* as an endophyte. Some other fungal strains, FS1, FS2, FS3, and FS4, were isolated from the soil sample. One study reported different agro residues such as groundnut oil, cattle feed, oil cake, wheat bran, etc., as a source for L-glutaminase production by non-pathogenic strain *Pseudomonas stutzeri* PIMS6 (Athira *et al.,* 2014). L-Glutaminase from *Bacillus cereus* RN-6 was isolated from the forest soil (Nagaraju *et al.,* 2019).

Further media and cultural conditions were optimized for increasing enzyme production. Bacterial strains isolated from brackish water samples for L-glutaminase production were screened on plate assay for isolation. The formation of pink zones around the colonies showed positive results for L-glutaminase production (Ibrahim *et al.,* 2020). L-glutaminase was produced by terrestrial fungal strain *Fusarium oxysporum* (Hamed Al-Wasify, 2016).

For bacterial screening, isolated bacterial strains were inoculated on Glutamine Salt plates were incubated in a thermal incubator for 3days for bacteria at 37°C and 7days for fungi at 30°C. According to various studies, high temperature has harmful effects on the production of enzymes compared to low temperature (Satish *et al.,* 2018). The color around the colony was observed as an indication for glutamine producers. Further screening was done in the presence and absence of glutamine, which is used as a substrate. Bacterial strains RSHG1, RSHU1, RSHQ1, RSHU3, and RSHS3, change color in media only in the presence of substrate. Bacterial screening using a substrate is then carried out in liquid media.

Liquid media in all the selected strains showed positive results only with the substrate. However, all the strains showed no color change in the liquid media without glutamine. The phenol red has been used as an indicator for pH change used by most cases to observe the presence of glutaminase. Still, in some cases, the color indication is the presence of pink colonies as the indication of the production of L-glutaminase (Ibrahim *et al.,* 2020). Investigations of Aly (Aly *et al.,* 2017) indicated the glutaminase presence and activity in *Streptomyces* sp. Bacterial strains isolated from soil and aquatic samples were isolated, and screening was performed on minimal glutamine media with an indicator (phenol red). They isolated the bacteria that formed pink-red colour around the colonies due to ammonia production (Emelda, 2016).

In the bacterial screening with the substrate, all selected strains showed positive results. After point inoculation on glutamine salt agar plates, the maximum hydrolysis zone was observed with RSHU3 and the smallest with RSHU1. Fifteen marine isolated bacterial strains showed a change of color yellow to pink areas around the colonies with zones of hydrolysis of about 8.5–17 mm in diameter (Mostafa *et al.,* 2021).

Bacterial strain RSHU1 showed the largest zone, 34 mm. At the same time, RSHQ1 gave the minimum zone (16mm) in screening media when Cell-free supernatant of the selected bacterial isolates was used in the well diffusion assay. The isolation of glutaminases was from different microbial sources for restorative applications (Katikala *et al.,* 2009; Dubey *et al.,* 2015). Fungal *Epiccocum* sp. W1 strain showed a hydrolysis zone in different concentrations, while the

hydrolysis zone showed 17mm and 16.5mm values. Enzyme assay reaction mixture was incubated in the presence of supernatant was checked through the Nesslerization method to estimate ammonia released by the enzyme. The same procedure used to determine the activity of L-asparaginase was used to determine the glutaminase activity. But the buffer contains L-glutamine-Tris-HCl and Urea. Tris-HCl instead of L-asparagine-Tris-HCl (Ashok *et al.,* 2019). The quantitative analysis for L-glutaminase production was carried out using Nessler's method (Mostafa *et al.,* 2021).

The biochemical testing of the bacterial strains was performed to determine the morphological and physiological characteristics. Twenty-four biochemical tests were performed for all five selected bacterial strains. RSHG1 showed 41.6% positive results, RSHU1 was positive for 62.5% of tests, biochemical testing for RSHQ1 gave 41.6% positive results. RSHU3 showed results only for urease, gelatin hydrolysis, and arginine hydrolase (16.6% positive results) and negative for all the other biochemical testing. RSHS3 showed 29.1% positive results and negative results for other biochemical tests. *Bacillus subtilis* isolated for L-glutaminase production gave 50% tests positive when 34 biochemical tests were performed (Dubey *et al.,* 2015). The biochemical properties were carried out for *Pseudomonas* VJ6. A total of eight biochemical tests such as glucose, lactose, sucrose, indole, Voges-Proskauer, citrate utilization, catalase, and oxidase tests were performed showed 50% positive and 50% negative results. Out of these tests, glucose, citrate utilization, catalase, and oxidase tests were positive, while other tests were negative (Jyothi *et al.,* 2011).

For identification of bacterial isolates, 16S rRNA gene sequencing was peformed, and the evolutionary history was inferred using the Maximum Likelihood method based on the Tamura-Nei model (Tamura *et al.,* 1993) on Mega X software (Kumar *et al.,* 2018). The isolates were identified as *Bacillus subtilis* RSHU1, *Achromobacter xylosoxidans* RSHG*, Bacillus halotolerance* RSHQ1, *Stenotrophomonas maltophilia* RSHU3, and *Alcaligenes* sp. RSHS3. Bacterial strain *Bacillus subtilis* JK-79 was reported as L-glutaminase producer bacteria and identified using 16 S gene sequencing (Kiruthika and Murugesan, 2020). The marine isolated bacterial strain was noted and used for glutaminase production was identified as *Alcaligenes faecalis* KLU102 by 16S rRNA gene analysis (Pandian *et al.,* 2014). *Stenotrophomonas maltophilia* and *Achromobacter* species have been reported for L-glutaminase production (Pandian *et al.,* 2014; Blazek and Benbough 1981).

After screening and identification, isolated bacterial strains were optimized for incubation time, pH, temperature, carbon source, nitrogen source, and effects of inducers. For incubation time on glutamine salt medium *A. xylosoxidans* RSHG1, *B. subtilis* RSHU1, *B halotolerance*

RSHQ1, and *Alcaligenes* sp. RSHS3 produced the highest glutaminase 1.39, 3.6, 2.6, and 1.3U/mg respectively on the 3rd day, while *S. maltophilia* RSHU3 showed the most increased activity 3U/mg on 2nd day of incubation. Marine isolated *Bacillus subtilis,* L-glutaminase was maximally produced in submerged fermentation after 18 hours of incubation (Zhang *et al.,* 2019). The best glutaminase production was observed after 72 hours of incubation by *Pseudomonas*VJ-6 (Jyothi *et al.,* 2011).

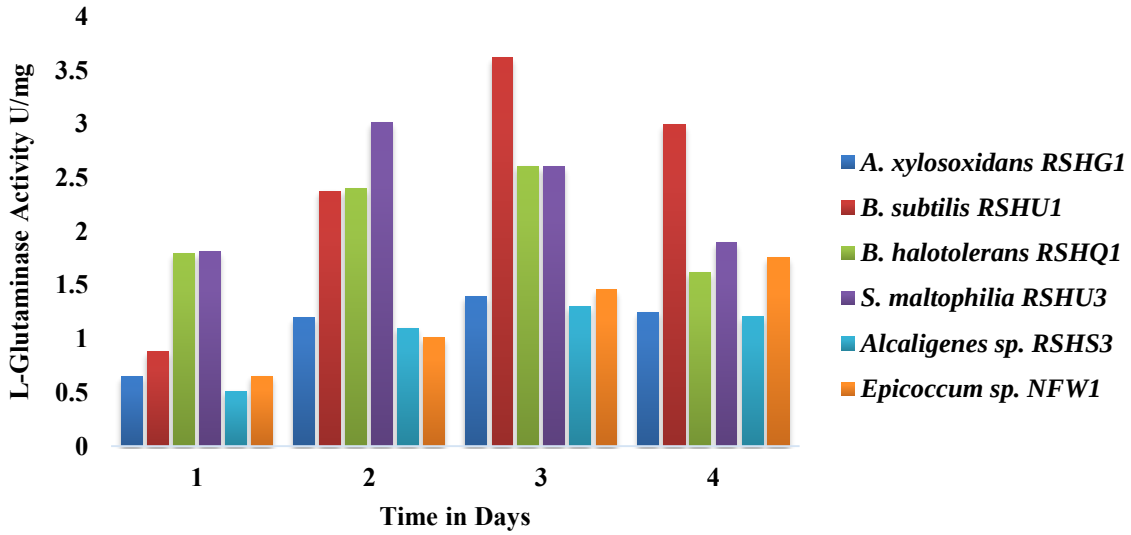

Figure 5.1. Effect of Incubation Time on L-Glutaminase production by Selected Microbial strains

The L-Glutaminase activity by *A. xylosoxidans* RSHG1, *B. subtilis* RSHU1, *B. halotolerance* RSHQ1, *S. maltophilia* RSHU3, and *Alcaligenes* sp. RSHS3 has monitored the highest activities at pH 9, 6, 7, 8, 8 showing more alkaline pH favoring production of the enzyme (Figure 5.2). L-Glutaminase production was noted best at pH 7 from the bacterial isolate *Bacillus* sp. screened from the forest soil (Nagaraju *et al.,* 2018). *Vibrio azureus* JK-79, screened and isolated from marine samples, showed the highest glutaminase production at pH8 (Kiruthika 2013). Soil isolated *Fusarium oxysporum* noted the maximum glutaminase production gained at pH 6 (Hamed and Al-Wasify, 2016).

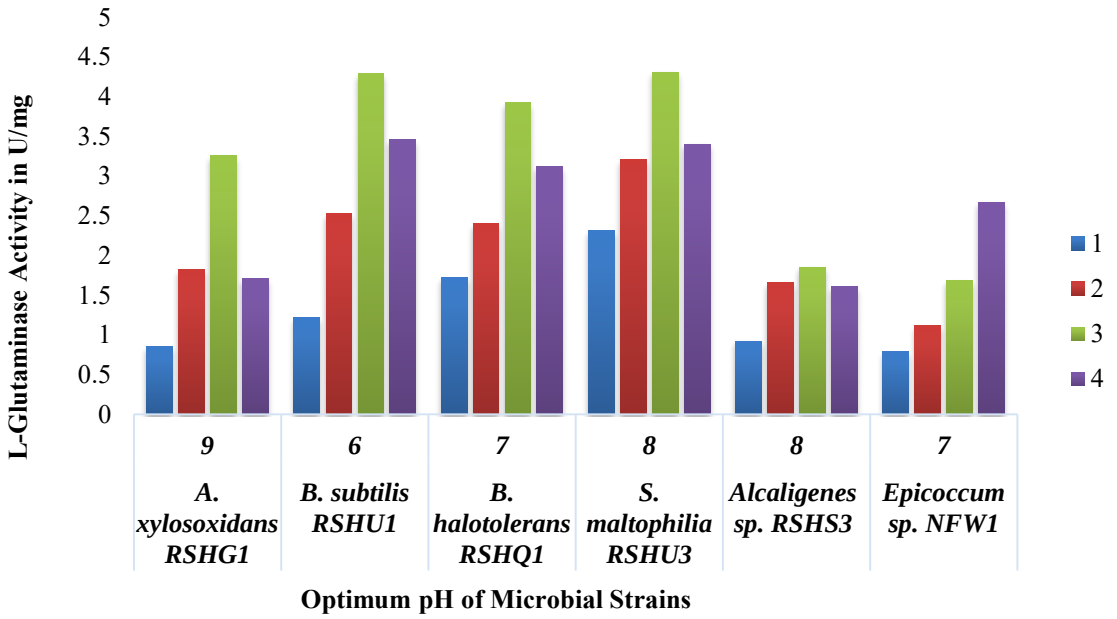

Figure 5.2. Optimum pH for L-Glutaminase Production by Selected Microbial Strains.

Production of L-glutaminase by selected bacterial strains was tested at 25°C, 30°C, and 37°C. L-Glutaminase activity was maximally produced at 37°C by *B subtilis* RSHU1 (4.62U/mg) and *B halotolerance* RSHQ1 3.89U/mg, at 30°C by *A. xylosoxidans* RSHG1 and *S. maltophilia* RSHU3 and at 25°C. Marine isolated *Bacillus subtilis* JK-79 showed the best glutaminase activity at 37°C (Kiruthika *et al.,* 2018). Bacterial strain *Pseudomonas* NS16 achieved the best glutaminase production at 35°C is (Al-Zahrani *et al.,* 2020). *Aspergillus fumigatus* WL002 showed maximum glutaminase production at 37°C (Dutta *et al.,* 2015). *Fusarium oxysporum* isolated from soil samples gained maximum activity at 35°C (Hamed and Al-Wasify, 2016). Endophytic fungi *Aspergillus sp*. ALAA-2000 showed the best glutaminase production at 27°C (Ahmed *et al.,* 2016).

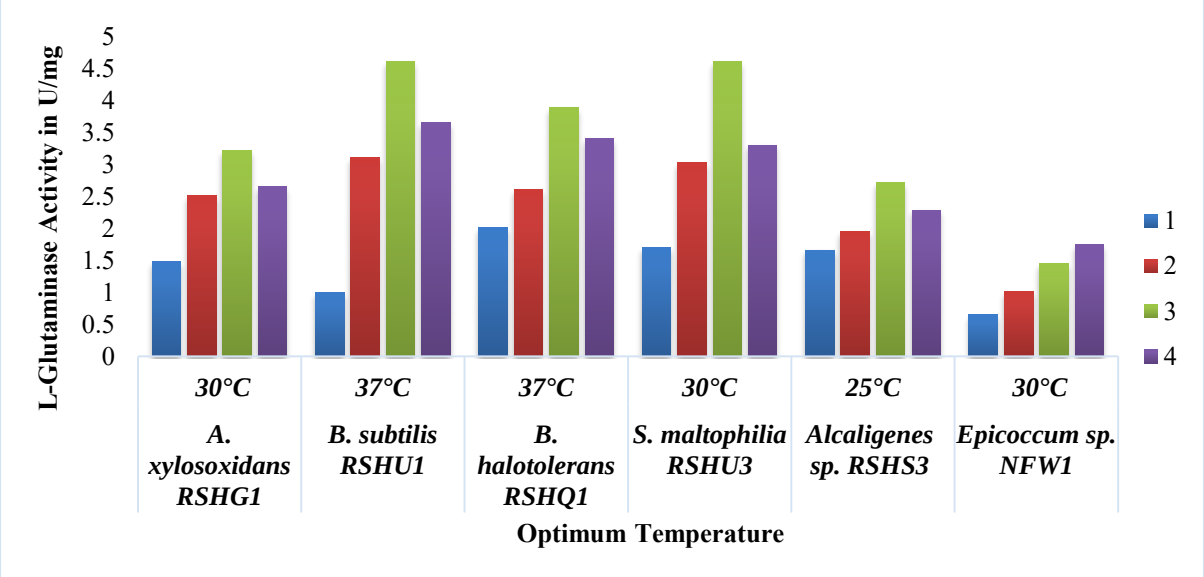

Figure 5.3. Optimum Temperature for L-Glutaminase Production by Selected Microbial Strains.

Different carbon sources were used to achieve the highest activities for glutaminase production when various carbon sources were tested. Glucose for *B. subtilis* RSHU1 and *Alcaligenes* sp. RSHS3, sorbitol for *A. xylosoxidans* RSHG1 and *S. maltophilia* RSHU3, sucrose for *B. halotolerance* RSHQ1 was noted. A study was conducted on the selection and isolation of L-glutaminase-producing soil bacteria with good glutaminase activities, and glucose was the best carbon source for glutaminase production by *Pseudomonas aurignosa* (Al-Zahrani *et al.,* 2020). 1.5% glucose was used as a carbon source for glutaminase production by *Halomonas meridian* that was isolated from the Red Sea (Mostafa *et al.,* 2021). *Zygosaccharomyces rouxii* showed maximum glutaminase production with sucrose as a carbon source (Iyer and Singhal, 2008; Figure 5.3)

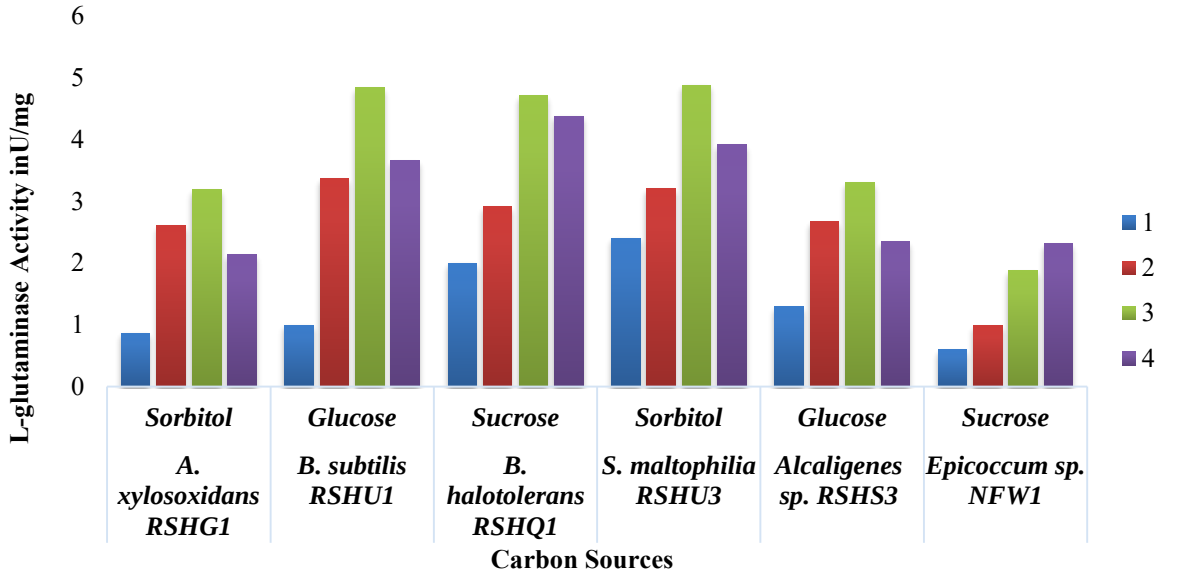

Figure 5.4. Carbon Sources used for maximum L-Glutaminase Production by Selected Microbial Strains.

The best nitrogen source was L-glutamine for *A. xylosoxidans* RSHG1 and *B. subtilis* RSHU1. *Pseudomonas aurignosa* gained the highest glutaminase production with glutamine among various nitrogen sources tested (Al-Zahrani *et al.,* 2020). The marine isolated bacterial strain *Bacillus subtilis* JK-79 showed the best glutaminase production with yeast extract as a nitrogen source (Kiruthika and Nachimuthu, 2014). The best inducer for glutaminase production by *A. xylosoxidans* RSHG1, *B. subtilis* U1, *B. halotolerance* RSHQ1, *S. maltophilia* RSHU3, and *Alcaligenes* sp. RSHS3 was L-glutamine. Glutaminase production was enhanced by glutamine in the case of *Bacillus subtilis* JK-79 and *Bacillus* sp. (Kiruthika and Nachimuthu, 2014; Sinha and Nigam 2016).

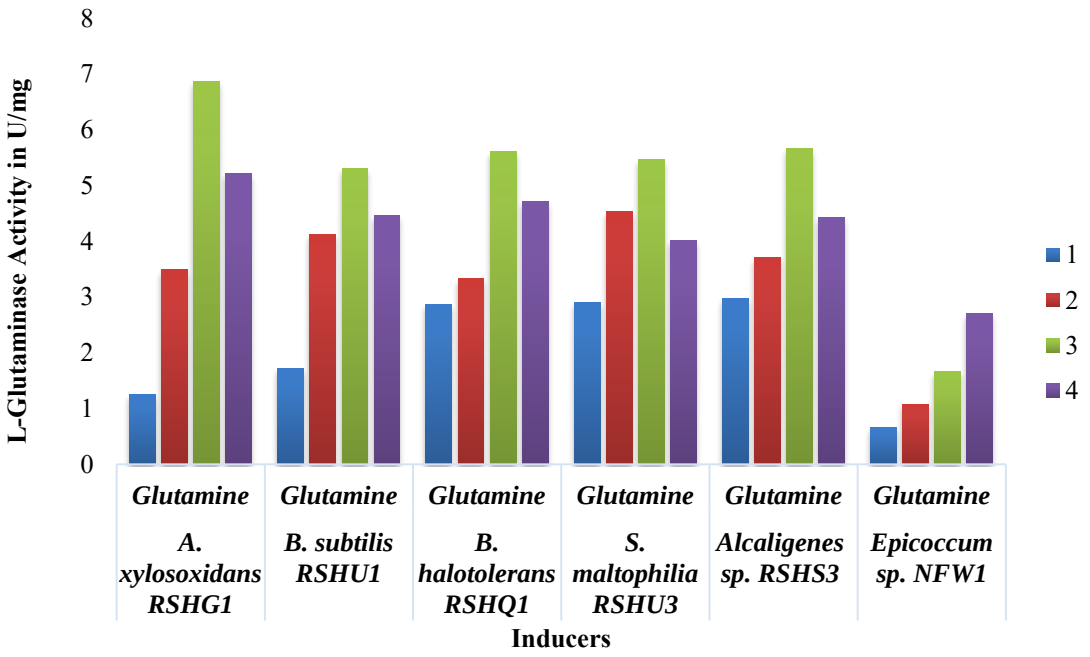

Figure 5.5. Inducers used for maximum L-Glutaminase Production by Selected Microbial Strains.

Fungal samples were screened from on glutamine salt agar of pH 6, and 3fungal samples showed positive screening, out of which the FS1 and FS3 showed better glutaminase production. But *Epicoccum* sp. NFW1 fungal strain taken from Microbiology lab Quaid-i-Azam University showed the best production. *Epicoccum* sp. NFW1 was grown on a glutamine salt broth of pH6, incubated at 30°C for 5days. The supernatant containing enzyme was collected at the regular intervals of 24hours and checked out from glutaminase production. Maximum activity was achieved on the 4th day of incubation on Glutamine salt broth. *Aspergillus flavus* L-glutaminase was maximally produced on the 5th day of incubation (Nathiya *et al.,* 2011). L-Glutaminase has also been isolated from terrestrial fungal strain *Fusarium oxysporum* using a submerged fermentation process. Maximum yield of enzyme achieved at pH 6, at 35°C, and with 0.025 % glutamine concentration and incubated for 7 days

(Hamed and Al-Wasify 2016). *Epicoccum* sp. W1 showed maximum production of L-glutaminase on Czapek Dox media. Fungi isolated from Egyptian soil was used to produce L-glutaminase on Czapek Dox media (Khalil *et al.,* 2020). L-Glutaminase produced by *Epicoccum* sp. W1 was maximally produced at pH 7 on the 4th day of incubation. *Aspergillus oryzae* NRRL 32657 showed maximum production at pH7 (Alian *et al.,* 2015). *Epicoccum* sp. NFW1 L-glutaminase gained maximum activity with 1% sucrose on the 4th day of incubation. L-glutaminase produced by terrestrial fungi *Fusarium oxysporum* showed maximum production with 1% sucrose (Hamed and Al-Wasify, 2016). *Epicoccum* sp. W1 L-glutaminase maximum production was observed with 2% L-glutamine as nitrogen and inducer source. L-Glutaminase *by Trichoderma koningii* showed maximum production with 2% glutamine (El-Sayed 2009). L-Glutaminase produced by *Trichoderma* sp. showed the highest production with 1% glutamine as an inducer (Olarewaju *et al.,* 2019).

L-Glutaminase by *A. xylosoxidans* RSHG1, *B. subtilis* RSHU1, *B. halotolerance* RSHQ1, *S. maltophilia* RSHU3, *Alcaligenes* sp. RSHS3, and *Epicoccum* sp. W1 was partially purified by using 70% ammonium sulfate saturation. (Awad *et al.,* 2019) partially purified glutaminase produced by *Streptomyces rochei* SAH2_CWMSG using 75% ammonium sulfate. L-glutaminase produced by *Pseudomonas* VJ-6 was partially purified using 80% ammonium sulfate (Jyothi *et al.,* 2011).

L-Glutaminase produced by *A. xylosoxidans* RSHG1 and *Epicoccum* sp. NFW1 was further purified on the gel filtration column with G-75 and *B. subtilis* RSHU1 on G-100. Awad (Awad *et al.,* 2019) purified glutaminase from *Streptomyces rochei* SAH2_CWMSG using gel filtration colum Sephadex G-100. L-Glutaminase produced by *Penicillium politans* NRC510 purified by using Sephadex G-100 gel filtration (Ali *et al.,* 2009).

RSHG1 L-glutaminase was purified to 19.6-fold with specific activity 35U/mg, and its molecular weight was 40KDa. RSHU1 L-glutaminase having specific activity 15U/mg was purified to 12.5-fold with a molecular weight of 60KDa. *Epicoccum* sp. NFW1 L-glutaminase was purified to 8.47-fold showed specific activity 19.5U/mg having a molecular weight of 38KDa approximately. *Streptomyces avermitilis* L-glutaminase molecular weight was 50KDa (Abdallah *et al.,* 2013). The marine species *Halomonas meridian* L-glutaminase molecular weight was 57kDa by the SDS-PAGE analysis (Mostafa *et al.,* 2021). The activity of partially purified L-glutaminase produced by endophytic isolate *Aspergillus* sp. was tested for incubation times showed the highest peak after 30minutes of incubation. The increasing time observed a decrease in activity (Ahmed *et al.,* 2016).

A. xylosoxidans RSHG1 L-glutaminase showed Vmax of the enzyme was 443.8U/mg, and Km was 0.235mM. L-glutaminase produced by *B. subtilis* RSHU1 achieved Vmax 522.5U/mg, and Km was 0.059mM. *B halotolerance* RSHQ1 Vmax and Km were 343.6U/mg and 0.37mM, respectively. Vmax and Km of partially purified L-glutaminase by *S. maltophilia* RSHU3 showed the Vmax value 351.6U/mg and lowest Km 0.166mM. *Alcaligenes* sp. RDHS3 L-glutaminase Vmax and Km were 232.9U/mg and 0.186mM, respectively. In comparison, the partially purified fungal L-glutaminase produced by *Epicoccum* sp. NFW1 showed Vmax and Km 450.4U/mg and 0.424mM, respectively. Km and Vmax of L-glutaminase produced by *Bacillus* sp. B12 was 0.4 mmol/L and 0.133mmol/min, respectively (Abdelhameed *et al.,* 2020). L-Glutaminase produced by *Hypocrea jecorina* was characterized for kinetic parameters, and Km and Vmax values achieved by Line Weaver Burk plot and Values were 0.491mM and 13.86 U/L, respectively (Bülbül and Karakuş, 2013). L-Glutaminase produced by soil isolated *Streptomyces* sp. showed Km value 2.8 mM, and V-max value was 7.57U/mL (Desai *et al.,* 2016).

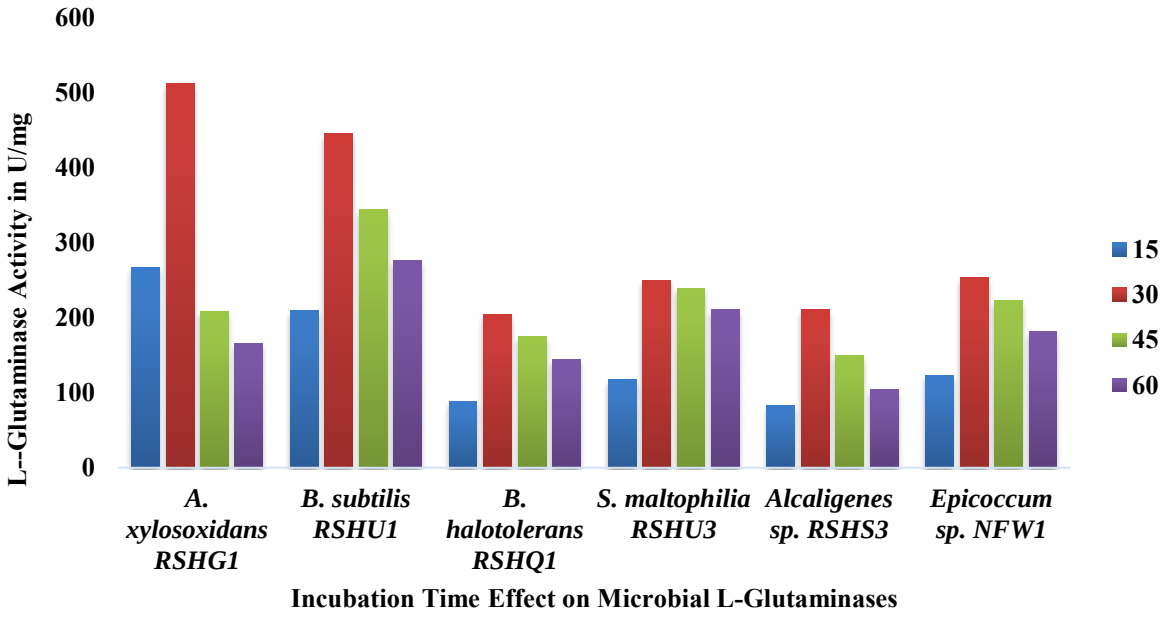

Figure 5.6. Effect of Incubation Time L-Glutaminase Activity by Selected Microbial Strains.

A. xylosoxidans RSHG1, *Alcaligenes* sp. RSHS3, and *Epicoccum* sp. NFW1 partially purified L-glutaminase showed the best activity at pH 7. While L-glutaminase produced by *B. subtilis* RSHU1, *B. halotolerance* RSHQ1, and S. *maltophilia* RSHU3 gained the highest activity at pH 6.5, 6, and 8, respectively (Figure 5.6). *Pseudomonas aeruginosa* isolated from Sangihe-Talaud Sea was used to produce L-glutaminase showed maximum activity at pH 7. L-glutaminase by *Streptomyces* sp. achieved the highest activity at pH 8 (Desai *et al.,* 2016). L-

Glutaminase from *vibrio* sp. M9 isolated from Mahabalipuram marine sediments showed maximum activity at pH 7 (Saravanan *et al.,* 2014). Generally, bacterial glutaminases function optimally at alkaline and neutral pH values, but there are some reports like glutaminases from *B. amyloliquefaciens* (Mao *et al.,* 2013) and *Bacillus* sp. LKG-01 (Kumar *et al.,* 2012) has acidic pH optima.

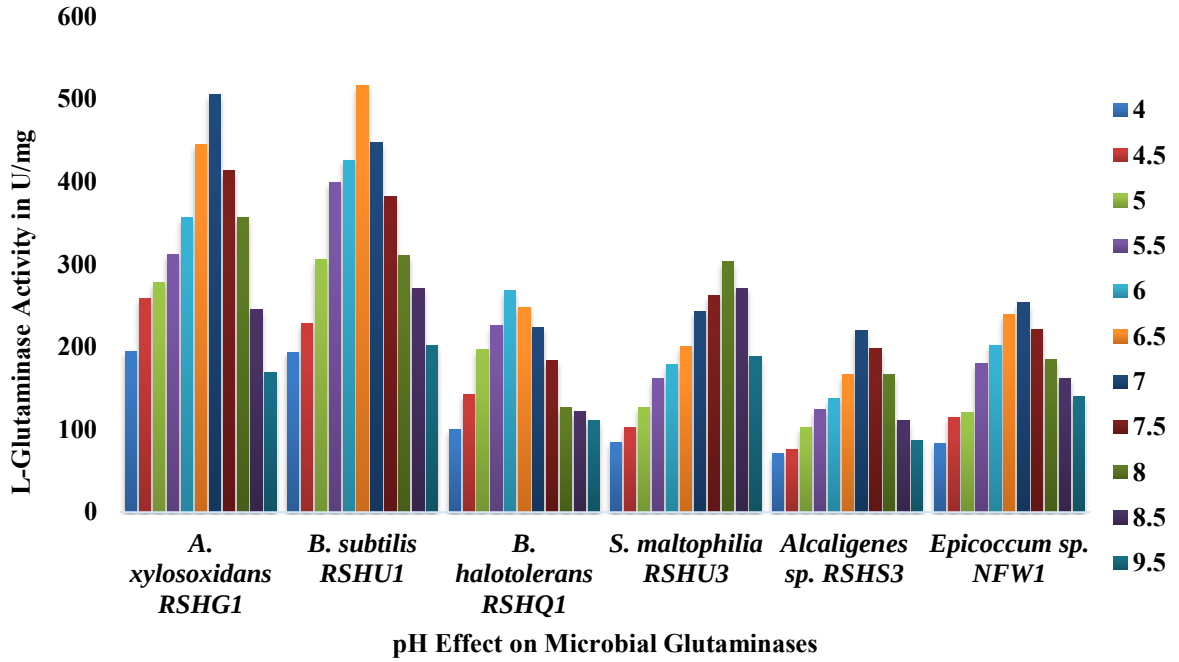

Figure 5.7. Effect of pH L-Glutaminase Activity by Selected Microbial Strains.

All the purified L-glutaminases produced by *A. xylosoxidans* RSHG1, *B. subtilis* RSHU1, and *Epicoccum* sp. NFW1 and partially purified *S. maltophilia* RSHU3, *Alcaligenes* sp. RSHS3, and *B halotolerance* RSHQ1 were studied for kinetic parameters. *A xylosoxidans* RSHG1, *B. subtilis* RSHU1, *S. maltophilia* RSHU3, *Alcaligenes* sp. RSHS3, and *Epicoccum* sp. W1 L-glutaminase achieved maximum activity at 40°C. In contrast, *B. halotolerance* RSHQ1 L-glutaminase gained the best activity at 50°C. L-glutaminase produced by *B. cereus* glutaminase gained the best activity at 40°C (Reda, 2015). L-Glutamiase produced by *Bacillus subtilis* NRRL achieved best activity at 40°C (El-Sousy *et al.,* 2021). Most bacterial glutaminases function optimally at mesophilic temperatures (20–45°C) like the present study; Still, some enzymes have a high temperature as optimum temperature; however, some glutaminases have high optimal temperatures, e.g., like enzymes from *Bacillus* sp. LKG-01 (70°C) (Kumar *et al.,* 2013) and *Stenotrophomonas maltophilia* (60°C) (Wakayama *et al.,* 2005). L-Glutaminase produced by *Streptomyces avermitilis* showed the highest activity at

30°C (Abdallah *et al.,* 2013). L-Glutaminase produced by *Cohnella* sp. A01 received the highest activity at 50 °C (Mosallatpour *et al.,* 2019).

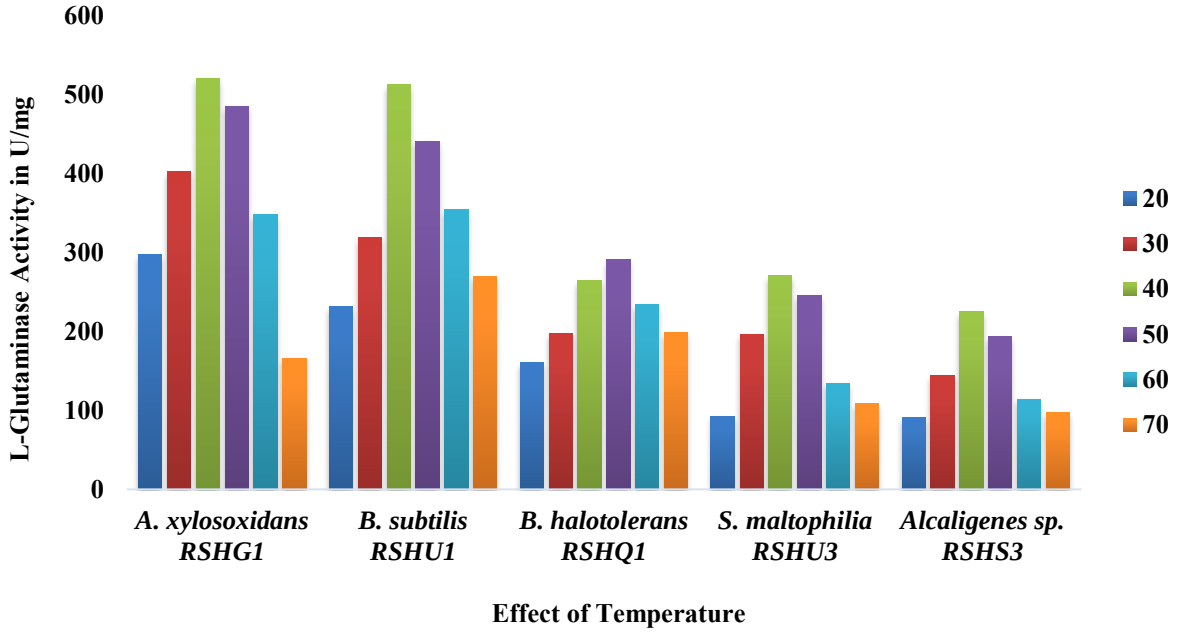

Figure 5.8. Effect of Temperature L-Glutaminase Activity by Selected Microbial Strains.

L-Glutaminase produced by *A. xylosoxidans* RSHG1 activity was increased using $CoCl_2$, $CaCl_2$, $BaCl_2$, $ZnSO_4$, $MgSO_4$, KCl, and NaCl and inhibited by EDTA, HgCl, $MnSO_4$, and $FeSO_4$. *B. subtilis* RSHU1 L-glutaminase activity was enhanced by $CoCl_2$, $CaCl_2$, $BaCl_2$, $MgSO_4$, KCl, $ZnSO_4$, and NaCl and decreased by EDTA, HgCl, $MnSO_4$, and $FeSO_4$. L-glutaminase produced by *B. halotolerance* RSHQ1 showed increased activity with $CoCl_2$, $BaCl_2$, HgCl, $MgSO_4$, KCl, $ZnSO_4$, and NaCl, and reduction activity was observed with EDTA, $CaCl_2$, $MnSO_4$, and $FeSO_4$. *S. maltophilia* RSHU3 L-glutaminase achieved increased activity with $CoCl_2$, $CaCl_2$, $BaCl_2$, HgCl, KCl, $ZnSO_4$, and NaCl, and decreased activity was observed with EDTA, $MnSO_4$, $MgSO_4$, and $FeSO_4$. *Alcaligenes* sp. RSHS3 L-glutaminase activity was enhanced by $CoCl_2$, $CaCl_2$, $BaCl_2$, $MgSO_4$, KCl, and NaCl and was reduced by EDTA, HgCl, $ZnSO_4$, $MnSO_4$, and $FeSO_4$. *Epicoccum* sp. NFW1 L-glutaminase showed increased activity with $CoCl_2$, $CaCl_2$, $BaCl_2$, KCl, $ZnSO_4$, $MgSO_4$, and NaCl, while reducing activity was observed by EDTA, HgCl, $MnSO_4$, and $FeSO_4$. *B. cereus* glutaminase activity was enhanced by Mg^{2+}, Na^{+}, and Co^{2+} while inhibited by Ca^{+2}, Ba^{2+}, Fe^{3+}, Zn^{2+}, Cu^{+}, Hg^{2+}, and Cd^{2+} (Reda, 2015). L-glutaminase by *Bacillus* sp. B12 was activated by Mn^{2+}, Mg^{2+}, Ca^{+2}, and Na^{+} while no effect by K^{+}, Co^{2+}, and Ni^{2+} and activity was decreased by Hg^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+} (Abdelhameed *et al.,* 2020). The L-glutaminase produced by marine endophytic *Aspergillus* sp. activity was increased by Ba^{2+}, Ca^{2+}, Na^{+}, Co^{2+}, Mn^{2+}, and Mg^{2+} (Ahmed *et al.,* 2016).

Glutaminase produced by *Escherichia coli* is activated by divalent cations Mg^{2+}, Mn^{2+}, and Ca^{2+} (Prusiner and Stadtman, 1976). Salt-tolerant L-glutaminase produced by *Bacillus amyloliquefaciens* was partially inhibited when incubated with 1mM EDTA.

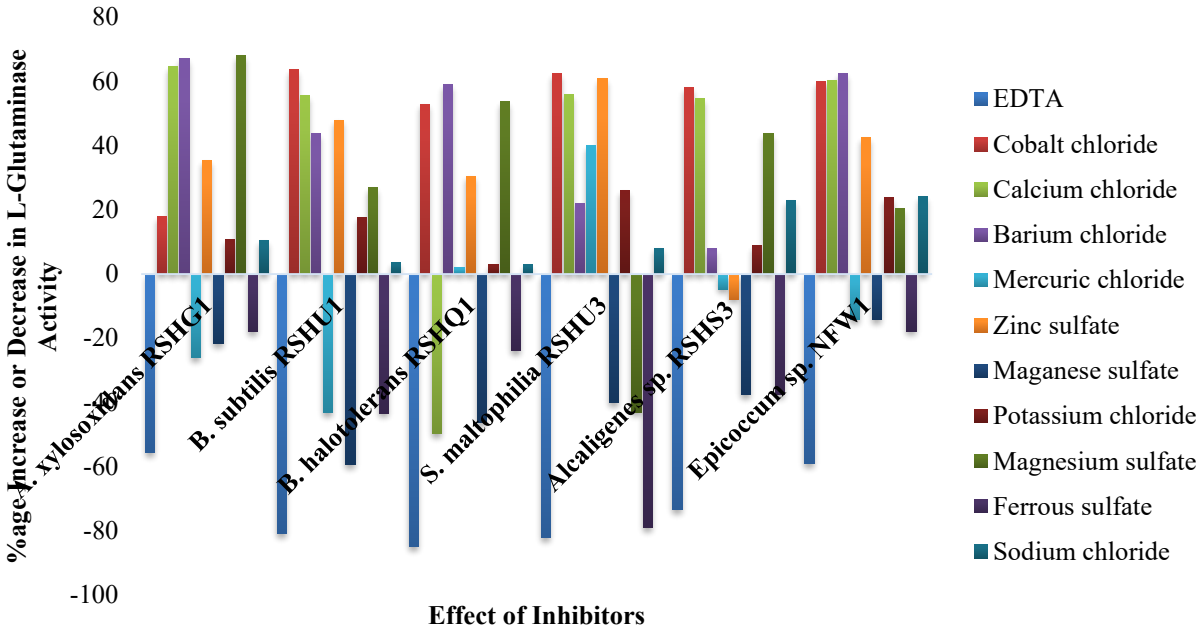

Figure 5.9. Effect of Inhibitors L-Glutaminase Activity by Selected Microbial Strains.

L-glutaminase by *A. xylosoxidans* RSHG1 activity was 64 % increased by 8 % NaCl. *B. subtilis* RSHU1 L-glutaminase activity was enhanced 65% by 16% NaCl. Partially purified L-glutaminase produced by *B. halotolerance* RSHQ1 achieved 53% increased activity with 16% NaCl. *S. maltophilia* RSHU3 L-glutaminase gained 32% increased activity with 12 % NaCl. *Alcaligenes* sp. RSHS3 L-glutaminase activity was improved 44% with 12% NaCl. *Epicoccum* sp. NFW1 L-glutaminase showed 64% increased activity with 16% NaCl. All L-glutaminases showed higher activities by using higher sodium chloride concentrations. L-Glutamine has lower solubility in water and buffers maybe this the reason that use of higher quantities of sodium chloride provides a hydrophobic environment and increases the activity of L-glutaminase. L-glutaminase producing marine bacteria from Sangihe-Talaud Sea, the enzyme was stable when NaCl solution was added up to 8% and began to decrease on the addition of NaCl solution of 16% and 20% (Chasanah *et al.,* 2013). L-glutaminase produced by *B. amyloliquefaciens* y-9 showed 68% increased activity with 20% NaCl (Mao *et al.,* 2013). L-Glutaminase by G1, after immobilization on agar enzyme, was stable for three weeks. Immobilization of glutaminase enzyme from *Hypocria jecorina* on polyacrylic acid (Karahan *et al.,* 2014). L-glutaminase produced by *Penicillium politans* NRC510 showed 75% increased activity by 15-30% NaCl (Ali *et al.,* 2009).

One of the important applications of L-glutaminases is in the food industry, especially fermented food, which requires high salt concentration salt-tolerant glutaminase is highly desirable. The most salt-tolerant glutaminases have been found in bacteria. Glutaminase I and II, constitutively produced by marine *M. luteus* K-3 in media containing 3% NaCl, performed optimally at NaCl concentrations of 8–16% (Moriguchi *et al.,* 1994) while glutaminase from Bacillus sp. LKG-01 showed a continuous increase in activities with up to 20% NaCl concentrations. up to 20% (w/v) and retained 95% of its activity at 30% NaCl concentration (Elshfei *et al.,* 2014). Purified glutaminase from *S. maltophilia* NYW-81 showed the highest salt tolerance among bacterial glutaminases and exhibited 86% of its original activity in the presence of 16% NaCl (Wakayama *et al.,* 2005) while *B. amyloliquefaciens* glutaminase showed 68% of the original activity in the presence of 20% NaCl (Mao *et al.,* 2013).

L-glutaminase activity is similar to the result obtained with the *P. brevicompactum* NRC 829 glutaminase. However, while the maximum activity of the former enzyme decreased to 80% at 25% NaCl concentration (Elshafei *et al.,* 2014), the latter retained 95% of its activity at 30% NaCl concentration (Elshafei *et al.,* 2014). Purified glutaminase from *S. maltophilia* NYW-81 possessed the highest salt tolerance among bacterial glutaminases and exhibited 86% of its original activity in the presence of 16% NaCl (Wakayama *et al.,* 2005). *B. amyloliquefaciens* glutaminase showed 68% of the original activity in the presence of 20% NaCl (Mao *et al.,*2013). The glutaminase from *A. oryzae* RIB40, different from the strain used in traditional food fermentations, is salt-tolerant (Masuo *et al.,* 2005).

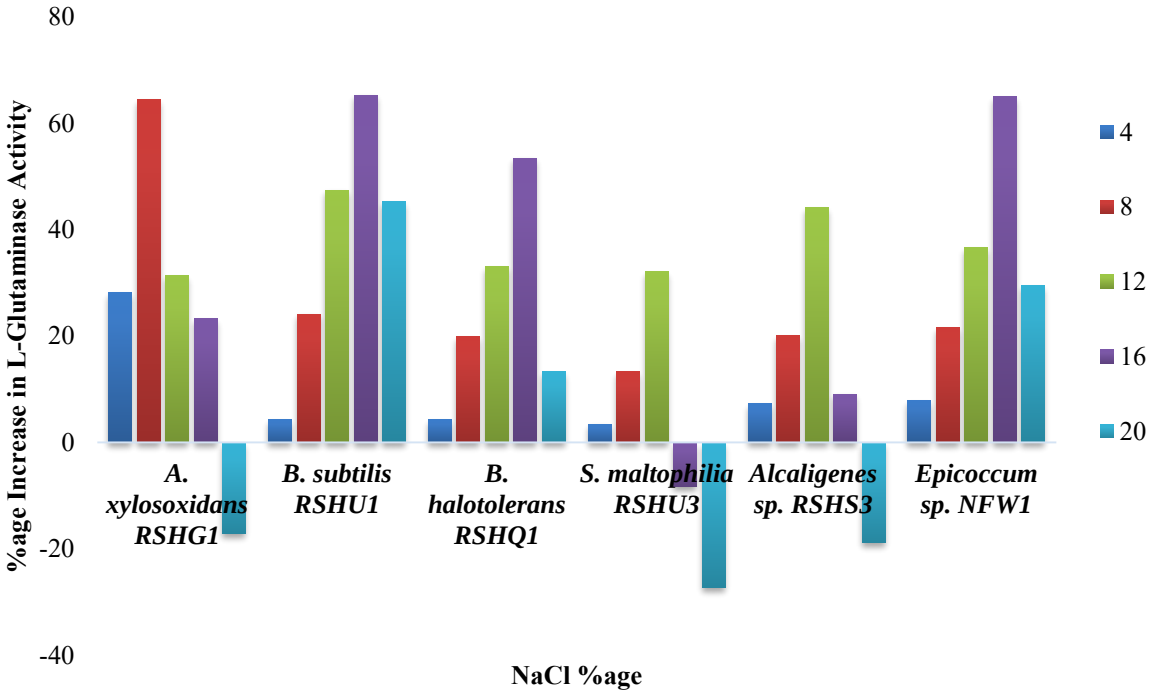

Figure 5.10. Effect of NaCl Concentration on L-Glutaminase Activity by Selected Microbial Strains.

L-Glutaminase by *A. xylosoxidans* RSHG1, *B. subtilis* U1, and *Epicoccum* sp. NFW1 was partially purified by 70% ammonium sulfate then all L-glutaminases were dialyzed and immobilized on 3.6% agar. Immobilized L-glutaminase was stable for 3weeks. L-glutaminase enzyme produced by *Hypocria jecorina* was immobilized on polyacrylic acid (Karahan *et al.,* 2014). *Bacillus subtilis* glutaminase was partially purified by 30% saturation of ammonium sulfate, then dialyzed and immobilized on Sephadex G-25, DEAE-cellulose, chitin powder, sawdust; the charcoal enzyme was more stable than free enzyme (Mahmod, 2016).

CONCLUSIONS

1. *A. xylosoxidans* RSHG1 L-glutaminase showed maximum production at pH9 *B. subtilis* RSHU1, gained the highest production at pH6, *B. halotolerans* RSHQ1 and *Epicoccum* sp. NFW1 L-glutaminase showed the best production at pH 7, *S. maltophilia* RSHU3, and *Alcaligenes* sp. RSHS3 maximum production at pH8.
2. *Alcaligenes* sp. RSHS3 achieved the highest L-glutaminase production at 25˚C, *A. xylosoxidans* RSHG1, *S. maltophilia* RSHU3, and *Epicoccum* sp. NFW1 showed maximum production at 30˚C and *B. subtilis* RSHU1 and *B. halotolerans* RSHQ1at 37˚C.
3. *A. xylosoxidans* RSHG1, *S. maltophilia* RSHU3 gained the best L-glutaminase production with sorbitol as a carbon source, *B. subtilis* RSHU1, and *Alcaligenes* sp. RSHS3 showed maximum production with glucose as a carbon source, *B. halotolerans* RSHQ1, and *Epicoccum* sp. NFW1 with sucrose as a carbon.
4. All selected strains showed L-glutaminase production with 1.5% L-glutamine as an inducer.
5. All glutaminases by selected strains showed maximum activity after incubation of 30minutes. Afterward, a reduction in enzyme activity was observed in all glutaminases.
6. *B. Subtilis* RSHU1 L-glutaminase showed the highest Vmax 522U/mg and best affinity (Km) for its substrate 0.0591mM.
7. *A. xylosoxidans* RSHG1, *Alcaligenes* sp. RSHS3, and *Epicoccum* sp. NFW1 L-glutaminases showed maximum activity at pH7 (neutral pH), *B Subtilis* RSHU1 at slight acidic pH6.5, *B. halotolerans* RSHQ1 at slight acidic pH6, and *S. maltophilia* RSHU3 at basic pH8.
8. All L-glutaminases gained the best activity at 40˚C except *B. halotolerans* RSHQ1 showed maximum activity at 50˚C.
9. EDTA, MnSO4, and FeSO4 inhibit all L-glutaminases. *A. xylosoxidans* RSHG1, *B. Subtilis* RSHU1, and *Epicoccum* sp. NFW1 was also inhibited by HgCl and activated by 20mM $CoCl_2$, $CaCl_2$, $BaCl_2$, $ZnSO_4$, KCl, $MgSO_4$, and NaCl. *B. halotolerans* RSHQ1 L-glutaminase was inhibited by CaCl2 and enhanced by $CoCl_2$, $BaCl_2$, $ZnSO_4$, KCl, $MgSO_4$, and NaCl. *S. maltophilia* RSHU3 L-glutaminase was additionally reduced by $MgSO_4$ and increased by $CoCl_2$, BaCl2, KCl, $ZnSO_4$, and NaCl. *Alcaligenes* sp. RSHS3 L-glutaminase showed an additional reduction in enzyme activity by $ZnSO_4$ and increased by $CoCl_2$, BaCl2, KCl, $MgSO_4$, and NaCl.

10. *A. xylosoxidans* RSHG1 L-glutaminase activity improved 64.4% by using 8% NaCl, afterward reduction in enzyme activity was monitored. *S. maltophilia* RSHU3 and *Alcaligenes* sp. RSHS3 L-glutaminase was enhanced using 12% NaCl with a 32% and 44% increase in activity, respectively. *B. Subtilis* RSHU1, *B halotolerans* RSHQ1, and *Epicoccum* sp. NFW1 L-glutaminase activity increased by 16% NaCl with 65%, 53%, and 64.9% enhanced enzyme activity.
11. The molecular weight of *A. xylosoxidans* RSHG1L-glutaminase was 40KDa *B. Subtilis* RSHU1 60, and *Epicocum* sp. NFW1 38KDa approximately. *A xylosoxidans* RSHG1, *B Subtilis* RSHU1, and *Epicocum* sp. W1 L-glutaminase was immobilized on 3.6% agar. The immobilized enzyme was stable for up to 3weeks.

Table 6.1 Conclusion in tabulated form

Microbial Strains	***A. xylosoxidans* RSHG1**	***B. Subtilis* RSHU1**	***B. halotolerans* RSHQ1**	***S. maltophilia* RSHU3**	***Alcaligenes* sp. RSHS3**	***Epicocum* sp. NFW1**
Microbial Sources	Expired glutamine sample	Soil Sample, University of Wah, Wah Cantt	Quetta soil	Soil sample, Quaid-i-Azam University, Islamabad	Taxila soil	Microbiology lab, Quaid-i-Azam University, Islamabad
Fermentation Conditions						
pH optimum for L-Glutaminase production	9	6	7	8	8	7
Temperature optimum for L-Glutaminase production	30˚C	37˚C	37˚C	30˚C	25˚C	30˚C
Carbon Sources for maximum Production	Sorbitol	Glucose	Sucrose	Sorbitol	Glucose	Sucrose
Inducer and Nitrogen source	L-glutamine	L-glutamine	L-glutamine	L-glutamine	L-glutamine	L-glutamine
L-Glutaminase Characterization						
Effect of Incubation Time	30min	30min	30min	30min	30min	30min
Vmax and Km	443U/mg & 0.235mM	522U/mg & 0.0591mM	343.6U/mg &0.37mM	351U/mg & 0.166mM	232U/mg & 0.186mM	450U/mg & 0.424mM
pH optimum	7	6.5	6	8	7	7
Temperature optimum	40˚C	40˚C	50˚C	40˚C	40˚C	40˚C
Inhibitors	EDTA, MnSO4, FeSO4 & HgCl	EDTA, MnSO4, FeSO4& HgCl	EDTA, MnSO4, FeSO4 & $CaCl_2$	EDTA, MnSO4, FeSO4 & $MgSO_4$	EDTA, MnSO4, FeSO4 & $ZnSO_4$	EDTA, MnSO4, FeSO4& HgCl
Activators	$CoCl_2$, $CaCl_2$, $BaCl_2$, $ZnSO_4$, KCl, $MgSO_4$, & NaCl	$CoCl_2$, $CaCl_2$, $BaCl_2$, $ZnSO_4$, KCl, $MgSO_4$, & NaCl	$CoCl_2$, $BaCl_2$, $ZnSO_4$, KCl, $MgSO_4$, & NaCl	$CoCl_2$, BaCl2, KCl, $ZnSO_4$, & NaCl	$CoCl_2$, BaCl2, KCl, $MgSO_4$, & NaCl	$CoCl_2$, $CaCl_2$, $BaCl_2$, $ZnSO_4$, KCl, $MgSO_4$, & NaCl
NaCl effect on L-glutaminase activity	64.4% increase by using 8% NaCl	65% increase with 16% NaCl	53% increase with 16% NaCl	32% increase with a 12% NaCl	44% increase with a 12% NaCl	64.9% increase with 16% NaCl
Molecular weight	40KDa	60KDa	-	-	-	38KDa
Immobilization on 3.6% agar	Stable up to 3weeks	Stable up to 3weeks	-	-	-	Stable up to 3weeks

FUTURE PROSPECTS

- Protein engineering could be done using Recombinant DNA technology and site-directed mutagenesis to explore new enzymes with improved properties required to apply the enzyme in diverse industries.
- Structural analysis of microbial L-glutaminases, including the elucidation of 3-D structures, prediction of active sites, identification, and interactions of catalytic residues, will aid in establishing structure–functional relationships of L-glutaminases from different sources.
- The application of L-glutaminases by isolated strains in the food industry as a flavour enhancing agent needs to be explored.
- Immobilized L-glutaminases on agar can be used in the food industry. For cancer treatment of L-glutaminase immobilization on nanoparticles could be studied for delivery.
- There is a prospect for glutaminase's in vivo therapeutic trials by selected strains for medical use like cancer therapy.
- Isolated L-glutaminases can be used to develop biosensors for monitoring the levels of L-glutamine in health and diagnosis.
- A genetic comparison of the L-glutaminase gene by all the selected strains could be made. Computational approaches should be used to get an insight into the structural-functional relationship of L-glutaminases.
- Isolated microbial strains can be genetically modified for L-glutaminases for industrial applications.
- Further enhancement of kinetic parameters and increased catalytic value of selected glutaminases for better therapeutic applications.

LIST OF PUBLICATIONS

Saleem, R.; Ahmed, S. Characterization of a New L-Glutaminase Produced by *Achromobacter xylosoxidans* RSHG1, Isolated from an Expired Hydrolyzed L-Glutamine Sample. Catalysts 2021, 11, 1262. https://doi.org/10.3390/catal11111262.

REFERENCES

Abdallah, N. A., Amer, S. K., & Habeeb, M. K. (2012). Screening of L-glutaminase produced by actinomycetes isolated from different soils in Egypt. *International Journal of ChemTech Research*, *4*(4), 1451-1460.

Abdallah, N.A., Amer, S.K., & Habeeb, M.K. (2013). Production, purification and characterization of L-glutaminase enzyme from *Streptomyces avermitilis*. *African Journal of Microbiol Research*, *7*, 1184-1190. https://doi.org/10.5897/AJMR2013.5367.

Abdelhameed, A., Alqaysi, R., & Hilal, H., Alharbi, N. (2020). Purification of L-Glutaminase from Bacillus sp. B12 and study its properties. *Indian Journal of Forensic Medicine and Toxicology, 14*(2), 1160-1166. https://doi.org/10.37506/ijfmt.v14i2.3063.

Abdumalikov, A., & Nikolaev, A. (1967). Separation and purification of glutaminase and deamidase of isoglutamine from yeast. *Biokhimiia (Moscow, Russia)*, *32*(4), 859-866.

Adegboye, M.F., Ojuederie, O.B., Talia, P.M. et al. (2021). Bioprospecting of microbial strains for biofuel production: metabolic engineering, applications, and challenges. *Biotechnology for Biofuels* 14, 5. https://doi.org/10.1186/s13068-020-01853-2.

Adeleke, B. S., Ojo, S. O., Oluwafemi, Y. D., & Olaniyi, O. O. (2017). Chemical mutagenesis of *Bacillus subtilis* for improved mannanase biosynthesis. *Journal of Advances in Microbiology*, 1-6.

Adrio, J. L., & Demain, A. L. (2014). Microbial enzymes: tools for biotechnological processes. *Biomolecules*, *4*(1), 117-139.

Ahmed, A. M. M., Taha, T. M., Abo-Dahab, N. F., & Hassan, F. S. (2016). Process optimization of L-glutaminase production; a tumour inhibitor from marine endophytic isolate *Aspergillus sp*. ALAA-2000. *Journal Microbial and Biochemical Technology*, 8, 256-267.10.4172/1948-5948.1000313.

Alaña, A., Gabilondo, A., Hernando, F., Moragues, M. D., Dominguez, J. B., Llama, M. J., & Serra, J. L. (1989). Pectin lyase production by a *Penicillium italicum* strain. *Applied and Environmental Microbiology*, *55*(6), 1612-1616.

Albayrak, D., & Karakuş, E. (2016). A novel glutamine biosensor based on zinc oxide nanorod and glutaminase enzyme from Hypocria jecorina. *Artificial cells, Nanomedicine, and Biotechnology*, *44*(1), 92–97. https://doi.org/10.3109/21691401.2014.913055.

Albrecht, J., & Norenberg, M. D. (2006). Glutamine: a Trojan horse in ammonia neurotoxicity. *Hepatology*, *44*(4), 788-794.

Albrecht, J., Sidoryk-Węgrzynowicz, M., Zielińska, M., & Aschner, M. (2010). Roles of glutamine in neurotransmission. *Neuron Glia Biology, 6*(4), 263–276. https://doi.org/10.1017/S1740925X11000093.

Alcaraz-Sandoval, V., Saucedo-Castañeda, G., & Huerta-Ochoa, S. (1997). Mannanase production by filamentous fungi in solid state fermentation. *In Advances in Solid State Fermentation* (pp. 311-319). Springer, Dordrecht.

Ali, T. H., Ali, N. H., Mohamed, L. A. (2009). Glutamine Amidohydrolase from *Penicillium politans* NRC 510. *Polish Journal of Food and Nutrition Sciences*, *59*(3), 211-217.

Alian, A. M., Bazaraa, W. A., El-Shimi, N. M., & Mohamed, R. M. (2015). Optimization of L-glutaminase synthesis by *Aspergillus oryzae* NRRL 32657 in submerged culture. *African Journal of Microbiology Research, 9*(9), 588-597. https://doi.org/10.5897/AJMR2014.7317.

Aly, M. M., Kadi, R. H., Alkhatib, M. H. & Wali, A.N. (2017). Production of the antitumor l-glutaminase enzyme from thermotolerant *Streptomyces sp.* D214, under submerged fermentation conditions. *Journal of Experimental Biology and Agriculture Sciences*, *5*(6), 878-885. https://doi.org/10.18006/2017.5(6).878.885.

Al-Zahrani, N., Al-Qahtani, S., Al-Sehri, W. (2020). Microbial genetics studies on L-glutaminase producer *Psuedomonas* NS16 isolated from eye contact lenses. *Journal of American Science*, 16(6)34-39. https://doi.org/10.7537/marsjas160620.04.

Amobonye, A., Singh, S., & Pillai, S. (2019). Recent advances in microbial glutaminase production and applications-a concise review. *Critical reviews in biotechnology*, *39*(7), 944–963. https://doi.org/10.1080/07388551.2019.1640659.

Anbu, P.; Gopinath, S. C. B.; Chaulagain, B.P. (2015). Thean-Hock Tang, Marimuthu Citartan, "Microbial Enzymes and Their Applications in Industries and Medicine 2014", *BioMed Research International*, vol. 2015, Article ID 816419, 3. https://doi.org/10.1155/2015/816419.

Aryuman, P., Lertsiri, S., Visessanguan, W., Niamsiri, N., Bhumiratana, A., & Assavanig, A. (2015). Glutaminase-producing *Meyerozyma (Pichia) guilliermondii* isolated from Thai soy sauce fermentation. *International Journal of Food Microbiology*, 192, 7–12. https://doi.org/10.1016/j.ijfoodmicro.2014.09.019.

Ashok, A., Doriya, K., Rao, J. V., Qureshi, A., Tiwari, A. K., & Kumar, D. S. (2019). Microbes producing L-asparaginase free of glutaminase and urease isolated from extreme locations of Antarctic soil and Moss. *Scientific reports*, *9*(1), 1-10.

Asli, M. S. (2010). A study on some efficient parameters in batch fermentation of ethanol using *Saccharomyces cerevesiae* SC1 extracted from fermented siahe sardasht pomace. *African Journal of Biotechnology*, *9*(20).

Athira, R. N., Elizebeth, T., Narendra, T., Sheik, T. A., Gupta, S. K., Chaudary, M., ... & Pramod, T. (2014). Investigation on the production of l-glutaminase from *pseudomonas stutzeri* strain under solid state fermentation using various agro residues. *Journal of Drug Delivery and Therapeutics*, *4*(2), 81-85. https://doi.org/10.22270/jddt.v4i2.814.

Awad, H.M., El-Deen, A.M.N., El-Sayed, & E.M., Hassabo, A.A. (2019). Biochemical studies and biological activities on L-glutaminase from rhizosphere soil *Streptomyces rochei* SAH2_CWMSG. *Egyptian Pharmaceutical Journal*, *18*(1), 27-41. https://doi.org/10.4103/epj.epj_32_18.

Bajar, S., Singh, A., & Bishnoi, N. R. (2020). Exploration of low-cost agro-industrial waste substrate for cellulase and xylanase production using Aspergillus heteromorphus. Appl Water Sci 10:153. .https://doi.org/10.1007/s13201-020-01236-w.

Balagurunathan, R., Radhakrishnan, M., & Somasundaram, S. T. (2010). L-Glutaminase producing actinomycetes from marine sediments–selective isolation, semi quantitative assay and characterization of potential strain. *Aust J Basic Appl Sci*, *4*(5), 698-705.

Balasubramanian, A. (2017). Soil Microorganisms. *Univ. Mysore*. 10.13140/RG.2.2.27925.12008.

Balasubramanian, N. (2015). Focus: A Multifaceted Battle Against Cancer: Introduction to Cancer Biology. *The Yale Journal of Biology and Medicine*, *88*(2), 201.

Bartholomew, H. P., Bradshaw, M., Jurick, W. M., 2nd, & Fonseca, J. M. (2021). The Good, the Bad, and the Ugly: Mycotoxin Production During Postharvest Decay and Their Influence on Tritrophic Host-Pathogen-Microbe Interactions. *Frontiers in Microbiology*, 12, 611881. https://doi.org/10.3389/fmicb.2021.611881.

Bazaraa, W., Alian, A., El-Shimi, N., & Mohamed, R. (2016). Purification and characterization of extracellular glutaminase from *Aspergillus oryzae* NRRL 32567. *Biocatalysis and Agricultural Biotechnology*. 6:76–81.

Bertola M, Ferrarini A, Visioli G. (2021) Improvement of Soil Microbial Diversity through Sustainable Agricultural Practices and Its Evaluation by -Omics Approaches: A Perspective for the Environment, Food Quality and Human Safety. *Microorganisms. 9*(7):1400. https://doi.org/10.3390/microorganisms9071400.

Bhardwaj, N., Kumar, B. & Verma, P. (2019). A detailed overview of xylanases: an emerging biomolecule for current and future prospective. *Bioresources. and Bioprocessing* 6, 40. https://doi.org/10.1186/s40643-019-0276-2.

Bilal, M., Zhao, Y., Noreen, S., Shah, S. Z. H., Bharagava, R. N., & Iqbal, H. M. (2019). Modifying bio-catalytic properties of enzymes for efficient biocatalysis: A review from immobilization strategies viewpoint. *Biocatalysis and Biotransformation*, *37*(3), 159-182.

Binod, P., Sindhu, R., Madhavan, A., Abraham, A., Mathew, A. K., Beevi, U. S., Sukumaran, R. K., Singh, S. P., & Pandey, A. (2017). Recent developments in l-glutaminase production and applications - An overview. *Bioresource Technology*, *245*(Pt B), 1766–1774. https://doi.org/10.1016/j.biortech.2017.05.059

Blazek, R., & Benbough, J. E. (1981). Improvement in the persistence of microbial asparaginase and glutaminase in the circulation of the rat by chemical modifications. *Biochimica et Biophysica Acta (BBA)-General Subjects, 677*(2), 220-224. https://doi.org/10.1016/0304-4165(81)90088-x.

Botman, D., Tigchelaar, W., & Van Noorden, C. J. (2014). Determination of phosphate-activated glutaminase activity and its kinetics in mouse tissues using metabolic mapping (quantitative enzyme histochemistry). *The journal of histochemistry and cytochemistry : official journal of the Histochemistry Society*, 62(11), 813–826. https://doi.org/10.1369/0022155414551177

Braux, A. S., Minet, J., Tamanai-Shacoori, Z., Riou, G., & Cormier, M. (1997). Direct enumeration of injured *Escherichia coli* cells harvested onto membrane filters. *Journal of microbiological methods*, *31*(1-2), 1-8.

Brown, G., Singer, A., Proudfoot, M., Skarina, T., Kim, Y., Chang, C., Dementieva, I., Kuznetsova, E., Gonzalez, C. F., Joachimiak, A., Savchenko, A., & Yakunin, A. F. (2008). Functional and structural characterization of four glutaminases from Escherichia coli and Bacillus subtilis. *Biochemistry*, *47*(21), 5724–5735. https://doi.org/10.1021/bi800097h

Bülbül, D., & Karakuş, E. (2013). Production and optimization of L-glutaminase enzyme from *Hypocrea jecorina* pure culture. *Preparative Biochemistry & Biotechnology*, 43(4), 385–397. https://doi.org/10.1080/10826068.2012.741641.

Castegna, A., & Menga, A. (2018). Glutamine Synthetase: Localization Dictates Outcome. *Genes, 9*(2), 108. https://doi.org/10.3390/genes9020108.

Castell, L. M., Newsholme, E. A., & Poortmans, J. R. (1996). Does glutamine have a role in reducing infections in athletes?. *European journal of applied physiology and occupational physiology*, *73*(5), 488-490.

Chandra, P., Enespa, Singh, R., & Arora, P. K. (2020). Microbial lipases and their industrial applications: a comprehensive review. *Microbial cell factories, 19*(1), 169. https://doi.org/10.1186/s12934-020-01428-8.

Chandrasekaran, M. (1997). Industrial enzymes from marine microorganisms: The Indian scenario. *Journal of Marine Biotechnology, 5*, 86-89

Chapman, J., Ismail, A.E., & Dinu, C.Z. (2018). Industrial Applications of Enzymes: Recent Advances, Techniques, and Outlooks. *Catalysts. 8,* 238. https://doi.org/10.3390/catal8060238

Charlton, B. G., Morris, J. B., & Williams, G. H. (1965). Experimental Study Of Spouting Beds Of Spheres (No. AERE-R--4852). *Atomic Energy Research Establishment, Harwell* (England).

Chasanah, E., Tambunan, U., & Yulianti, T. (2013). Screening and characterization of L-glutaminase produced by bacteria isolated from sangihe talaud sea. *Squalen Bulletin of Marine and Fisheries Postharvest and Biotechnology*, *7*(3), 115-122. https://doi.org/10.15578/SQUALEN.V7I3.6.

Chen, J., Zhou, L., Zhang, X., Lu, X., Cao, R., Xu, C., & Xu, G. (2012). Urinary hydrophilic and hydrophobic metabolic profiling based on liquid chromatography- mass spectrometry methods: Differential metabolite discovery specific to ovarian cancer. *Electrophoresis*, *33*(22), 3361-3369.

Chen, J.Q., & Russo, J. (2012). Dysregulation of glucose transport, glycolysis, TCA cycle and glutaminolysis by oncogenes and tumor suppressors in cancer cells. *Biochimica et Biophysica Acta*.1826, 370–384.

Chen, Q., Kirk, K., Shurubor, Y. I., Zhao, D., Arreguin, A. J., Shahi, I., Valsecchi, F., Primiano, G., Calder, E. L., Carelli, V., Denton, T. T., Beal, M. F., Gross, S. S., Manfredi, G., & D'Aurelio, M. (2018). Rewiring of Glutamine Metabolism Is a Bioenergetic Adaptation of Human Cells with Mitochondrial DNA Mutations. *Cell Metabolism, 27*(5), 1007–1025.e5. https://doi.org/10.1016/j.cmet.2018.03.002.

Chenthamara, D., Subramaniam, S., Ramakrishnan, S. G., Krishnaswamy, S., Essa, M. M., Lin, F. H., & Qoronfleh, M. W. (2019). Therapeutic efficacy of nanoparticles and routes of administration. *Biomaterials Research*, 23, 20. https://doi.org/10.1186/s40824-019-0166-x.

Chiu, D. K. C., Tse, A. P. W., Law, C. T., Xu, I. M. J., Lee, D., Chen, M., ... & Wong, C. C. L. (2019). Hypoxia regulates the mitochondrial activity of hepatocellular carcinoma cells through HIF/HEY1/PINK1 pathway. *Cell death & disease*, *10*(12), 1-16. https://doi.org/10.1038/s41419-019-2155-3.

Choat, H. M., Martin, A., Mick, G. J., Heath, K. E., Hubert, M. T., McGwin Jr, G., & McCormick, K. L. (2019). Effect of gamma aminobutyric acid (GABA) or GABA with glutamic acid decarboxylase (GAD) on the progression of type 1 diabetes mellitus in children: Trial design and methodology. *Contemporary clinical trials*, 82, 93-100.

Choi, Y. K., and Park, K. G. (2018). Targeting Glutamine Metabolism for Cancer Treatment. *Biomolecules & Therapeutics*, *26*(1), 19–28. https://doi.org/10.4062/biomolther.2017.178

Chou, C. C., Yu, R. C., & Tsai, C. T. (1993). Production of glutaminase by *Actinomucor elegans*, *Actinomucor taiwanensis* and *Aspergillus oryzae*. *Journal-Chinese Agricultural Chemical Society*, *31*, 78-78.

Cluntun, A. A., Lukey, M. J., Cerione, R. A., & Locasale, J. W. (2017). Glutamine Metabolism in Cancer: Understanding the Heterogeneity. *Trends in Cancer, 3*(3), 169–180. https://doi.org/10.1016/j.trecan.2017.01.005

Cook, W. R., Hoffman, J. H., & Bernlohr, R. W. (1981). Occurrence of an inducible glutaminase in *Bacillus licheniformis*. *Journal of Bacteriology*, 148(1), 365–367. https://doi.org/10.1128/jb.148.1.365-367.1981.

Cruzat, V., Macedo Rogero, M., Noel Keane, K., Curi, R., & Newsholme, P. (2018). Glutamine: Metabolism and Immune Function, Supplementation and Clinical Translation. *Nutrients*, 10(11), 1564. https://doi.org/10.3390/nu10111564

Curi, R., Newsholme, P., Procopio, J., Lagranha, C., Gorjão, R., & Pithon-Curi, T. C. (2007). Glutamine, gene expression, and cell function. *Frontiers in bioscience: a journal and virtual library*, 12, 344–357. https://doi.org/10.2741/2068.

Curthoys, N. P., & Gstraunthaler, G. (2001). Mechanism of increased renal gene expression during metabolic acidosis. *American Journal of Physiology-Renal Physiology*, *281*(3), F381-F390.

Curthoys, N. P., & Watford, M. (1995). Regulation of glutaminase activity and glutamine metabolism. *Annual review of nutrition*, *15*(1), 133-159.

Daikhin, Y., & Yudkoff, M. (2000). Compartmentation of brain glutamate metabolism in neurons and glia. *The Journal of nutrition*, *130*(4), 1026S-1031S.

Dang, C. V., O'Donnell, K. A., Zeller, K. I., Nguyen, T., Osthus, R. C., & Li, F. (2006, August). The c-Myc target gene network. *In Seminars in cancer biology* (Vol. 16, No. 4, pp. 253-264). Academic Press.

De Melo, M. P., Pithon-Curi, T. C., & Curi, R. (2004). Indole-3-acetic acid increases glutamine utilization by high peroxidase activity-presenting leukocytes. *Life sciences*, *75*(14), 1713-1725.

De Souza, H. M., Borba-Murad, G. R., Ceddia, R. B., Curi, R., Vardanega-Peicher, M., & Bazotte, R. B. (2001). Rat liver responsiveness to gluconeogenic substrates during insulin-induced hypoglycemia. *Brazilian Journal of Medical and Biological Research, 34*(6), 771-777.

Dean, J. F., & Anderson, J. D. (1991). Ethylene biosynthesis-inducing xylanase: II. purification and physical characterization of the enzyme produced by *Trichoderma viride*. *Plant physiology*, *95*(1), 316-323.

DeBerardinis, R. J., & Cheng, T. (2010). Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. *Oncogene, 29*(3), 313–324. https://doi.org/10.1038/onc.2009.358.

DeBerardinis, R. J., Mancuso, A., Daikhin, E., Nissim, I., Yudkoff, M., Wehrli, S., & Thompson, C. B. (2007). Beyond aerobic glycolysis: transformed cells can engage in glutamine metabolism that exceeds the requirement for protein and nucleotide synthesis. *Proceedings of the National Academy of Sciences*, *104*(49), 19345-19350.

DeBerardinis, V., Vallenet, D., Castelli, V., Besnard, M., Pinet, A., Cruaud, C., ... & Durot, M. (2008). A complete collection of single- gene deletion mutants of *Acinetobacter baylyi* ADP1. *Molecular systems biology*, *4*(1), 174.

Desai, S. S., Chopra, S. J., & Hungund, B. S. (2016). Production, purification and characterization of L-Glutaminase from *Streptomyces* sp. isolated from soil. *Journal of Applied and Pharmaceutical Science*, *6*(7), 100-105. https://doi.org/10.7324/JAPS.2016.60715.

De-Souza, D. A., & Greene, L. J. (2005). Intestinal permeability and systemic infections in critically ill patients: effect of glutamine. *Critical care medicine*, *33*(5), 1125-1135.

Dhiman, S., Mukherjee, G., & Singh, A. K. (2018). Recent trends and advancements in microbial tannase-catalyzed biotransformation of tannins: a review. *International Microbiology*, *21*(4), 175-195.https://doi.org/10.1007/s10123-018-0027-9.

Di Luccio, M., Capra, F., Ribeiro, N. P., Vargas, G. D., Freire, D. M., & De Oliveira, D. (2004). Effect of temperature, moisture, and carbon supplementation on lipase production by solid-state fermentation of soy cake by *Penicillium simplicissimum*. *Applied biochemistry and biotechnology, 113*(1), 173-180.

Doelle, H. W., Mitchell, D. A., & Rolz, C. E. (Eds.). (1992). Solid substrate cultivation. Springer.

Dominati, E., Patterson, M., & Mackay, A. (2010). A framework for classifying and quantifying the natural capital and ecosystem services of soils. *Ecological economics*, *69*(9), 1858-1868.

Dubey, R., Paul, A., & Prity, N. (2015). Isolation, production and screening of anticancer enzyme L-Glutaminase from *Bacillus Subtilis*. *International Journal of Pharmacy and Biological Sciences*, *5*(2), 96-105.

Dueramae, S., Varichanan, P., & Enomoto, T. (2021). Rapid Screening of Cultural Parameters for Extracellular Halophilic Glutaminase Production from *Tetragenococcus Muriaticus* FF5302 using the Plackett-Burman's Experimental Design. Walailak *Journal of Science and Technology (WJST), 18*(9), 9317-7.

Durai, S., Selvaraj, B., Manikkam, R., & Ramasamy, B. (2014). Production and optimization of L-glutaminase from *Vibrio* sp. M9 isolated from Mahabalipuram marine sediments. *World Journal of Pharmaceutical Research, 3*(2), 2117-2126.

Durante, W. (2019). The emerging role of l-glutamine in cardiovascular health and disease. *Nutrients, 11*(9), 2092. doi:10.3390/nu11092092.

Dutta, S., Ghosh, S., & Pramanik, S. (2015). L-asparaginase and L-glutaminase from *Aspergillus fumigatus* WL002: Production and some physicochemical properties. *Applied Biochemistry and Microbiology, 51*(4), 425-431. https://doi.org/10.1134/S0003683815040067.

Ehrensvard, G., Fischer, A. & Stjernholm, R. (1949) Protein metabolism of tissue cells in vitro. The chemical nature of some obligate factors of tissue cell nutrition. *Acta Physiologica Scandinavica*. 18:218.

Ejaz, U., Sohail, M., & Ghanemi, A. (2021). Cellulases: From Bioactivity to a Variety of Industrial Applications. *Biomimetics*, *6*(3), 44.https://doi.org/10.3390/biomimetics6030044.

El Enshasy, H. A., Elsayed, E. A., Suhaimi, N., Abd Malek, R., & Esawy, M. (2018). Bioprocess optimization for pectinase production using *Aspergillus niger* in a submerged cultivation system. *BMC Biotechnology*, *18*(1), 1-13. https://doi.org/10.1186/s12896-018-0481-7.

El-Asmar, F. A., Greenberg, D. M., & Amand, G. S. (1966). Studies on the mechanism of inhibition of tumor growth by the enzyme glutaminase. *Cancer research*, *26*(1), 116-122.

El-Sayed A. S. (2009). L-glutaminase production by *Trichoderma koningii* under solid-state fermentation. *Indian journal of microbiology*, *49*(3), 243–250. https://doi.org/10.1007/s12088-009-0020-2.

El-Sousy, S. M., Easa, S. M., Hassan, A. A., & Ismail, A. M. S. (2021). Production of a bacterial extracellular L-glutaminase possessing high antioxidant capability. *Egyptian Pharmaceutical Journal*, *20*(1), 59.

Elshafei, A. M., Hassan, M. M., Ali, N. H., Abouzeid, M. A. E., Mahmoud, D. A., & Elghonemy, D. H. (2014). Purification, kinetic properties and antitumor activity of L-glutaminase from Penicillium brevicompactum NRC 829. *Microbiology Research Journal International*, *4*(1), 97-115. https://doi.org/10.9734/BMRJ/2014/5098

Elzainy, T. A., & Ali, T. H. (2006). Detection of the antitumor glutaminase-asparaginase in the filamentous fungi. *Journal of Applied Sciences*, *6*(1), 1389-1395.

Emelda, J. Isolation and Screening of L-asparaginase and L-glutaminase Producing Bacteria and their Antimicrobial Potential from Environmental Sources. *Journal of Pharmaceutical and Biological Sciences* **2016**, *11*(3), 47-53. https://doi.org/10.9790/3008-1103024753.

Eremenko, V. V., Zhukov, A. V., & Nikolaev, A. (1975). Asparaginase and glutaminase activity in *Pseudomonas fluorescens* in continuous cultivation. *Mikrobiologiia*, *44*(4), 615-620.

Falcone P, Serafin-Fracassini D, Del-Duca S (1993) Comparative studies of transglutaminase activity and substrates in different organs of *Helianthus tuberosus*. J Plant Physiol 142:263–273.

Fan, J., Meng, Q., Guo, G., Xie, Y., Li, X., Xiu, Y., ... & Ma, L. (2010). Effects of glutamine added to enteral nutrition on Peyer's patch apoptosis in severely burned mice. *Burns*, *36*(3), 409-417.

Fatima, N., Kondratyuk, T. P., Park, E. J., Marler, L. E., Jadoon, M., Qazi, M. A., ... & Pezzuto, J. M. (2016). Endophytic fungi associated with Taxus fuana (West Himalayan Yew) of Pakistan: potential bio-resources for cancer chemopreventive agents. *Pharmaceutical Biology*, *54*(11), 2547-2554.

Felsenstein, J. (1985). Confidence limits on phylogenies: a justification. *Evolution*, *39*, 783-791.

Felsher, D. W. (2010). MYC inactivation elicits oncogene addiction through both tumor cell–intrinsic and host-dependent mechanisms. *Genes & cancer*, *1*(6), 597-604.

Ferreira, F. V., Herrmann-Andrade, A. M., Binolfi, A., Calabrese, C. D., Mac Cormack, W. P., & Musumeci, M. A. (2021). Characteristics of a Cold-Adapted L-glutaminase with Potential Applications in the Food Industry. *Applied Biochemistry and Biotechnology,* 1-18. DOI: 10.1007/s12010-021-03596-8.

Fortier, L. C., & Moineau, S. (2009). Phage production and maintenance of stocks, including expected stock lifetimes. In *Bacteriophages* (pp. 203-219). Humana Press.

Fuchs, B. C., & Bode, B. P. (2006). Stressing out over survival: glutamine as an apoptotic modulator. *Journal of Surgical Research, 131*(1), 26-40.

Fukushima, Y., & Motai, H. (1990). Continuous conversion of glutamine to glutamate by immobilized glutaminase-producing yeast. *Journal of Fermentation and Bioengineering*, *69*(3), 189-191.

Fullstone, G., Wood, J., Holcombe, M., & Battaglia, G. (2015). Modelling the transport of nanoparticles under blood flow using an agent-based approach. *Scientific Reports*, *5*(1), 1-13. https://doi.org/10.1038/srep10649.

Fulop, N., Zhang, Z., Marchase, R. B., & Chatham, J. C. (2007). Glucosamine cardioprotection in perfused rat hearts associated with increased O-linked N-acetylglucosamine protein modification and altered p38 activation. *American Journal of Physiology-Heart and Circulatory Physiology*, *292*(5), H2227-H2236.

Fung, M. K. L., & Chan, G. C. F. (2017). Drug-induced amino acid deprivation as strategy for cancer therapy. *Journal of hematology & oncology, 10*(1), 1-18. https://doi.org/10.1186/s13045-017-0509-9

Gaglio, D., Metallo, C. M., Gameiro, P. A., Hiller, K., Danna, L. S., Balestrieri, C., ... & Chiaradonna, F. (2011). Oncogenic K- Ras decouples glucose and glutamine metabolism to support cancer cell growth. *Molecular systems biology*, *7*(1), 523.

Gaglio, D., Soldati, C., Vanoni, M., Alberghina, L., & Chiaradonna, F. (2009). Glutamine deprivation induces abortive s-phase rescued by deoxyribonucleotides in k-ras transformed fibroblasts. *PloS one*, *4*(3), e4715.

Ganbarov, K. G., Kulieva, N. A., & Muradov, P. Z. (2001). Biosynthesis of Pectinase by Fungi of the Genera *Bjerkanderaand Coriolusduring* Solid-Phase Fermentation. *Applied Biochemistry and Microbiology*, *37*(6), 593-595.

Gao, P., Tchernyshyov, I., Chang, T. C., Lee, Y. S., Kita, K., Ochi, T., ... & Dang, C. V. (2009). c-Myc suppression of miR-23a/b enhances mitochondrial glutaminase expression and glutamine metabolism. *Nature*, *458*(7239), 762.

Garcia, C., Pithon-Curi, T. C., De Lourdes Firmano, M., Pires De Melo, M., Newsholme, P., & Curi, R. (1999). Effects of adrenaline on glucose and glutamine metabolism and superoxide production by rat neutrophils. *Clinical science*, *96*(6), 549-555.

Gargova, S., & Sariyska, M. (2003). Effect of culture conditions on the biosynthesis of *Aspergillus niger* phytase and acid phosphatase. *Enzyme and Microbial Technology*, *32*(2), 231-235.

Gerber, H. P., Seipel, K., Georgiev, O., Höfferer, M., Hug, M., Rusconi, S., & Schaffner, W. (1994). Transcriptional activation modulated by homopolymeric glutamine and proline stretches. Science (New York, N.Y.), 263(5148), 808–811. https://doi.org/10.1126/science.8303297

Giordano, C., Esposito, R., Mazzola, G., Vecchio, G., Pluvio, M., Cirillo, D., Capasso, G. B., & Buonanno, G. (1981). L-glutaminase and L-asparaginase by extracorporeal route in acute lymphoblastic leukemia therapy. *The International journal of artificial organs, 4*(5), 244–248.

Gomri, M.A.; Rico-Díaz, A.; Escuder-Rodríguez, J.-J.; El Moulouk Khaldi, T.; González-Siso, M.-I.; Kharroub, K. (2018) Production and Characterization of an Extracellular Acid Protease from *Thermophilic Brevibacillus sp*. OA30 Isolated from an Algerian Hot Spring. *Microorganisms*, 6, 31. https://doi.org/10.3390/microorganisms6020031.

Gonzalez Herrera, K. N., Lee, J., & Haigis, M. C. (2015). Intersections between mitochondrial sirtuin signaling and tumor cell metabolism. *Critical Reviews in Biochemistry and Molecular Biology, 50*(3), 242-255.

Greenberg, D. M., Blumenthal, G., & Ramadan, M. E. A. (1964). Effect of administration of the enzyme glutaminase on the growth of cancer cells. *Cancer research*, *24*(6 Part 1), 957-963.

Gross, M. I., Demo, S. D., Dennison, J. B., Chen, L., Chernov-Rogan, T., Goyal, B., ... & Bennett, M. K. (2014). Antitumor activity of the glutaminase inhibitor CB-839 in triple-negative breast cancer. *Molecular cancer therapeutics, 13*(4), 890-901.

Gstraunthaler, G., Holcomb, T., Feifel, E., Liu, W., Spitaler, N., & Curthoys, N. P. (2000). Differential expression and acid-base regulation of glutaminase mRNAs in gluconeogenic LLC-PK1-FBPase+ cells. *American Journal of Physiology-Renal Physiology*, *278*(2), F227-F237.

Gudmundsdottir, H., Wells, A. D., & Turka, L. A. (1999). Dynamics and requirements of T cell clonal expansion in vivo at the single-cell level: effector function is linked to proliferative capacity. *The Journal of Immunology*, *162*(9), 5212-5223.

Gulve, E. A., Cartee, G. D., Youn, J. H., & Holloszy, J. O. (1991). Prolonged incubation of skeletal muscle increases system A amino acid transport. *American Journal of Physiology-Cell Physiology*, *260*(1), C88-C95.

Gutman, A. B., & Yue, T. F. (1965). Urinary Ammonium Excretion in Primary Gout. The Journal of clinical investigation, 44(9), 1474–1481. https://doi.org/10.1172/JCI105254.

Guzman, L. A., Labhasetwar, V., Song, C., Jang, Y., Lincoff, A. M., Levy, R., & Topol, E. J. (1996). Local intraluminal infusion of biodegradable polymeric nanoparticles. A novel approach for prolonged drug delivery after balloon angioplasty. *Circulation, 94*(6), 1441–1448. https://doi.org/10.1161/01.cir.94.6.1441.

Hamed, S., & Al-wasify, R. S. (2016). Production and optimization of L-glutaminase from a terrestrial fungal *Fusarium oxysporum*. *International Journal of PharmTech Research*, *9*(4), 233-241.

Hanahan, D., & Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *cell, 144*(5), 646-674.

Hansen, M. B., Nielsen, S. E., & Berg, K. (1989). Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. *Journal of immunological methods, 119*(2), 203-210.

Hassan, M. E., Yang, Q., Xiao, Z., Liu, L., Wang, N., Cui, X., & Yang, L. (2019). Impact of immobilization technology in industrial and pharmaceutical applications. 3 *Biotechnology, 9*(12), 440. https://doi.org/10.1007/s13205-019-1969-0.

Häussinger, D. (1989). Glutamine metabolism in the liver: overview and current concepts. *Metabolism-Clinical and Experimental*, *38*(8), 14-17.

Haüssinger, D. (1990). Nitrogen metabolism in liver: structural and functional organization and physiological relevance. *Biochemical Journal*, *267*(2), 281-290.

Heine, U. L., Munoz, E. F., Flanders, K. C., Ellingsworth, L. R., Lam, H. Y., Thompson, N. L., & Sporn, M. B. (1987). Role of transforming growth factor-beta in the development of the mouse embryo. *The Journal of Cell Biology*, *105*(6), 2861-2876.

Hemraj, V., Diksha, S., & Avneet, G. (2013). A review on commonly used biochemical test for bacteria. *Innovare J Life Sci, 1*(1), 1-7.

Hensley, C. T., Wasti, A. T., & DeBerardinis, R. J. (2013). Glutamine and cancer: cell biology, physiology, and clinical opportunities. *The Journal of clinical investigation*, 123(9), 3678–3684. https://doi.org/10.1172/JCI69600.

Hertz, L., & Rothman, D. L. (2017). Glutamine-Glutamate Cycle Flux Is Similar in Cultured Astrocytes and Brain and Both Glutamate Production and Oxidation Are Mainly Catalyzed by Aspartate Aminotransferase. *Biology, 6*(1), 17. https://doi.org/10.3390/biology6010017.

Hiatt, R. A., & Breen, N. (2008). The social determinants of cancer: a challenge for transdisciplinary science. *American journal of preventive medicine*, *35*(2), S141-S150.

Holcenberg, J. S.; Borella, L. D.; Camitta, B. M.; Ring, B. J. (1979). Human pharmacology and toxicology of succinylated *Acinetobacter* glutaminase-asparaginase. *Cancer Research*, 39(8), 3145–3151.

Houfani, A. A., Větrovský, T., Navarrete, O. U., Štursová, M., Tláskal, V., Beiko, R. G., ... & Jorquera, M. A. (2019). Cellulase− hemicellulase activities and bacterial community composition of different soils from Algerian Ecosystems. *Microbial ecology*, 77(3), 713-725. https://doi.org/10.1007/s00248-018-1251-8.

Hughes, D. E., & Williamson, D. H. (1952). Some properties of the glutaminase of *Clostridium welchii*. *Biochemical Journal*, *51*(1), 45-55.

Hugouvieux-Cotte-Pattat, N., Condemine, G., & Shevchik, V. E. (2014). Bacterial pectate lyases, structural and functional diversity. *Environmental microbiology reports, 6*(5), 427–440. https://doi.org/10.1111/1758-2229.12166.

Ibrahim, Z. K. M., Ahmed, T. T. M., & Salah, M. G. (2020). Isolation, Screening and Characterization of L-Glutaminase Producing Bacteria from Brackish Water. *International Journal of Progressive Sciences and Technologies*, *22*(1), 227-231. http://dx.doi.org/10.52155/ijpsat.v22.1.2057.

Illanes, A. (2008). Enzyme biocatalysis. *Principles and Applications. Editorial Springer-Verlag New York Inc., United States*, 1-56.

Imada, A., Igarasi, S., Nakahama, K., & Isono, M. (1973). Asparaginase and glutaminase activities of micro-organisms. *Microbiology*, *76*(1), 85-99.

Irajie, C., Mohkam, M., Nezafat, N., Hosseinzadeh, S., Aminlari, M., & Ghasemi, Y. (2016). In silico analysis of glutaminase from different species of *Escherichia* and *Bacillus*. *Iranian Journal of Medical Sciences, 41*(5), 406.

Ito, K., Koyama, Y., & Hanya, Y. (2013). Identification of the glutaminase genes of *Aspergillus sojae* involved in glutamate production during soy sauce fermentation. *Bioscience, Biotechnology, and Biochemistry*, *77*(9), 1832–1840. https://doi.org/10.1271/bbb.130151.

Ito, K., Umitsuki, G., Oguma, T., Koyama, Y. (2011). Salt-Tolerant and Thermostable Glutaminases of *Cryptococcus* Species Form a New Glutaminase Family. *Bioscience, Biotechnology, and Biochemistry*, 75. 1317-24. 10.1271/bbb.110092.

Ito, K.; Matsuyama, A. (2021) Koji Molds for Japanese Soy Sauce Brewing: Characteristics and Key Enzymes. *Journal of Fungi*. *7*(8):658. https://doi.org/10.3390/jof7080658

Itoh, T., Hoshikawa, Y., Matsuura, S. I., Mizuguchi, J., Arafune, H., Hanaoka, T. A., ... & Kyotani, T. (2012). Production of l-theanine using glutaminase encapsulated in carbon-coated mesoporous silica with high pH stability. *Biochemical Engineering Journal*, *68*, 207-214.

Iwasa, T., Fujii, M., & Yokotsuka, T. (1987). On the glutaminase produced by *Cryptococcus albidus* ATCC 20293. Purification and some properties of the enzyme. *Nippon Shoyu Kenkyusho Zasshi, 13*, 205-210.

Iyengar, P., Wardak, Z., Gerber, D. E., Tumati, V., Ahn, C., Hughes, R. S., ... & Pulipparacharuvil, S. (2018). Consolidative radiotherapy for limited metastatic non–small-cell lung cancer: a phase 2 randomized clinical trial. *JAMA oncology*, *4*(1), e173501-e173501.

Iyer, P. V., & Singhal, R. S. (2009). Screening and selection of marine isolate for L-glutaminase production and media optimization using response surface methodology. *Applied Biochemistry and Biotechnology, 159*(1), 233-250.

Iyer, P., & Singhal, R. S. (2008). Production of glutaminase (EC 3.2.1.5) from *Zygosaccharomyces rouxii*: Statistical optimization using response surface methodology. *Bioresource Technology*, *99*(10), 4300-4307.

Iyer, P., & Singhal, R. S. (2010). Production of glutaminase (E.C. 3.2.1.5) from *Zygosaccharomyces rouxii* in solid-state fermentation and modeling the growth of Z. rouxii therein. *Journal of Microbiology and Biotechnology, 20*(4), 737–748.

Jäättelä, M., Wissing, D., Kokholm, K., Kallunki, T., & Egeblad, M. (1998). Hsp70 exerts its anti- apoptotic function downstream of caspase- 3- like proteases. *The EMBO journal, 17*(21), 6124-6134.

Jagannathan, S. V., Manemann, E. M., Rowe, S. E., Callender, M. C., & Soto, W. (2021). Marine Actinomycetes, New Sources of Biotechnological Products. *Marine Drugs*, 19(7), 365. https://doi.org/10.3390/md19070365

Janssen, P. H. (2006). Identifying the dominant soil bacterial taxa in libraries of 16S rRNA and 16S rRNA genes. *Applied and Environmental Microbiology*, *72*(3), 1719-1728.

Jenkinson, D. S. (1966). Studies on the decomposition of plant material in soil: II. Partial sterilization of soil and the soil biomass. *Journal of Soil Science*, *17*(2), 280-302.

Jenstad, M., & Chaudhry, F. A. (2013). The amino acid transporters of the glutamate/GABA-glutamine cycle and their impact on insulin and glucagon secretion. *Frontiers in Endocrinology*, 4, 199.

Jesuraj SAV, Sarker MMR, Ming LC, Praya SMJ, Ravikumar M, Wui WT (2017) Enhancement of the production of L-glutaminase, an anticancer enzyme, from *Aeromonas veronii* by adaptive and induced mutation techniques. *PLoS ONE 12*(8): e0181745. https://doi.org/10.1371/journal.pone.0181745.

Jesuraj, S. A. V., ravikumar, M., sarker, M. M. R., & praya, S. M. (2015). Apoptotic Induction On Ht29 Cells By Immobilized L-Glutaminase. Pharmanest : *An International Journal Of Advances In Pharmaceutical Sciences*. 6. 2960-2963.

Jiang, Y., Li, Y., & Zhu, B. (2015). T-cell exhaustion in the tumor microenvironment. *Cell Death & Disease*, *6*(6), e1792.

Jobmann, M., & Rafler, G. (2002). Submicronparticles from biodegradable polymers. *International Journal of Pharmaceutics*, 242(1-2), 213-217.

Jones, E. G., Stanley, S. J., & Pinruan, U. (2008). Marine endophyte sources of new chemical natural products: a review. *Botanica Marina* 51(3): 163-170

Juhasz, T., Szengyel, Z., Reczey, K., Siika-Aho, M., & Viikari, L. (2005). Characterization of cellulases and hemicellulases produced by *Trichoderma reesei* on various carbon sources. *Process Biochemistry*, *40*(11), 3519-3525.

Jyothi, H., Kumar, S., & Vandana, R. (2011). Production of L-glutaminase by Pseudomonas VJ-6. *Research Journal of Biotechnology* 2011, 6(3):42-49.

Kalsoom, M., Rehman, F. U., Shafique, T. A. L. H. A., Junaid, S. A. N. W. A. L., Khalid, N., Adnan, M., ... & Ali, H. (2020). Biological importance of microbes in agriculture, food and pharmaceutical industry: A review. *Innovare Journal of Life Sciences,8*(6),1-4.

Kamini, N. R., Mala, J. G. S., & Puvanakrishnan, R. (1998). Lipase production from *Aspergillus niger* by solid-state fermentation using gingelly oil cake. *Process Biochemistry*, 33(5), 505-511

Karahan, M., Karakuş, E., Bülbül, D., & Atacı, N. (2014). Immobilization of glutaminase enzyme from *Hypocria jecorina* on polyacrylic acid: preparation and biochemical characterization. *Artificial cells, nanomedicine, and biotechnology*, *42*(4), 262-267.https://doi.org/10.3109/21691401.2013.808646.

Karakuş, E., & Pekyardımcı, Ş. (2012). Comparison of covalent and noncovalent immobilization of malatya apricot pectinesterase (*Prunus armeniaca* L.). *Artificial Cells, Blood Substitutes, and Biotechnology, 40*(1-2), 132-141. 10.3109/10731199.2011.611471.

Karakuş, E., Pekyardımcı, Ş., & Kiliç, E. (2013). Potentiometric glucose determination in human serum samples with glucose oxidase biosensor based on Iodide electrode. *Applied Biochemistry and Microbiology, 49*(2), 194-198. 10.1134/S0003683813020051.

Karim, G. F., & Thalij, K. M. (2016). Determination of L-Glutaminase Activity by Some Bacterial Species. *International Journal of Current Microbiology and Applied Sciences, 5*(4), 218-225. http://dx.doi.org/10.20546/ijcmas.2016.504.027

Kashyap, P., Sabu, A., Pandey, A., Szakacs, G., & Soccol, C. R. (2002). Extra-cellular L-glutaminase production by *Zygosaccharomyces rouxii* under solid-state fermentation. *Process Biochemistry*, *38*(3), 307-312.

Katikala, P. K., Bobbarala, V., Tadimalla, P., & Guntuku, G. S. (2009). Screening of L-glutaminase producing marine bacterial cultures for extracellular production of L-glutaminase. *International Journal of ChemTech Research*, *1*(4), 1232-1235.

Katt, W. P., Lukey, M. J., & Cerione, R. A. (2017). A tale of two glutaminases: homologous enzymes with distinct roles in tumorigenesis. *Future Medicinal Chemistry, 9*(2), 223–243. https://doi.org/10.4155/fmc-2016-0190.

Katunuma, N., Katsunuma, T., Towatari, T., & Tomino, I. (1973). The Enzymes of Glutamine Metabolism. *by S. Prusiner and ER Stadtman, Academic Press, New York*, 227-275.

Kaur, G., Thompson, L. A., & Dufour, J. M. (2014, June). Sertoli cells–immunological sentinels of spermatogenesis. In *Seminars in cell & developmental biology* (Vol. 30, pp. 36-44). Academic Press.

Kaushal, G. P., Zeng, Y. U. C. H. E. N. G., & Elbein, A. D. (1993). Biosynthesis of glucosidase II in suspension-cultured soybean cells. *Journal of Biological Chemistry*, *268*(19), 14536-14542.

Keerthi, T. R., Suresh, P. V., Sabu, A., Rajeevkumar, S., & Chandrasekaran, M. (1999). Extracellular production of L-glutaminase by alkalophilic *Beauveria bassiana* BTMF S10 isolated from marine sediment. *World Journal of Microbiology and Biotechnology*, *15*(6), 751-752.

Khalil, M. S., Moubasher, M. H., Mokhtar, M., & Michel, M. M. (2020). Evaluation of antitumor activity of fungal L-glutaminase produced by egyptian isolates. *Letters in Applied NanoBioScience*, *9*, 924-930. https://doi.org/10.33263/LIANBS91.924930.

Kimura, R. E., LaPine, T. R., & Gooch, W. M. (1988). Portal venous and aortic glucose and lactate changes in a chronically catheterized rat. *Pediatric research*, *23*(2), 235-240.

Kiruthika, J., & Murugesan, S. (2020). Studies on optimization of L-glutaminase production under submerged fermentation from marine *Bacillus subtilis* JK-79. *African Journal of Microbiology Research, 14*(1), 16-24. https://doi.org/10.5897/AJMR2019.9107.

Kiruthika, J., & Saraswathy, N. (2013). Production of L-glutaminase and its optimization from a novel marine isolate *Vibrio azureus* JK-79. *African Journal of Biotechnology, 12*(50), 6944-6953.

Kiruthika, J., & Saraswathy, N. (2014). Isolation and characterization of a novel L-glutaminase producing marine *Bacillus subtilis* JK-79. *Asian Journal of Microbiology Biotechnology and Environmental Science, 16*(3), 601-610.

Kiruthika, J., Saraswathy, N., & Murugesan, S. (2018). Maximizing L-glutaminase production from marine *Bacillus subtilis* JK-79 under solid state fermentation. *African Journal of Biotechnology*, *17*(9), 288-305. https://doi.org/10.5897/AJB2017.16015.

Klimberg, V. S., & McClellan, J. L. (1996). Glutamine, cancer, and its therapy. *The American Journal of Surgery, 172*(5), 418-424. https://doi.org/10.1016/s0002-9610(96)00217-6.

Krieg, N. R., & Holt, J. G. (1984). Eds Bergey's Manual of Systematic Bacteriology Vol. 1. *Williams and Wilkins, Baltimore*.

Kujawa, J., Głodek, M., Li, G., Al-Gharabli, S., Knozowska, K., & Kujawski, W. (2021). Highly effective enzymes immobilization on ceramics: Requirements for supports and enzymes. *The Science of the Total Environment, 801*, 149647. https://doi.org/10.1016/j.scitotenv.2021.149647.

Kumar, A., & Singh, S. (2013). Directed evolution: tailoring biocatalysts for industrial applications. *Critical Reviews in Biotechnology*, *33*(4), 365-378.

Kumar, L., Singh, B., Adhikari, D. K., Mukherjee, J., & Ghosh, D. (2012). A temperature and salt-tolerant L-glutaminase from gangotri region of uttarakhand himalaya: enzyme purification and characterization. *Applied Biochemistry and Biotechnology*, *166*(7), 1723-1735.

Kumar, S. R., & Chandrasekaran, M. (2003). Continuous production of L-glutaminase by an immobilized marine Pseudomonas sp BTMS-51 in a packed bed reactor. *Process Biochemistry*, *38*(10), 1431-1436.

Kumar, S., Stecher, G., Li, M., Knyaz, C. (2018). Tamura, K. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. *Molecular Biology and Evolution*, *35*(6), 1547-1549. https://doi.org/10.1093/molbev/msy096.

Kumar, S.R.; and Chandrasekaran, M. (2003). Continuous production of L-glutaminase by an immobilized marine *Pseudomonas sp* BTMS-51 in a packed bed reactor. *Process Biochemistry*, *38*(10), 1431-1436. https://doi.org/10.1016/S0032-9592(03)00035-9

Kumar, V., Ahluwalia, V., Saran, S., Kumar, J., Patel, A. K., & Singhania, R. R. (2021). Recent developments on solid-state fermentation for production of microbial secondary metabolites: Challenges and solutions. *Bioresource Technology*, 323, 124566. https://doi.org/10.1016/j.biortech.2020.124566.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *nature*, *227*(5259), 680-685. https://doi.org/10.1038/227680a0.

Lagranha, C. J., Doi, S. Q., Pithon-Curi, T. C., Curi, R., & Sellitti, D. F. (2008). Glutamine enhances glucose-induced mesangial cell proliferation. *Amino acids*, *34*(4), 683-685.

Lane, D., & Levine, A. (2010). p53 Research: the past thirty years and the next thirty years. *Cold Spring Harbor Perspectives in Biology*, *2*(12), a000893.

Lauzier, B., Vaillant, F., Merlen, C., Gélinas, R., Bouchard, B., Rivard, M. E., ... & Chatham, J. C. (2013). Metabolic effects of glutamine on the heart: anaplerosis versus the hexosamine biosynthetic pathway. *Journal of Molecular and Cellular Cardiology*, *55*, 92-100. https://doi.org/10.1016/j.yjmcc.2012.11.008.

Le, B., & Yang, S. H. (2019). Microbial chitinases: properties, current state and biotechnological applications. *World Journal of Microbiology & Biotechnology, 35*(9), 144. https://doi.org/10.1007/s11274-019-2721-y.

Lebedeva, Z. I., Berezov, T. T., & Orekhovich, V. N. (1981). Glutamin (asparagin) ase from *Pseudomonas aurantiaca* BKMB-548. *Biokhimiia (Moscow, Russia)*, *46*(1), 85-91.

Lemieux, R. M., Popovici-Muller, J., Salituro, F. G., Saunders, J. O., Travins, J., & Yan, S. (2018). *U.S. Patent No. 10,011,574*. Washington, DC: U.S. Patent and Trademark Office.

Li, N., Zhang, Y., Ye, Q., Zhang, Y., Chen, Y., Chen, X., ... & Ying, H. (2012). Effect of ribose, xylose, aspartic acid, glutamine and nicotinic acid on ethyl (S)-4-chloro-3-hydroxybutanoate synthesis by recombinant *Escherichia coli*. *Bioresource Technology*, *118*, 572-575.

Li, X., Zhu, H., Sun, W., Yang, X., Nie, Q., & Fang, X. (2021). Role of glutamine and its metabolite ammonia in crosstalk of cancer-associated fibroblasts and cancer cells. *Cancer Cell International*, *21*(1), 1-13. https://doi.org/10.1186/s12935-021-02121-5.

Li, Y., Peer, J., Zhao, R., Xu, Y., Wu, B., Wang, Y., ... & Zheng, J. (2017). Serial deletion reveals structural basis and stability for the core enzyme activity of human glutaminase 1

isoforms: relevance to excitotoxic neurodegeneration. *Translational Neurodegeneration*, *6*(1), 1-10. https://doi.org/10.1186/s40035-017-0080-x.

Liang, Y., Li, M., Liu, Z., Li, Y., Wang, L., & Song, L. (2021). The glutaminase (CgGLS-1) mediates anti-bacterial immunity by prompting cytokine synthesis and hemocyte apoptosis in Pacific oyster Crassostrea gigas. Scientific reports, 11(1), 1-14.

Lister, A., Bourgeois, S., Imenez Silva, P. H., Rubio-Aliaga, I., Marbet, P., Walsh, J., Shelton, L. M., Keller, B., Verrey, F., Devuyst, O., Giesbertz, P., Daniel, H., Goldring, C. E., Copple, I. M., Wagner, C. A., & Odermatt, A. (2018). NRF2 regulates the glutamine transporter Slc38a3 (SNAT3) in kidney in response to metabolic acidosis. *Scientific Reports, 8*(1), 5629. https://doi.org/10.1038/s41598-018-24000-2.

Liu, J., Marchase, R. B., & Chatham, J. C. (2007). Glutamine-induced protection of isolated rat heart from ischemia/reperfusion injury is mediated via the hexosamine biosynthesis pathway and increased protein O-GlcNAc levels. *Journal of Molecular and Cellular Cardiology*, *42*(1), 177-185.

Liu, S., Cui, T., & Song, Y. (2020). Expression, homology modeling and enzymatic characterization of a new β-mannanase belonging to glycoside hydrolase family 1 from *Enterobacter aerogenes* B19. *Microbial Cell Factories*, *19*(1), 1-19. https://doi.org/10.1186/s12934-020-01399-w.

Lo, M., Wang, Y. Z., & Gout, P. W. (2008). The x cystine/glutamate antiporter: a potential target for therapy of cancer and other diseases. *Journal of Cellular Physiology*, *215*(3), 593-602.

L. liger, J. (2000). Function and importance of glutamate for savory foods. *The Journal of Nutrition*, 130(4), 915S-920S.

López-Garzón, C. S., & Straathof, A. J. (2014). Recovery of carboxylic acids produced by fermentation. *Biotechnology Advances*, *32*(5), 873-904.

Low, S. Y., Rennie, M. J., & Taylor, P. M. (1994). Sodium-dependent glutamate transport in cultured rat myotubes increases after glutamine deprivation. *The FASEB Journal*, *8*(1), 127-131.

Lu, X., Poon, T. C. W., & Zhang, H. (2020). Mass production of active recombinant *Chryseobacterium proteolyticum* protein glutaminase in *Escherichia coli* using a sequential dual expression system and one- step purification. *IUBMB life*, 72(11), 2391-2399.

M Orabi, H., El-Fakharany, E. M., Abdelkhalek, E. S., & Sidkey, N. M. (2021). L-asparaginase and L-glutaminase: sources, production, and applications in medicine and industry. *Journal of Microbiology, Biotechnology and Food Sciences*, 179-190.

MacFaddin, J. F. (2000). Biochemical tests for identification of medical bacteria.

Madden, S. K., de Araujo, A. D., Gerhardt, M., Fairlie, D. P., & Mason, J. M. (2021). Taking the Myc out of cancer: toward therapeutic strategies to directly inhibit c-Myc. *Molecular Cancer*, *20*(1), 1-18.https://doi.org/10.1186/s12943-020-01291-6.

Maharem, T. M., Emam, M. A., & Said, Y. A. (2020). Purification and characterization of l-glutaminase enzyme from camel liver: Enzymatic anticancer property. *International Journal of Biological Macromolecules, 150*, 1213–1222. https://doi.org/10.1016/j.ijbiomac.2019.10.131.

Mahmod, M. (2016). Immobilization of Bacillus subtilis glutaminase on different supportis. *Journal of Nutritional Health & Food Engineering. 5*(4):668-670. DOI: 10.15406/jnhfe.2016.05.00179.

Mao, Y., Xi, L., & LiYun, Z. (2013). Production of a novel salt-tolerant L-glutaminase from *Bacillus amyloliquefaciens* using agro-industrial residues and its application in Chinese soy sauce fermentation. *Biotechnology, 12*(1), 25-35. https://doi.org/10.3923/biotech.2013.25.35.

Masuo, N., Yoshimune, K., Ito, K., Matsushima, K., Koyama, Y., & Moriguchi, M. (2005). *Micrococcus luteus* K-3-type glutaminase from *Aspergillus oryzae* RIB40 is salt-tolerant. *Journal of Bioscience and Bioengineering*, *100*(5), 576-578.

Matés, J. M., Di Paola, F. J., Campos-Sandoval, J. A., Mazurek, S., & Márquez, J. (2020). Therapeutic targeting of glutaminolysis as an essential strategy to combat cancer. *Seminars in Cell & Developmental Biology, 98*, 34–43. https://doi.org/10.1016/j.semcdb.2019.05.012.

Mateus, I., Feijó, M., Espínola, L. M., Vaz, C. V., Correia, S., & Socorro, S. (2018). Glucose and glutamine handling in the Sertoli cells of transgenic rats overexpressing regucalcin: plasticity towards lactate production. *Scientific Reports*, *8*(1), 1-13.

Mazat, J. P., & Ransac, S. (2019). The Fate of Glutamine in Human Metabolism. The Interplay with Glucose in Proliferating Cells. *Metabolites, 9*(5), 81. https://doi.org/10.3390/metabo9050081

McNerney, M. E., Brown, C. D., Wang, X., Bartom, E. T., Karmakar, S., Bandlamudi, C., ... & Anastasi, J. (2013). CUX1 is a haploinsufficient tumor suppressor gene on chromosome 7 frequently inactivated in acute myeloid leukemia. *Blood, The Journal of the American Society of Hematology*, *121*(6), 975-983.

Meena, B., Anburajan, L., Sathish, T., Raghavan, R. V., Dharani, G., Vinithkumar, N. V., & Kirubagaran, R. (2015). L-Asparaginase from *Streptomyces griseus* NIOT-VKMA29: optimization of process variables using factorial designs and molecular characterization of L-asparaginase gene. *Scientific Reports*, *5*, 12404.

Mehrotra, P. J. research, c., 2016. *Biosensors and their Applications–A review, 6*(2), 153-159.https://doi.org/10.1016/j.jobcr.2015.12.002.

Meynial-Denis, D. (2016). Glutamine metabolism in advanced age. *Nutrition Reviews, 74*(4), 225-236. https://doi.org/10.1093/nutrit/nuv052.

Michaelidis, T. M., Tzimagiorgis, G., Moschonas, N. K., & Papamatheakis, J. (1993). The human glutamate dehydrogenase gene family: gene organization and structural characterization. *Genomics*, *16*(1), 150-160.

Miura, S., Arimura, T., Itoda, N., Dwiarti, L., Feng, J. I. N., Bin, C. U. I. H., & Okabe, M. (2004). Production of L -Lactic Acid from Corncob. *Journal of Bioscience and Bioengineering, 97*(3), 153-157.

Monier, J. M., Demanèche, S., Delmont, T. O., Mathieu, A., Vogel, T. M., & Simonet, P. (2011). Metagenomic exploration of antibiotic resistance in soil. *Current Opinion in Microbiology*, *14*(3), 229-235.

More, S. S., Swamy, R., Mohan, N., Navyashree, M., Janardhan, B., & Niyonzima, F. N. (2018). Purification and characterization of anti-cancer L-glutaminase of *Bacillus cereus* strain LC13. *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences, 88*(2), 695-705.

Moriguchi, M., Sakai, K., Tateyama, R., Furuta, Y., & Wakayama, M. (1994). Isolation and characterization of salt-tolerant glutaminases from marine *Micrococcus luteus* K-3. *Journal of Fermentation and Bioengineering*, *77*(6), 621-625.

Mosallatpour, S., Aminzadeh, S., Shamsara, M., & Hajihosseini, R. (2019). Novel halo-and thermo-tolerant *Cohnella* sp. A01 L-glutaminase: heterologous expression and biochemical characterization. *Scientific Reports, 9*(1), 1-14. https://doi.org/10.1038/s41598-019-55587-9.

Mostafa, Y. S., Alamri, S. A., Alfaifi, M. Y., Alrumman, S. A., Elbehairi, S. E. I., Taha, T. H., & Hashem, M. (2021). L-Glutaminase Synthesis by Marine *Halomonas meridiana* Isolated from the Red Sea and Its Efficiency against Colorectal Cancer Cell Lines. *Molecules*, *26*(7), 1963. https://doi.org/10.3390/molecules26071963.

Mu, W., Zhang, T., & Jiang, B. (2015). An overview of biological production of L-theanine. *Biotechnology Advances*, *33*(3-4), 335–342.

Muthuvelayudham, R., & Viruthagiri, T. (2013). Medium optimization for production of L-Glutaminase (EC 3.5. 1.2) by Streptomyces griseus under submerged fermentation. *International Journal of Science and Engineering Applications*, *2*(1), 1-5.

Muthuvelayudham, R., & Viruthagiri, T. (2013). Medium optimization for production of L-Glutaminase (EC 3.5. 1.2) by Streptomyces griseus under submerged fermentation. *International Journal of Science and Engineering Applications, 2*(1), 1-5.

Nagaraju, K., & Ram, M. R. (2018). Studies on optimization of L-glutaminase from *Bacillus endophyticus* RN-7. *International Journal of Pharmacy and Biological Sciences, 8*(3), 1146-1153.

Nagaraju, K., & Ram, M. R. (2019). *Bacillus cereus* Rn-6, Potential L-Glutaminase Producing Bacteria from Forest Soils. *Life Science Informatics Publications, 5*(4), 134-142.

Nagendra Prabhu, G. (1997). L glutaminase production by marine *Vibrio costicola* under solid state fermentation.

Nakadai, T., & Nasuno, S. (1989). Use of glutaminase for soy sauce made by Koji or a preparation of proteases from *Aspergillus oryzae*. *Journal of Fermentation and Bioengineering*, *67*(3), 158-162.

Nandakumar, M. P., Thakur, M. S., Raghavarao, K. S. M. S., & Ghildyal, N. P. (1999). Studies on catabolite repression in solid state fermentation for biosynthesis of fungal amylases. *Letters in Applied Microbiology*, *29*(6), 380-384.

Nandakumar, R., Yoshimune, K., Wakayama, M., & Moriguchi, M. (2003). Microbial glutaminase: biochemistry, molecular approaches and applications in the food industry. *Journal of Molecular Catalysis B: Enzymatic, 23*(2-6), 87–100.doi:10.1016/s1381-1177(03)00075-4

Nathiya, K., Nath, S. S., Angayarkanni, J., & Palaniswamy, M. (2011). Optimised production of L-glutaminase: A tumour inhibitor from *Aspergillus flavus* cultured on agroindustrial residues. *African Journal of Biotechnology*, *10*(63), 13887-13894. https://doi.org/10.5897/ajb11.1251.

Neermann, J., & Wagner, R. (1996). Comparative analysis of glucose and glutamine metabolism in transformed mammalian cell lines, insect and primary liver cells. *Journal of Cellular Physiology*, *166*(1), 152-169.

Negi, S., & Banerjee, R. (2010). Optimization of culture parameters to enhance production of amylase and protease from *Aspergillus awamori* in a single fermentation. *African Journal of Biochemistry Research*, *4*(3), 73-80.

Newsholme, E. A., Crabtree, B., & Ardawi, M. S. M. (1985). The role of high rates of glycolysis and glutamine utilization in rapidly dividing cells. *Bioscience Reports*, *5*(5), 393-400.

Newsholme, P. (2001). Why is L-glutamine metabolism important to cells of the immune system in health, postinjury, surgery or infection?. *The Journal of nutrition*, *131*(9), 2515S-2522S.

Newsholme, P., Lima, M. M. R., Procopio, J., Pithon-Curi, T. C., Bazotte, R. B., & Curi, R. (2003). Glutamine and glutamate as vital metabolites. *Brazilian Journal of Medical and Biological Research, 36*(2), 153-163.https://doi.org/10.1590/s0100-879x2003000200002.

Newsholme, P., Procopio, J., Lima, M. M., Pithon-Curi, T. C., & Curi, R. (2003). Glutamine and glutamate--their central role in cell metabolism and function. *Cell Biochemistry and Function, 21*(1), 1–9. https://doi.org/10.1002/cbf.1003.

Nieminen, A. I., Eskelinen, V. M., Haikala, H. M., Tervonen, T. A., Yan, Y., Partanen, J. I., & Klefström, J. (2013). Myc-induced AMPK-phospho p53 pathway activates Bak to sensitize mitochondrial apoptosis. *Proceedings of the National Academy of Sciences*, *110*(20), E1839-E1848.

Nikulin, M., & Švedas, V. (2021). Prospects of Using Biocatalysis for the Synthesis and Modification of Polymers. *Molecules*, *26*(9), 2750. https://doi.org/10.3390/molecules26092750

Oehler, R., Pusch, E., Dungel, P., Zellner, M., Eliasen, M. M., Brabec, M., & Roth, E. (2002). Glutamine depletion impairs cellular stress response in human leucocytes. *British Journal of Nutrition, 87*(S1), S17-S21.

Olarewaju, M. O., & Nzelibe, H. C. (2019). Statistical optimization of Lâ€“glutaminase production by *Trichoderma* species under solid state fermentation using African locust beans as substrate. *African Journal of Biochemistry Research, 13*(6), 73-81.

Olenchock, B. A., Moslehi, J., Baik, A. H., Davidson, S. M., Williams, J., Gibson, W. J., ... & Kelekar, A. (2016). EGLN1 inhibition and rerouting of α-ketoglutarate suffice for remote ischemic protection. *Cell*, *164*(5), 884-895.

Orabi, H., El-Fakharany, E., Abdelkhalek, E., & Sidkey, N. (2020). Production, optimization, purification, characterization, and anti-cancer application of extracellular L-glutaminase produced from the marine bacterial isolate. *Preparative Biochemistry & Biotechnology, 50*(4), 408–418. https://doi.org/10.1080/10826068.2019.1703193.

Padma, I., & Singhal, R. (2010). Isolation, screening, and selection of an L-glutaminase producer from soil and media optimization using a statistical approach. *Biotechnology and Bioprocess Engineering, 15*(6), 975-983. https://doi.org/10.1007/s12257-009-0187-8.

Pandey, A., Soccol, C. R., & Larroche, C. (Eds.). (2008). *Current developments in solid-state fermentation*. Springer Science & Business Media.

Pandian, S. R. K., Deepak, V., Sivasubramaniam, S. D., Nellaiah, H., & Sundar, K. (2014). Optimization and purification of anticancer enzyme L-glutaminase from *Alcaligenes faecalis* KLU102. *Biologia, 69*(12), 1644-1651. https://doi.org/10.2478/s11756-014-0486-1

Papandreou, I., Cairns, R. A., Fontana, L., Lim, A. L., & Denko, N. C. (2006). HIF-1 mediates adaptation to hypoxia by actively downregulating mitochondrial oxygen consumption. *Cell Metabolism*, *3*(3), 187-197.

Paquette, J. C., Guérin, P. J., & Gauthier, E. R. (2005). Rapid induction of the intrinsic apoptotic pathway by l- glutamine starvation. *Journal of cellular physiology, 202*(3), 912-921.

Parker, J. H., Smith, G. A., Fredrickson, H. L., Vestal, J. R., & White, D. C. (1982). Sensitive assay, based on hydroxy fatty acids from lipopolysaccharide lipid A, for Gram-negative bacteria in sediments. *Applied and Environmental Microbiology*, *44*(5), 1170-1177.

Partanen, K., Jalava, T., & Valaja, J. (2007). Effects of a dietary organic acid mixture and of dietary fibre levels on ileal and faecal nutrient apparent digestibility, bacterial nitrogen flow, microbial metabolite concentrations and rate of passage in the digestive tract of pigs. *Animal*, *1*(3), 389-401.

Patel, N. Y., Baria, D. M., Yagnik, S. M., Rajput, K. N., Panchal, R. R., & Raval, V. H. (2021). Bio-prospecting the future in perspective of amidohydrolase L-glutaminase from marine habitats. *Applied Microbiology and Biotechnology*, 1-16. https://doi-org.libproxy.viko.lt/10.1007/s00253-021-11416-6.

Patra, J. K., Das, G., Fraceto, L. F., Campos, E., Rodriguez-Torres, M., Acosta-Torres, L. S., Diaz-Torres, L. A., Grillo, R., Swamy, M. K., Sharma, S., Habtemariam, S., & Shin, H. S. (2018). Nano based drug delivery systems: recent developments and future prospects. *Journal of Nanobiotechnology, 16*(1), 71. https://doi.org/10.1186/s12951-018-0392-8

Pavezzi, F. C., Gomes, E., & da Silva, R. (2008). Production and characterization of glucoamylase from fungus *Aspergillus awamori* expressed in yeast *Saccharomyces cerevisiae* using different carbon sources. *Brazilian journal of microbiology, 39*(1), 108–114. https://doi.org/10.1590/S1517-838220080001000024.

Penninckx, M. J., & Jaspers, C. J. (1985). Caractérisation d'une activité γ-glutamylarylamidase-glutaminase chez *Saccharomyces cerevisiae*. *Biochimie, 67*(9), 999-1006. https://doi.org/10.1016/s0300-9084(85)80294-7.

Petersen, S. B. (2005). Protein engineering: Design and engineering on the nano scale. *Applied Biocatalysis*, *second ed. Amsterdam: Harwood Academic Publications*, 263-310.

Pike, L. S., Smift, A. L., Croteau, N. J., Ferrick, D. A., & Wu, M. (2011). Inhibition of fatty acid oxidation by etomoxir impairs NADPH production and increases reactive oxygen species resulting in ATP depletion and cell death in human glioblastoma cells. *Biochimica et Biophysica Acta (BBA)-Bioenergetics*, *1807*(6), 726-734.

Powlson, D. S., & Jenkinson, D. S. (1976). The effects of biocidal treatments on metabolism in soil—II. Gamma irradiation, autoclaving, air-drying and fumigation. *Soil Biology and Biochemistry*, *8*(3), 179-188.

Powlson, D. S., Hirsch, P. R., & Brookes, P. C. (2001). The role of soil microorganisms in soil organic matter conservation in the tropics. *Nutrient Cycling in Agroecosystems*, *61*(1-2), 41-51.

Prabhu, G. & Chandrasekaran M. (1999). Purification and characterization of an anti-cancer enzyme produced by marine *Vibrio Costicola* under a novel solid state fermentation process. *Arquivos de Biologia e Technologia. 42.* 363-367. 10.1590/S1516-89131999000300015.

Prabhu, G. N., & Chandrasekaran, M. (1995). Polystyrene—an inert carrier for Lxxx-glutaminase production by marine *Vibrio costicola* under solid-state fermentation. *World Journal of Microbiology and Biotechnology, 11*(6), 683-684. https://doi.org/10.1007/BF00361017.

Prabhu, G. N., & Chandrasekaran, M. (1999). Purification and characterization of an anti-cancer enzyme produced by marine *Vibrio Costicola* under a novel solid state fermentation process. *Brazilian Archives of Biology and Technology, 42*, 363-368.

Prakash, P. J., Poorani, E., & Anantharaman, P. (2010). Effect of media composition on L-glutaminase production from lagoon Vibrio sp. SFL-2. *International Journal of Biotechnology & Biochemistry*, *6*(5), 769-783.

Priyanka, S., Jayashree, M., Shivani, R., Anwesha, S., & Rao, K. B. (2019). Characterisation and identification of antibacterial compound from marine *actinobacteria*: In vitro and in silico analysis. *Journal of Infection and Public Health*, *12*(1), 83-89. https://doi.org/10.1016/j.jiph.2018.09.005.

Priyodip, P., Prakash, P. Y., & Balaji, S. (2017). Phytases of Probiotic Bacteria: Characteristics and Beneficial Aspects. *Indian Journal of Microbiology, 57*(2), 148–154. https://doi.org/10.1007/s12088-017-0647-3.

Prusiner, S. T. A. N. L. E. Y., & Stadtman, E. R. (1976). Regulation of glutaminase B in Escherichia coli. III. Control by nucleotides and divalent cations. *Journal of Biological Chemistry*, *251*(11), 3463-3469.

Qing, G., Li, B., Vu, A., Skuli, N., Walton, Z. E., Liu, X., ... & Simon, M. C. (2012). ATF4 regulates MYC-mediated neuroblastoma cell death upon glutamine deprivation. *Cancer Cell, 22*(5), 631-644.

Raizel, R., & Tirapegui, J. (2018). Role of glutamine, as free or dipeptide form, on muscle recovery from resistance training: A review study. *Nutrire, 43*(1), 1-10. https://doi.org/10.1186/s41110-018-0087-9.

Rajendra, D., Anubrata, P., & Neha, P., (2015). From, L. G. Isolation, Production & Screening Of Anti-Cancer Enzyme. *Research article Biological Sciences,* 5, 96-105.

Rao, R., & Samak, G. (2012). Role of glutamine in protection of intestinal epithelial tight junctions. *Journal of epithelial biology & pharmacology, 5*(Suppl 1-M7), 47. doi:10.2174/1875044301205010047.

Rashmi, A. M., Gopinath, S. M., Kumar, K., & Murthy, N. T. P. (2012). Optimization of submerged fermentation process for L-Glutaminase produced by *Pseudomonas aeruginosa* BGNAS-5. *International journal of latest research in science and technology, 1*(4), 308-310.

Raveendran, S., Parameswaran, B., Ummalyma, S. B., Abraham, A., Mathew, A. K., Madhavan, A., Rebello, S., & Pandey, A. (2018). Applications of Microbial Enzymes in Food Industry. *Food technology and biotechnology*, *56*(1), 16–30. https://doi.org/10.17113/ftb.56.01.18.5491

Reda F. M. (2015). Kinetic properties of Streptomyces canarius L- Glutaminase and its anticancer efficiency. *Brazilian journal of microbiology: publication of the Brazilian Society for Microbiology*, *46*(4), 957–968. https://doi.org/10.1590/S1517-838246420130847

Roberts, J., & McGregor, W. G. (1991). Inhibition of mouse retroviral disease by bioactive glutaminase-asparaginase. *Journal of General Virology, 72*(2), 299-305.

Roberts, J., Holcenberg, J. S., & Dolowy, W. C. (1970). Antineoplastic activity of highly purified bacterial glutaminases. *Nature*, *227*(5263), 1136-1137.

Roberts, J., Holcenberg, J. S., & Dolowy, W. C. (1972). Isolation, crystallization, and properties of *Achromobacteraceae* glutaminase-asparaginase with antitumor activity. *Journal of Biological chemistry*, *247*(1), 84-90.

Roberts, J., MacAllister, T. W., Sethuraman, N., & Freeman, A. G. (2001). *U.S. Patent No. 6,312,939*. Washington, DC: U.S. Patent and Trademark Office.

Robinson, T., Singh, D., & Nigam, P. (2001). Solid-state fermentation: a promising microbial technology for secondary metabolite production. *Applied Microbiology and Biotechnology*, *55*(3), 284-289.

Rogeri, P. S., Gasparini, S. O., Martins, G. L., Costa, L. K. F., Araujo, C. C., Lugaresi, R., ... & Lancha Jr, A. H. (2020). Crosstalk between skeletal muscle and immune system: which roles do IL-6 and glutamine play?. *Frontiers in Physiology*, 11, 1286. https://doi.org/10.3389/fphys.2020.582258.

Rubi, B., Ishihara, H., Hegardt, F. G., Wollheim, C. B., & Maechler, P. (2001). GAD65-mediated glutamate decarboxylation reduces glucose-stimulated insulin secretion in pancreatic beta cells. *Journal of Biological Chemistry*, *276*(39), 36391-36396.

Sabu, A. (2003). Sources, properties and applications of microbial therapeutic enzymes.

Sabu, A. (2003). Sources, Properties and Applications of Microbial Therapeutic Enzymes. *Indian Journal of Biotechnology. 2.* 334-341.

Sabu, A., Chandrasekaran, M., & Pandey, A. (2000). Biopotential of microbial glutaminases. *Chimica oggi*, *18*(11-12), 21-25.

Sabu, A., Keerthi, T. R., Kumar, S. R., & Chandrasekaran, M. (2000). L-Glutaminase production by marine Beauveria sp. under solid state fermentation. *Process Biochemistry*, *35*(7), 705-710.

Sabu.A, (2003). Sources, properties and applications of microbial therapeutic enzymes. *Indian Journal of Biotechnology. 2*:334-341.

Sajitha, N., Vasuki, S., & Suja, M. (2014). Antibacterial and antioxidant activities of L-glutaminase from seaweed endophytic fungi *Penicillium citrinum*. *World Journal of Pharmaceutical Sciences, 3*(4), 682-695.

Sánchez-Rodríguez, C., Bauer, S., Hématy, K., Saxe, F., Ibáñez, A. B., Vodermaier, V., ... & Neumetzler, L. (2012). Chitinase-like1/pom-pom1 and its homolog CTL2 are glucan-interacting proteins important for cellulose biosynthesis in Arabidopsis. *The Plant Cell*, *24*(2), 589-607.

Sarada, K.V., (2013). Production and Applications Of L-Glutaminase Using Fermentation Technology. *Asia-Pacific Journal of Research. 1*, 1–4

Sathish, T., and Prakasham, R.S. (2010). Enrichment of glutaminase production by *Bacillus subtilis* RSP-GLU in submerged cultivation based on neural network - Genetic algorithm approach. *Journal of Chemical Technology and Biotechnology. 85*. 50-58. 10.1002/jctb.2267.

Sathish, T., Kezia, D., Bramhachari, P. V., & Prakasham, R. S. (2018). Multi-objective based superimposed optimization method for enhancement of L-glutaminase production by *Bacillus subtilis* RSP-GLU. *Karbala International Journal of Modern Science*, *4*(1), 50-60.

Sattar, H., Aman, A., & Qader, S. (2018). Agar-agar immobilization: An alternative approach for the entrapment of protease to improve the catalytic efficiency, thermal stability and recycling efficiency. *International journal of biological macromolecules*, *111*, 917–922. https://doi.org/10.1016/j.ijbiomac.2018.01.105

Sato, I., Kobayashi, H., Hanya, Y., Abe, K., Murakami, S., Scorzetti, G., & Fell, J. W. (1999). *Cryptococcus nodaensis* sp nov, a yeast isolated from soil in Japan that produces a salt-tolerant and thermostable glutaminase. *Journal of Industrial Microbiology and Biotechnology*, *22*(3), 127-132.

Schoolwerth, A. C. (1991). Regulation of renal ammoniagenesis in metabolic acidosis. *Kidney International*, *40*(5), 961-973.

Schousboe, A., Scafidi, S., Bak, L. K., Waagepetersen, H. S., & McKenna, M. C. (2014). Glutamate metabolism in the brain focusing on astrocytes. *Advances in Neurobiology, 11*, 13–30. https://doi.org/10.1007/978-3-319-08894-5_2.

Sefcick, A., Anderton, D., Byrne, J. L., Teahon, K., & Russell, N. H. (2001). Naso-jejunal feeding in allogeneic bone marrow transplant recipients: results of a pilot study. *Bone Marrow Transplantation*, *28*(12), 1135-1139.

Selvakumar, P., Ashakumary, L., & Pandey, A. (1998). Biosynthesis of glucoamylase from *Aspergillus niger* by solid-state fermentation using tea waste as the basis of a solid substrate. *Bioresource Technology*, *65*(1-2), 83-85.

Seth, M., & Chand, S. (2000). Biosynthesis of tannase and hydrolysis of tannins to gallic acid by *Aspergillus awamori*—optimisation of process parameters. *Process Biochemistry*, *36*(1-2), 39-44.

Shah, A. M., Wang, Z., & Ma, J. (2020). Glutamine metabolism and its role in immunity, a comprehensive review. *Animals*, *10*(2), 326. https://doi.org/10.3390/ani10020326.

Shah, L., Nadeem, M. S., Khan, J. A., Zeyadi, M. A., Zamzami, M. A., & Mohammed, K. (2019). Recombinant l-glutaminase obtained from *Geobacillus thermodenitrificans* DSM-465: characterization and in silico elucidation of conserved structural domains. *RSC Advances, 9*(8), 4258-4267.

Shah, R., & Chen, S. (2020). Metabolic Signaling Cascades Prompted by Glutaminolysis in Cancer. *Cancers, 12*(9), 2624. https://doi.org/10.3390/cancers12092624.

Shariatpanahi, Z. V., Eslamian, G., Ardehali, S. H., & Baghestani, A. R. (2019). Effects of Early Enteral Glutamine Supplementation on Intestinal Permeability in Critically Ill Patients. Indian journal of critical care medicine : peer-reviewed, official publication of *Indian Society of Critical Care Medicine, 23*(8), 356–362. https://doi.org/10.5005/jp-journals-10071-23218.

Sherr, C. J. (2000). The Pezcoller lecture: cancer cell cycles revisited. *Cancer Research*, *60*(14), 3689-3695.

Shi, D., Caldovic, L., & Tuchman, M. (2018). Sources and Fates of Carbamyl Phosphate: A Labile Energy-Rich Molecule with Multiple Facets. *Biology*, 7(2), 34. https://doi.org/10.3390/biology7020034.

Shindia AA, Kkalaf SA and El-Sayed ASA (2007) L-glutaminase production by some filamentous fungi. *Bull Faculty of Science Zagazig Univ Egypt* 29:99–111.

Shiraishi, K., & Yokoyama, M. (2019). Toxicity and immunogenicity concerns related to PEGylated-micelle carrier systems: a review. *Science and Technology of Advanced Materials, 20*(1), 324–336. https://doi.org/10.1080/14686996.2019.1590126.

Shuler, M. L. (1992). Bioprocess enzineering. Prentice-Hall, 412-420.

Sibson, N. R., Mason, G. F., Shen, J., Cline, G. W., Herskovits, A. Z., Wall, J. E., ... & Shulman, R. G. (2001). In vivo13C NMR measurement of neurotransmitter glutamate cycling, anaplerosis and TCA cycle flux in rat brain during [2- 13C] glucose infusion. *Journal of neurochemistry*, *76*(4), 975-989.

Silva, E. M., & Yang, S. T. (1998). Production of Amylases from Rice by Solid- State Fermentation in a Gas− Solid Spouted- Bed Bioreactor. *Biotechnology Progress, 14*(4), 580-587.

Simon, C., & Daniel, R. (2011). Metagenomic analyses: past and future trends. *Applied and environmental microbiology*, *77*(4), 1153-1161.

Singh, P., & Banik, R. M. (2013). Biochemical characterization and antitumor study of L-glutaminase from *Bacillus cereus* MTCC 1305. *Applied Biochemistry and Biotechnology, 171*(2), 522–531. https://doi.org/10.1007/s12010-013-0371-3.

Sinha, S., & Nigam, V. K. (2016). Production and Characterization of L-Glutaminase By *Bacillus Sp*. *International Journal of Pharmaceutical Sciences and Research*, *7*(4), 1620-1626.

Sivakumar, K., Sahu, M. K., Manivel, P. R., & Kannan, L. (2006). Optimum conditions for L-glutaminase production by actinomycete strain isolated from estuarine fish, Chanos chanos (Forskal, 1775). *Indian Journal of Experimental Biology, 44*(3), 256–258.

Skobe, M., Rockwell, P., Goldstein, N., Vosseler, S., & Fusenig, N. E. (1997). Halting angiogenesis suppresses carcinoma cell invasion. *Nature medicine*, *3*(11), 1222-1227.

Solovyeva, I. V., Ananjin, V. M., Boev, A. V., & Okunev, O. N. (1997). The controlled biosynthesis of cellobiase by *Aspergillus* fungi. *Process Biochemistry*, *32*(1), 21-28.

Son, J., Lyssiotis, C. A., Ying, H., Wang, X., Hua, S., Ligorio, M., ... & Kang, Y. A. (2013). Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature*, *496*(7443), 101-105.

Son, J., Lyssiotis, C. A., Ying, H., Wang, X., Hua, S., Ligorio, M., ... & Kimmelman, A. C. (2013). Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature*, 496(7443), 101-105.

Sookoian, S., & Pirola, C. J. (2012). Alanine and aspartate aminotransferase and glutamine-cycling pathway: their roles in pathogenesis of metabolic syndrome. *World Journal of Gastroenterology: WJG*, *18*(29), 3775.

Soren, J. P., Paul, T., Banerjee, A., Mondal, K. C., & Mohapatra, P. K. D. (2020). Exploitation of agricultural waste as sole substrate for production of bacterial L-glutaminase under submerged fermentation and the proficient application of fermented hydrolysate as growth promoting agent for probiotic Organisms. *Waste and Biomass Valorization*, *11*(8), 4245-4257. https://doi.org/10.1007/s12649-019-00761-3.

Souza, P. M. D., & Magalhães, P. D. O. (2010). Application of microbial α-amylase in industry-A review. *Brazilian Journal of Microbiology, 41*, 850-861. https://doi.org/10.1590/S1517-83822010000400004.

Spiers, A. S., & Wade, H. E. (1976). Bacterial glutaminase in treatment of acute leukaemia. *British medical journal, 1*(6021), 1317–1319. https://doi.org/10.1136/bmj.1.6021.1317.

Spiridonov, N. A., & Wilson, D. B. (1998). Regulation of biosynthesis of individual cellulases in *Thermomonospora fusca*. *Journal of Bacteriology*, *180*(14), 3529-3532.

Springer, Dordrecht. Miura, S., Arimura, T., Itoda, N., Dwiarti, L., Feng, J. B., Bin, C. H., & Okabe, M. (2004). Production of L-lactic acid from corncob. *Journal of Bioscience and Bioengineering*, 97(3), 153-157.

Stumvoll, M., Perriello, G., Meyer, C., & Gerich, J. (1999). Role of glutamine in human carbohydrate metabolism in kidney and other tissues. *Kidney International*, *55*(3), 778-792.

Sugiyama, S. I. (1984). Selection of micro-organisms for use in the fermentation of soy sauce. *Food Microbiology*, *1*(4), 339-347.

Suzuki, S., Tanaka, T., Poyurovsky, M. V., Nagano, H., Mayama, T., Ohkubo, S., ... & Sugano, S. (2010). Phosphate-activated glutaminase (GLS2), a p53-inducible regulator of glutamine metabolism and reactive oxygen species. *Proceedings of the National Academy of Sciences*, *107*(16), 7461-7466.

Tachiki, T., Yamada, T., Mizuno, K., Ueda, M., Shiode, J., & Fukami, H. (1998). γ-Glutamyl Transfer Reactions by Glutaminase from *Pseudomonas nitroreducens* IFO 12694 and Their Application for the Syntheses of Theanine and γ-Glutamylmethylamide. *Bioscience, Biotechnology, and Biochemistry, 62*(7), 1279–1283. https://doi.org/10.1271/bbb.62.1279.

Tambasco-Studart, M., Tews, I., Amrhein, N., & Fitzpatrick, T. B. (2007). Functional analysis of PDX2 from Arabidopsis, a glutaminase involved in vitamin B6 biosynthesis. *Plant Physiology*, *144*(2), 915-925.

Tamura, K., & Nei, M. (1993). Estimation of the number of nucleotide substitutions in the control region of mitochondrial DNA in humans and chimpanzees. *Molecular Biology and Evolution, 10*(3), 512-526.

Tamura, K., Nei, M., & Kumar, S. (2004). Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences*, *101*(30), 11030-11035.

Tanaka, S., Robinson, E. A., Appella, E., Miller, M., Ammon, H. L., Roberts, J., ... & Wlodawer, A. (1988). Structures of amidohydrolases. Amino acid sequence of a glutaminase-asparaginase from *Acinetobacter glutaminasificans* and preliminary crystallographic data for an asparaginase from *Erwinia chrysanthemi*. *Journal of Biological Chemistry, 263*(18), 8583-8591.

Tannen, R. L., & Sahai, A. (1990). Biochemical pathways and modulators of renal ammoniagenesis. *Mineral and electrolyte metabolism*, *16*(5), 249-258.

Teja, D. D., Devi, V. S., Harsha, N., Vishala, S. S., & Lakshmi, P. S. (2014). Production of L-glutaminase from marine ecosystems and optimal conditions for maximal production by actinomycetes. *International Journal of Advanced Research*, *2*(1), 485-491.

Thammarongtham, C., Turner, G., Moir, A. J., Tanticharoen, M., & Cheevadhanarak, S. (2001). A new class of glutaminase from *Aspergillus oryzae*. *Journal of molecular microbiology and biotechnology*, *3*(4), 611-618.

Tullimilli, A., Sankar, J., Bondili, P. B., & Ramachandra, R. (2014). Studies on isolation, screening and molecular characterization of L-glutaminase producing marine isolates. *IJSID, 4* (1): 33-44.

Unissa, R., Sudhakar, M., Reddy, A. S. K., & Sravanthi, K. N. (2014). A review on biochemical and therapeutic aspects of glutaminase. *International Journal of Pharmaceutical Sciences and Research*, 5(11), 4617-4634.

Van Oort, M. (2010). Enzymes in bread making. *Enzymes in food technology*, 103.

Viniegra-González, G., Favela-Torres, E., Aguilar, C. N., de Jesus Rómero-Gomez, S., Dıaz-Godınez, G., & Augur, C. (2003). Advantages of fungal enzyme production in solid state over liquid fermentation systems. *Biochemical Engineering Journal, 13*(2-3), 157-167. 10.1016/S1369-703X(02)00128-6.

Vita, M., & Henriksson, M. (2006). The Myc oncoprotein as a therapeutic target for human cancer. *Seminars in Cancer Biology*, *16*(4), 318–330. https://doi.org/10.1016/j.semcancer.2006.07.015.

Vo, T. D., Sulaiman, C., Tafazoli, S., Lynch, B., Roberts, A., & Chikamatsu, G. (2020). Safety assessment of glutaminase from *Aspergillus niger*. Food science & nutrition, 8(3), 1433-1450. https://doi.org/10.1002/fsn3.1426

Wade H.E. 1980. Synthesis and functions of microbial asparaginases and glutaminases, pp. 563–575. In: Payne J.W. (ed.), Microorganisms and Nitrogen Sources, John Wiley and Sons, New York.

Wade, H. E., & Payne, J. W. (1980). Synthesis and functions of microbial asparaginases and glutaminases. *Microorganisms and nitrogen sources: Transport and utilization of amino acids, peptides, proteins and related substances. Wiley Interscience, Chichester*, 563-575.

Wakayama, M., Yamagata, T., Kamemura, A., Bootim, N., Yano, S., Tachiki, T., Yoshimune, K., & Moriguchi, M. (2005). Characterization of salt-tolerant glutaminase from *Stenotrophomonas maltophilia* NYW-81 and its application in Japanese soy sauce fermentation. *Journal of Industrial Microbiology & Biotechnology, 32*(9), 383–390. https://doi.org/10.1007/s10295-005-0257-7.

Wang, L., & Yang, S. T. (2007). Solid state fermentation and its applications. *Bioprocessing for value-added products from renewable resources*, 465-489.

Wang, L., Zhang, S., & Wang, X. (2021). The metabolic mechanisms of breast cancer metastasis. *Frontiers in Oncology*, *10*, 2942. https://doi.org/10.3389/fonc.2020.602416.

Wang, Y. T., Yu, L., Zhu, Z. Q., Zhang, J., Zhu, J. Z., & Fan, C. H. (2009). Improved enzyme immobilization for enhanced bioelectrocatalytic activity of glucose sensor. *Sensors and Actuators B: Chemical*, *136*(2), 332-337.

Wang, Y., Bai, C., Ruan, Y., Liu, M., Chu, Q., Qiu, L., Yang, C., & Li, B. (2019). Coordinative metabolism of glutamine carbon and nitrogen in proliferating cancer cells under hypoxia. *Nature Communications, 10*(1), 201. https://doi.org/10.1038/s41467-018-08033-9.

Wang, Z., Liu, F., Fan, N., Zhou, C., Li, D., Macvicar, T., ... & Zhao, Y. (2020). Targeting Glutaminolysis: New Perspectives to Understand Cancer Development and Novel Strategies for Potential Target Therapies. *Frontiers in Oncology*, *10*, 2321. https://doi.org/10.3389/fonc.2020.589508

Warburg, O., Wind, F., & Negelein, E. (1927). The metabolism of tumors in the body. Journal of General Physiology, 8(6), 519-530.

Watford, M. (2015). Glutamine and glutamate: Nonessential or essential amino acids?. *Animal Nutrition, 1*(3), 119-122. https://doi.org/10.1016/j.aninu.2015.08.008.

Westermark, U. K., Wilhelm, M., Frenzel, A., & Henriksson, M. A. (2011, October). The MYCN oncogene and differentiation in neuroblastoma. In *Seminars in Cancer Biology* (Vol. 21, No. 4, pp. 256-266). Academic Press.

Wilhelm, B., Meinhardt, A., & Seitz, J. (1996). Transglutaminases: purification and actuivity assays. *Journal of Chromatography B: Biomedical Sciences and Applications*, *684*(1-2), 163-177.

Windmueller, H. G., & Spaeth, A. E. (1978). Identification of ketone bodies and glutamine as the major respiratory fuels in vivo for postabsorptive rat small intestine. *Journal of Biological Chemistry*, *253*(1), 69-76.

Winnock, F., Ling, Z., De Proft, R., Dejonghe, S., Schuit, F., Gorus, F., & Pipeleers, D. (2002). Correlation between GABA release from rat islet β-cells and their metabolic state. *American Journal of Physiology-Endocrinology and Metabolism*.

Wise, D. R., & Thompson, C. B. (2010). Glutamine addiction: a new therapeutic target in cancer. *Trends in Siochemical sciences, 35*(8), 427–433. https://doi.org/10.1016/j.tibs.2010.05.003.

Wise, D. R., DeBerardinis, R. J., Mancuso, A., Sayed, N., Zhang, X. Y., Pfeiffer, H. K., ... & Thompson, C. B. (2008). Myc regulates a transcriptional program that stimulates mitochondrial glutaminolysis and leads to glutamine addiction. *Proceedings of the National Academy of Sciences*, *105*(48), 18782-18787.

Wlodawer, A., Roberts, J., & Holcenberg, J. S. (1977). Characterization of crystals of L-glutaminase-asparaginase from *Acinetobacter glutaminasificans* and *Pseudomonas* 7A. *Journal of Molecular Biology*, *112*(3), 515-519.

Woraharn, S., Lailerd, N., Sivamaruthi, B. S., Wangcharoen, W., Sirisattha, S., & Chaiyasut, C. (2014). Screening and kinetics of glutaminase and glutamate decarboxylase producing lactic acid bacteria from fermented Thai foods. *Food Science and Technology (Campinas), 34*, 793–799. doi:10.1590/1678-457X.6519

Wright, P. A., & Knepper, M. A. (1990). Phosphate-dependent glutaminase activity in rat renal cortical and medullary tubule segments. *American Journal of Physiology-Renal Physiology*, *259*(6), F961-F970.

Wu, B., Liu, J., Zhao, R., Li, Y., Peer, J., Braun, A. L., Zhao, L., Wang, Y., Tong, Z., Huang, Y., & Zheng, J. C. (2018). Glutaminase 1 regulates the release of extracellular vesicles during neuroinflammation through key metabolic intermediate alpha-ketoglutarate. *Journal of neuroinflammation, 15*(1), 79. https://doi.org/10.1186/s12974-018-1120-x.

Yamamoto, S. (1974). Production of glutaminase by *Aspergillus sojae.*

Yamamoto, S., & Hirooka, H. (1974). Production of glutaminase by *Aspergillus sojae*. *Journal of Fermentation Technology (Japan)*.

Yang, L., Venneti, S., & Nagrath, D. (2017). Glutaminolysis: A Hallmark of Cancer Metabolism. *Annual review of biomedical engineering, 19*, 163–194. https://doi.org/10.1146/annurev-bioeng-071516-044546.

Yang, Y. Z., Ding, S., Wang, Y., Li, C. L., Shen, Y., Meeley, R., ... & Tan, B. C. (2017). Small kernel2 encodes a glutaminase in vitamin B6 biosynthesis essential for maize seed development. *Plant physiology, 174*(2), 1127-1138.

Yano, T., Ito, M., Tomita, K., & Kumagai, H. (1988). Purification and properties of glutaminase from *Aspergillus oryzae. Journal of Fermentation Technology, 66*(2), 137-143.

Ye, M; Liu, X.; and Zhao, L. (2013) Production of a Novel Salt-tolerant L-glutaminase from *Bacillus amyloliquefaciens* Using Agro-industrial Residues and its Application in Chinese Soy Sauce Fermentation. *Biotechnology, 12*: 25-35.

Yin, C., Ying, L., Zhang, P. C., Zhuo, R. X., Kang, E. T., Leong, K. W., & Mao, H. Q. (2003). High density of immobilized galactose ligand enhances hepatocyte attachment and function. *Journal of Biomedical Materials Research Part A: An Official Journal of The Society for Biomaterials, The Japanese Society for Biomaterials, and The Australian Society for Biomaterials and the Korean Society for Biomaterials*, *67*(4), 1093-1104.

Ying, H., Kimmelman, A. C., Lyssiotis, C. A., Hua, S., Chu, G. C., Fletcher-Sananikone, E., ... & DePinho, R. A. (2012). Oncogenic Kras maintains pancreatic tumors through regulation of anabolic glucose metabolism. *Cell, 149*(3), 656-670.

Yoo, H. C., Yu, Y. C., Sung, Y., & Han, J. M. (2020). Glutamine reliance in cell metabolism. *Experimental & Molecular Medicine, 52*(9), 1496-1516. https://doi.org/10.1038/s12276-020-00504-8

Zaak, H., Peirce, S., De Albuquerque, T. L., Sassi, M., & Fernandez-Lafuente, R. (2017). Exploiting the versatility of aminated supports activated with glutaraldehyde to immobilize β-galactosidase from *Aspergillus oryzae*. *Catalysts, 7*(9), 250. https://doi.org/10.3390/catal7090250.

Zang, X., Liu, M., Fan, Y., Xu, J., Xu, X., & Li, H. (2018). The structural and functional contributions of β-glucosidase-producing microbial communities to cellulose degradation in composting. *Biotechnology for Biofuels, 11*(1), 1-13. https://doi.org/10.1186/s13068-018-1045-8.

Zeller, K. I., Zhao, X., Lee, C. W., Chiu, K. P., Yao, F., Yustein, J. T., ... & Fu, Y. (2006). Global mapping of c-Myc binding sites and target gene networks in human B cells. *Proceedings of the National Academy of Sciences*, *103*(47), 17834-17839.

Zhang, X., Xu, Z., Liu, S., Qian, K., Xu, M., Yang, T., ... & Rao, Z. (2019). Improving the production of salt-tolerant glutaminase by integrating multiple copies of mglu into the protease and 16S rDNA genes of *Bacillus subtilis* 168. *Molecules*, 24(3), 592. https://doi.org/10.3390/molecules24030592

Zhao, Y., Chen, X. Q., & Du, J. Z. (2009). Cellular adaptation to hypoxia and p53 transcription regulation. *Journal of Zhejiang University. Science. B, 10*(5), 404–410. https://doi.org/10.1631/jzus.B0820293.

Zhdanov, A. V., Waters, A. H., Golubeva, A. V., Dmitriev, R. I., & Papkovsky, D. B. (2014). Availability of the key metabolic substrates dictates the respiratory response of cancer cells to the mitochondrial uncoupling. *Biochimica et Biophysica Acta (BBA)-Bioenergetics, 1837*(1), 51-62.

Zhou, M., Guan, W., Walker, L. D., Mezencev, R., Benigno, B. B., Gray, A., ... & McDonald, J. F. (2010). Rapid mass spectrometric metabolic profiling of blood sera detects ovarian cancer with high accuracy. *Cancer Epidemiology and Prevention Biomarkers*, *19*(9), 2262-2271.

Zhou, M., Zhao, Y., Ding, Y., Liu, H., Liu, Z., Fodstad, O., ... & Tan, M. (2010). Warburg effect in chemosensitivity: targeting lactate dehydrogenase-A re-sensitizes taxol-resistant cancer cells to taxol. *Molecular cancer, 9*(1), 1-12.

Zouhri, S. A. M. I. R. A., Saadi, S., Elmouki, I., Hamdache, A., & Rachik, M. (2013). Mixed immunotherapy and chemotherapy of tumors: optimal control approach. *International Journal of Computer Science Issues (IJCSI), 10*(4), 81.

.

16s rDNA Sequences of Selected Bacterial Strains

1. RSHU1 Sequence

GCTTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCAC
TTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCAC
CAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCC
GCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTT
TTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATA
GGGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGC
CAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGG
CGTAAAGGGCTCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCCCCCGGC
TCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGG
AGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAA
CACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGA
AAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
ACGATGAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTA
ACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAA
AGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGA
AGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGA
GATAGGACGTCCCCTTCGGGGCAGAATGACAGGTGGTGCATGGTTGTCGT
CAGCTCGGGTCGGGAGATGTTGGGTTAAGTCCCCCACCAACCCACCCTTG
ATCTTATTGCCAGCATTCGTTGGCGCTCTAAGGTGACTGCCGGTTACAACC
GGAAGAA

2. Sequence for RSHG1

TAGCGGGGGATAACTACTCAAAAGCGTATCTAATACCGCATACGCCCTAC
GGAGGGCAAAGCATGGGGCATCGCAAGACCTTGCACTATTGGAGCGGCC
GATATCGGATTAGCTAGTTGGTGGGGTAACGGCTCACCGAGGCAACGATC
CGTAGCTGGTTTGATAGGACGACCGTCCACTCTGGGACTGAGACACGGCC
CATACTCCTACGGGAGGCAGCACTGGGGAATTTTGGACAATGGGGGAAAC
CCTGATCCAGCCATCCCGCGTGTGCGATGAAGGCCTTCAGGTTGTAAAGC
ACTTTTGGCAGGAAAGAAACGTCGCGGGTTAATACCCCACGAAACTGACG

GTACCTGCAAAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAAT
ACGTAGGGTGCAAGCGTTAATCGGAATTACTGCGCGTATAGCGTGCGCAG
GCGGTTCGGAAAGAAAGATGTGAAATCCCAGAGCTTAACTTTGGAACTGC
ATTTTTAACTACCGGGCTATAGTGTGTCAGAGGGAGGTGGAAATTCCGCG
TGTAACACTGAAATGCGTAGATATGCCGAGGAACACCTATGGCGAAAGCA
ACCTCCGGGGATACCCCGACCCTCATGCACGAAAGCCTGTGGTAGCCAAC
AGGGATTAAATCCCCTCTGTAGGCCACACCCCCAAACGATGTCCGCTATTT
GTTGGGCCCTCGGGTGTTGGGTACCCCCCGAAAACGCGAAGGGCGCCCTA
GAGGAAGAC

3. Sequence for RSHQ1

CCCGAGGCGCGTGCCTACTAATGCAAGTCGTGCGAGGCNNAATGGAGGA
GCTTGCTCCCTGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACC
TGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGC
TTGTTTGAACCGCATGGTTCAAACATAAAAGGTGGCTTCGGCTACCACTTA
CAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAATGGCTCACCAA
GGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACT
GAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGC
AATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTC
GGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGG
CGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAG
CAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGT
AAAGGGCTCGCAGGCGGTTCCTTAAGTCTGATGTGAAAGCCCCCGGCTCA
ACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGA
GTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGAACACC
AGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGC
GTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
GAGTGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCA
TTAAGCACTCCGCCTGGCGAGTACGGTCGCAGGACTGAAACTCAAAGGAA
TTGACGGGGGCCCGCACAACCGGTGGAGCATGTGGTTTAATTCAAAGCAA
CGCCAAGAACCTTACCAGGTCTTGGCATCCTCTGACAATCCTAGAGATAG
GACGTCCGCTTCGGGGGCAGAACGACAGTTGTTGCAGGTTGTCTTNNCTC
CCGTCCGAGAAGTTGAGTTAATGCCCACCTCCACCCCACGCCTGGATTTAT
TTCCAGNATTCATTTGGCNNNCGAAAAGTANTCCGGTGCCACCCNNAAAA
AGGGGGGNTGGGTGCAATCATCTGGCGCTTGNCCCNCCTNCNCCGTGCTC

TATTGTGTAANNNAGGGNGACAAACCCCGCGTNAACCACCCCCACTTTTT
TTTTTTTATCACCCCNTTTTTTNTGAAGGGGGGGGAANNATGACGGCGGG
GAAGTTTCNNNTTNNCCCCCGGCCCCCCTTTNNCCCCCGGGGAGATTTTGT
GGCTCTATGTATGGGGGGAGGTTGGGGCNNAAAAAGAAAAAAAAAAAAA
AAA

4. Sequence for RSHU3

GGAATCTACTCTGTCGTGGGGGATAACGTAGGGAAACTTACGCTAATACC
GCATACGACCTACGGGTGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTG
AATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGG
CGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAG
ACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAAT
GGGCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTCGGG
TTGTAAAGCCCTTTTGTTGGGAAAGAAATCCAGCTGGCTAATACCCGGTT
GGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCAGCAGC
CGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGGGCGTAAAG
CGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAAAGCCCTGGGCTCAACCT
GGGAACTGCAGTGGATACTGGGCGACTAGAATGTGGTAGAGGGTAGCGG
AATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCAGGAGGAACATCCATG
GCGAAGGCAGCTACCTGGACCAACATTGACACTGAGGCACGAAAGCGTG
GGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATGCG
AACTGGATGTTGGGTGCAATTTGGCACGCAGTATCGAAGCTAACGCGTTA
AGTTCGCCGCCTGGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATT
GAAGGGGCCCCGCACAAGCGGTGGAGTATGTGGTTTAATTTCTATGCAAC
GCAAGAACCTTACTTGGCCTTGACATGTCGGGAACTTTCCAAAGATGGAT
TGGTGCCTTCGGGAATCGAACCCAAGGCTGATGGCTGTGTCAGCTCTTCG
CAATGTGGGGTGAATTCCCCCACCGA

5. Sequence for RSHS3

GGATCGCAAGACCTCTCACTATTGGAGCGGCCGATATCGGATTAGCTAGT
TGGTGGGGTAAAGGCTCACCAAGGCAACGATCCGTAGCTGGTTTGAGAGG
ACGACCAGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATTTTGGACAATGGGGGAAACCCTGATCCAGCCATCCC
GCGTGTATGATGAAGGCCTTCGGGTTGTAAAGTACTTTTGGCAGAGAAGA

AAAGGCATCCCCTAATACGGGATGCTGCTGACGGTATCTGCAGAATAAGC
ACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGT
TAATCGGAATTACTGGGCGTAAAGCGTGTGTAGGCGGTTCGGAAAGAAAG
ATGTGAAATCCCAGGGCTCAACCTTGGAACTGCGTTTTTAACTGCCGAGCT
AGAGTATGTCATAGGGGGGTAGAATTCCACGTGTAGCAGTGAAATGCGTA
AATATGTGGAGGAATACCGATGGCGAACGCGGCCGCCTGGATAATACTGA
CGCTCAGACACGAAAGCGTCGGAGGAGACACCATTAGATACGCTGGTAGT
CCACACCCTCAACGATGTCAACTGCCTCACGGGGGCGGTACCCTCCTTAG
CACAGCTCACCTCGCAGTGAAACAACTGGGTTAGGGGTCGCGCCCTGGTT
CACACTATTTGTTTGCAAACCCGCCCACCACGTGTGGCGCGATTATTTCAA
ATA

Turnitin Originality Report

PRODUCTION, PURIFICATION, AND CHARACTERIZATION OF MICROBIAL L-GLUTAMINASE by Rabia Saleem .

From DRSM (DRSM L)

- Processed on 12-Nov-2021 09:51 PKT
- ID: 1700451939
- Word Count: 32127

Similarity Index
13%
Similarity by Source

Internet Sources:
8%
Publications:
11%
Student Papers:
2%

Focal Person (Turnitin)
Quaid-i-Azam University
Islamabad

DR. SAFIA AHMED
PROFESSOR
Department of Microbiology
Quaid-i-Azam University
Islamabad

sources:

1 2% match (Internet from 23-Jun-2016)
http://mdpi.com/1422-0067/16/9/22830/htm

2 1% match (publications)
Kiruthika Jambulingam, Saraswathy Nachimuthu, Murugesan Saranya. "Maximizing L-glutaminase production from marine Bacillus subtilis JK-79 under solid state fermentation", African Journal of Biotechnology, 2018

3 < 1% match (Internet from 13-Jun-2018)
http://shodhganga.inflibnet.ac.in/bitstream/10603/2190/11/11_chapter%202.pdf

4 < 1% match ()
James, Donny Lawrence. "Biochemical Dechlorination of Hexachloro-1,3-butadiene"

5 < 1% match ()
Macedo, Sara, Cabral, Cátia, Novais, António, Teixeira, Mónica, Knock, Anna. "Terlipressin-related Ischaemic Necrosis of the Skin: A Rare Complication", 'SMC Media', 2019

6 < 1% match (publications)
Nidhi Y. Patel, Dhritiksha M. Baria, Shivani M. Yagnik, Kiransinh N. Rajput, Rakesh R. Panchal, Vikram H. Raval. "Bio-prospecting the future in perspective of amidohydrolase L-glutaminase from marine habitats", Applied Microbiology and Biotechnology, 2021

7 < 1% match (publications)
Hanaa Orabi, Esmail El-Fakharany, Eman Abdelkhalek, Nagwa Sidkey. "Production, optimization, purification, characterization, and anti-cancer application of extracellular L-glutaminase produced from the marine bacterial isolate", Preparative Biochemistry & Biotechnology, 2019

8 < 1% match (publications)
Hanaa Orabi, Esmail El-Fakharany, Eman Abdelkhalek, Nagwa Sidkey. "Production, optimization, purification, characterization, and anti-cancer application of extracellular L-glutaminase produced from the marine bacterial isolate", Preparative Biochemistry & Biotechnology, 2019

9 < 1% match (Internet from 15-Jul-2020)
https://deepblue.lib.umich.edu/bitstream/handle/2027.42/99873/mnbrown_1.pdf?sequence=1

10 < 1% match (publications)
Parameswaran Binod, Raveendran Sindhu, Aravind Madhavan, Amith Abraham et al. "Recent

catalysts

MDPI

Article

Characterization of a New L-Glutaminase Produced by *Achromobacter xylosoxidans* RSHG1, Isolated from an Expired Hydrolyzed L-Glutamine Sample

Rabia Saleem [1,2] and Safia Ahmed [1,*]

[1] Department of Microbiology, Quaid-i-Azam University, Islamabad 45320, Pakistan; rabiasaleem804@yahoo.com
[2] Department of Biosciences, University of Wah, Quaid Avenue, Wah Cantt 47040, Pakistan
* Correspondence: sahmed@qau.edu.pk; Tel.: +92-51-9064-3009

Abstract: As significant biocatalyst, L-glutaminases find potential applications in various fields, from nourishment to the pharmaceutical industry. Anticancer activity and flavor enhancement are the most promising applications of L-glutaminases. In this study, L-glutaminase was isolated and purified from an old glutamine sample. A selected bacterial isolate was characterized taxonomically by morphological characters, biochemical testing and 16S rDNA sequence homology testing. The taxonomical characterization of the isolate identified it as *Achromobacter xylosoxidans* strain RSHG1. The isolate showed maximum enzyme production at 30 °C, pH 9, with Sorbitol as a carbon source and L-Glutamine as a nitrogen and inducer source. L-Glutaminsae was purified by using column chromatography on a Sephadex G-75. The enzyme has a molecular weight of 40 KDa, pH optimal 7 and is stable in the pH range of 6–8. The optimum temperature for the catalyst was 40 °C and stable at 35–50 °C. The kinetic studies of the purified L-glutaminase exhibited Km and Vmax of 0.236 mM and 443.8 U/mg, respectively. L-Glutaminase activity was increased when incubated with 20 mM $CaCl_2$, $BaCl_2$, $ZnSO_4$, KCl, $MgSO_4$ and NaCl, whereas EDTA, $CoCl_2$, HgCl, $ZnSO_4$ and $FeSO_4$ decreased the activity of the enzyme. The addition of 8% NaCl enhanced the glutaminase activity. L-Glutaminase immobilized on 3.6% agar was stable for up to 3 weeks.

Keywords: L-glutaminase production; optimization; characterization; molecular identification; *Achromobacter xylosoxidans* RSHG1

Citation: Saleem, R.; Ahmed, S. Characterization of a New L-Glutaminase Produced by *Achromobacter xylosoxidans* RSHG1, Isolated from an Expired Hydrolyzed L-Glutamine Sample. *Catalysts* **2021**, *11*, 1262. https://doi.org/10.3390/catal11111262

Academic Editor: Francisco Plou

Received: 31 August 2021
Accepted: 10 October 2021
Published: 20 October 2021

1. Introduction

Biocatalysts play a crucial role in maintaining and sustaining the food, chemical, agriculture and cosmetic industries as commercially economic products. Biocatalysts are involved in all the biochemical processes within the cells and living organisms. Moreover, they accelerate the rate of a chemical reaction without being a part of the end product [1–3]. Enzymatic processes are eco-friendly, which reduces the risk of byproducts that are toxic for the human health and the environment [4]. Due to these features, biocatalysts play a vital role in the pharmaceutical industry [2]. L-Glutaminases are amidohydrolases, belong to the hydrolase class of enzymes and cleave L-glutamine into L-glutamic acid and ammonia. This enzyme plays a significant role in nitrogen metabolism at the cellular level. This enzyme is ubiquitous and present in both microorganisms (bacteria, fungi and yeast) and macro-organisms (animals and plants) [5–7]. The probable sources may include animals, plants, bacteria, actinomycetes, yeast and fungi [7]. Numerous bacteria are involved in extracellular and intracellular glutaminases production, such as *Bacillus* sp., *Pseudomonas*, *Actinobacterium* sp. and *E. coli* [7,8]. Important fungal glutaminases are *Aspergillus* sp. and *Trichoderma* sp. [9]. Different species of actinomycetes also produce L-glutaminase. L-Glutaminase production and the extraction from plants are not well studied. Due to complex organization, animals are not well known for L-glutaminase isolation from animal