

Republic of Iraq

Ministry of Higher Education and scientific Research

Univercity of Al-Qadisiyah

College of Science

Department of Biology



Potential Pharmaceutical Properties For Fungal L-asparaginase Isolated From *Aspergillus Niger*

A Thesis

**Submitted to the Council of College of Science, University of Al-Qadisiyah in Partial Fulfillment of the Requirements the Degree of
Doctor of Philosophy Science**

By

Noor Abdul Khudhur Salal

**B.Sc.Biology-College of Science – University of Al-Qadisiya -
2016 M.Sc.Biology-College of Science-University of
Al-Mustansiriya-2020**

Supervision by

Prof .Dr. Neeran Obied Jasim

1447A.H

2025AD

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

« وَإِنْ لَبِثَ الْإِنْسَانُ إِلَّا مَا سَعَى (٣٩) وَإِنْ لَسَعِبَهُ لَسَوْفَ يَرْجِعُ (٤٠) ثُمَّ

يُجْزَاءُ الْجَزَاءَ الْإِوْفَى (٤١) »

صَلَّى اللَّهُ عَلَى الْعَلَاءِ الْعَظِيمِ

(٣٩, ٤٠, ٤١ : النجم)

Dedication

To First and foremost, to Allah — thank You for Your constant guidance, hidden mercy, and the strength You granted me when I needed it most.

To..... Our first teacher who taught us the purpose of life is the Prophet Muhammad (may God bless him and his family)

To..... my beloved parents — your endless love and prayers shaped every step I took. This accomplishment is yours as much as it is mine.

To..... the soul of my dear sister(huda)

To..... My lovely Brothers and my sister— my steady support and silent strength, thank you for standing by me through every challenge and joy.

TO..... My lovely Friends... Thank you for your support

TO.....Prof.Dr. Neeran Obied Jasim Thank you for your insightful guidance, kind patience, and constant support, which played a vital role in the success of this work.With all my gratitude and respect.

NOOR

Acknowledgments

*First of all, I thank Allah, the almighty, who granted guidance and help to carry out the present research. I would like to express my thanks and full appreciation and deep gratitude to my supervisor(**Prof .Dr. Neeran Obied Jasim**) for the guidance, correction, and teaching me of his abundance of humanity, high morals, and high level his provision of valuable guidance and information contributed to enriching the subject of our study.*

*Thank you very much to the Dean of Science College (**Prof. salwan ali ahead**)and Head of the Biology Department(**Dr.laith sareaa muter**). extend all thanks and gratitude to the Bagdad laboratory in Diwaniyah **especially (Prof. Dr. Aws rasul Hussein)***

Thanks from the heart to my father my power source, and my family for their endurance and patience in completing my study career and my arrival at this stage and a very grateful to the faculty member and every one who help and encouragement me.

NOOR

Summary

Summary

This study investigated the production, purification, and biomedical applications of L-asparaginase from *Aspergillus niger*, isolated from soil. production was tested extracellular L-asparaginase by two methods on the solid asparagine agar medium as a primary screening processes for asparaginase production. six *A. niger* isolates with isolates with maximum ability of formation yellow zone around the colony wells were selected for secondary screening. significant L-asparaginase activity was evidenced by hydrolysis zones (4.5–5 cm). Secondary screening via submerged fermentation and Nesslerization revealed the highest enzyme activity (2.811 U/mg) in one isolate (*A. niger* SAP-1) Purification of this enzyme by gel filtration chromatography achieved a 6.3-fold increase in specific activity (630 U/mg) with a 35.5% yield. HPLC analysis of the L-asparaginase, it was shown that *A. niger* produced L-asparaginase. The L-asparaginase showed peak shapes and relative retention times (4.15 min) that were identical to the L-asparaginase reference substance.

Results of anticancer activity of showed the purified enzyme exhibited potent anticancer activity in MTT assays, showing concentration-dependent cytotoxicity against A375 melanoma (49.38% viability at 400 µg/mL) and HepG2 hepatocellular carcinoma cells (65.01% viability at 400 µg/mL), while sparing normal human dermal fibroblasts (HdFn).

Isolation and identification results revealed that from 300 specimens obtained from patients with different infection, which identified fungal infections 205(68.5%) were the predominant, followed by bacterial infections 77 (25.5%), and mixed bacterial-fungal infections 18(6.0%). Epidemiological data on the distribution of bacterial pathogens showed that *Streptococcus pneumoniae* was the dominant strain at 29.89%, followed by *Klebsiella pneumoniae* (23.39%), *Staphylococcus epidermidis* (15.58%), *Acinetobacter baumannii* (12.98%), and

Summary

Pseudomonas aeruginosa (11.68%). while distribution of fungal pathogens reveals *Microsporum* spp. (43.9%) and *Trichophyton* spp. (42.93%) dominating the spectrum, collectively accounting for nearly 90% of cases, while *Aspergillus niger* (5.3%) and *Coccidioides immitis* (4.8%) .

Results of antimicrobial of L-asparaginase show demonstrated efficacy against fungal (*Candida albicans*, *Rhizopus* sp.) and bacterial pathogens (*Staphylococcus epidermidis*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*), with optimal inhibition at low concentrations (e.g., 0.015 µg/mL for *P. aeruginosa*)

Synergistic antioxidant effects were observed when L asparaginase was combined with superoxide dismutase (SOD) or glutathione peroxidase, enhancing oxidative stress in target cells. These findings highlight *A. niger* SAP-1-derived L-asparaginase as a promising therapeutic agent for cancer and infections, with selective cytotoxicity, antimicrobial properties, and synergistic antioxidant potential. The study underscores the enzyme's clinical relevance amid rising antimicrobial resistance and cancer prevalence.

List of contents

CONTENTS	Page No.
Summary	I
List of contents	III
List of tables	VII
List of figures	VIII
List of abbreviation	IX
<i>Chapter one</i>	
1. Introdaction	1
1.1 Aim of the study	3
<i>Chapter Two</i>	
2. Literature review	4
2.1 Microbial secondary metabolites	4
2.1.1 Fungal Secondary Metabolites	6
2.2 <i>Aspergillus niger</i>	7
2.3 L-asparaginase	9
2.3.1 Sources of L-asparaginase	11
2.3.2 Mechanism cation of Asparaginase	12
2.4 Fermentation techniques for L-Asparaginase production	13
2.4.1 Submerged fermentation	14
2.5 Optimum conditions of producing L-Asparaginase	15
2.5.1 Optimum culture medium	15
2.5.2 Carbon and Nitrogen sources	15
2.5.3 Initial pH	17
2.5.4 Temperature	17
2.5.5 Incubation period	18
2.6 Purification of microbial L-asparaginase	18
2.7 Pharmaceutical character of L-asparaginase	20
2.8 L-asparaginase characterization	21
2.8.1 pH effects on L-asparaginase activity and stability	21
2.8.2 Temperature effects on L-asparaginase activity and stability	22

List of Contents

2.8.3 Metal Ion Effect	23
2.9 Cancer	23
2.9.1 Risk factors for cancer	24
2.9.2 Exploring the Mechanisms and Treatments of Cancer	24
2.9.3 Skin cancer	26
2.9.4 Liver cancer	27
2.10 Cell line	29
2.10.1 Exploring the Relationship Between Skin Cancer and Cell Line	30
Chapter Three	
3. Materials and Methods	34
3.1 Materials	34
3.1.1 Equipment and apparatus	34
3.1.2 Chemical and biological materials	35
3.1.3 Kits Table	36
3.1.4 Media	37
3.2 Methods	37
3.2.1 Preparation Media	37
3.2.1.1 Culture media	37
3.2.1.2 Asparagine Medium for primary screening	38
3.2.2 Sterilization techniques	38
3.2.3 Maintenance of fungi isolates	39
3.2.4 Maintenance of bacteria isolates	39
3.2.5 Solutions and Stains	40
3.2.5.1 Lacto phenol-cotton blue	40
3.2.5.2 Gram stain	40
3.2.6 Fungal strain	40
3.2.6.1.1 Dilution method	40
3.2.7 Screening of <i>Aspergillus niger</i> isolated for L-Asparaginase production	41
3.2.7.1 Primary Screening of Asparagine production((Qualitative method- solid medium)	41
3.2.7.2 Quantitative secondary screening (Quantitative screening)	41
3.2.8 Determination of L-asparaginase activity	42

List of Contents

3.2.8.1 Buffers, solutions and reagents	42
3.2.8.1.1 Nessler's Reagent	42
3.2.8.1.2 Potassium Phosphate Buffer (0.05 M)	42
3.2.8.1.3 Solution of L-asparagine (200 mM, pH: 8.0)	42
3.2.8.1.4 Trichloroacetic Acid Solution (1.5 M)	43
3.2.9 Preparation of NH ₄ Cl standard curve	43
3.2.10 Asparaginase activity measurement	44
3.2.11 Determination of protein concentration	45
3.2.11.1 Buffers and solutions	45
3.2.11.1.1 Tris-HCL buffer 0.02 M, pH 8.0	45
3.2.11.1.2 Coomassie Brilliant-Blue G-250 solution	46
3.2.11.1.3 Standard Protein Solution	46
3.2.11.1.4 The Bradford assay's standard curve preparation	46
3.2.11.2 Determination of protein concentration	47
3.2.12 Determination of specific activity	48
3.2.13 Extraction of crude L-Asparaginase	48
3.2.14 Partial purification of L-asparaginase	49
3.2.14.1 Gel Filtration chromatography	49
3.2.14.1.1 Buffer and solution	49
3.2.14.1.2 Tris-HCL buffer (0.05M, pH 8.0)	49
3.2.14.1.3 Preparation Sephadex G-150 gel	49
3.2.14.1.4 Separation of Enzyme through Gel Filtration G-150 Column	49
3.2.15 HPLC analysis	50
3.2.16 Biological properties of asparaginase	50
3.2.16.1 Cytotoxic Effect of Purified L-ASNase	50
3.2.16.1.1 Cell line maintenance	50
3.2.16.1.2 MTT Protocol (Cytotoxicity assay)	51
3.2.17 anti-microbial activates	52
3.2.17.1 Collection of specimens	52
3.2.18 Mycological examination	53
3.2.18.1 Direct microscopic examination	53
3.2.18.2 Cultivation of fungal	53
3.2.18.3 Morphology phenotypic examination	53

List of Contents

3.2.19 Bacterial identification	54
3.2.19.1 Cultural and morphological identification	54
3.2.19.2 Microscopic examination	54
3.2.19.3 Identification of bacteria by Vitek -2 system	54
3.2.20 MIC (Minimum Inhibitory Concentration) Protocol	55
3.2.21 Estimation of the effect of enzyme on antioxidants	56
3.2.21.1 The effect of L-asparaginase on superoxide dismutase(SOD) by ELISA	56
3.2.21.2 The effect of L-asparaginase on glutathione (GSH) by ELISA	57
3.2.22 Analysis of statistics	59
Chapter Four	
4. Results and Discussions	60
4. 1 Isolation of <i>Aspergillus niger</i>	60
4. 2 Screening of <i>Aspergillus niger</i> isolates for L- asparaginase production	60
4.2.1 Primary Screening of Asparagine production	60
4.2.2 Secondary screening (Quantitative screening) Enzyme production using submerged fermentation	62
4.3 Purification of L-asparaginase	64
4.3.1 Gel Filtration Chromatography	65
4.4 HPLC analysis	67
4.5 Biological properties of asparaginase	69
4.5.1 Cytotoxic Effect of Purified L-ASNase Using MTT Assay	69
4.5.2 Antimicrobial activates	74
4.5.2.1 Isolation and identification	74
4.5.2.2 Bacterial identification	76
4.5.2.3 Fungi isolation	81
4.5.3 L-asparaginase as anti-microbial activity	85
4.5.3.1 The Minimum inhibition concentration of L-asparaginase (Antifungal activity)	85
4.5.3.2 The Minimum Inhibition concentration of. L- asparaginase (Antibacterial activity)	87
4.5.4 Antioxidant activity	93
4.5.4.1 Antioxidant effect of L-Asparaginase Combined with	93

List of Contents

Superoxide Dismutase (SOD)	
4.5.4.2 The Antioxidant effect of L-Asparaginase Combined with Glutathione (GSH)	95
Chapter Five	
Conclusions	98
Recommendation	99
REFERENCES	
References	100
Appendix	137
Abstract (Arabic)	i

List of Table

Title	Page No.
Table 3-1: Equipment and apparatus used in this study	34
Table 3-2: Chemicals, and biological martial used in Study	35
Table 3-3: Contents of the Kit Package used in Study	36
Table 3-4: List of diagnostic media used in this study	37
Table 3-5: Preparation of Bovine serum albumin concentrations	47
Table 4-1: particular activity of isolates of Aspergillus niger producing L-asparaginase on the submerged medium	64
Table 4-2: Purification steps of asparaginase from isolate A. niger SAP-1	67
Table 4-3: Effect of L- ASNase on A375	71
Table 4-4: Effect of L- ASNase on HepG2	73
Table 4-5: distribution of patients according to site of infection	75
Table 4-6: Frequencies of Fungal and Bacterial infections.	76
Table 4-7: Frequencies of bacterial infections	78
Table 4-8: Frequencies of Fungal infections	82

List of Contents

List of Figures

Title	Page No.
Figure 2-1: Schematic diagram representing integration of primary and secondary metabolism	5
Figure 2-2: <i>Aspergillus niger</i> . (A) on PDA medium; (B) arising conidiophore from a thick walled foot cell, 250X; (C) septate hyphae, 250X; (D) detailed structure of conidiophores, 400X.	9
Figure 2-3: Antineoplastic action of L-ASNase	11
Figure 2-4: Mechanism of action of asparaginase	13
Figure 2-5: bioreactor used in submerged fermentation	14
Figure 2-6: Cross section of skin	32
Figure 3-1: Has shown the subjects groups for the present study	33
Figure 3-2: A standard curve for ammonium chloride is used to determine the concentration of ammonium ions (NH_4^+) in a sample	44
Figure 3-3: BSA standard curve performed by Bradford method	48
Figure 4-1: <i>Aspergillus niger</i> SAP-1	60
Figure 4-2: <i>A. niger</i> L-asparaginase activity on asparagine plate agar medium after 24hrs. at. 7°C	61
Figure 4-3: Gel filtration chromatography was used to purify L-asparaginase from <i>A. niger</i> SAP-1	66
Figure 4-4: HPLC Analysis of of l-asparagine (Standard)	68
Figure 4-5: HPLC Analysis of of l-asparagine (Submerged Fermentation)	68
Figure 4-6: A375 cells and HdFn cell survival curve (mean $\pm$ SD%) following L-ASNase treatment using MTT in vitro assay at 37 °C, 5% CO ₂ for 24 hours	71
Figure 4-7: HepG2 cells and HdFn cell survival curve (mean $\pm$ SD%) following L-ASNase treatment using MTT in vitro assay at 37 °C, 5% CO ₂ for 24 hours	73
Figure 4-8: bacterial species that isolated in this study	80
Figure 4-9: fungal species	84
Figure 4-10: Effect L-asparaginase on <i>Candida albicans</i> , (Control) It is the control group, (G1 to G8) they are candida groups at different concentrations	86

List of Contents

Figure 4-11: Effect L-asparaginase on <i>Rhizopus</i> , (Control) It is the control group, (G1 to G8) they are <i>Rhizopus</i> groups at different concentrations	87
Figure 4-12: Effect L-asparaginase on <i>Staphylococcus epidermidis</i> , (Control) It is the control group, (G1 to G8) they are groups at different concentrations	88
Figure 4-13: Effect L-asparaginase on <i>A. baumannii</i> , (Control) It is the control group, (G1 to G8) they are groups at different concentrations	90
Figure 4-14: Effect L-asparaginase on <i>P. merbalis</i> , (Control) It is the control group, (G1 to G8) they are groups at different concentrations	91
Figure 4-15: Effect L-asparaginase on <i>Pseudomonas aeruginosa</i> , (Control) It is the control group, (G1 to G8) they are groups at different concentrations	93
Figure 4-16: Effect L-asparaginase on anti-oxidant superoxide dismutase SOD, (Control) It is the control group, (G1 to G4) they are SOD groups at different concentrations	94
Figure 4-17: Effect L-asparaginase on anti-oxidant glutathione GSH, (Control) It is the control group, (G1 to G4) they are GSH groups at different concentrations	96

List of Abbreviation

Abbreviation	Title
BA	Blood agar
BHIA	Brain heart infusion agar
BHIB	Brain heart infusion broth
BRCA1	Breast Cancer 1 Gene
BSA	Bovine serum albumin
Caspases	Cysteine aspartic protease
CRAB	Carbapenem-Resistant <i>Acinetobacter baumannii</i>
CWA	Cell wall–anchored
DFI	Diabetic Foot Infection
D.W	Distilled Water
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-Linked Immunosorbent Assay

List of Contents

EPR	Enhanced Permeability and Retention Effect
ER	Estrogen Receptor
ERBB2	Erythroblastic Oncogene B Receptor tyrosine-protein kinase 2
g/L	Gram per Liter
G1	Gap1 (cell cycle phase)
GRAS	Generally Recognized as Safe
GSH	Glutathione
HCC	Hepatocellular Carcinoma
HER2	Human Epidermal Growth Factor receptor
HPLC	High-Performance Liquid Chromatography
Hrs.	Hours
IC50	Half-maximal inhibitory concentration
ID GNB	Identification Gram Negative Bacteria
ID GPB	Identification Gram Positive Bacteria
IHC	Immunohistochemically
Ipa	Invasion plasmid antigen
L-ASNase	L-asparaginase
M	Molar
MCF-7	Michigan Cancer Foundation-7 (breast cancer cell line)
MDR	Multidrug-Resistant
Mg	Milligram
Min.	Minute
MSA	Mannitol salt agar
MSCRAMMs	Microbial surface component recognizing adhesive matrix molecules
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye
O.D	Optical Density
OFAT	Optimization by one factor at a time
PBPs	Penicillin-Binding Proteins
pH	Potential Hydrogen

List of Contents

Rpm	Revolutions per Minute
RPMI-1640	Roswell Park Memorial Institute-1640 (cell culture medium)
SDS-PAGE	Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis
SH-PEG-COOH	Poly(ethylene glycol) 2-Mercaptoethyl Ether Acetic Acid
SMF	Submerged fermentation
SOD	Superoxide Dismutase
Spp.	Species (plural)
SSF	Solid-State fermentation
TBE buffer	Tris-Borate EDTA Buffer
TCA	Trichloroacetic acid
TP53 (p53)	Tumor Protein p53
Tris	Tris(hydroxymethyl)aminomethane
TSS	Toxic shock syndrome
U	Units
UV-VIS	Ultraviolet/visible spectrophotometer
V/V	Volume to volume
VP	Voges-Proskauer (test)
W/V	Weight per volume
W/W	Weight to weight
WRL-68	Human hepatic cell line

Chapter one

Introduction

1. Introduction

Secondary metabolism represents a crucial biochemical process in microorganisms, enabling the production of specialized metabolites with diverse pharmaceutical, agricultural, and industrial applications (Wadhwa *et al.*, 2024). Unlike primary metabolism, which supports growth and reproduction, secondary metabolism generates non-essential compounds such as antibiotics, pigments, and enzymes that enhance survival and ecological competitiveness (Brito-Lopez *et al.*, 2025). Recent advances in genomics and synthetic biology have revealed the complex regulatory networks governing these pathways, particularly in fungi like *Aspergillus sp.* and *Streptomyces sp.*, which are prolific producers of bioactive metabolites (Aggarwal & Kumari, 2024; Zhgun, 2023), microbial secondary metabolites account for over 50% of FDA-approved drugs, underscoring their therapeutic significance (Patridge *et al.*, 2016). Innovations in metabolic engineering and fermentation technologies have further optimized yield and purity, addressing challenges in scalability and cost-effectiveness (Xu *et al.*, 2023). For instance, secondary metabolism in *Aspergillus niger* plays a pivotal role in producing bioactive compounds with applications in medicine, agriculture, and industry (Wadhwa *et al.*, 2024). This filamentous fungus synthesizes diverse secondary metabolites, including enzymes like L-asparaginase, pigments, and immunomodulatory, leveraging its robust metabolic pathways and adaptability to extreme environmental conditions (Aggarwal & Kumari, 2024; Salazar-Hamm & Torres-Cruz, 2024).

Recent advances in genetic engineering and omics technologies have elucidated the regulatory mechanisms behind these metabolites, enabling targeted overproduction through CRISPR-based strain optimization and co-cultivation strategies (Brito-Lopez *et al.*, 2025; Xu *et al.*, 2023). Notably, *A. niger*'s status

underscores its suitability for industrial enzyme production, such as citric acid and anticancer L-asparaginase, which are critical in pharmaceuticals (Haque *et al.*, 2024; Tosta Pérez *et al.*, 2023)

L-asparaginase (L-ASNase) is an amid hydrolase enzyme and a common enzyme found in bacteria, yeast, fungus, and plants. L-asparaginase converts circulating asparagine to ammonia and L-aspartate, preventing neoplasm protein synthesis. Therefore, it has been utilized and investigated extensively because of its relevant potential as an oncological agent and as an acrylamide mitigation agent in the food sector (Singh *et al.*, 2019). Asparaginase received increased attention in recent years for its anti-carcinogenic potential. Cancer cells differentiate themselves from normal cells in diminished expression of l-asparagine (Bhutada and Dahikar, 2017).

L-asparaginase may also be found in fungi and bacteria. Bacterial asparaginase has a number of negative side effects that frequently limit its use. This obstacle calls for a search for new sources of L-asparaginase. Compared to bacteria, fungi are more closely related to humans. As a result, there will be fewer chances of an immunological reaction against fungal asparaginase. Because fungal asparaginase is produced extracellularly and is an extremely simple extracellular enzyme to purify, it has gained significance. (Batool, *et; al.*, 2018).

Several techniques have been documented for the production and optimization of L-asparaginase from diverse microorganisms, such as submerged fermentation (SMF) and solid state fermentation (SSF). Enzymes can be produced constitutively or after induction, and the conditions of the reaction differ depending on the organism. (Muneer, *et.al.*, 2020). L-asparaginase activity has been assayed using a variety of techniques. The amount of ammonia or aspartic acid released during the reaction, or the asparagine's disappearance, can be used to measure L-

asparaginase activity. L-asparaginase activity has been estimated using both quantitative and qualitative methods.

1.1 Aim of the study

The production, isolation, and purification of L-Asparaginase, a metabolic product of one fungal species (*Aspergillus niger*), as a natural source, and an assessment of its antimicrobial, antioxidant, and anticancer properties are the goals of this study. However, to achieve such goals the following steps were suggested:

1. Isolation of *Aspergillus niger* from soil.
2. Qualitative detection of L-asparaginase enzyme production by *Aspergillus niger* using plating method.
3. Detection of the enzyme quantitatively using submerged cultures.
4. Purification the produced enzyme using the available chromatographic methods such as gel filtration.
5. Detection of active compounds in L-asparaginase enzyme using HPLC
6. Study of the toxic effect of the partially purified enzyme against some cancer cells
7. Testing the effectiveness of L-asparaginase enzyme as antimicrobials
8. Testing the effectiveness of L-asparaginase enzyme as antioxidant

Chapter two

Literature review

2. Literature Review

2.1 Microbial secondary metabolites

Currently referred to as specialized metabolites, many of them have shown significant impacts on our society's health, nutrition, and economy. They frequently have peculiar structures (Brito-Lopez *et al.*, 2025). Usually produced by secondary metabolism, these substances have a low molecular weight and do not depend on the primary microbial development phase. They include compounds with hormonal action or particular effects on the metabolism of fats and carbohydrates, as well as antibiotics, pigments, poisons, enzyme inhibitors and immunological modulators, (Hashem, 2025).

Some have already shown promise as diuretics, laxatives, antioxidants, antivirals, antitumor agents, vaso-relaxants, or contractants. Others serve as insecticides, colorants, or growth enhancers for plants and animals (Jan *et al.*, 2020). About 53% of FDA-approved drugs derived from natural products come from microorganisms. (Patridge *et al.*, 2016).

Secondary metabolites have significant applications in biomedicine. The overproduction of these microbial products can be achieved through various methods, including the use of elicitors, genetic and metabolic engineering, ribosomal engineering, and techniques to amplify secondary metabolites via biosynthetic genes. This also involves inactivating competing pathways, disrupting regulatory genes, manipulating secondary mechanisms, expressing suitable heterologous proteins, and employing combinatorial biosynthesis. (Nafiseh & Mohammad, 2013).

Due to their diverse communities and metabolic versatility, microbes have been selected for the large-scale production of bioactive compounds (Maithani *et al.*, 2022).

Many studies have highlighted the production of microbial bioactive compounds and their economic significance. For instance, marine fungi have been identified for their potential to produce anticancer compounds (Pattnaik *et al.*, 2022). *Streptomyces heliomyces* for the production of actinomycin (Abu Zaid *et al.*, 2021), Co-cultivating fungi and bacteria has resulted in the production of new secondary metabolites (Xu *et al.*, 2023). Various factors influence the production of bioactive compounds in microbes. The distinction between primary and secondary metabolites can be ambiguous, as many intermediates in primary metabolism overlap with those in secondary metabolism (Figure 2-1) (Karlsson *et al.*, 2017). Although amino acids are typically classified as primary metabolites, they can also be regarded as secondary metabolites. In contrast, sterols are considered secondary metabolites (Zhgun, 2023).

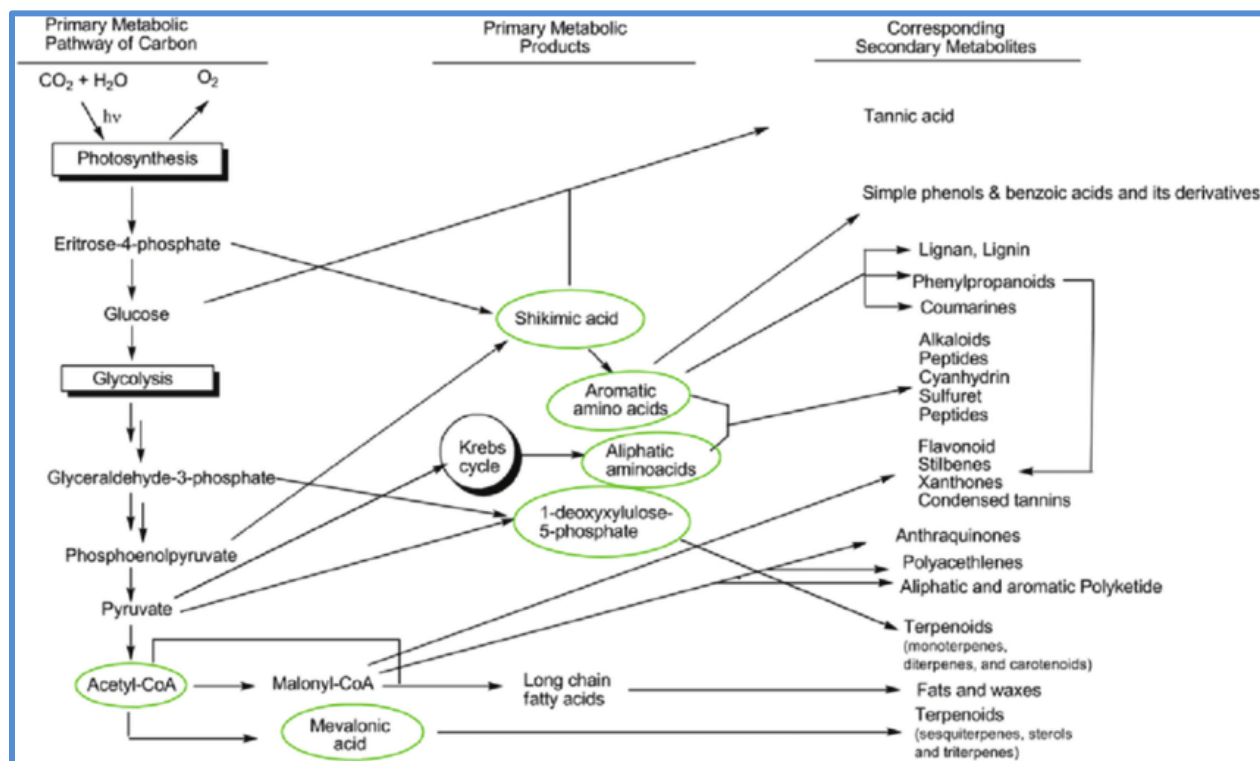


Figure (2-1): Schematic diagram representing integration of primary and secondary metabolism (El-Naggar & El-Shweihy, 2020)

2.1.1 Fungal Secondary Metabolites

Fungi are remarkable for their capacity to produce a wide range of natural products known as fungal secondary metabolites, which display various biological activities (Naranjo-Ortiz & Gabaldón, 2020). Consequently, researchers from various fields collaborate to understand fungal secondary metabolites in terms of their regulation, function, and applications, while also evaluating their toxicity(Wadhwa *et al.*, 2024).

Endophytic fungi are a complex group of microorganisms that infect healthy tissues with no apparent symptoms and inhabit totally or partially the cells of their host plants(Akram *et al.*, 2023). Endophytic fungus Endophytic fungus have become common source of bioactive products with a wide range of potential biological activities (SEM) (Kandasamy & Kathirvel, 2023).

Fungal secondary metabolites are natural products possessing specific biological activities commonly produced by endophytic filamentous fungus as the main source of these materials(Du *et al.*, 2020). During the last 2 decades, an enormous number of medicinally valuable bioactive molecules having antibiotic, insecticide, cytotoxic, and anticancer are derived from endophytic fungi (Nischitha & Shivanna, 2021). Among the most prevalent classes of fungal bioactive chemicals include lignin, phenylpropanoids, phenols, lactones, quinolones, isocoumarins, alkaloids, terpenoids, and steroids (Conrado *et al.*, 2022). *Trichoderma* species exhibit antibacterial activity against various microorganisms and they are associated with several hosts(Mukhopadhyay & Kumar, 2020). *Aspergillus niger* is a well-known fungus, isolated from different sources such as food, nuts and soil. To the biotechnological industry it represents a rich source due

to its great number of secondary metabolites with cytotoxic and immunomodulatory activities in relation to cancer cells (Devi *et al.*, 2020).

2.2 *Aspergillus niger*

A. niger is thermophilic, and it can grow at temperatures ranging between 6 and 47°C, with optimal growth range of 35–37°C. Its water activity limit to grow is from 0.88 in comparison with other *Aspergillus* species. Furthermore, *A. niger* can grow in a wide pH ranges, from 1.4 to 9.8. These modifications, together with the general penchant for abundant production of conidiospore, can shed light on the ubiquity of the species, especially in tropical and subtropical conditions (Salazar-Hamm & Torres-Cruz, 2024). *Aspergillus niger* is a filamentous fungus that resembles tiny plants due to its filamentous hyphae. Their growth is initially white, but after a few days, they turn black and produce conidial spores, according to macroscopic observation. Radial fissures are created by the colonies' pale yellow edges. Its conidia and conidiophores are smooth-colored. Protrusions from a septate and hyaline hyphae are known as conidiophores. The conidial heads split into columns (biseriate) and appear radial. The phialides on the conidiophores are supported by sterile cells called metulae that are produced by the conidiophore vesicle. Conidiophores are hyaline, smooth, and 400–3000 µm long. (Bennett, 2007) (Figure 2-2)

One of the most important biotechnological microorganisms, *Aspergillus niger*, is used since 160 years to produce extracellular food enzymes. A number of these enzymes are considered as GRAS (Generally Recognized as Safe) by the US Food & Drug Administration. *A. niger* is also employed for waste management and biotransformation. It has become an important transformation host for the production of food enzymes over the last 20 years (Aggarwal & Kumari, 2024).

Aspergillus species are abundant in nature and produce large quantities of asexual, haploid conidia, which are highly hydrophobic and easily airborne. Generally speaking, *Aspergillus niger* is a common, non-pathogenic fungus found in nature. Every day, humans are being exposed to the spores without showing any signs of illness. Rarely, though, *A. niger* can colonize the human body as an opportunist invader, especially in people receiving immunosuppressive therapy or with a history of serious illness (Rinker et al., 2024).

James Currie's (1917) discovery, which demonstrated that *Aspergillus niger* could biosynthesize huge amounts for citric acid by cultivating it in solutions of sugar at low pH, gave the fungus's productive metabolism considerable commercial significance. Pfizer founded the biotechnology industry in 1919 as a result of this groundbreaking study, which showed how environmental & nutritional factors directly affect citric acid yield (Haque *et al.*, 2024).

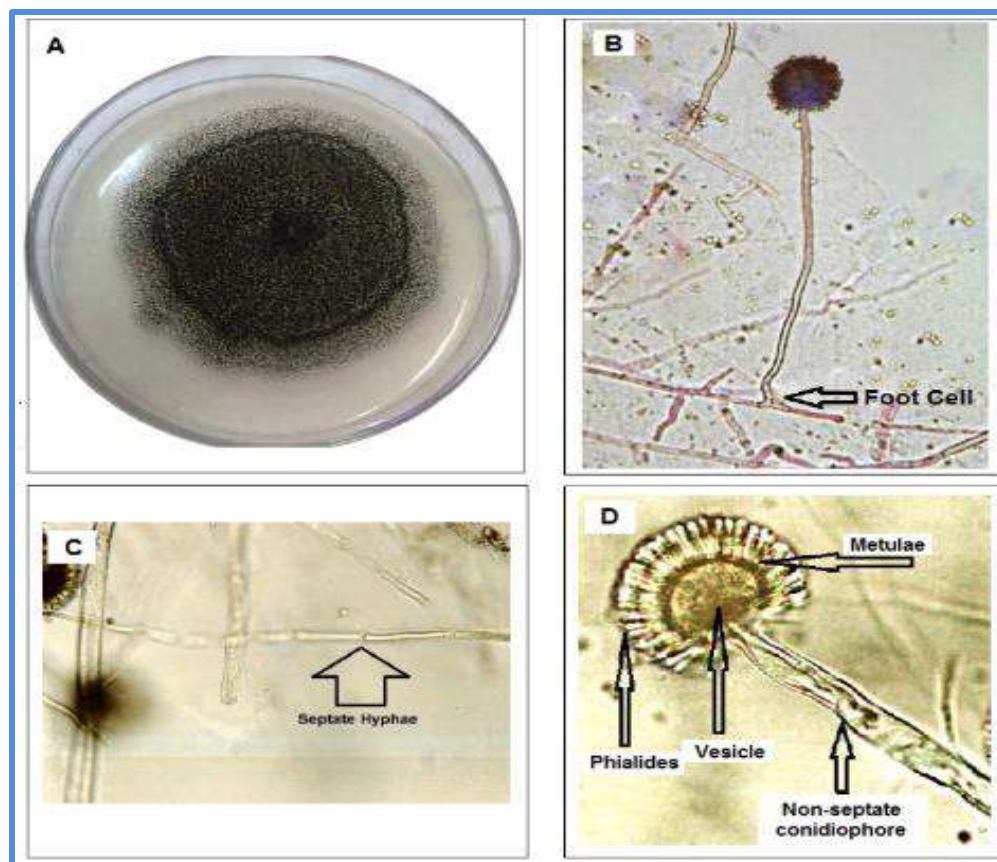


Figure (2-2): *Aspergillus niger*. (A) on PDA medium; (B) arising conidiophore from a thick walled foot cell, 250X; (C) septate hyphae, 250X; (D) detailed structure of conidiophores, 400X. (Abou-Bakr,et.al.,2013)

2.3 L-asparaginase

The amid hydrolase enzyme L-asparaginase (EC 3.5.1.1) catalyzes the breakdown of L-asparagine into L-aspartic acid and ammonia (Lubkowski & Wlodawer, 2021). The substrates and products of the enzyme reaction play essential roles in various metabolic processes across all organisms, from bacteria to mammals (Nüse *et al.*, 2023). L-asparaginase is widely reported in bacteria, yeast, fungi, and plants, with *Escherichia coli* strains serving as the main source for medical L-asparaginase (Castro *et al.*, 2021). L-asparaginase significantly contributes to the global supply of medicinal enzymes used in anti-leukemia and

anti-lymphoma treatments (Al-Dulimi, 2021) In patients treated with L-asparaginase, L-asparagine is rapidly depleted from the blood, converted into L-aspartate and ammonia. This cascade of reactions has a devastating effect on cell proliferation, particularly in cancer cells that rely on L-asparagine for growth (Al-Dulimi, 2021).

L-asparagine is an amino acid that cells need continuously to construct proteins. Healthy cells can produce L-asparagine using L-asparagine synthetase. When L-asparagine is hydrolyzed, it leads to the accumulation of L-aspartic acid and ammonia, which stunts the development of cancer cells. Additionally, the activity of L-glutaminase, another function of L-asparaginase, catalyzes the hydrolysis of L-glutamine—the sole nitrogen donor for L-asparagine synthesis—contributing to its antileukemic action. L-asparaginase, a bacterial enzyme used in treating acute lymphoblastic leukemia, is currently derived from either *Escherichia coli* or *Erwinia carotovora* (Tosta Pérez *et al.*, 2023). (Figure 2-3).

The antineoplastic action of L-asparaginase extends beyond its medical applications; microbial L-asparaginases are also marketed to reduce acrylamide production in food. Swedish scientists found that overcooking starch-rich foods, such as potatoes, rice, and cereals, leads to the formation of acrylamide (G. P. Yadav *et al.*, 2022).

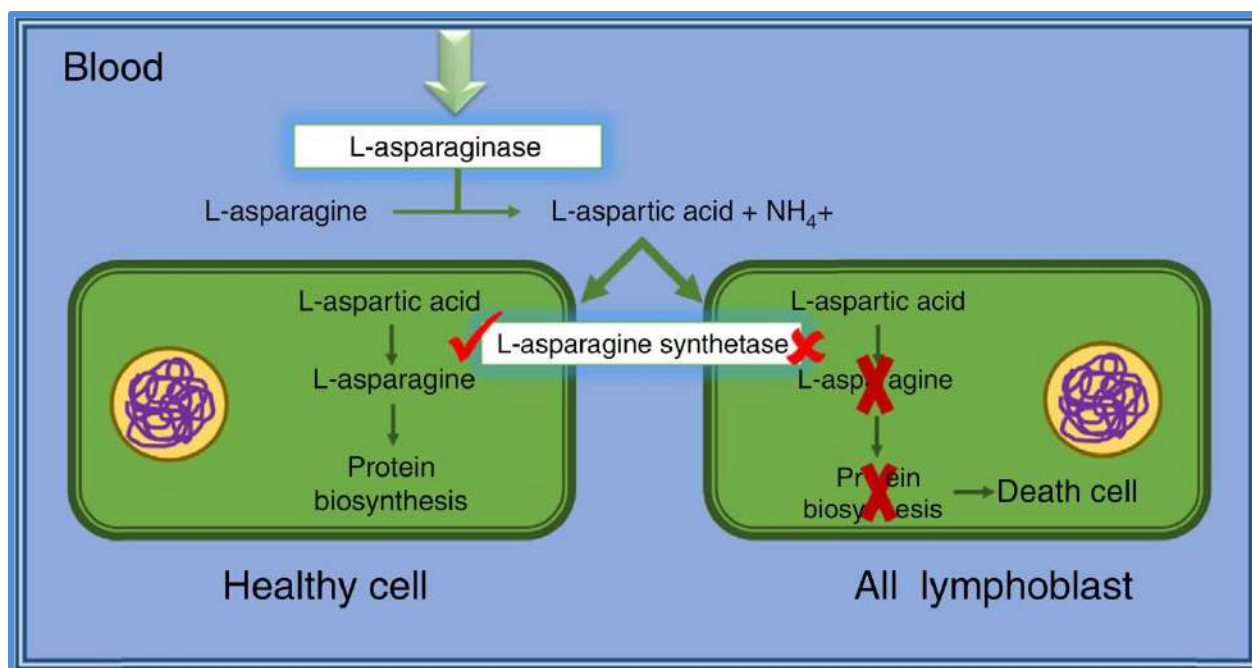


Figure (2-3): Antineoplastic action of L-ASNase (Ali *et al.*, 2016)

2.3.1 Sources of L-asparaginase

L-asparaginases is an enzyme expressed and generated by several of microorganisms (Muneer *et al.*, 2020). The production of L asparaginase has been studied in *Serratia marcescens* (Castro *et al.*, 2021). *Erwinia carotovora*, *Escherichia coli*, *Enterobacter aerogenes*, *Pseudomonas aeruginosa*, *Bacillus subtilis* (Ковалевський, 2024). *Aspergillus tamari*, *Aspergillus niger*, *Erwinia aroideae* , *Vibrio succinogenes*, *Pseudomonas stutzeria*, *Staphylococcus aureus* and *Aspergillus terreus*(Castro *et al.*, 2021). with different sources of carbon and nitrogen in both fermentative and aerobic settings. L-asparaginase has been found in a variety of yeasts, fungi, and algae. L-asparaginase is produced by various microorganisms. Since the 1970s, numerous microbial strains from different fungi and bacteria have been identified for their potential to produce asparaginase(Muneer *et al.*, 2020).

2.3.2 Mechanism cation of Asparaginase

An enzyme in the amidase family, breaks down the amide link between the ammonia and the aspartic acid in L-asparagine (SALAMI, 2021). The fact of lymphocytic leukemia cells are unable to produce asparagine and must get it from an outside source is the basis for asparaginase's mode of action. When asparagine levels in the blood and surrounding tissue drop suddenly, the development of these cells is critically affected (Yuan *et al.*, 2024). In the first step, the enzyme's nucleophile binds to the carbon atom of an amide substrate through a strong hydrogen bond with a crucial neighboring residue. This interaction generates an acyl-enzyme intermediate via a tetrahedral transition state (Klebe, 2025).

In the process, the oxygen atom of the amide group acquires a negative charge. This charge is stabilized through interactions with nearby hydrogen bond donors, which help maintain the stability of the transition state during the enzymatic reaction (Liu *et al.*, 2022). The constellation of those hydrogen bond donors, typically consisting of main-chain N–H groups, is referred to as the "oxyanion hole. The enzymatic process is continued in the second step of the reaction when an active water nucleophile targets the ester's carbon atom. Similar to the preceding stage, this stage also entails stabilizing the transition status through interactions (Chen *et al.*, 2022). (Figure 2-4).

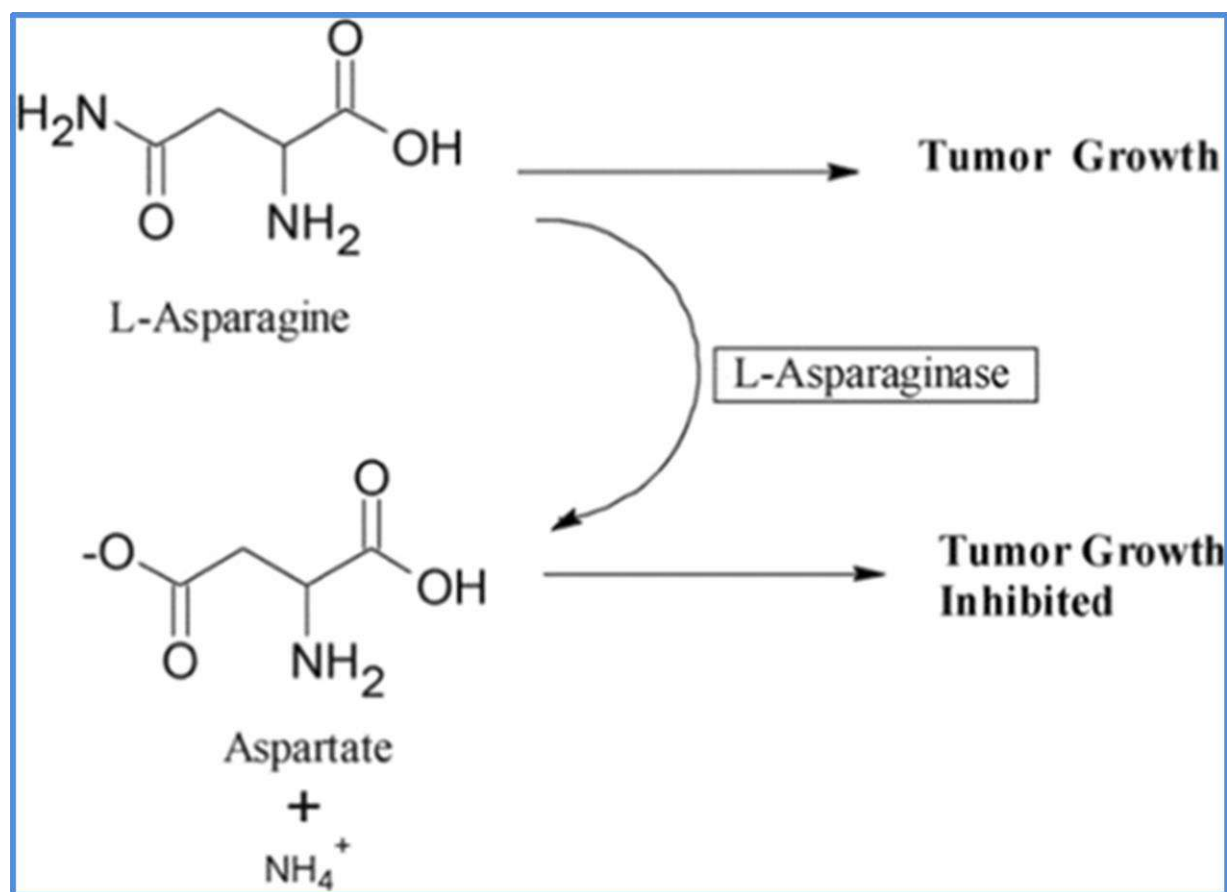


Figure (2-4): Mechanism of action of asparaginase (Osama *et al.*, 2023)

2.4 Fermentation techniques for L-Asparaginase production

The technique of producing enzymes for industrial purposes could be described as fermentation. It included enzyme-generating microorganism (bacteria and fungi). Two significant types of fermentation used to produce enzymes were submerged fermentation and solid- state fermentation (Amaya-Chantaca *et al.*, 2022). In general, L-Asparaginase was produced by submerged fermentation, but the latter had been utilized to a lower extent to produce these enzymes (Castro *et al.*, 2021).

2.4.1 Submerged fermentation

This technique involved growing selected microorganisms, like bacteria and fungi in closed vessels containing a rich fermentation medium of nutrients and a high concentration of oxygen (closed vessels) (Figure 2-5). In fermentation, microorganisms decompose nutrients and release the desired enzymes into the production medium. This process is vital for generating various products, such as alcohols, organic acids, and enzymes, which have numerous applications in food, pharmaceuticals, and other industries (Patel *et al.*, 2023) . The culture of microorganisms in high oxygen concentrated liquid nutrient broth had been submerged fermentation (SMF) (Premalatha *et al.*, 2023). It was primarily involved producing liquid secondary metabolites; this fermentation would produce enzymes of industrial importance. One of its most common advantages, the purification of desired products is easy although SMF relatively advanced its high-cost technique(Slak *et al.*, 2022) .

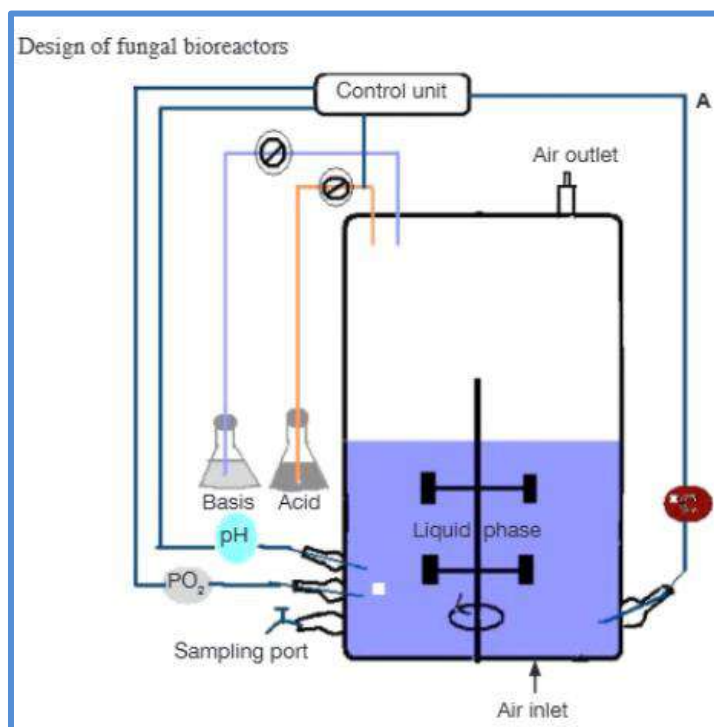


Figure (2-5): bioreactor used in submerged fermentation (Musoni,et.al.,2015)

2.5 Optimum conditions of producing L-Asparaginase

2.5.1 Optimum culture medium

The optimization process is crucial in the industrial production of metabolites. microbes can be cultured in a suitable biochemical and biophysical environment, with the culture medium providing the necessary nutrients. Given the unique requirements of different microbial strains, a wide variety of culture media have been developed, each tailored for specific purposes and uses. This diversity ensures optimal growth and productivity for the desired metabolites (Abhini *et al.*, 2022). In commercial practice, optimizing medium composition is essential to maintain a balance among the various components in production media. This optimization helps minimize the number of unutilized components remaining at the end of fermentation, enhancing efficiency and reducing costs. Optimization of nutrient levels and ratios allows producers to attain maximum microbial propagation and production of metabolites (Yadav *et al.*, 2024).

2.5.2 Carbon and Nitrogen sources

The culture media used for enzyme production include carbonaceous sources, nitrogen sources and other substances that enhance the production process. It has been shown that carbon source acts not only as an inducer of growth of microorganisms but also as an influence of enzyme formation in many aspects. The effect on the growth rate and yield for certain enzyme can be different with the different carbon source, which suggests the importance of the carbon source selection for the best performance (Fu *et al.*, 2022). They may either induce or repress production, depending on the nature of microorganism, the carbon source employed, and its concentration in the production medium. Various microorganisms have different responses, and it is vital to adjust these factors to

maximize enzyme production (Ma *et al.*, 2022). As carbon sources, glucose, mannose, maltose, fructose, lactose are generally employed. Nevertheless, high levels of glucose and sucrose may repress L-asparaginase production by preventing the induction of production process. This underscores the importance of regulating the carbon source level to achieve the maximum of enzymatic yields (Ma *et al.*, 2022). It is possible to solve the problems of the enzyme production when polymeric substrate is used, for example cellulose and starch. These substrates would have a slower sugar release rate, causing microbial growth and enzyme synthesis to occur gradually. Use of such substrates can lead to higher enzyme titer in addition to less inhibition which commonly accompanies monosaccharides (Chand *et al.*, 2020).

However, maltose was proved to be the best carbon source for the production of L-asparaginase from amongst the carbon sources screened where this enzyme was produced in considerable amounts. *S. albidoflavus* also reported to produce maximum when maltose was used as only carbon source. Glucose, fructose, galactose, mannitol, and arabinose on the other hand were screened and proved to be less efficient in L-asparaginase synthesis signifying that appropriate (or) correct choice of carbon source could be crucial for fine enzyme formation (Castro *et al.*, 2021). The nitrogen sources of media containing organic or inorganic nitrogen compounds such as amino acids, proteins, nucleic acids, yeast extract, peptone, urea, $(\text{NH}_4)_2\text{SO}_4$, and NaNO_3 are important for the growth of microorganisms and the production of enzyme. The added nitrogen sources of these nutrients have the role of improving the general metabolism and activity of the involved microorganisms in the production of enzymes (Salih, 2022).

2.5.3 Initial pH

Potential hydrogen (pH) being a critical factor directly associates with the proliferation of microorganisms and breeding in the culture medium as well as the generation of enzymes by these microorganisms (Wang, *et al.*, 2020). Enzyme formation in micro-organisms is very much affected by the pH of the medium. Typically, near neutral pH is the ideal for growth and manufacturing in many experiment species. But some microbes grow better in more acidic or alkaline environments and they tend to like specific pH levels that boost the production of the enzymes. These pH requirements are important to know in order to optimize enzyme yields in a culture of microorganisms (Elkhateeb & Fadel, 2022). Any deviation from the balance of H^+ and OH^- can disrupt an enzyme's H bonds and ionic bonds, and thus interfere with the arrangement of the enzyme's active site. Optimum pH for L-asparaginase production by *Streptomyces fradiae* is 8.0. This emphasizes the importance of maintaining proper pH for enzyme stability and activity in production (Soliman *et al.*, 2020).

2.5.4 Temperature

Microorganisms grow and produce enzymes according to the temperature effect on cellular metabolisms. The temperature that is optimal for growth of the microorganism may not be optimal for production of the enzyme, such that the best temperature for optimization of production of the enzyme might not be even the same for divergent strains of the identical species. This highlights the importance of careful temperature selection to obtain high enzyme yields along with healthy microbial growth (Patel *et al.*, 2023). Incubation temperature influences the metabolism of microorganisms. The best temperature for L-asparaginase production differs widely in the strains. A rise or fall above or below the optimum

temperature can result in a gradual reduction in the manufacture of proteins. In most of the strains, the optimal temperature for L-asparaginase production is approximately 37°C, and maintaining the desired temperature is crucial for maximum enzyme production (Muneer *et al.*, 2020).

2.5.5 Incubation period

Incubation time is a very sensitive factor in enzyme production. Inducible enzymes are usually released relatively quickly into the culture medium and the constitutive enzymes are made at a steady rate. Constitutive enzymes, however, may hinder most inducible enzymes after brief incubation. This interaction suggests that the time of incubation needs to be optimized for maximum production of the target enzymes (Cheng *et al.*, 2020). Incubation time was required to reach maximum enzyme production (Boudrant *et al.*, 2020). The L-Asparaginase produced from the *Pseudomonas pseudoalcaligenes* had a range of incubation time between 24 to 96 hrs.; bacterial culture was inoculated in nutrient broth and incubated at 37°C, rpm. Maximum L-Asparaginase production was reached by 24hrs of incubation (Boudrant *et al.*, 2020).

2.6 Purification of microbial L-asparaginase

For the measurement of physiological activity, the enzyme must also be purified and concentrated to a suitable extent. The purity of the enzyme should be high enough to allow accurate determination of the enzyme's conformation and shape. This will provide more consistent results for its function and eventual applications (Kilikian *et al.*, 2024). When these enzymes are purified, their enzymatic study as kinetic and thermodynamic parameters, as well as their reaction mechanisms, is simplified. L-asparaginase specifically is of such significance due to its anti-carcinogenic and other medicinal properties.

Knowledge of these aspects may render it further usable therapeutically and effective against different disorders (Muneer *et al.*, 2020). An enzyme has to be purified to create vital formulations for industrial and medical purposes (Meghwanshi *et al.*, 2020). It is also possible to purify L-asparaginase from crude microbial extracts, with slight modifications according to whether the origin is bacteria or fungus. The purification of an enzyme generally represents a series of steps based on the biological and physicochemical characteristics of the enzyme in order to use it effectively for therapeutic applications (Meghwanshi *et al.*, 2020).

It is necessary to separate (or extract) proteins when purifying proteins of interest from biological samples: living organisms can only produce proteins. It is desired that this protein of interest be separated from non-protein materials as well as irrelevant proteins. Enzyme separation is the essential step through the activity and structure of enzyme to overnight the isolated enzyme competes for medicinal and clinical uses (Meghwanshi *et al.*, 2020). For example, asparaginase from *Pisum sativum* was purified about 1328-fold with the 1 % activity recovery by anion exchange chromatography with LH 20 and DEAE-Sephacel, and gel filtration chromatography using Sepharose and Sephacryl S-200. The molecular weight of asparaginase was estimated to be 69,000 Daltons by Sephacryl S-200 chromatography and native SDS-PAGE in relation to BSA (Cunha, 2021). (Aisha *et al.*, 2025) purified asparaginase from *Withania somnifera* by several purification steps and obtained a high specific activity of 1540 U/mg, purification fold of 13.14. Similarly, (El-Naggar & El-Shweihy, 2020) showed that asparaginase generated by *Streptomyces* sp. strain PDK2 could be purified by dialysis, precipitation of ammonium sulfate (45–85%), and gel filtration used Sephadex G-

50 and G-200. This purification produced a yield of just 2.18% and a maximal specific activity from 63.0 U/mg, with an 82.9-fold purification.

2.7 Pharmaceutical character of L-asparaginase

Asparaginase has gained popularity in the pharmaceutical and food industries due to its significant therapeutic applications. This enzyme is crucial in treating various cancers, including acute lymphocytic leukemia, Hodgkin disease, acute myelomonocytic leukemia, chronic lymphocytic leukemia, lymph sarcoma, reticulosarcoma, and melanomasarcoma(Parmar *et al.*, 2021). One key feature of asparaginase is that acute lymphoblastic leukemia cells, along with some other tumor cells, cannot synthesize the non-essential amino acid asparagine, while normal cells can. Consequently, leukemic cells depend on circulating asparagine, which is critical for their survival(Yuan *et al.*, 2024).

Asparaginase catalyzes the conversion of L-asparagine to aspartic acid and ammonia, effectively depriving leukemic cells of asparagine, leading to cell death (Chen & Zhang, 2024). Further research highlights the importance of asparagine for brain development and function and its role in protein function. Additionally, asparaginase has applications in biosensors; an Indian team developed a novel diagnostic biosensor to detect L-asparagine in leukemia cells using this enzyme(Nunes *et al.*, 2021). However, asparaginase can also lead to coagulopathy by decreasing protein synthesis, including coagulation factors, which may result in bleeding or thrombotic events. Advances in biotechnology have enabled the production of recombinant, immobilized, and modified asparaginase from microorganisms, enhancing its activity compared to wild-type sources (Wang *et al.*, 2021). In the food industry, asparaginase is used to reduce acrylamide levels in

bread by degrading L-asparagine, the precursor to acrylamide, prior to baking(Tafuri, 2023).

2.8 L-asparaginase characterization

2.8.1 pH effects on L-asparaginase activity and stability

The optimum pH for asparaginase activity and stability is crucial, as enzymes are proteins sensitive to pH changes. Each enzyme has a specific pH range where it operates most effectively, influenced by several factors:(Lukić *et al.*, 2021): (1) Binding of the enzyme to substrate. (2) Catalytic activity of the enzyme. (3) Ionization of the substrate. (4) Variation of protein structure. The detection of optimum pH for enzyme stability consider a necessary task to provide a suitable environment for enzyme storage (Morellon-Sterling *et al.*, 2022).

The optimum pH of 8.5 for asparaginase derived from *Withania somnifera* resembles that of *Escherichia coli* (Morellon-Sterling *et al.*, 2022). Siddalingeshwara and Lingappa (2011) investigated the optimum pH of purified L-asparaginase from *Pectobacterium carotovorum* MTCC 1428, finding it to be 9.0 over a range of pH values from 4.0 to 11.0. Additionally, (Ariaeenejad *et al.*, 2023). reported an optimal pH of 8.4 for partially purified L-asparaginase from a locally isolated strain of *E. coli*. Furthermore, L-asparaginase isolated from *Enterobacter cloacae* also exhibited an optimum pH of 8.5(SALAMI, 2021). In another study, maximum enzyme activity for L-asparaginase produced from bacterial soil was recorded at pH 8 (Haji Mirghasemi *et al.*, 2023).

For at least 24 hours of incubation, the purified L-asparaginase maintained 100% activity in this pH. On the other hand, after 24 hours, *Trichoderma birdie's* L-asparaginase maintained 82% of its activity (Elshafei & El-Ghonemy, 2021), while the enzyme from *Mucor hiemalis* maintained stability for only 4 hours

before activity began to decline (Hameed *et al.*, 2020). Notably, L-asparaginase of *Fusarium culmorum* ASP-87 is a viable candidate for therapeutic usage due to its stability at alkaline pH(El-Gendy *et al.*, 2021).

2.8.2 Temperature effects on L-asparaginase activity and stability

Most enzymes in nature are characterized by their susceptibility to high temperatures, which can impact their secondary, tertiary, and quaternary structures, leading to denaturation and loss of activity (Selvaraj *et al.*, 2022). Functional studies of enzyme temperature-related properties have established the concept of an "enzyme temperature optimum," which arises from a complex interplay between activity and thermal stability, influenced by assay duration. As a result, measuring enzyme temperature adaptation may have limited significance (Kroll, 2020).

The stability of asparaginase from *S. aureus* was maximum at 40°C and further deduction showed inactivity and on the rise in temperature above 50°C for two hours, and showed deactivated the protein after 2 hours of incubation(Zhang *et al.*, 2020). Explain the enzyme's temperature characteristics. For native enzymes, the maximal activity occurs at 50°C. According to (Bhosale *et al.*, 2022) the bacterial L-asparaginase, showing maximum activity under 37°C at optimized conditions. In general, other studies have been mentioned that most asparaginases reach maximum activity at 37°C(Muneer *et al.*, 2020). According to reports, *Penicillium brevicompactum* NRC 829's L-asparaginase can remain stable at 37°C for up to an hour(Job *et al.*, 2025). L-asparaginase of *Fusarium culmorum* ASP 87, in contrast, exhibits considerable thermostability, which is important for its possible uses in the pharmaceutical sector. This thermal stability could enhance its effectiveness as a therapeutic agent (Al Yousef, 2022).

2.8.3 Metal Ion Effect

Metal ions are essential for the catalytic action of certain enzymes, playing a critical role in various biochemical processes. They contribute to catalysis by attracting or donating electrons, facilitating substrate binding through coordination links, and stabilizing the enzyme's tertiary and quaternary structures. In metallo enzymes, the removal of the metal component typically results in a loss of enzymatic activity. Examples of such enzymes include: Catalase, Peroxidase and Cytochrome C. Additionally, magnesium is necessary for enzymes that utilize ATP as a cofactor. The active form of ATP is the ATP-Mg²⁺ complex, where Mg²⁺ binds to the negatively charged phosphate group of ATP, enhancing its reactivity and stability (Maake, 2022). The effects of various metal ions on enzyme activity were assessed by preincubating the enzyme alone with 10 mM metal ions for 15 minutes before adding the substrate. In this context, the enzyme's activity measured in the absence of metal ions was set at 100%. This approach allows for the evaluation of how different metal ions influence enzymatic activity compared to the baseline established without these ions (Gulcin & Alwasel, 2022).

2.9 Cancer

Cancer is a difficult disease that has the potential to spread to the body's other organs as well as spread locally. In the next 20 to 40 years, it is expected that the number of cancer cases would more than quadruple globally, and the primary cause of death to surpass heart disease (ReFaey *et al.*, 2021). Additionally, a growing issue in an aging population is the treatment of cancer which is crucial in emerging nations (Lemij *et al.*, 2023). By the year 2030, the International Agency for Research on Cancer (IARC) estimates that there will be 26 million new instances of cancer and 17 million cancer-related deaths (Bizuyehu *et al.*, 2024).

As a result of population growth, aging, an increase in the frequency of unhealthy behaviors, and particular risk factors like smoking, cancer incidence rates have been rising in the majority of countries since 1990. This constitutes an increasing threat to public health on a global scale(Salam *et al.*, 2023). 17.5 million new cases of cancer have been reported globally since 2005, a 33 percent rise (Workie *et al.*, 2023).

2.9.1 Risk factors for cancer

In Iraq, the frequency of cancer risk factors, including diabetes, obesity, smoking and poor diet, has recently increased without enough preventative measures being implemented. Iraq has high incidence rates for bladder, lung, and breast cancer in addition to the rising frequency of many other cancers (Hussain & Lafta, 2021). With the aid of accurate and quantitative assessments of the burden of cancer, policymakers and health management may more effectively prioritize diseases and distribute resources. If the time of the deployment of a control measure is indicated, they can also establish what factors contributed to a drop or rise in the incidence of cancer and how this affected the illness rate (Ji *et al.*, 2023).

2.9.2 Exploring the Mechanisms and Treatments of Cancer

The exploration of cancer mechanisms and treatments has garnered significant attention in recent years, particularly in understanding the intricate biological processes that underpin tumor development and metastasis(Eryılmaz *et al.*, 2025). offer a thorough review that brings out the complexity of cancer genesis, underlining the crucial importance of cell adhesion and metastasis(Yayan *et al.*, 2024). Their findings explain how alterations in adhesion pathways provide the tumor cells with a means to override standard cellular constraints and elongate, thereby enhancing their invasive potential. The authors discuss the significant

adhesion molecules that facilitate cancer cell adhesion to the ECM including integrins, cadherins and selectins. In addition to promoting tumor growth & local invasion, this crosstalk prepares cancer cells to be amenable to metastasis, which is a critical stage in the progression of the disease(Liu *et al.*, 2024), (Yayan *et al.*, 2024)

The authors illustrate the ways in which these same interactions enable not only local tumor growth, but also the spread of cancer to distant metastatic sites. The reviewers present a detailed description of these adhesion molecules as well as potential therapeutic strategies that target blockage, which eventually might improve patient survival.

Overall, in the context of the mechanisms of cancer, the literature clearly indicates that it is important to understand the relationship between cellular adhesion and metastasis. The results shown by (Shi *et al.*, 2024) are also significant on an philosophical-emergent (sic) level because they may open the gate for novel therapies that will change the face of cancer treatment again also for oncology patients.

2.9.3 Skin cancer

The body of literature concerning skin cancer suggests an increasing awareness of the problem of skin cancer and the five modes of cancer prevention on the part of target populations. In the study by (OKUYAN, 2023), the authors discuss the complications of melanoma, the most life-threatening skin cancer especially in young people which women under the age of 40 are the most vulnerable on suffering from it SENT. They highlight the critical importance of early detection and the role of risk factors such as sun exposure and genetic predisposition. The review underscores the necessity of increasing awareness about warning signs of melanoma to ensure timely intervention, as early-stage melanoma is often curable. The authors also discuss the anatomical prevalence of melanoma, noting common areas such as the legs, neck, and face, and emphasize that individuals with fair skin are at a heightened risk. This foundational understanding of melanoma sets the stage for subsequent research on preventative measures(Trager *et al.*, 2022).

Building on this knowledge, (Thoonen *et al.*, 2020) explore the effectiveness of environmental interventions aimed at preventing skin cancer among children and adolescents. Their systematic review evaluates various strategies, particularly those promoting sun protection behaviors and reducing ultraviolet radiation (UVR) exposure. The authors find that while interventions such as free sunscreen distribution have been implemented, their effectiveness remains inconsistent. Notably, evidence supporting shade provision as a significant factor in encouraging shade-seeking behavior emerges as a key finding(Roky *et al.*, 2025) . This study highlights the urgent need for effective environmental strategies in light of the alarming rise in melanoma and non-melanoma skin cancer (NMSC) incidences, particularly since the early 1980s. The authors point out that early childhood sun

exposure and sunburns are critical risk factors for developing melanoma later in life, thereby reinforcing the importance of preventive measures targeting younger demographics(Djavid *et al.*, 2021). Together, these articles illustrate a comprehensive view of skin cancer, from understanding its pathophysiology and risk factors to evaluating preventative interventions. They collectively emphasize the need for a multifaceted approach to combat the rising incidence of skin cancer, particularly through early detection and effective environmental strategies aimed at vulnerable populations(Kadry *et al.*, 2024).

2.9.4 Liver cancer

Males are over twice as likely as females to have liver cancer worldwide. While South-Central as well as Western Asia, as well as Northern and Eastern Europe, have lower rates, Southeast and East Asia, as well as Middle & Western Africa, have the highest rates (Mohammadian & Mohammadian-Hafshejani, 2020). Variations in exposures to environmental pathogens and hepatitis viruses are reflected in these regional variances in the prevalence of liver cancer. About 60% to liver cancer cases in developing nations are caused by a hepatitis B virus, or HBV, infection, whereas roughly 33% are caused by a hepatitis C virus, (HCV), infection(Amponsah-Dacosta, 2021).

The obesity epidemic as well as rising transmission of HCV rates, especially among injecting drug users, are probably to blame for the rising prevalence of liver cancer in the US and Central Europe(McGlynn *et al.*, 2021). The bulk of liver cancer cases in the USA as well as other low-risk Western nations are thought to be caused by alcohol-related cirrhosis & nonalcoholic fatty liver, both of which are linked to obesity(Chrysavgis *et al.*, 2022). In contrast, certain traditionally high-risk locations have seen a decline in liver cancer incidence rates, possibly as a

result of the HBV vaccine (Roberts *et al.*, 2021). For instance, Taiwan's 1984 universal newborn hepatitis immunization program has dramatically decreased the incidence of liver cancer in children ages 6 to 19, with those who received the vaccination at birth having an age- & sex-adjusted absolute risk of 0.31(Garcia, 2023). For individuals who do not respond to Sorafenib treatment, there is currently no conventional second-line chemotherapeutic drug. Since advanced carcinoma of the liver (HCC) is typically thought of as a tumor that is resistant to chemotherapy, chemotherapy hasn't been utilized frequently for individuals with this type of cancer.

This dearth of viable alternatives emphasizes the necessity of innovative therapeutic approaches and medications to enhance these patients' results(Shannon *et al.*, 2022), Most individuals with severe underlying hepatic impairment do not tolerate systemic chemotherapy effectively. Some people, especially those with non-cirrhotic livers, may still benefit from it, though. Chemotherapy may be a viable therapeutic option for certain individuals with advanced liver cancer in these situations because the possible advantages may exceed the dangers(Calistri *et al.*, 2021).

For advanced forms of hepatocellular carcinoma (HCC), doxorubicin has been the most researched single-agent chemotherapeutic drug available. At dosages of 75 mg/m², it has demonstrated an objective response rate of 20% or less(Sasaki *et al.*, 2022). To increase efficacy, several combined chemotherapy regimes have been tried, but the outcomes have typically been unsatisfactory(Bailly *et al.*, 2020). Generally speaking, only medically qualified patients with sufficient liver function should receive cytotoxic chemotherapy(Hallemeier *et al.*, 2023). The literature surrounding liver cancer reveals a pressing public health issue, particularly as the global incidence is

projected to surpass one million cases by 2025. The article by (Yang *et al.*, 2020) provides a comprehensive bibliometric and visual analysis of the risk factors associated with primary liver cancer (PLC), emphasizing the importance of understanding these factors in order to effectively screen high-risk individuals and implement preventive measures.

The authors describe chronic hepatitis B and C infections, alcoholism, non alcoholic fatty liver disease, and aflatoxin as the main identified risk factors for hepatocellular carcinoma (HCC). They also raise smoking and diabetes as risk factors that should be investigated further (Durlach *et al.*, 2022).

The revelations from (Yang *et al.*, 2020), and highlight the urgent need for population-based strategies which can focus attention on screening and prevention, particularly in vulnerable populations. Reading further into the literature, we see that not only is it crucial to tackle these risk factors for prevention of liver cancer, but for treatment, should (and we believe will) come from understanding the disease's etiology in more nuanced detail ever that will inform potential treatment modalities (Wang *et al.*, 2021).

2.10 Cell line

Investigation of cell lines is an important part of biomedical research, specifically in the field of cancer research, wherein establishment and description of these lines can strongly impact the report of the experimental results. (Zuñiga Martinez *et al.*, 2022), where the authors give a general overview of the development, authentication, and characterization process of cervical cancer cell lines. Ethical issues that researchers must adhere to and the need for informed consent from biological material donors and approved by research ethics committees (Gefenas *et al.*, 2022) are also stressed in this article. These ethical

frameworks must be upheld if scientific research is to remain credible and accountable. Technological advances have also been emphasized in the article, as they allow investigators to use more sophisticated techniques to confirm the authenticity and growth properties of cell lines. The implications of these technology advances are considerable, increasing confidence in experimental data generated from cell lines. Henrik Larsson *et al.* (2020) provided an extremely comprehensive review of the methods and ethics behind cervical cancer cell lines and paved the way for further work in this critical area. The authors highlight several limitations of pre-clinical oncology models which rely on cancer cell lines which do not appear to mimic human tumors often. They also argue that these models do not accurately replicate the tumor microenvironment, and the variations in results can be attributed to heterogeneity in cancer cell lines.

This diversity is further compounded by the influence of culture conditions and passage history of cell lines, and can lead to large fluctuations in genetic content, transcriptomic profile and drug response in commonly used cell lines(Larsson *et al.*, 2020). Furthermore, the authors advocate for patient-derived biopsies to capture a more advancing time-course view of tumor evolutionary status. But they acknowledge that these tissue samples still offer only a partial picture of the life history of the tumor. In order to address these issues, they advocated for development of robust experimental models to better reflect the complexity of cancer initiation, progression, and metastasis (Sajjad *et al.*, 2021).

2.10.1 Exploring the Relationship Between Skin Cancer and Cell Line

Human skin derived cell lines provide indispensable resources in dermatological research, which offer invaluable data and knowledge to hard work

on the biology of human skin and various skin diseases. The complex multi-layered structure of human skin which is mainly involving keratinocytes, fibroblasts and other important types of cells, presents limitations that hinder the full investigation of skin functions and diseases patterns(Kamil *et al.*, 2024). Advances in cell culture technology, including the development of three-dimensional (3D) cell culture models, have recently led to improved quality of models that more closely replicate the microanatomy (structure) of human tissue such as skin (Hoyle *et al.*, 2022).

This advance is key to supporting researchers with the production of reliable and reproducible skin equivalents that can then be used for a variety of applications in clinical and cosmetic research industry(Roger *et al.*, 2019). As they faithfully recapitulate some of the layered architecture of the stratified epithelium and cell differentiation, 3D skin tissue model has been widely employed to study not only skin biology, but also to test the safety and efficacy of cosmetic products, especially after the ban on animal testing 4,5,6) The investigation of cancer biology through the prism of cell lines has received increased attention in the past few years with a particular interest in the context of the study of enigmatic diseases such as cancer of skin (Figure 2-6).

In a pivotal study, (Hynds *et al.*, 2018) delve into the genetic and epigenetic diversity that exists both between and within patient tumors, illuminating the intricate landscape of cancer research. They argue that the pre-clinical oncology pipeline, which heavily relies on human cancer model systems includin and colleagues, reveal that widely used cancer cell lines exhibit significant heterogeneity in genetics and therapeutic responses. This variability underscores the importance of recognizing that cell lines are not static entities; rather, they evolve over time due to ongoing mutational processes and chromosomal

instability. Such dynamics can lead to divergent experimental outcomes, raising critical questions about the reliability of these models in recapitulating the complexities of human cancer. g cell lines, is critical for studying tumor biology and evaluating therapeutic efficacy. testing in the European Union. Among the notable(Cini & Borragán, 2022).

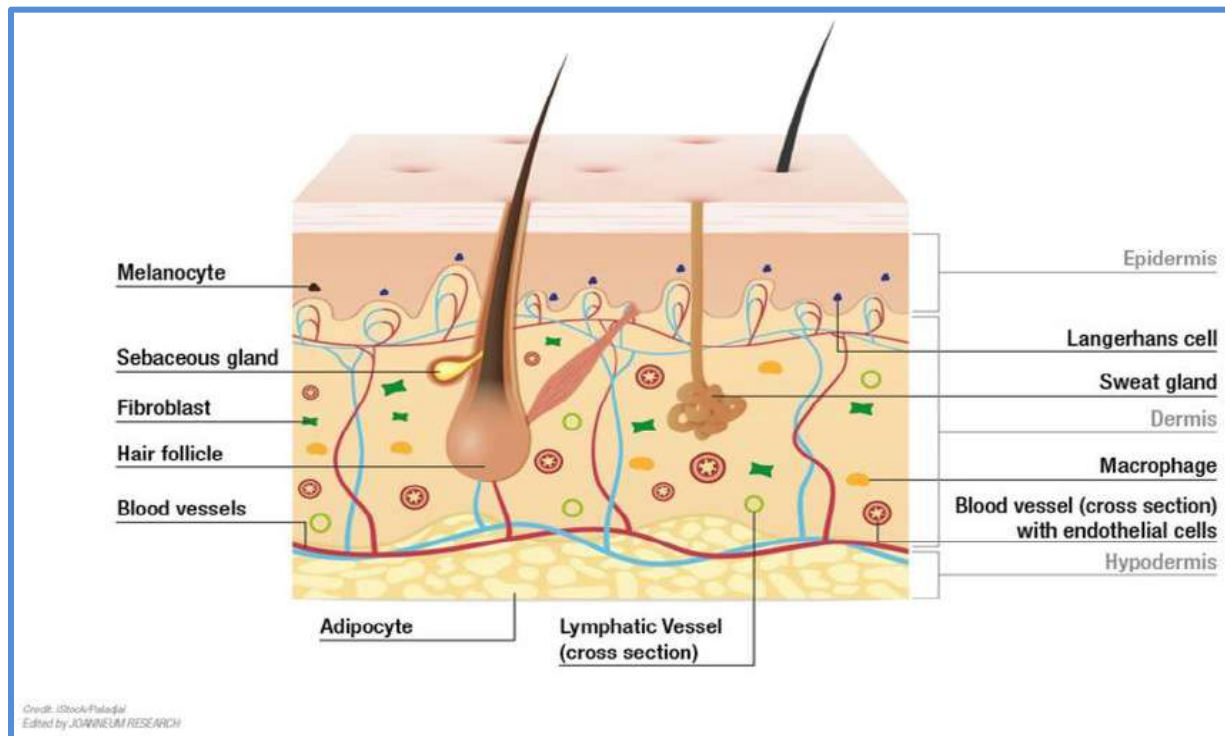


Figure (2-6): Cross section of skin (Alsharabasy, 2015)

The epidermis primarily consists of keratinocytes, along with non-epithelial cells such as Langerhans cells, melanocytes, and Merkel cells. Beneath the epidermis, the dermis contains skin appendages—including sweat glands, sebaceous glands, and hair follicles—as well as blood vessels and nerve endings(Vargas, 2021).

Chapter three

Materials and Methods

Design of study

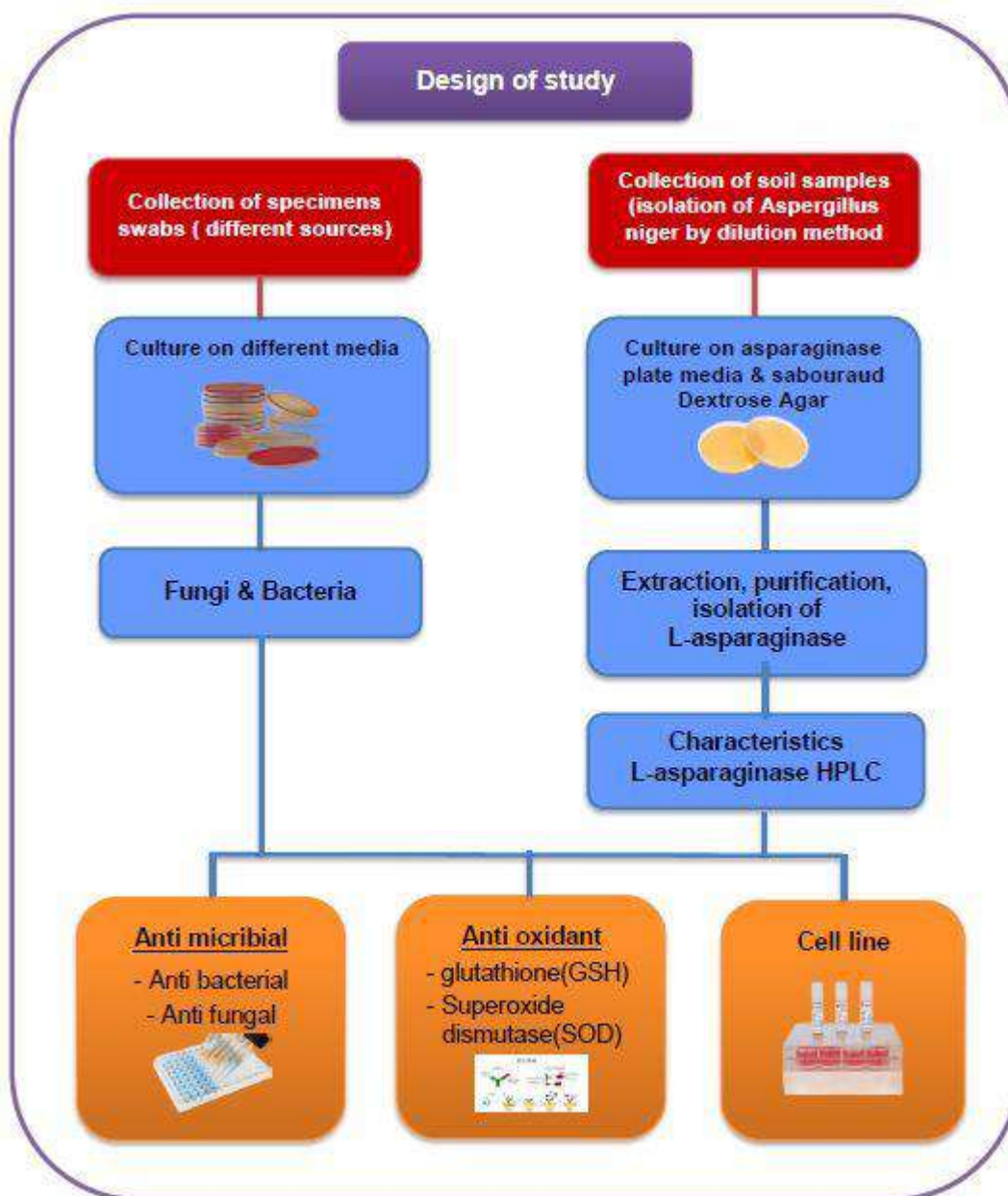


Figure (3-1): has shown the subjects groups for the present study

3- Material and Methods

3.1 Materials

3.1.1 Equipment and apparatus

The following tools and devices were utilized during the study, as shown in the (Table 3-1).

Table (3-1): Equipment and apparatus used in this study

Equipment and apparatus	Company	Origin
Autoclave	Hirayama Co.	Japan
Balance	A.N.D.	Japan
Biological Hood	CLASS II-A2	Turkey
Centrifuge	Gemmy	Germany
CO2 Incubator	Gallenkamp	England
Dialysis Tubes	Hi-media	India
Flame Burner	Rashmi Scientific	India
Incubator	Memmert	Germany
Magnetic stirrer and hot plate	Scientific Industries	Mumbai
Micropipettes	Volac	Germany
Microscope	Novex	Holland
Millipore filter units	Gema medical	Spain
pH-meter	Crimson	USA
Refrigerator	Koncored	Koria
Sensitive balance	Sartorius	Germany
Shaker incubator	Adolf Kuner	Germany
UV-VIS spectrophotometer	Apel	Germany
Vacuum pump	FED-REP	Germany
Vortex	Scientific industries	Japan
Water bath	Gemmy	Taiwan
Water Distiller	G.L.F.	Germany

3.1.2-Chemical and biological materials

The following chemicals, and biological material that were used throughout this study are listed in (Table 3-2).

Table (3-2):Chemicals, and biological material used in Study

Chemicals & Biological Material	Manufacturing Company	Origins
Coomassie blueG-250	Fluka	Switzerland
Ferric Sulfate (FeSO_4),	BDH	England
Ammonium sulfate,	BDH	England
Manganese Sulfate (MnSO_4),	BDH	England
Mercuric chloride (HgCl_2),	BDH	England
Calcium Chloride (CaCl_2),	BDH	England
Potassium Chloride (KCl),	BDH	England
Zinc Sulphate (ZnSO_4),	BDH	England
Copper Sulphate (CuSO_4),	BDH	England
Glycerol	Oxoid	England
L-asparagine	Pharmacia	Sweden
Normal saline solution	Inventor	Iraq
Phenol cotton blue stain	CDH	India
Phenol red	Buchs SG	Switzerland
Potassium hydroxide (KOH), ethyl alcohol ,	ROMIL purechem	UK

Sodium Di- hydrogen phosphate (NaH ₂ PO ₄),	Merck	Germany
Di Sodium hydrogen phosphate (Na ₂ HPO ₄),	Merck	Germany
Potassium Di hydrogen phosphate (KH ₂ PO ₄),	Merck	Germany
Di-Potassium Hydro Phosphate (K ₂ HPO ₄),	Merck	Germany
Hydrogen peroxide (H ₂ O ₂)	Merck	Germany
Sodium hydroxide,	Sigma	USA
Tris-HCl ,	Sigma	USA
Tris- base,	Sigma	USA
Bovine Serum Albumin,	Sigma	USA
Gram Stain Kit	Sigma	USA
Sodium hypochlorite	Fas	Iraq

3.1.3 Kits Table

Table (3-3): Contents of the Kit Package Used in Study.

Kits	Company	Origin
enzyme-linked immunosorbent assay (ELISA)	Bio tek	U.S.A
Vitek 2 compact	Bio Merieux	France
High-Performance Liquid chromatography (HPLC)	Vicam	Germany

3.1.4. Media

All of the culture media used in this study to find and identify bacteria and fungi are shown in (Table 3-4).

Table (3-4): List of diagnostic media used in this study

Culture Media	Company	Origin
Blood agar base (BA)	Hi-media	India
Muller-Hinton Broth		
MacConkey agar		
Nutrient agar		
Nutrient broth		
Sabouraud Dextrose Agar		
Agar-agar		

3-2-Methods

3.2. 1 Preparation of Media

3.2.1.1 Culture media

Each medium utilized in this investigation was prepared in accordance with the manufacturer's instructions and autoclaved for 15 minutes at 15 psi to sterilize it. The medium of agar was cooled to 45–50°C, then poured into sterile petri dishes & incubated at 37°C for 24 hours. After that, the media were kept at 4°C in the refrigerator.

3. 2. 1.2 Asparagine Medium for primary screening (Sharma *et al.*, 2017)

The following ingredients were dissolved in 950 milliliters of distilled water to create the L- asparaginase plate media. 10 grams of asparagine, 2 grams of glucose, 1.52 grams of KH_2PO_4 , 0.52 grams of KCl , 0.52 grams of MgSO_4 , 0.03 grams of FeSO_4 , 0.03 grams of ZnSO_4 , 0.05 grams of CuSO_4 , and 20 grams of agar were added. The volume was next added to 1 liter, properly mixed, then the pH was set. to 7.0. The solution was autoclaved to 15 minutes in 15 pressure and 121°C to sterilize it. 0.09 grams of phenol red was then added to the filtered, sterilized medium once it had cooled to 45–50°C. The mixture was dispensed into sterilized petri dishes and incubated for 24 hours at 37°C before being stored at 4°C in the refrigerator.

3.2.2- Sterilization techniques**1. Auto cleaving (wet heating)**

It sterilized buffers , solutions and culture media at 121°C for 15 min in autoclave, which was cooled to room temperature.

2. Sterilization by dry heat

Glassware and other objects were sanitized for two hours at 180 degrees Celsius in an electric oven

3. Filtration (membrane sterilization).

After dissolving Phenol red in the D.W. a Millipore filter unit were used to ensure they were clean and safe.

3.2.3 Maintenance of fungi isolates (Talukdar et al., 2020)

The fungi isolates were grown in special 20 ml bottles filled with Saproduct Dextrose Agar medium at an angle, and placed in the refrigerator at 4°C. For two months after that, they were re-cultivated on Saproduct Dextrose Agar medium in a Petri dish, and the process was repeated again.

3.2.4. maintenance of bacteria isolates (Tuttle *et al.*, 2021)

The maintenance of bacterial isolates was conducted using the following modified methods:

- **Short-Time Maintenance**

The bacterial isolate was grown on a nutrient agar plate for 24 hours at 37°C. After that, the plates were parafilm-sealed and kept at 4°C in a refrigerator until when they were needed. Every four weeks, the chosen isolate was subcultured to maintain it.

- **Mid- Time Maintenance**

One colony of the bacterial isolate was cultivated on the nutrient-agar slant. The tubes were kept for between one and three months after being incubated for 24 hours at 37°C.

- **Long-Time Maintenance (15%) Glycerol**

The isolates of bacteria were grown in nutrient broth for 24 hours at 37°C. After incubation, 0.5 ml of the culture was transferred to an Eppendorf tube and well combined with 0.5 ml from sterile glycerol. The tubes were parafilm-sealed and stored in a freezer at -20°C.

3.2.5 Solutions and Stains

3.2.5.1 Lacto phenol-cotton blue

To facilitate the fungi's microscopic visualization. (Ajayi & Olatunji, 2019)

Phenol crystals 20g (Heat the phenol crystals until they dissolve, then add the remaining ingredients.

Lactic acid 20ml

Glycerol 40ml

Distilled water 20ml

3.2.5.2 Gram staining (Tripathi & Sapra, 2020)

1. **Primary Stain:** Crystal violet (1%)
2. **Mordant:** Gram's iodine solution
3. **Decolorizer:** Acetone-alcohol (or 95% ethanol)
4. **Counterstain:** Safranin (0.5%) or carbol fuchsin

3.2.6 Fungal strain

Aspergillus niger, SAP-1 By using the dilution method, it was isolated from soil. Using the micro and macro characters mentioned in (Barwant & Lavhate, 2020) the isolates were identification. At 28°C, this fungus was kept on potato dextrose agar (PDA).

3.2.6.1.1 Dilution method

One gram of solid was suspended in 9 ml of distilled water in a sterile tube, and the mixture was stirred for 20 min. Serial dilutions were prepared at 10^{-2} , 10^{-3} , 10^{-4} , and 10^{-5} . The tube were then incubated for three days at the dark at 28°C (Chauhan *et al.*, 2020).

3.2.7 Screening of *Aspergillus niger* isolated for L-Asparaginase production

3.2.7.1 Primary Screening of Asparagine production((Qualitative method-solid medium)

Asparaginase production was qualitatively screened using the procedure outlined by (El-Naggar & El-Shweihi, 2020), with some modifications. The specifics of the modifications can be detailed further if needed. involves Phenol red serves as a pH indicator and 0.5% L-asparagine solution, used as the sole source of both carbon and nitrogen A change in the pH of the medium causes its color to transition from yellow to pink, which indicates that the colony is producing extracellular L-asparaginase., since L-asparaginase disassembles the amide bond. bond in L-asparagine and releases ammonia. With very little agar medium, the fungal isolate was streaked in small petri dishes. Following two Upon incubation at 37°C, the plates became pink, signifying a successful reaction (Freitas *et al.*, 2021).

3.2.7.2-Quantitative secondary screening (Quantitative screening)

A. niger SAP-1 screening for the the L asparaginase manufacturing process by prepared submerged medium in accordance with (10 gram asparagine, 2 gram glucose, 1.52 g (KH₂PO₄), 0.52 gram (KCl), 0.52 gram (Mg SO₄), 0.03 gram (FeSO₄), 0.03 gram (Zn SO₄), and 0.05 gram (Cu SO₄)) were dissolved in 950 L of distilled water to create the asparaginase plate medium (g/L). The volume was then filled to 1 L, mixed well, and the pH was brought down to 7.0.. Finally, the media was autoclaved at 121°C for 15 minutes at 15 psi to sterilize it. After transferring 50 ml of media into a 250 ml Erlenmeyer conical flask, 2% (3x10¹¹ cells/mL) was added as an 5 days culture. The flasks were then placed in a rotary shakers incubator that was placed to 150 rpm and 37 °C.

Each flask's culture was incubated for 7 days before being centrifuged for ten minutes at 10,000 rpm. The asparaginase activity was measured using the clear supernatant as a crude enzyme (El-Gendy *et al.*, 2021).

3.2.8 Determination of L-asparaginase activity

The method described by (Lenicek Krleza *et al.*, 2024) was employed to measure the L-asparaginase enzyme's activity, as indicated below:

3.2.8.1 Buffers, solutions and reagents

3.2.8.1.1 Nessler's Reagent

A solution was prepared by mixing 4 grams of NaOH in 20 mL of water, 6 grams of KI in 10 mL of water, and 2 grams of HgCl₂ in 40 mL of water. Then we added water to make a total of 100 mL (Varghese *et al.*, 2023).

3.2.8.1.2 Potassium Phosphate Buffer (0.05 M)

Two solutions were combined to create it. To make Solution A, potassium dihydrogen phosphate (0.05 M), 0.68 grams of KH₂PO₄ were dissolved in 100 milliliters of purified water. To make Solution B, K₂HPO₄ (0.05 M), 0.87 g of K₂HPO₄ was dissolved in 100 mL of D.W. To bring the pH of this buffer to [8.0].94.7 ml of solution B was mixed with (5.3) ml of solution A. Asparagine is prepared as a substrate using this buffer(Varghese *et al.*, 2023).

3.2.8.1.3 Solution of L-asparagine (200 mM, pH: 8.0)

A 2.64 gram L-asparagine solution was prepared in 100 mL (KH₂PO₄) buffer (from 3.2.8.1.2), filtered, and kept at 4°C(Yang *et al.*, 2020).

3.2.8.1.4 Trichloroacetic Acid Solution (1.5 M)

A solution of trichloroacetic acid was prepared by dissolve 24.5 grams of trichloroacetic acid in 100 milliliters of D.W. The solution was completely mixed until complete dissolution(Nouri, & Khaleghian, 2020)

3.2.9 -Preparation of NH₄Cl standard curve(A. Wang et al., 2025)

In order to construct a standard curve of ammonium chloride for an asparaginase assay, serial dilutions ranging from 100 to 500 micromolar were prepared from a 0.5 millimolar stock solution of ammonium chloride. One milliliter aliquots of each dilution were added to one milliliter of enzyme supernatant and incubated at 37 degrees Celsius for thirty-minutes. A wavelength for 625 nanometers was then used to measure each sample's absorbance. A blank sample was prepared by adding one milliliter of Nessler's reagent to one milliliter of distilled water in lieu of the enzyme supernatant. A standard curve was generated by plotting the concentration of ammonium chloride in micromolar units against the corresponding absorbance at 625 nanometers, as depicted in (Figure 3-1).

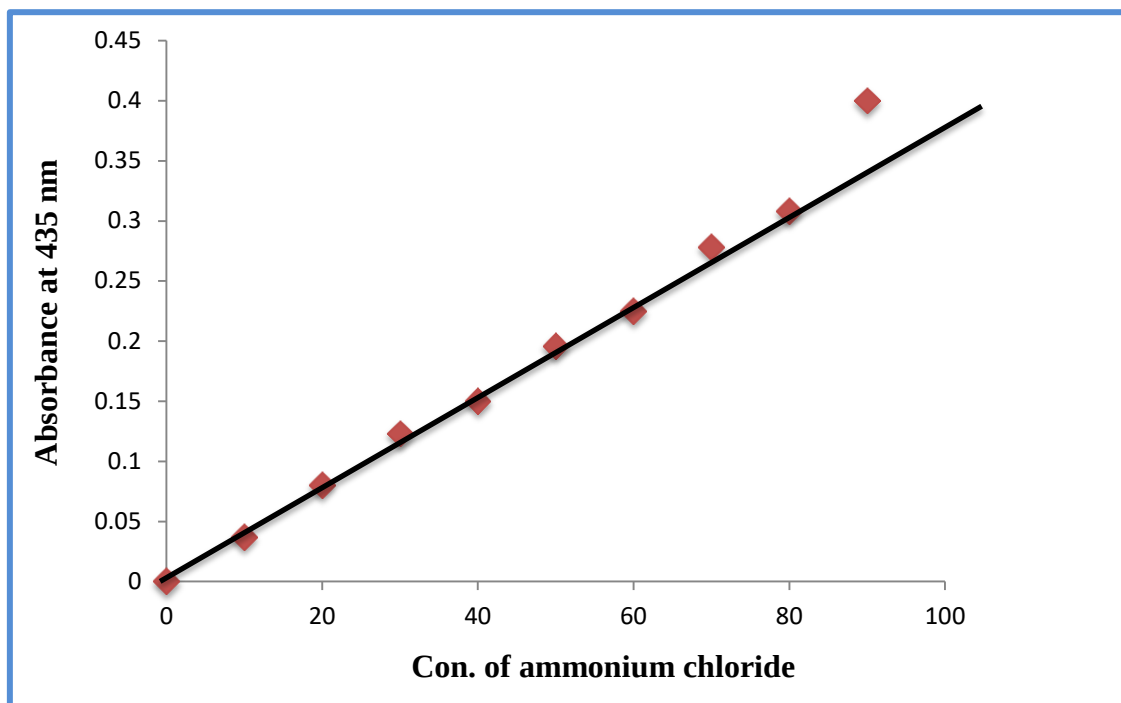


Figure (3-2): A standard curve for ammonium chloride is used to determine the concentration of ammonium ions (NH_4^+) in a sample

3.2.10 Asparaginase activity measurement

Asparaginase activity was assessed using the Nesslerization process, which relies on the transformation of L-asparagine into L-aspartate and ammonia, with a maximum absorption at 625 nm, as per(Chand *et al.*, 2020), with some modifications.

- One milliliter of crude L-ASNase was added to the test tube, then 1 ml of L-asparagine prepared in (3.2.8.1.3) was then added with gentle mixing, thereafter placed in incubator at 37°C for 30 min.
- After incubation, 1ml of trichloroacetic acid prepared in (3.2.8.1.4) was applied to the solution mixture to stop reaction. The mixture was placed in centrifuge 10,000 rpm for 10 minutes.
- L-ASNase activity was measured within the supernatant using the

direct Nesslerization process prepared in (3.2.8.1.1) for each sample by mixing 1ml of Nessler reagent and 1ml of enzyme supernatant with well shaking.

- Then the mixture was placed in incubator at 37°C for 30 min. The optical density was measured at 625nm.
- For preparation of blank, 1 ml of Nessler reagent was added to 1 ml of reaction mixture above except add 1ml of trichloroacetic acid before adding 1ml of the crude enzyme. One L-ASNase unit was defined as the enzyme amount that releases 1μmol of ammonia per minute at the experimental conditions according to below formula:

$$\text{Enzyme activity (U/ml)} = \frac{Ab}{\text{Slope}} / (T \times C)$$

Where:

$\frac{Ab}{\text{slope}}$: is the Concentration of ammonia,

T: is the Time of reaction, 30 min.

C: is the Constant, (14)

Ab: Absorption

3.2.11 Determination of protein concentration

The Bradford method was employed to determine the protein concentration, and the results are as follows: -

3.2.11.1 Buffers and solutions

3.2.11.1.1 Tris-HCL buffer 0.02 M, pH 8.0 (Wang et al., 2022)

0.3 grams of Tris-HCl was dissolved in 90 mL from distilled water to create the buffer. After adjusting the pH to 8.0, distilled water was added to the final volume to reach 100 mL.

3.2.11.1.2 Coomassie Brilliant-Blue G-250 solution

The dye was made by mixing 0.1 grams of powdered Coomassie blue with 50 milliliters from 95% ethanol. Stirring on ice, 100 mL in 85% (phosphoric acid) was then added. Distilled water was used to get the volume down to 1000 mL. Before being used, the stain was kept in a dark container in 4°C after being thrice filtered with Whatman filtering paper No.1.

3.2.11.1.3 Standard Protein Solution

0.1 grams of bovine serum albumin (BSA), were dissolved to 100 milliliters from distilled water to create a stock solution with a concentration in 1 mg/mL. Then, using the method provided, this solution was utilized to create various protein concentrations starting at 0.1 , to 1.0 mg/mL for concentration-dependent protein concentration estimation

3.2.11.1.4 The Bradford assay's standard curve preparation

Using the Bradford assay technique, three duplicate serial a values (0.1-1.0) mg/ml in the (BSA) solution to stock (3.2.11.1.3) were created in order to create a BSA standard curve (Table 3-5).

$$\text{Protein concentration (mg/ml)} = \frac{\text{Ab}}{\text{Slope}} \times C$$

Where:

Ab: Absorption

C : is the Constant (0.001)

Table (3-5): Preparation of Bovine serum albumin concentrations

Dilution of enzyme	Con. 1	Con. 2	Con. 3
0	0.5	0.55	0.57
10	1.87	1.8	1.9
20	2.92	2.86	2.91
30	3.6	3.54	3.8
40	2.95	2.86	3.1
50	3.83	3.58	4.01
60	4.24	4.22	4.4
70	4.81	4.82	5.2
80	5.68	5.7	6
90	6.38	6.34	6.42

***Con: concentration**

3.2.11.2 Determination of protein concentration

- Crude enzyme (0.1 mL) was mixed with 0.4 mL of Tris-HCl buffer (as described in section 3.2.11.1.1) in a test tube.
- To each tube, 2.5 mL of Coomassie Brilliant Blue G-250 solution (prepared as per section 3.2.11.1.2) was added, and the mixture was allowed to stand at room temperature for 5 minutes.
- The blank was prepared by adding 2.5 mL of Coomassie Brilliant Blue G-250 to 0.5 mL of Tris-HCl buffer.
- The absorbance of the colorimetric products was measured at 595 nm

using a spectrophotometer

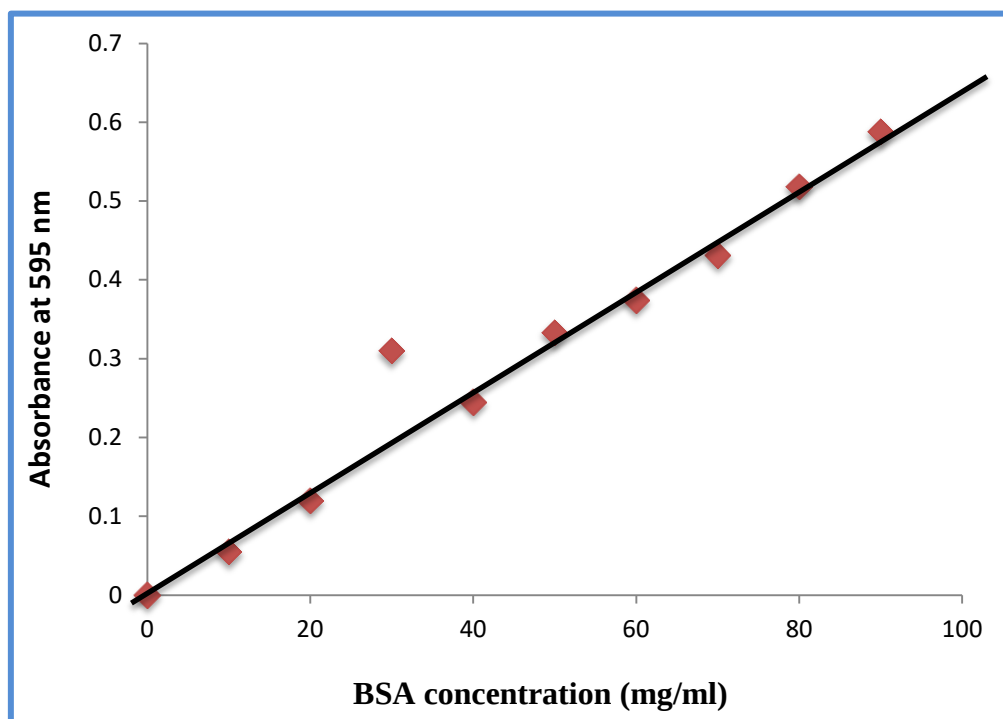


Figure (3-3): BSA standard curve performed by Bradford method

3.2.12 Determination of specific activity (Polouckova, 2021)

The following formula was used to determine an enzyme's specific activity:

$$\text{Specific activity (U/mg protein)} = \frac{\text{Enzyme activity (U/ml)}}{\text{Protein concentration (mg/ml)}}$$

3.2.13 Extraction of crude L-Asparaginase

L-Asparaginase enzyme was extracted from the selected *A. niger* eleventh isolate strain after grown in optimum production medium the cells were separated by centrifuge at 10,000 rpm at 10 min. Then the supernatant (crude L-Asparaginase) was taken to measure the enzyme activity (3.2.9), protein concentration (3.2.11) and specific activity (3.2.12) to use for further purification experiments.

3.2.14 Partial purification of L-asparaginase

L-asparaginase was isolated by gel filtration chromatography and Sephadex G-150.

3.2.14.1 Gel Filtration chromatography (Ishak *et al.*, 2021)

3.2.14.1.1 Buffer and solution

3.2.14.1.2 Tris-HCL buffer (0.05M, pH 8.0)

To create this buffer, 450 milliliters of distilled water were mixed with 3.9 grams of Tris-HCL, and the pH was adjusted to 8.0. Finish the volume to 500 ml with D.W.

3.2.14.1.3 Preparation Sephadex G-150 gel

Following the manufacturer's recommendations (Pha-Sweden), the column was prepared as well as packed. About 20 g for Sephadex G150 was dissolved in 500 mL for distilled water at 90°C and allowed to swell for three hours in gentle agitation. The gel was then placed within a cold environment in 4°C overnight. The gel was twice washed in 0.05 M Tris-HCl buffer, which was made in accordance with section(3.2.14.1.2). Following the washing, it was then resuspended in the same type of buffer, degassed by using a vacuum pump and carefully packed to a column of glass (1.6 × 21 cm). This column was then equilibrated using the same Tris-HCl buffer.

3.2.14.1.4 Separation of Enzyme through Gel Filtration G-150 Column

The crude enzyme produced in (3.2.13) were run through a (1.6 × 21) centimeter Sephadex G-150 column., using Tris-HCl buffer prepared in (3.2.14.1.2), collecting 3 mL to each portion at a flow rate that is 20 mL/h. At a wavelength of 280 nm, the protein concentration in each fraction was calculated. After measuring the enzyme activity for these fractions, effective

fractions were gathered. Enzyme activity and protein content were estimated, and the volume of every effective fraction was measured. For use in further tests, this amount was concentrated, divided up into tubes, frozen, & kept in storage.

3.2.15 HPLC analysis

Using a carrier phase made up of methanol, acetonitrile, with 5% formic acid with the ratios of 20:60:20 with a rate of flow of 1 mL/min, the procedure outlined by (Joseph *et al.*, 2022) was used. To separate the amino acids, a C18-NH₂ (250 mm × 4.6 mm) columns was used. Detection was carried out with a fluorescence detector set to excitation at 445 nm and emission at 465 nm. The amino acids were analyzed using the (Aristoy & Toldrá, 2024) software.

3.2.16 Biological properties of asparaginase

3.2.16.1 Cytotoxic Effect of Purified L-ASNase

The possible cytotoxic effects were investigated using an in vitro approach. L-asparaginase was prepared in various concentrations, and its cytotoxicity was tested on tumor cell lines, specifically HepG2 (hepatocellular carcinoma) and A375 (melanoma). The normal cell line HdFn was used as a control for comparison.

3.2.16.1.1 Cell line maintenance

This done was according to (Christian, 2022), Once the cells in the vessel formed a confluent monolayer, the following protocol was executed:

- 1- Aspirate the growth medium and wash the cell sheet with PBS.

- 2- Add 2 to 3 mL of trypsin/versine solution to the cells. Gently rock the vessel to ensure the monolayer is fully covered. Incubate at 37°C for 1 to 2 minutes, until the cells detach from the vessel.
- 3- Add fresh complete RPMI medium (15-20 mL) and disperse the cells into the growth medium by pipetting.
- 4- Redistribute the cells at the required concentration into culture vessels, flasks, or plates as needed, and incubate at 37°C in a 5% CO₂ incubator. Cell concentration was determined by counting the cells using a hemocytometer.

3.2.16.1.2 MTT Protocol (Cytotoxicity assay)

The MTT 3-(4,5-Dimethylthiazol-2-yl)-2,5-Di-phenyltetrazolium Bromide ready for use kit was used to assess the cytotoxic effects of LASNase on HepG2 Hepatocellular , A375 melanoma cell and normal cell line (HdFn).

MTT solution: 1ml×10 vials, and the solubilization solution: 50ml×2 bottles

A) Procedure

1. The HepG2 Hepatocellular , A375 melanoma cell and normal cell line (HdFn). 96 flat-well micro-titer plates were used to cultivate 1×10^4 cells mL⁻¹. Total culture media was utilized in a final volume of 200µl per well, and sterilized Para film was used to gently cover and shake the microplate gently after that incubated to 24hr, at 37°C, 5% CO₂.
2. The medium was withdrawn after incubation, and 200 µL of a 2-fold serial dilution of L-asparaginase (at concentrations of 25, 50, 100, 200, and 400 µg/mL) was added to the wells. Control cells were those that had not been treated (The cells treated with serum-free medium). Each

concentration and control were tested in triplicate. Then plate was kept at 37°C and 5% CO₂, the plates were incubated for 48hr

3. 10µl from MTT solution was added to every well after exposure. The plate was incubated for another 4hr at 37°C with 5% CO₂. The media was removed carefully, and each well-received 100µl from solubilization solution for 5 min.
4. The absorbance was calculated using an ELISA plate reader at a wavelength of 575 nm. For each cell line, the optical density data were evaluated statistically to estimate the required concentration to cause a 50% drop in cell viability to every cell line

3.2.17 Anti-microbial activities

3.2.17.1 Collection of specimens

300 specimens were collected for this study (male and female) their ages ranged between (6 to 85 years old) who suffered from different disease including (sputum, swab(Diabetic foot) dermatitis , hair and pulmonary), distributed between (115 from the dermatitis, 10 from ear, ,55from swab, 80 from sputum and 40 from pulmonary. Specimens were collected from Al-Diwaniyah Teaching Hospital and Outpatient clinics, during the periods (December 2023 – September 2024). The isolates were identified by VITK2 Compact card system.

The specimens included : sputum , swabs hair , skin and ear. The affected area was cleaned with 70% ethyl alcohol to remove dirt and environmental contaminants, such as bacteria, and allowed to dry completely. The periphery of the infection was scraped with a sterile scalpel to obtain the skin sample, while hair samples were collected using sterile scissors. The samples were

placed on folded paper to prevent bacterial contamination by keeping the specimens dry. The paper was then folded and secured with a paper clip(Wang *et al.*, 2021).

3.2.18 Mycological identification

3.2.18.1 Direct microscopic examination

The diagnosis of fungi is based on the demonstration of fungal components (branching and septate hyphae and spore) by direct microscopic inspection of the nails and skin and KOH (potassium hydroxide) mounting. The specimens were put on a clean slide flooded with drops of 10 % KOH heated at 30 °C for 5 to 10 minutes, after which the slide was allowed to cool, and the cover slip was positioned and viewed under a low (10X) and high (40X) magnification microscope(Mason & Raymond, 2022).

3.2.18.2 Cultivation of fungi

The standard medium for the isolation of the human pathogenic fungi from clinical specimens is Sabouraud Dextrose Agar (SDA) with 250 mg/l chloramphenicol to suppress the development of rapidly dividing bacteria. each specimen was incubated at 28°C. Cultures were firstly examined after 7 days, then twice weekly for at least 3-4weeks before being considered negative (Sadiq et al., 2024). The colony character, growth rate, surface and reverse color, and colony uniformity were all examined under the microscope. Originally, lacto phenol cotton blue was utilized for microscopic examination of positive fungal colonies (Putriningsih & Arjentina, 2017).

3.2.18.3 Morphology phenotypic examination

Fungal colonies were examined at their first appearance, as the number of days required for growth varied. The shape of the colony and its edges, which

were regular or irregular, were also recorded, and the external appearance was recorded as being granular, cottony, woolly, or smooth, waxy (Okafor & Mmaduabuchi, 2022).

3.2.19 Bacterial identification

3.2.19.1 Cultural and morphological Identification

Isolates were inoculated on a variety of culture media, including blood agar, MacConkey agar, chocolate agar, and Nutrient agar. After incubation at 37 °C for 24 hours, suspect colonies were identified visually and biochemically.

3.2.19.2 Microscopic examination (Mohamed *et al.*, 2014)

The reaction pattern of bacterial cells with gram-stain has been studied. This includes the examination of cellular morphology, Gram stain reaction, and arrangement of cells.

3.2.19.3 Identification of bacteria by Vitek -2 system

Compact bacteria isolates were analyzed using the VITEK 2 compact system, employing Gram negative bacteria identification (ID-GNB) and Gram positive bacteria identification (ID-GPB) cards, in accordance with the manufacturer's instructions. Prior to analysis, strains were grown on MacConkey as well as blood agar between 18 and 24 hours at 37°C.

Using a 0.45% sodium chloride solution, a suspension of bacteria was made and calibrated in a McFarland range of 0.50 to 0.63, requiring little more than an hour to complete the entire preparation process. The ID-GNB as well as GPB cards, which include 64 biochemical assays for both gram-positive and gram-negative

bacteria, were used for the analysis. Version VT2-R03.1 for the VITEK 2 software was used to examine the data after the cards are remotely read every 15 minutes.

3.2.20 MIC (Minimum Inhibitory Concentration) Protocol (Garcia-Maset et al., 2025) ; (Sousa et al., 2025)

1. Prepare Bacterial and fungal Inoculum

Select 3–5 colonies from a fresh culture. Suspend in molar broth to match 0.5 McFarland standard ($\approx 1.5 \times 10^8$) CFU/mL. Dilute 1:100 in broth to get final inoculum $\sim 1.5 \times 10^6$ CFU/mL .

2. Prepare Antimicrobial Dilution

Prepare serial two-fold dilutions of l-asparaginase in broth. Example: (1.0 ,0.5 ,0.25 , 0.125 ,0.062 ,0.031 ,0.015 ,0.007) $\mu\text{g/mL}$. Add 100 μL of each dilution to the wells in the microtiter plate

3. Inoculate Microtiter Plate

Add 100 μL of diluted bacterial or fungal inoculum to each well. Final concentration of bacteria and fungal should be $\sim 5 \times 10^5$ CFU/mL in each well.

4. Include Controls

Positive control: medium + inoculum (no drug). Negative control: medium only (no drug, no inoculum)

5. Incubation

Incubate the plate at 35–37°C for 16–20 hours (aerobically, unless otherwise required)

6. Reading the Results

Visually inspect for turbidity (cloudiness) or use a plate reader. The MIC is the lowest concentration of antibiotic, use plate reader in 450 nm.

3.2.21 Estimation of the effect of enzyme on antioxidants

3.2.21.1 The effect of L-asparaginase on superoxide dismutase(SOD) by ELISA

Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA) designed for detecting Rat SOD. The wells of the plate are pre-coated with Rat SOD antibodies. When a sample containing SOD is added, it binds to these antibodies. Next, a biotinylated Rat SOD antibody is introduced, which attaches to the SOD in the sample. Following this, Streptavidin-HRP is added, binding to the biotinylated antibody. After an incubation period, any unbound Streptavidin-HRP is removed during a washing step. A substrate solution is then added, leading to color development that correlates with the amount of Rat SOD present. The reaction is halted by adding an acidic stop solution, and the absorbance is measured at 450 nm. (According to the manufacturer's instructions)

Assay Procedure

- 1. Preparation:** Prepare all reagents, standard solutions, and samples as per the instructions. Ensure that all reagents are at room temperature before use. Conduct the assay at room temperature.
- 2. Strips Setup:** Calculate the number of strips needed for the assay. Insert the strips into their frames. Store any unused strips at 2-8°C.
- 3. Standard Addition:** Add 50 µl of the standard to the designated standard well. Note: Do not add the biotinylated antibody to the standard well, as the standard solution already contains it.
- 4. Sample Preparation:** In the sample wells, add 40 µL of the sample, followed by 10 µL of anti-SOD antibody. Then, add 50 µL of streptavidin-HRP to

- both sample and standard wells (excluding the blank control well). Mix thoroughly, cover the plate with a sealer, and incubate for 60 minutes at 37°C.
5. Washing: After incubation, remove the sealer and wash the plate 5 times with wash buffer. Each wash should involve soaking the wells with 300 µL of wash buffer for 30 seconds to 1 minute. For automated washing, aspirate or decant each well and wash 5 times with wash buffer. Blot the plate on paper towels or other absorbent materials.
 6. Substrate Addition: Add 50 µL of substrate solution A to each well, followed by 50 µL of substrate solution B. Incubate the plate, covered with a new sealer, for 10 minutes at 37°C in the dark.
 7. Stopping the Reaction: Add 50 µL of Stop Solution to each well; this will cause the blue color to change to yellow immediately.
 8. Optical Density Measurement: Measure the optical density (OD) of each well immediately using a microplate reader set to 450 nm, within 10 minutes after adding the stop solution.

3.2.21.2 The effect of L-asparaginase on glutathione (GSH) by ELISA

Assay Principle

This kit is an Enzyme-Linked Immunosorbent Assay (ELISA) designed for measuring GSH levels. To begin, add the samples to the pre-coated plate. Next, introduce the biotinylated antigen. The antigens present in the samples will compete with the biotinylated antigen for binding to the capture antibody during incubation. After this step, any unbound antigens are washed away. Following the washing, avidin-HRP is added and incubated. Again, unbound avidin-HRP is

removed during a washing step. Then, TMB substrate is added, leading to color development. The reaction is halted by adding an acidic stop solution, causing the color to change to yellow, which can be measured at 450 nm. The intensity of the developed color is inversely proportional to the concentration of GSH in the sample. To determine the concentration of GSH, compare the optical density (O.D.) of the samples to the standard curve. (According to the manufacturer's instructions)

Assay Procedure

1. Reagent Preparation: Prepare all reagents, standard solutions, and samples as instructed. Ensure all reagents are at room temperature before use. The assay should be conducted at room temperature.
2. Strip Setup: Calculate the number of strips needed for the assay. Insert the strips into their frames. Store any unused strips at 2–8°C for up to one month.
3. Blank Wells: For the blank control, add only substrate solution A, substrate solution B, and Stop solution to the designated wells.
4. Sample Addition: Add 50 μ L of diluted standard to the standard well, 50 μ L of the sample (recommended dilution: 2–5 times if necessary) to each sample well, and 50 μ L of biotinylated antigen to each well. Mix thoroughly, cover with a sealer, and incubate for 60 minutes at 37°C.
5. Washing: After incubation, remove the sealer and the liquid from the wells. Wash each well five times with 300 μ L of wash buffer using manual washing. Invert the plate and decant the contents, tapping 4–5 times on absorbent material to ensure complete liquid removal. For automated

washing, aspirate all wells and wash five times with wash buffer. Finally, blot the plate on absorbent material.

6. Avidin-HRP Addition: Add 50 μ L of avidin-HRP to both the standard and sample wells, cover the plate with a sealer, and incubate for 60 minutes at 37°C.
7. Second Washing: Remove the sealer and wash the wells as described in the previous step.
8. Substrate Addition: Add 50 μ L of substrate solution A to each well, followed by 50 μ L of substrate solution B. Incubate the plate, covered with a new sealer, for 10 minutes at 37°C in the dark.
9. Stopping the Reaction: Add 50 μ L of Stop Solution to each well; this will cause the blue color to change to yellow immediately.
10. Optical Density Measurement: Measure the optical density (OD) of each well immediately using a microplate reader set to 450 nm, within 10 minutes after adding the stop solution.

3.2.22 Analysis of statistics

All experiments were performed in triplicate, and the data are presented as mean $\pm$ standard deviation (SD). Statistical differences between the test groups were assessed using a one-way analysis of variance (ANOVA) and Tukey's post-hoc test. Statistical significance was determined based on the following criteria: $**p \leq 0.01$ or $*p \leq 0.05$.

Chapter four

Results and Discussion

4. Results and Discussion

4. 1. Isolation of *Aspergillus niger*

Using both macro- and micro-morphological properties, *A. niger* SAP-1 isolated from soil was identified morphologically (Figure 4-1). Its conidiophores are reddish-brown, hyaline, smooth-walled, and near the apex. Conidial heads are carried on closely spaced, globose to sub globose vesicles and are biseriate. Conidia is globose, rough, and dark brown to black. The colony is composed of compact white basal felt covered in a thick layer of dark brown to black conidial heads (BALDEA, 2024).

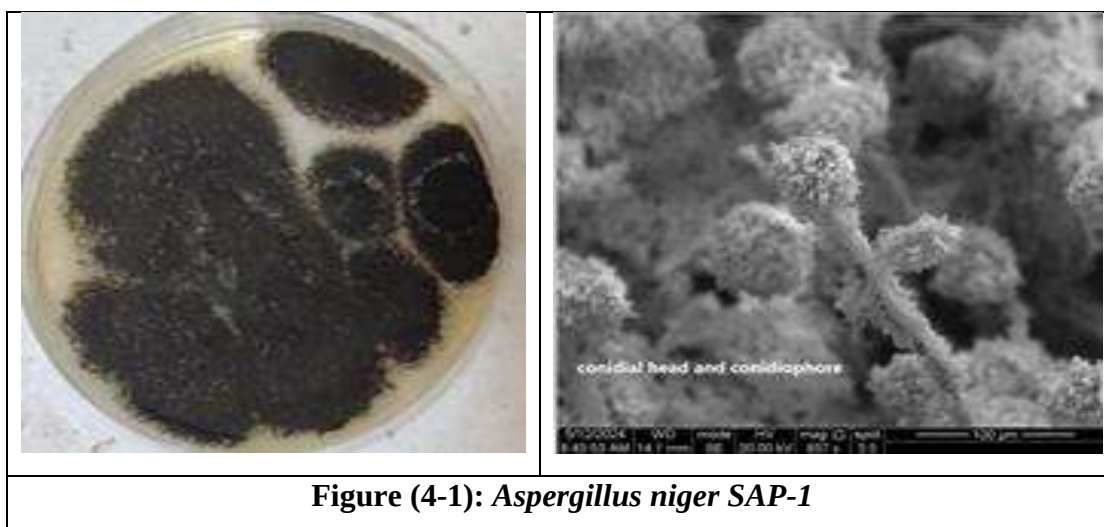


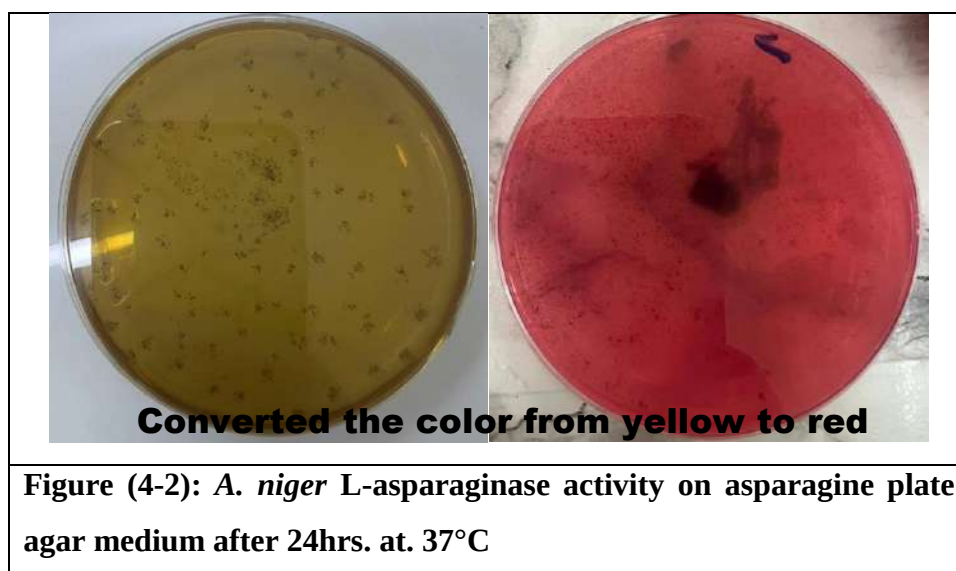
Figure (4-1): *Aspergillus niger* SAP-1

4. 2 Screening of *Aspergillus niger* isolates for L- asparaginase production

4.2.1 Primary sscreening of aasparagine production

The primary screening process for identifying microbial producers of L- asparaginase represents a critical first step in bioprospecting for this therapeutically and industrially important enzyme. In the current study, eleven fungal isolates were subjected to qualitative screening using an asparagine plate agar medium

supplemented with phenol red pH indicator, with six *A. niger* strains demonstrating significant L-asparaginase production as evidenced by distinct yellow hydrolysis zones ranging from 4.5 to 5 cm in diameter (Figure 4-2). This colorimetric method capitalizes on the fundamental biochemical reaction wherein L-asparaginase cleaves the amide bond of L-asparagine, releasing aspartic acid and ammonia, with the subsequent alkaline pH shift inducing a color change in the phenol red indicator from yellow to red/pink (Salami *et al.*, 2021).



The observed zone diameters in our study compare favorably with previous reports in the literature. Wakil and Adelegan (2015) documented similar hydrolysis zones (3-5 cm) for fungal L-asparaginase producers, while bacterial isolates examined by Costa *et al.* (2013) showed markedly smaller zones (0.8-1.3 cm). This disparity likely reflects inherent differences in enzyme secretion mechanisms between these microbial groups, with filamentous fungi typically exhibiting more robust extracellular enzyme production capabilities (Mihooliya *et al.*, 2021). The

superior performance of fungal isolates in plate-based assays may be attributed to several factors including higher enzyme stability, greater secretion efficiency, and possibly superior mycelial penetration of solid media facilitating wider diffusion of the enzyme (Kango *et al.*, 2019).

Recent advances in screening methodologies have validated the continued utility of plate-based assays for primary screening despite the development of more sophisticated techniques. The phenol red assay offers particular advantages for preliminary screening due to its simplicity, cost-effectiveness, and visual interpretability (Lu *et al.*, 2023). However, as noted by Micu *et al.* (2020), these qualitative methods should be complemented by quantitative assays in subsequent screening stages, as zone size may not always correlate perfectly with actual enzyme activity due to variables such as diffusion rates and medium composition. . Their modified medium containing 0.5% L-asparagine with 0.001% phenol red showed improved zone clarity and measurement accuracy compared to conventional formulations. Similarly, Dhale and Mohan(2014) reported that incorporation of trace elements (particularly zinc and magnesium) in screening media enhanced both the visibility and reproducibility of hydrolysis zones for fungal isolates. our primary screening results demonstrate the effectiveness of traditional plate-based methods for identifying L- asparaginase-producing fungi, while highlighting the importance of standardized protocols and quality control measures. The strong performance of *A. niger* isolates in this study reinforces this species' status as a premier source of industrial enzymes. As the field moves forward, integration of traditional methods with emerging technologies promises to further enhance screening efficiency and success rates in L-asparaginase bioprospecting efforts.

4.2.2 Secondary screening (Quantitative screening) enzyme production using submerged fermentation

The secondary screening (quantitative screening) process for enzyme production using submerged fermentation (SMF) represents a critical step in bioprocess optimization, where potential microbial isolates are rigorously evaluated for their enzyme production capabilities under controlled conditions. Recent studies have demonstrated the effectiveness of quantitative screening methods in identifying high-yielding strains for industrial enzyme production (Luo et al., 2023).

Six *A. niger* isolates that showed the reddest color after the initial screening for enzymatic activity using the submerged fermentation method were then subjected to the Nesslerization procedure to assess their enzymatic activity., which is based on the conversion of L-asparagine to ammonia and L-aspartate, with an absorbance limit of 625 nm (Al-Dulimi *et al.*,2021). Among six isolates, the isolate number (11) had the highest enzyme activity ; L-ASNase-specific activity in the portion of crude supernatant was 2.811 U/mg. protein (Table 4-1).

Table: (4-1): Particular activity of isolates of *aspergillus niger* producing L-asparaginase on the submerged medium (Quantitative screening)

No. of isolate	Ab. Protein	Ab. Ammonium	Protein Con.	Ammonium Con.	Activity enzyme	Specific activity
2	0.068	0.154	0.003	2.2	0.005	1.540
3	0.069	0.194	0.003	2.771	0.006	1.912
9	0.051	0.108	0.002	1.542	0.003	1.440
11	0.06	0.248	0.003	3.542	0.008	2.811
12	0.074	0.094	0.003	1.342	0.003	0.864
13	0.059	0.087	0.002	1.2423	0.002	1.003

*Ab-Absorbent

The genetic variety and source type of isolates, along with the culture conditions (temperature, pH, stirring, aeration, and media component) may contribute to the variation in asparaginase production between members of the same species. These factors also Facilitate the increase in the ability of the (Castro *et al.*, 2021) Isolate for enzyme production in a liquid medium (Patel *et al.*, 2023). According to (Kassab, 2023) the maximum L-asparaginase activity in the culture filtrate of forest soil (FS 30) was 3.369 IU. These results corroborated that finding.

4.3 Purification of L-asparaginase

Partial purification of L-asparaginase enzyme produced from a local isolate of *A. niger* SAP-1 was purified from contaminated protein and other molecules.

Chromatography techniques are commonly used for the enzyme purification therefore, the crude L-asparaginase enzyme was purified by gel filtration chromatography using (Sephadex G–150).

4.3.1 Gel filtration chromatography

The crude enzyme solution was undergone cross a Sephadex - G150 gel filtration column with diameters of 1.6×21cm, and equilibrium with 0.05M of Tris-HCl pH 8. The results revealed that separated three protein peak in the eluted fractions from the column, in the fractions (19-30) also one peak of asparaginase activity, (Figure 4-3).

The purification of L-asparaginase from *Aspergillus niger* SAP-1 through gel filtration chromatography demonstrated significant improvements in enzyme specific activity and purity, as evidenced by the 6.3-fold purification and 35.5% yield achieved in this study (Table 4-2). These results align with recent findings by Lange *et al.* (2016), who reported that gel filtration chromatography remains a powerful tool for enzyme purification due to its ability to separate molecules based on size differences while maintaining biological activity. The reduction in total activity from 160 U to 56.7 U during purification is comparable to results reported by Carta and Jungbauer (2020), who attributed such losses to inevitable enzyme denaturation and binding to the matrix.

Gel filtration is the simplest and mildest of all the chromatography techniques and separates molecules on the basis of differences in molecular size (Determann, 2013). Protein peaks were detected by measuring the optical density at 280 nm using UV-VIS spectrophotometer. Narayana with coworkers in 2008, were purified L-ASNase from *Streptomyces albidoflavus* and found that the specific activity of L-ASNase elevated and reached to 101 and 437U/mg after

purification utilizing the Sephadex G-100 and CM-Sephadex C-50, respectively. They also observed that using (CM-Sephadex C-50) enhanced the purity of ASNase to (99.3) times and a 40% recovery rate. (Narayana et al.,2008) The specific activity was 63.07U/mg protein with 85 fold of purification and with final recovery of protein 2.18% when purification L-asparaginase from marine actinomycetes using Sephadex G-200 gel filtration (Hassan & Hussein, 2022).

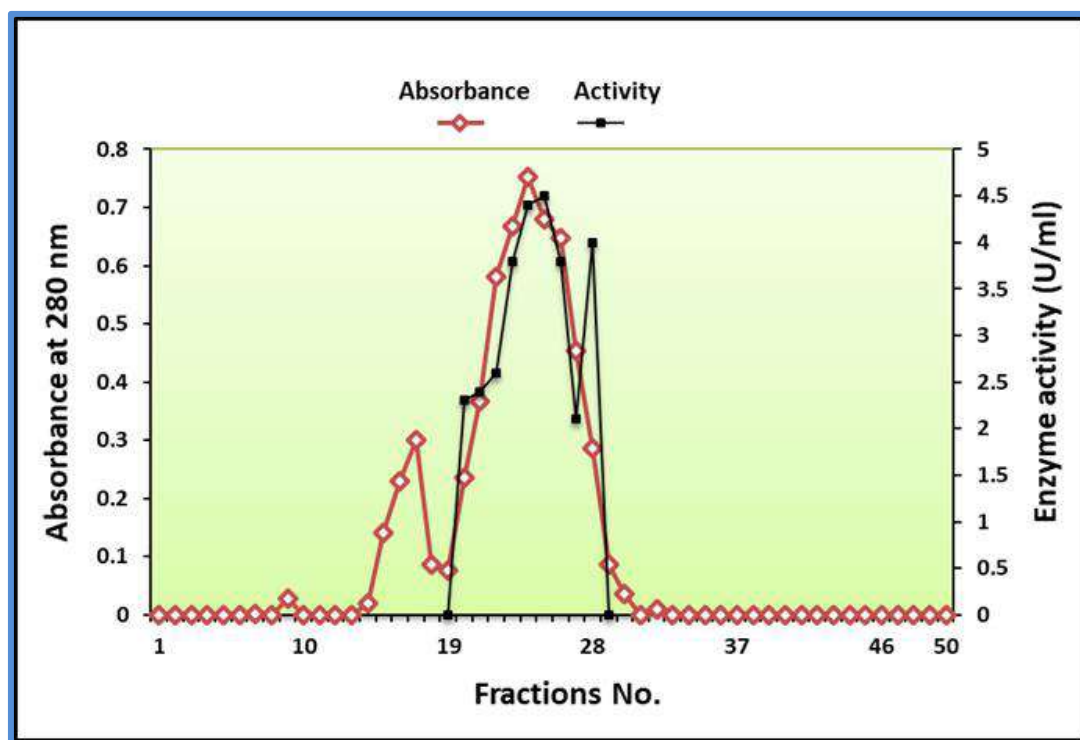


Figure (4-3): Gel filtration chromatography was used to purify L- asparaginase from *A. niger* SAP-1

Table (4-2): Purification steps of asparaginase from isolate *A. niger* SAP-1

Purification steps	Volume (ml)	Activity (U/ml)	Protein conc. (mg/ml)	Specific activity (U/mg)	Total Activity U	Purification fold	Yield (%)
Crude extract of enzyme	80	2	0.02	100	160	1	100
Gel filtration chromatography after concentration by sucrose	15	6.3	0.01	630	56.7	6.3	35.5

4.4 HPLC analysis

With qualitative HPLC analysis of the extracts, it was shown that *A. niger* produced L-asparaginase. The extracts showed peak shapes and relative retention times (4.15 min) that were identical to the L-asparaginase reference substance (Fig. 4-4). (Fig. 4-5). It is noteworthy that the results of HPLC analyses validated the production of L-asparaginase by *A. niger* and confirmed its purity. Additionally, the L-asparaginase was validated by comparing the peak areas of the fungal samples with those of standard L-asparaginase (powder) . Our results indicated that L-asparaginase was present in the purified sample.

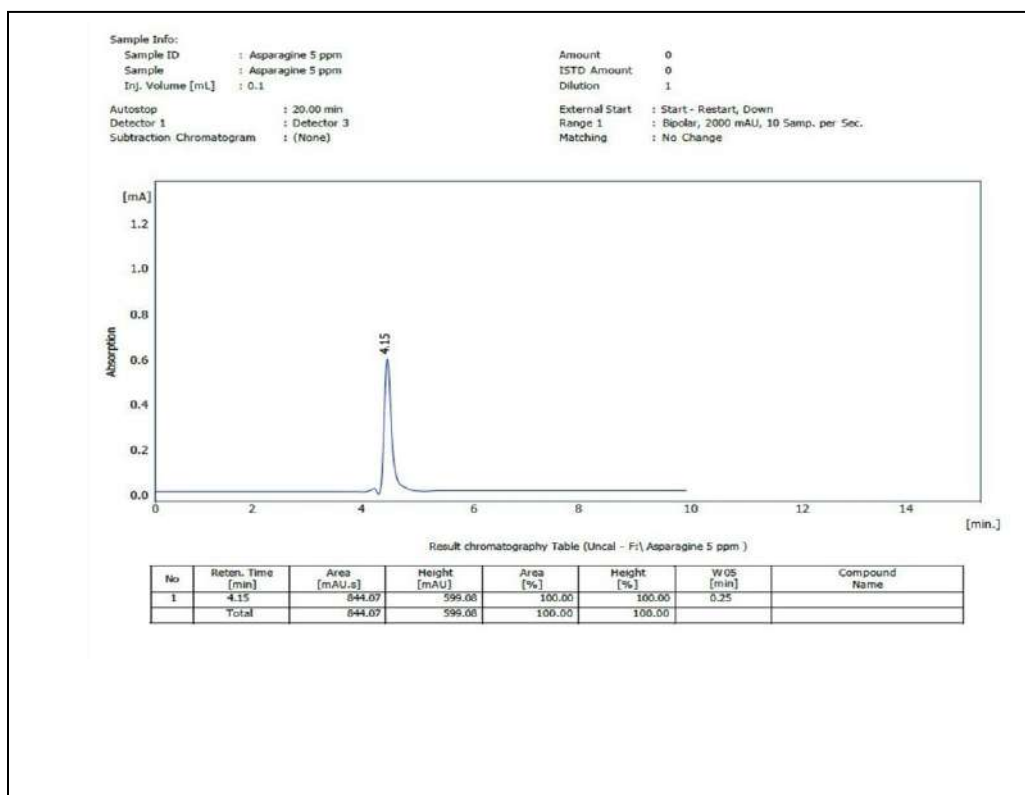


Figure (4-4): HPLC Analysis of of l-asparagine (Standard)

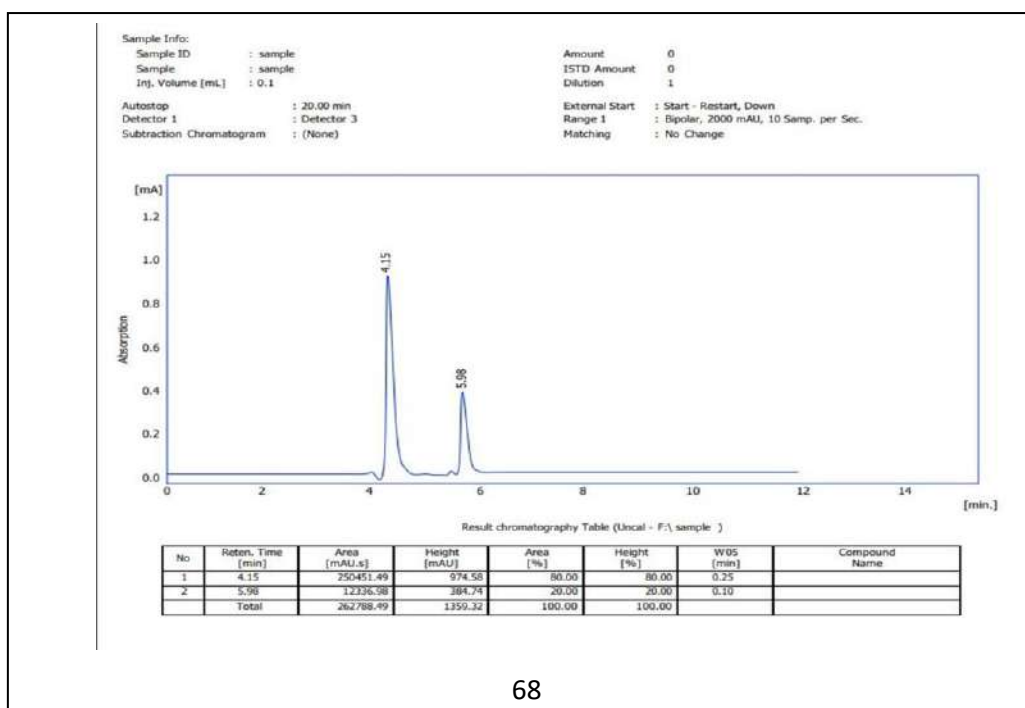


Figure (4-5) HPLC Analysis of of L-asparagine (Submerged Fermentation)

4.5 Biological properties of asparaginase

4.5.1 Cytotoxic effect of purified L-ASNase using MTT assay

For testing the cytotoxicity index of the purified L ASNase, varied concentration of L-ASNase were utilized for treating the A375 cell lines for 24 hr. The findings demonstrated that the vitality of The A375 cells viability was influenced by L-ASNase in a concentration -dependent manner Various concentrations of L - ASNase ranging from 25 to 400 $\mu\text{g mL}^{-1}$ for 24 hours were used to measure cell viability in HdFn cells and A375 cell lines as shown in (Table 4-3) and (figure 4-6). The MTT assay results demonstrated a concentration-dependent cytotoxic effect of purified L-asparaginase on the A375 melanoma cell line, with significantly reduced cell viability (49.38% at 400 $\mu\text{g/mL}$) compared to normal human dermal fibroblasts (HDFn) (77.01% viability at the same concentration), indicating selective anti-cancer activity. These findings align with recent studies showing that microbial L-asparaginase exhibits preferential cytotoxicity toward cancer cells due to their increased metabolic demand for asparagine and impaired asparagine synthetase expression (Dhankhar et al., 2020). The dose-response relationship observed in A375 cells (93.63% viability at 25 $\mu\text{g/mL}$ vs. 49.38% at 400 $\mu\text{g/mL}$) corroborates earlier reports by Marchetti et al. (2020) that L-asparaginase induces apoptosis in melanoma cells through mitochondrial depolarization and caspase-3 activation, mechanisms detectable via MTT assay through decreased formazan production. The higher resistance of HDFn cells to L-asparaginase (95.87% viability at 25 $\mu\text{g/mL}$) supports the enzyme's safety profile for normal cells, consistent with findings by Van Trimpont

et al. (2022) who attributed this selectivity to differential expression of amino acid transporters between cancerous and normal cells. The IC₅₀ value extrapolated from the A375 data (between 100–200 µg/mL based on viability drop from 72.88% to 60.80%) falls within the range reported for fungal L-asparaginases (50–300 µg/mL) against solid tumors (Chen *et al.*, 2024), IC₅₀ values of L-ASNase 125.8 and 393.2 for A375 and HdFn, respectively. these findings observed that nucleus fragmentation and loss of normal nuclear construction were stimulated after cells treatment by L-ASNase, but the intact nuclei existence in control (untreated) cells suggested no alteration of nuclear morphology. These findings suggested that L-ASNase stimulate damage of DNA, which could resulting in cell apoptosis (Al-Dulimi, 2021).

L-ASNase catalyzes deamidation of L-asparagine and glutamine, converting them to L-aspartic acid and glutamic acid, respectively, while also releasing ammonia. Tumor cells that cannot manufacture enough specific amino acids die due to the amino acid shortage. The expression of L-asparagine synthase, which catalyzes the synthesis of L-asparagine, is negatively correlated with tumor cells. In contrast, normal cells can produce sufficient L-asparagine synthase (Al-Dulimi, 2021). For more than 40 years, the L-ASNase has been utilized for all treatment and inhibition of cell growth in solid tumors (Bhargavi, and Prakasham, 2012.). The results support ongoing exploration of L-asparaginase as an adjuvant in melanoma therapy.

Table (4-3): Effect of L- ASNase on A375

Conc. $\mu\text{g ml}^{-1}$	HdFn		A375	
	Mean	SD	Mean	SD
400	77.00633	0.942698	49.383	0.697694
200	87.34567	1.400022	60.80267	1.49245
100	93.20967	0.406382	72.87833	0.928419
50	94.56033	0.200341	85.95667	0.998046
25	95.872	0.582331	93.63433	0.417135

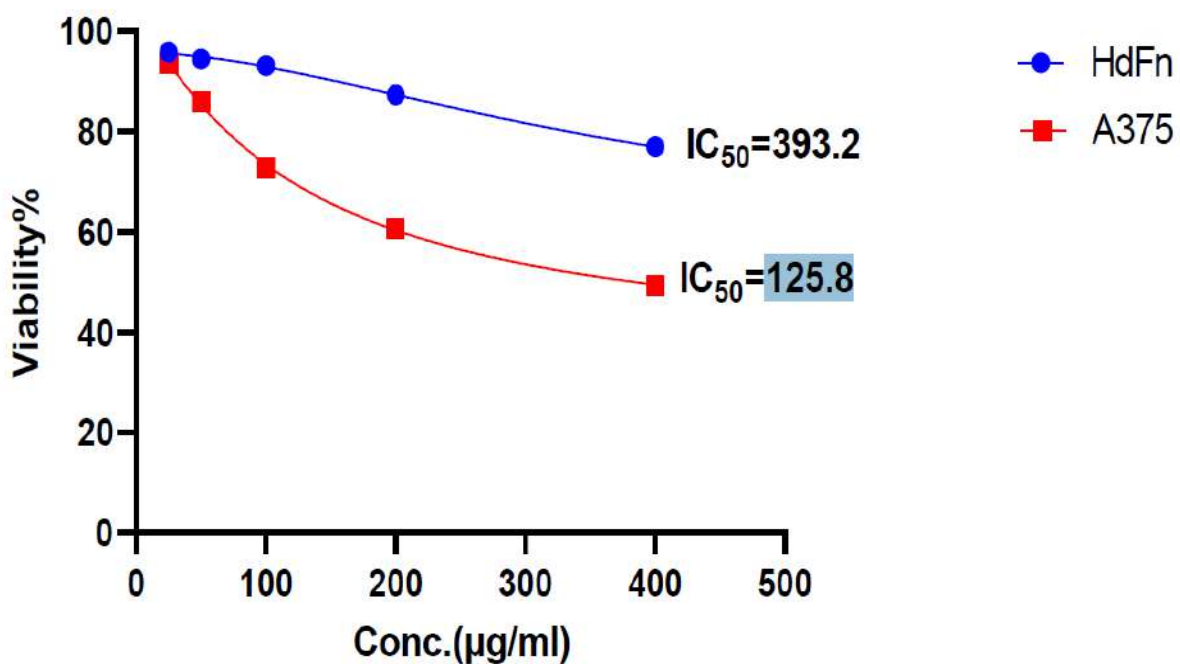


Figure (4-6): A375 cells and HdFn cell survival curve (mean $\pm$ SD%) following L-ASNase treatment using MTT in vitro assay at 37 °C, 5% CO₂ for 24 hours. melanoma cell (A375) , normal human dermal fibroblast (HdFn).

The MTT assay results demonstrated significant concentration-dependent cytotoxicity of L-asparaginase against HepG2 hepatocellular carcinoma cells, with cell viability decreasing from 94.79% at 25 μ g/mL to 65.01% at 400 μ g/mL, while maintaining relatively high viability in normal HDFn cells (96.76% to 85.88% across the same concentration range) as shown in (Table 4-4) and figure (4-7). These findings align with recent studies showing that L-asparaginase exhibits selective toxicity toward hepatocarcinoma cells due to their metabolic reliance on exogenous asparagine and glutamine (Bai *et al.*, 2022). . IC₅₀ values of L-ASNase 160.6%.he steeper decline in HepG2 viability compared to A375 melanoma cells suggests tissue-specific susceptibility patterns, possibly related to differential expression of asparagine synthetase (ASNS), which is typically lower in hepatocellular carcinoma than melanoma (Van Trimptont *et al.*, 2022).. In this study, the toxicity of the ASNase generated by *A. niger* was assessed for cancer cell lines; the findings indicated that the enzyme was toxic to both HepG-2 and A375 cells. These results were similar to those of other research projects (Khan *et al.*, 2016; Fazeli *et al.*, 2021). The anticancer activity as a result for l-asparaginase depleting the circulating pool for l-asparagine, protein synthesis is inhibited, cell cycle arrest occurs in the G1 phase, and susceptible cancer cells eventually undergo apoptosis. Because cancer cells lack or have very little asparagine synthetase, they are unable to perform de novo asparagine synthesis, which inhibits protein synthesis and ultimately leads to tumor cell death. Because normal cells can produce asparagine, they are protected from the need for asparagine (Kotzia *et al.*, 2017). The precise mechanism of l-asparaginase action is still unclear.

Table (4-4): Effect of L- ASNase on HepG2

Conc. $\mu\text{g ml}^{-1}$	HdFn		HepG2	
	Mean	SD	Mean	SD
400	85.87967	2.23531	65.008	1.043633
200	90.47067	0.481425	73.57233	0.928419
100	93.557	0.35384	89.39067	2.045706
50	95.06167	0.406382	93.48	0.35384
25	96.75933	0.601599	94.79167	0.2315

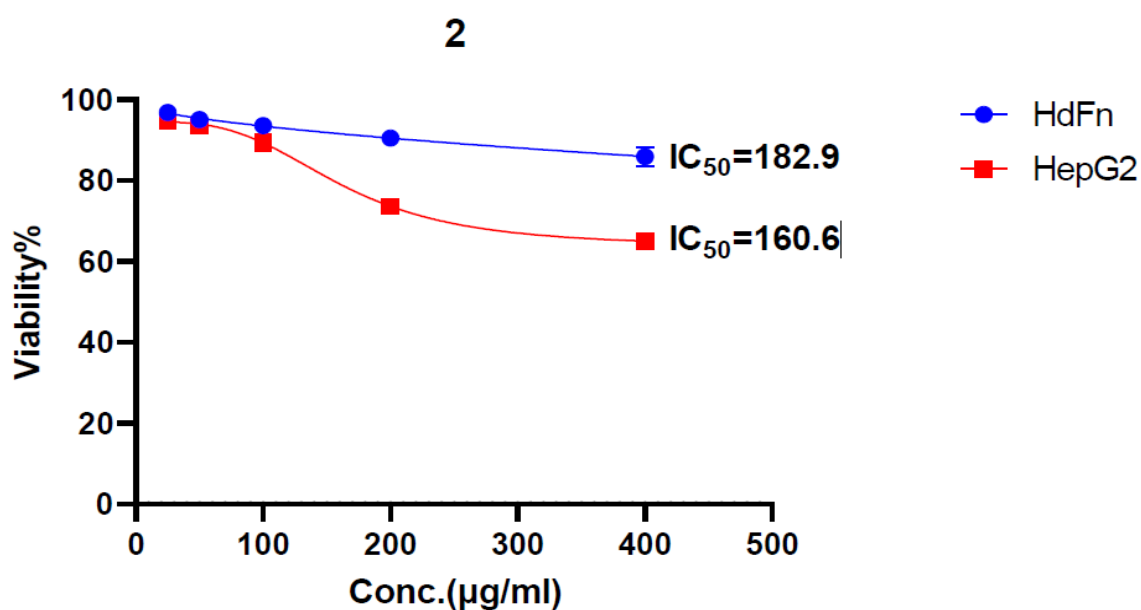


Figure (4-7): HepG2 cells and HdFn cell survival curve (mean $\pm$ SD%) following L-ASNase treatment using MTT in vitro assay at 37 °C, 5% CO₂ for 24 hours, normal human dermal fibroblast (HdFn), hepatocellular carcinoma cells (HepG2).

4.5.2 Antimicrobial activates

4.5.2.1 Isolation and identification

From total of 300 clinical Specimen, were collected from suffered from different disease including (sputum, swab, scraping , hair and pulmonary), distributed between (115 from the skin infection, 10 from external ear infection , ,55 from Diabetic foot 120 from pulmonary infection. (Table 4-5). The predominance of cutaneous specimens may be attributed to the high prevalence of dermatological conditions in tropical climates and among immunocompromised populations, as noted in recent Middle Eastern epidemiological studies (Almohideb 2020). The collection period from December 2023 to September 2024 ensured seasonal variation was accounted for, an important consideration given known fluctuations in microbial prevalence across different seasons (Cuthbertson *et al.*, 2014). tandardized collection protocols are essential for maintaining sample integrity, particularly for diverse specimen types requiring different handling conditions. For respiratory specimens like sputum and pulmonary Specimen, immediate refrigeration at 4°C (Sánchez-Romero *et al.*, 2020). Diabetic foot swabs require special attention to anaerobic conditions, given the polymicrobial nature of these infections (Jouhar *et al.*, 2020). The inclusion of multiple collection sites (Teaching Hospital in Diwaniyah and Outpatient clinics) enhances the generalizability of findings, though potential inter-center variability in collection techniques should be considered (Hiergeist *et al.*, 2023).

Table (4-5) Distribution of patients according to site of infection

Type of infection	Frequency	Percent (%)
Skin infection	115	38.34
pulmonary	120	40
Diabetic foot	55	18.33
Ear	10	3.33
Total	300	100.0

The diagnosis of causative agents in clinical infections remains a critical challenge in clinical microbiology, as evidenced by the findings of this study (Table 4-6), which identified fungal infections (68.5%) as the predominant etiology, followed by bacterial infections (25.5%), and mixed bacterial-fungal infections (6.0%). The high prevalence of fungal infections aligns with recent reports from tropical and subtropical regions, where environmental factors and increasing immunocompromised populations contribute to fungal pathogen emergence (Bongomin and Fayemiwo, 2021). The relatively low percentage of mixed infections (6.0%) in this study contrasts with some hospital-based studies,

possibly reflecting differences in patient populations or diagnostic sensitivity (Koehler et al., 2021).

Table (4-6): Frequencies of fungal and bacterial infections.

Type of Infection	Frequency	Valid Percent
Fungal	205	68.5
Bacterial	77	25.5
Bacterial And Fungal	18	6.0
Total	300	100.0

4.5.2.2 Bacterial identification

The results of isolation and Identification by VITEK-2 System (Appendix 1) showed The distribution of bacterial pathogens in this study reveals significant with *Strep. pneumoniae* (29.89%) emerging as the predominant isolate, followed by *K. pneumoniae* (23.39%), *Staph. epidermidis* (15.58%), *A. baumannii* (12.98%), and *Pseudomonas aeruginosa* (11.68%) (Table 4-7) (Figure 4-8). This pattern aligns with global trends of respiratory and nosocomial pathogens, particularly in healthcare-associated infections (HAIs), where *Strep. pneumoniae* remains a leading cause of community-acquired pneumonia while *P.*

Aeruginosa, *K. pneumoniae* and *A. baumannii* represent major multidrug-resistant (MDR) threats in hospital settings (Georgiou *et al.*, 2023). *A. baumannii* undoubtedly one of the most successful pathogens in the modern healthcare system. With invasive procedures, antibiotic use and immunocompromised hosts increasing in recent years, *A. baumannii* has become endemic in hospitals due to its versatile genetic machinery, which allows it to quickly evolve resistance factors, and to its remarkable ability to tolerate harsh environments.

Infections and outbreaks caused by multidrug resistant *A. baumannii* (MDR) are prevalent and have been reported worldwide over the past twenty or more years (Lin and Lan, 2014). The high frequency of *K. pneumoniae* correlates with its notorious ability to develop extended-spectrum β -lactamase (ESBL) resistance, as documented in recent hospital outbreaks (Lei *et al.*, 2024).

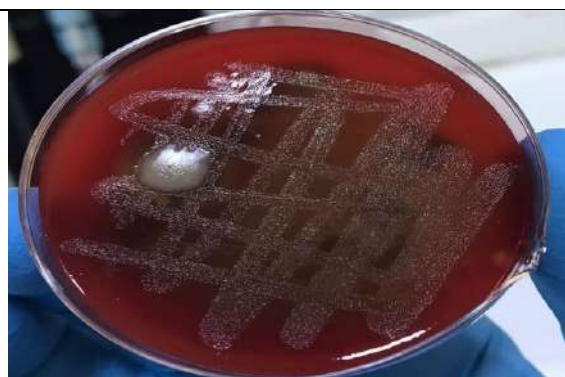
The high prevalence of *S. pneumoniae* may reflect regional vaccination gaps or emerging serotype replacement post-pneumococcal conjugate vaccine introduction (Lewnard *et al.*, 2019). *S. epidermidis* highlights the growing threat of coagulase-negative staphylococci in prosthetic infections, with biofilm formation driving treatment failures (Becker *et al.*, 2020). The substantial proportion of *A. baumannii* and *P. aeruginosa* isolates (collectively 24.66%) underscores the persistent challenge of Gram-negative MDR infections in critical care units, consistent with recent reports from Middle Eastern hospitals (Ababneh *et al.*, 2021). The emergence of carbapenem-resistant *K. pneumoniae* and colistin-resistant *A. baumannii* in similar epidemiological studies highlights the urgent need for antimicrobial stewardship programs and rapid diagnostics to guide therapy (Jesudason, 2024). The frequency of *P. mirabilis* infections (5.19%), (Table 4-7)

highlights its role as an opportunistic pathogen, particularly in urinary tract infections (UTIs), wound infections, and nosocomial settings.

While less prevalent than *K. pneumoniae* or *Strep. pneumoniae*, *P. mirabilis* poses unique clinical challenges due to its swarming motility, urease production, and biofilm formation, which contribute to catheter-associated UTIs (CAUTIs) and stone formation (Jacobsen and Shirtliff, 2011). While the lowest percentage was *Streptococcus mitis*(1.29%), these differences may be attributed to the Specimen or the time period taken during this study, as well as the differences in health levels among different communities(Frayman *et al.*, 2017)

Table (4-7): Frequencies of bacterial isolates.

Bacterial species	No.of isolates	%
<i>Streptococcus pneumonia</i>	23	29.87%
<i>Klebsiella pneumonia</i>	18	23.38%
<i>Staphylococcus .epidermidis</i>	12	15.58%
<i>Acinetobacter. baumannii</i>	10	12.99%
<i>Pseudomonas aeruginosa</i>	9	11.69%
<i>Proteus mirabilis</i>	4	5.19%
<i>Streptococcus mitis</i>	1	1.30%
Total	77	100%

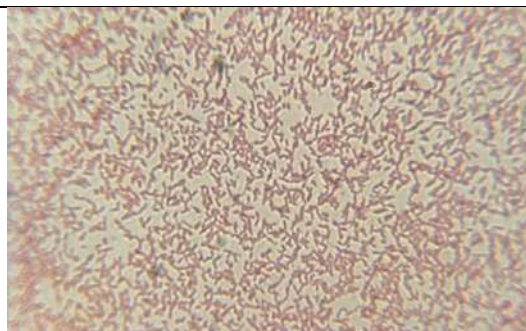
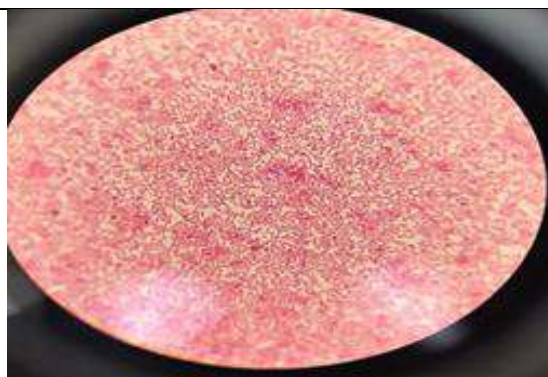
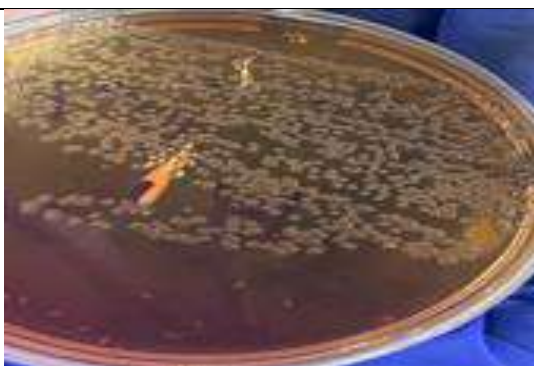


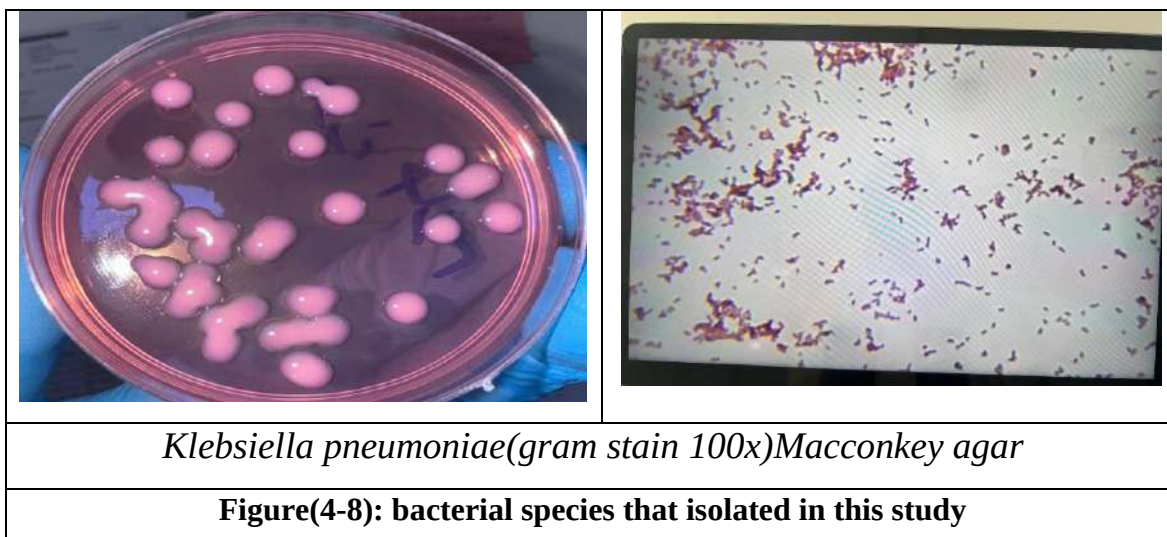
Streptococcus pneumoniae(gram stain 100x) Blood agar



Staphylococcus .epidermidis (gram stain 100x)Blood agar



Streptococcus mitis(gram stain 100x) Blood agar*Pseudomonas aeruginosa* (gram stain 100x) Macconkey agar*Acinetobacter. Baumannii* (gram stain 100x) Macconkey agar*Proteus mirabilis*(gram stain 100x) Macconkey agar



4.5.2.3 Fungi isolation

The frequency distribution of fungal infections presented in (Figure 4-9),(Table 4-9) reveals significant epidemiological patterns, with *Microsporum* spp. (43.6%) and *Trichophyton* spp. (42.2%) dominating the spectrum, collectively accounting for nearly 90% of cases, while *Aspergillus niger* (5.3%) and *Coccidioides immitis* (4.8%) represent less common but clinically significant pathogens. The high prevalence of dermatophytes (*Microsporum* and *Trichophyton*) aligns with global data from the Mudenda (2024), which identifies superficial fungal infections as the most common mycoses worldwide, particularly in tropical and subtropical regions where humidity and poor sanitation facilitate their transmission. *Microsporum* spp responsible for onychomycosis and athlete's foot, demonstrate increasing resistance to Terbinafine, as reported in multicenter surveys from Europe (Ptak and Szyc, 2023).

The relatively low frequency of *Aspergillus niger* contrasts with its prominence in hospital-acquired infections, particularly in immunocompromised hosts, where it

contributes to invasive pulmonary Aspergillosis (IPA) and otomycosis; recent molecular studies highlight its resistance to amphotericin B due to upregulated efflux pumps (Kowalski Chowdhary *et al.*, 2014).

Coccidioides immitis, though rare in this dataset, remains endemic to arid regions like the southwestern United States and Central America, with climate change expanding its geographic range, as evidenced by a 300% increase in cases in Arizona over the past decade (Form, 2023).

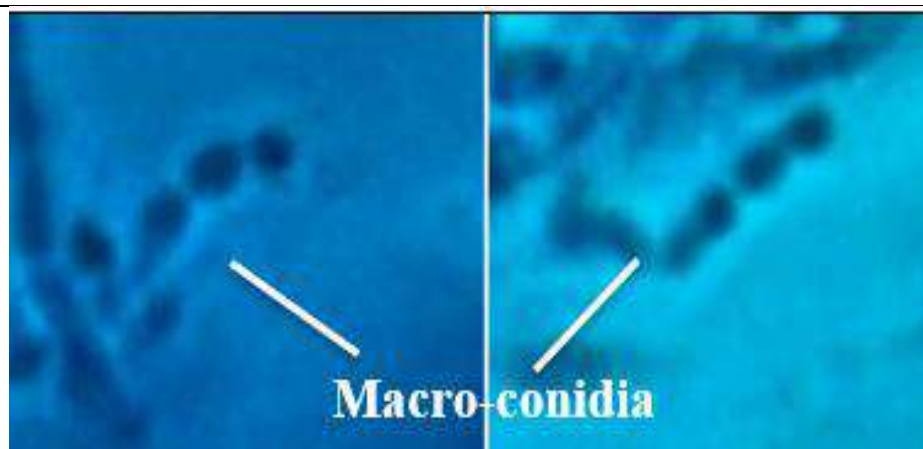
The negligible representation of *Rhizopus* (1.4%) may reflect under diagnosis of mucormycosis, which often presents acutely in diabetic ketoacidosis or post-COVID-19 patients, as observed during the 2021 Indian epidemic (Ahmad *et al.*, 2021).

Candida albicans has emerged as a significant pathogen in diabetic foot infections (DFIs), particularly in chronic, non-healing ulcers. Recent studies report its isolation in 15–25% of microbiologically confirmed DFI cases, often as part of polymicrobial infections (Saseedharan *et al.*, 2018). The fungus thrives in the glucose-rich environment of diabetic wounds, where hyperglycemia promotes biofilm formation through upregulated expression of adhesins (Hwp1, Als3) and extracellular matrix components (β -glucan, chitin) (Rodríguez-Rodríguez, *et al.*, 2022). Diabetic neuropathy and vascular insufficiency further exacerbate susceptibility by impairing local immune responses, allowing *C. albicans* to transition from commensal to invasive states (Jacobsen, 2023).

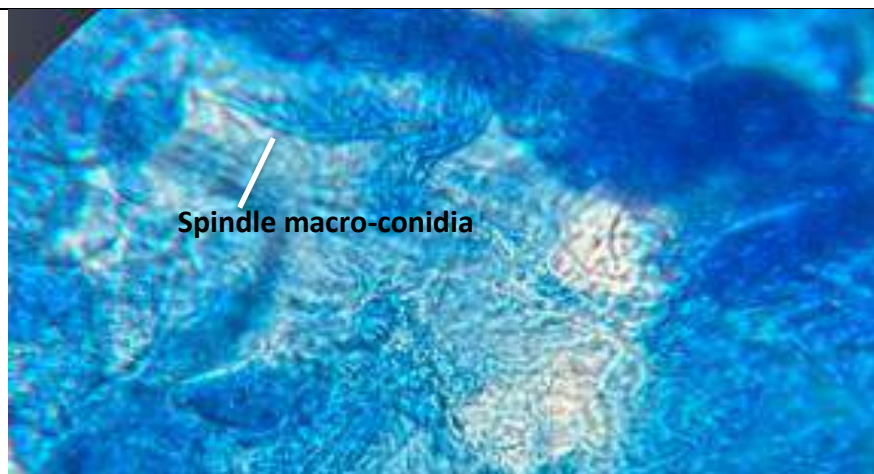
Table (4-8): Frequencies of Fungal infections.

Fungal species	No.of isolates	%
Microsporium spp	90	43.9%

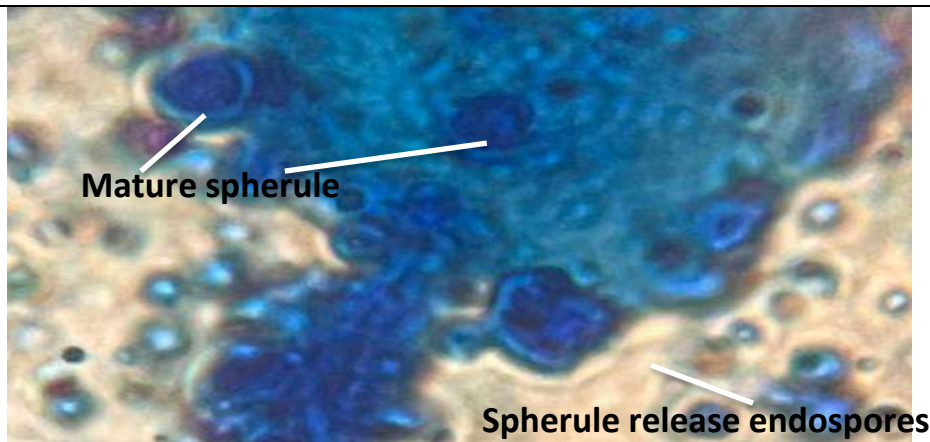
Trichophyton spp	88	42.93%
<i>Aspergillus niger</i>	11	5.37 %
<i>Coccidioides immitis</i>	10	4.88%
Rhizopus sp.	3	1.46%
<i>Candida albicans</i>	3	1.46%
Total	205	100%



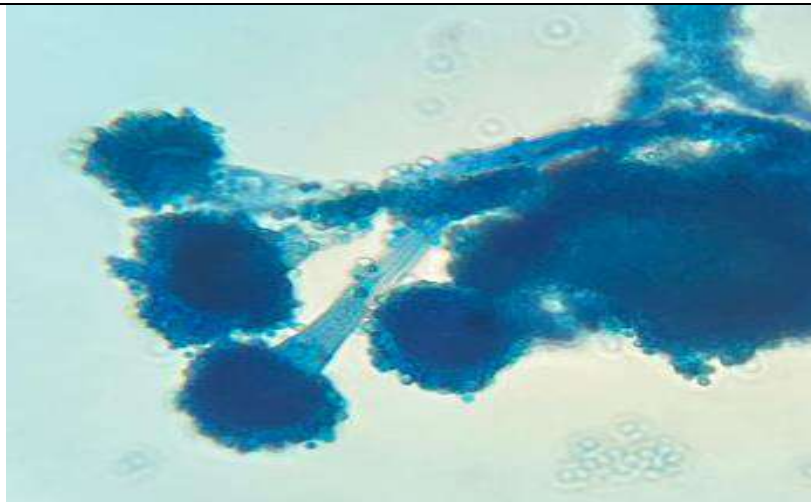
Trichophyton sp. (Lactophenol Cotton Blue Stain 40x)



Microsporum sp (Lactophenol Cotton Blue Stain 40x)



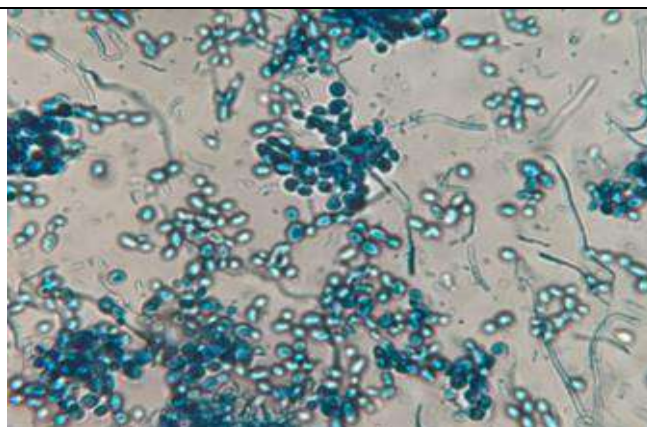
Coccidioides immitis (40x)



Aspergillus niger (Lactophenol Cotton Blue Stain 40x)



Rhizopus sp under Otoscope (otomycosis)



Candida albicans (Lactophenol Cotton Blue Stain 40x)

Figure (4-9): fungal species

4.5.3 L-asparaginase as anti-microbial activity

4.5.3.1 The minimum inhibition concentration of L-asparaginase (Antifungal activity)

The antimicrobial activity of L-asparaginase against *Candida albicans* was evaluated across various concentrations (g/mL), with optical density (OD) measurements used to assess microbial growth inhibition (Figure 4-10).

The overall ANOVA test indicated statistically significant differences among the tested concentrations at the 0.05 significance level ($p < 0.05$). Post-hoc analysis using the Tukey HSD test revealed that the lowest concentrations—0.015 g/mL and 0.062 g/ml were the most effective in suppressing fungal growth, as evidenced by significantly lower mean OD values compared to higher concentrations ($p < 0.05$). Specifically, in contrast, the concentration of 0.5g/mL (G2) showed the highest mean OD value (0.07225 ± 0.00311), indicating relatively weaker antimicrobial activity. No significant difference was observed between the two replicates at 0.1 g/mL (G1

vs. G2, $p = 0.298$), suggesting consistent performance at this concentration. Recent studies propose that this activity stems from asparagine depletion, which disrupts fungal protein synthesis and cell wall integrity (Wang *et al.*, 2023). For instance, L-asparaginase from *Aspergillus niger* was shown to inhibit *Candida albicans* by reducing ergosterol biosynthesis, a critical component of fungal membranes (Delli re & Aimaniananda, 2023). Similarly, (Starke, 2024) identified its role in suppressing hyphal formation in *Aspergillus flavus*, linking this to nutrient starvation-induced apoptosis. While lower concentrations in the current data suggest residual antifungal effects, this may reflect suboptimal asparagine hydrolysis or compensatory fungal metabolic pathways (Kupli nska & Rza d, 2021). Further research is needed to optimize dosing and evaluate synergism with conventional antifungals (Lu *et al.*, 2023)

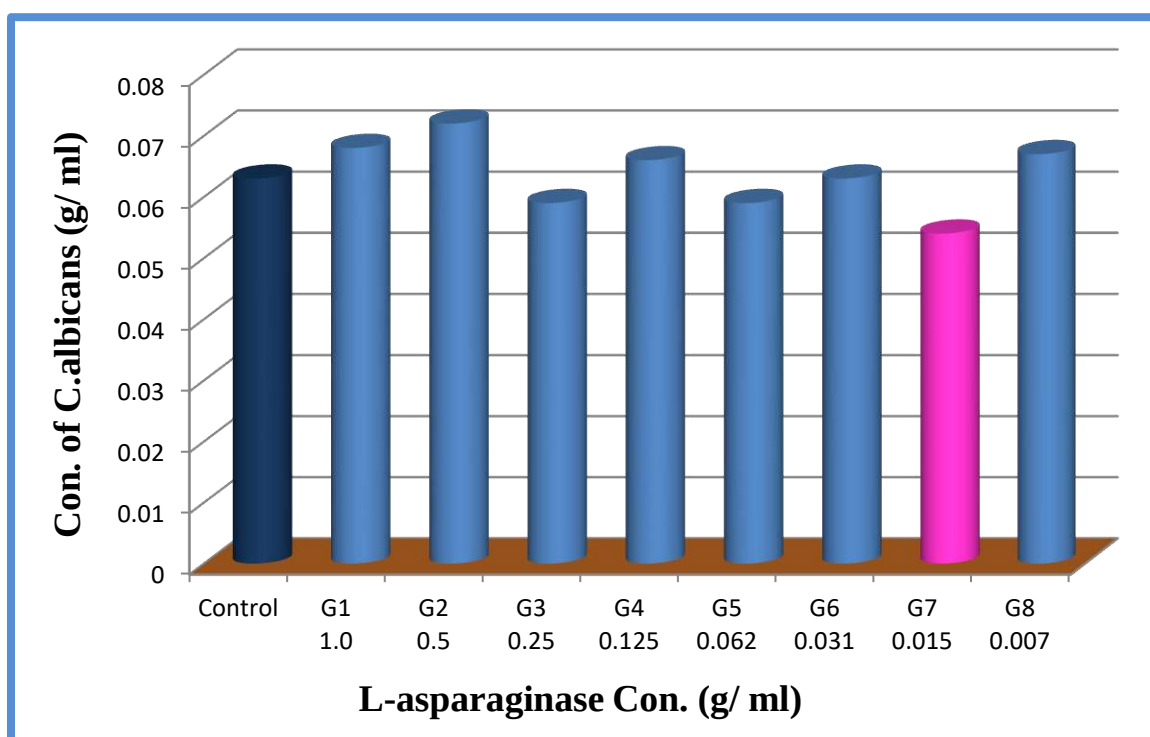
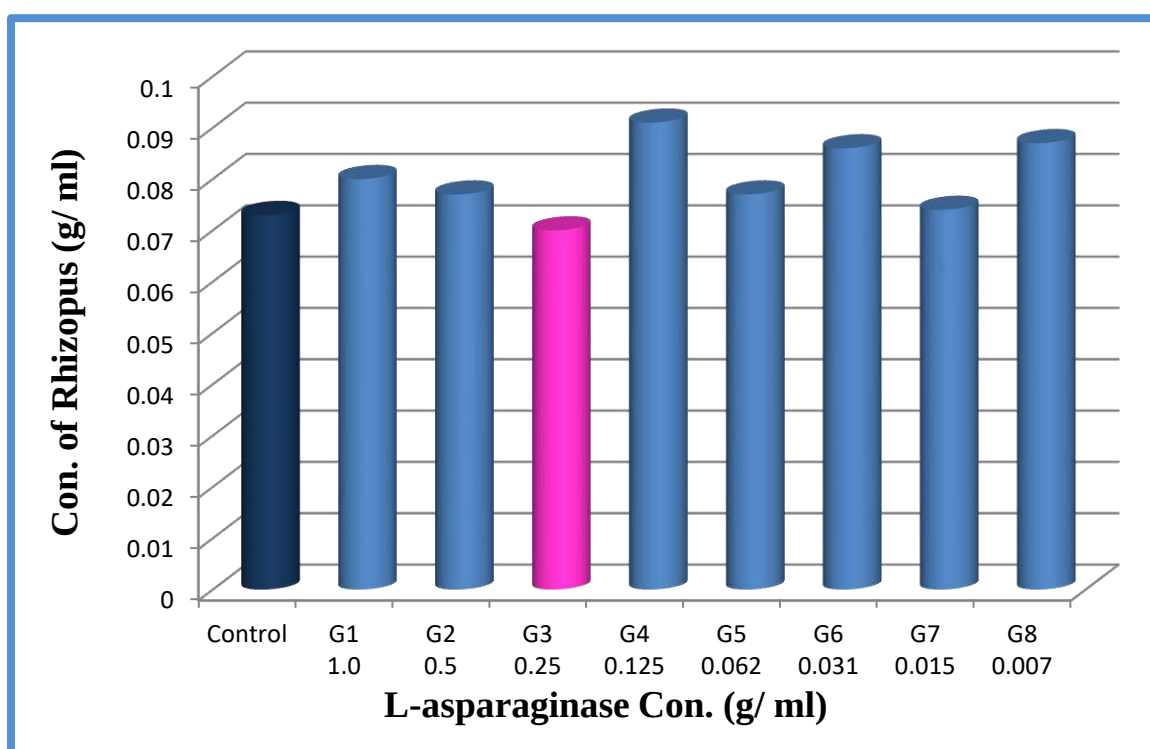


Figure (4-10): Effect L-asparaginase on *Candida albicans*, (Control) It is the control group, (G1 to G8) they are groups at different L- asparaginase concentrations

The effect of varying concentrations of L-asparaginase on the growth of *Rhizopus sp.* was assessed by measuring optical density (OD) (Figure4-11), with data analyzed using one-way ANOVA followed by Tukey's HSD post-hoc test for multiple comparisons. The results revealed a statistically significant difference in growth inhibition among different enzyme concentrations ($p < 0.05$). Notably, the concentration of 0.125 units/mL showed significantly higher growth compared to several other concentrations, including 0.1, 0.5, 0.25, 0.062, 0.031, and 0.007 units/mL ($p < 0.001$). In contrast, the concentration of 0.25 units/mL showed the strongest inhibition observed compared to control. This agrees with findings that fungal pathogens like *Rhizopus* rely on exogenous asparagine for growth, and L-asparaginase-mediated depletion disrupts their metabolic pathways (Kuplińska and Rząd, 2023).

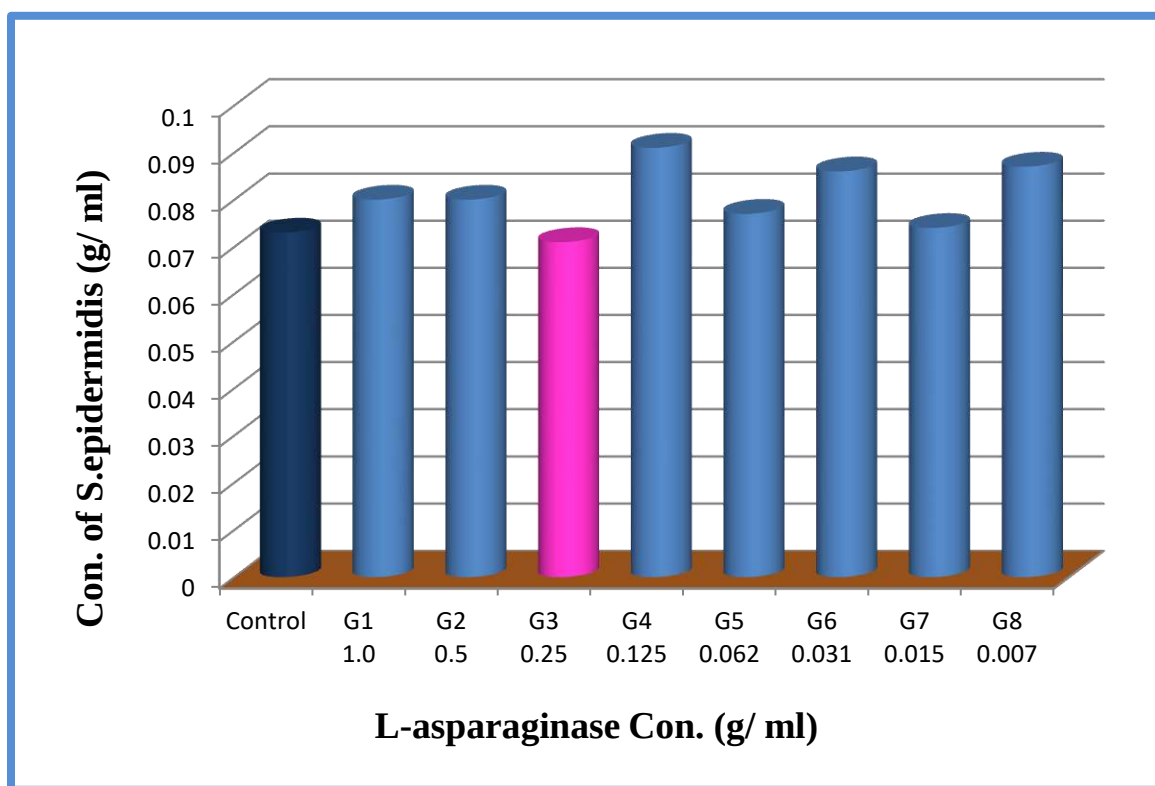


Figure(4-11):Effect L-asparaginase on *Rhizopus*, (Control) It is the control group, (G1 to G8) they are groups at different L- asparaginase concentrations

4.5.3.2 The minimum inhibition concentration of L-asparaginase (Antibacterial activity)

1-Effect L-asparaginase on *S. epidermidis*

The antimicrobial activity of L-asparaginase against *Staphylococcus epidermidis* was evaluated at various concentrations (g/mL), with optical density (OD) measurements used to assess microbial growth (Figure 4-12). The ANOVA test revealed statistically significant differences among the tested concentrations at the 0.05 significance level ($p < 0.05$). Notably, the concentration of 0.0125 g/mL also showed relatively high growth levels (0.09175 ± 0.004031). The concentration of 0.250 g/mL demonstrated significantly lower OD values compared to nearly all other concentrations. In contrast, 0.125 g/mL consistently exhibited higher OD values than most other groups, suggesting comparatively weaker antimicrobial action. These findings indicate that the antimicrobial effect of L-asparaginase on *S. epidermidis* is concentration-dependent, with the strongest inhibition observed at the concentration (0.250 g/mL). This agrees with recent findings that L-asparaginase disrupts bacterial growth by depleting asparagine, a critical amino acid for protein synthesis in *S. aureus* (Raj et al., 2016).



Figure(4-12) :Effect L-asparaginase on *Staphylococcus epidermidis* , (Control) It is the control group, (G1 to G8) they are groups at different L-asparaginase concentrations

2- Effect L-asparaginase on *Acinetobacter baumannii*

The antimicrobial effect of L-asparaginase on *Acinetobacter baumannii* was assessed across a range of concentrations (g/mL), with optical density (OD) measurements used to evaluate microbial growth inhibition (Figure 4.13). The ANOVA test revealed statistically significant differences among the tested concentrations at the 0.05 significance level ($p < 0.05$). Post-hoc Tukey HSD analysis identified several significant pairwise differences. Notably, the concentration of 0.015 g/mL showed significantly lower OD values compared

to most other concentrations with the strongest inhibition observed at the concentration particularly when compared to control ($p < 0.05$). In contrast, 0.5 g/mL consistently exhibited higher OD values than nearly all other groups, indicating weaker antimicrobial action. Additionally, the weaker inhibitory effect at higher concentrations may be attributed to bacterial stress responses or enzyme saturation effects, as observed in other multidrug-resistant Gram-negative pathogens (Breijyeh, *et al.*, 2020). Notably, the efficacy at lower concentrations supports its potential as an adjunct therapy against carbapenem-resistant *A. baumannii* (CRAB), which poses a significant clinical challenge (El-Naggar, *et al.*, 2022). These findings suggest that the antimicrobial effect of L-asparaginase on *A. baumannii* is concentration-dependent, with maximum

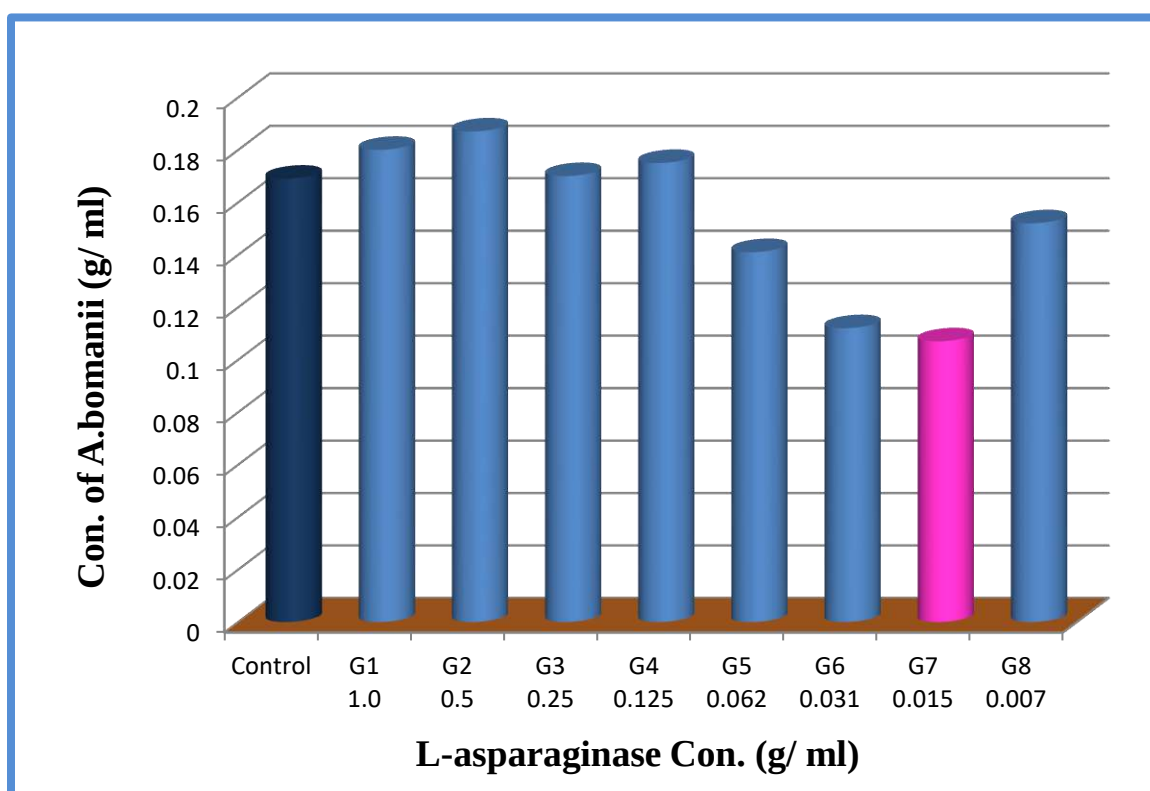
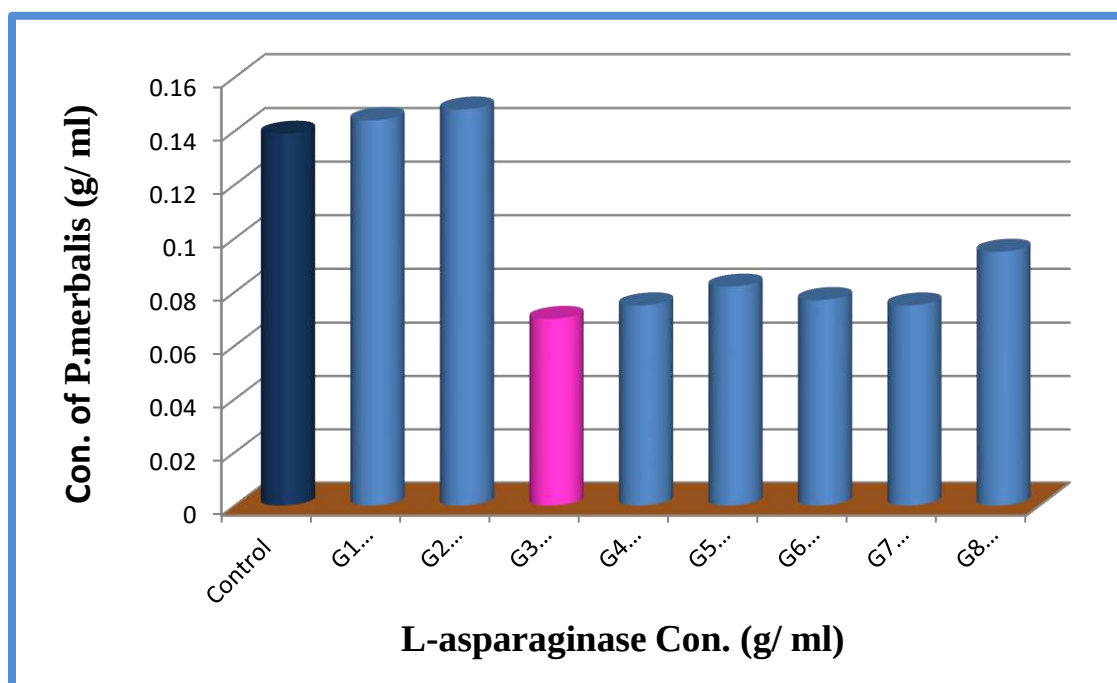


Figure (4-13): Effect L-asparaginase on *A. baumannii*, (Control) It is the control group, (G1 to G8) they are groups at different L- asparaginase concentrations

3- Effect L-asparaginase on *Proteus mirabilis*

The antimicrobial activity of L-asparaginase on *Proteus mirabilis* was evaluated across various concentrations (g/mL), with optical density (OD) measurements used to assess microbial growth inhibition (Figure 4-14). A one-way ANOVA revealed statistically significant differences among the tested concentrations at the 0.05 significance level ($p = 0.001$), indicating a concentration-dependent inhibitory effect. The overall mean OD value across all groups was 0.08804 ± 0.02283 , with the lowest mean OD observed at 0.25 g/mL (0.07075 ± 0.00222), suggesting the highest antimicrobial efficacy at this concentration this finding supports recent research demonstrating that L-asparaginase effectively targets urease-producing pathogens like *P. mirabilis* by depleting asparagine, a crucial amino acid for bacterial metabolism and biofilm formation (Yuvarajan *et al.*, 2022). Post-hoc analysis using the Tukey HSD test confirmed that 0.25 g/mL significantly reduced microbial growth compared to all other concentrations (mean difference range: 0.04475–0.06900; $p < 0.05$). In contrast, higher OD values were recorded at 0.5 g/mL (0.1485 ± 0.00112), indicating relatively lower antimicrobial activity at this concentration may reflect the adaptive metabolic flexibility of *Proteus* species, which can utilize alternative nitrogen sources under nutrient stress (Allocati *et al.*, 2003). These results are particularly relevant given the rising incidence of multidrug-resistant *P. mirabilis* infections in clinical settings, suggesting L-asparaginase could serve as a potential adjunct therapy (Morgan *et al.*, 2023). These findings suggest that the antimicrobial effect of L-asparaginase on *P. mirabilis* is most pronounced at concentration of 0.25 g/mL, making it the most effective in suppressing microbial growth under the conditions of this study.



Figure(4-14): Effect L-asparaginase on *P merbalis*, (Control) It is the control group, (G1 to G8) they are groups at different L- asparaginase concentrations

4- Effect L-asparaginase on *pseudomonas aeruginosa*

The antimicrobial effect of L-asparaginase on *Pseudomonas aeruginosa* was assessed across a range of concentrations using non-parametric analysis due to the lack of normality in the data distribution (Figure 4-15). The Kruskal-Wallis test revealed statistically significant differences among the tested concentrations at the 0.05 significance level ($p = 0.001$), indicating a concentration-dependent inhibitory effect of L-asparaginase. The median optical density (OD) values, expressed as median $\pm$ interquartile range (IQR), varied significantly across groups. The lowest median OD was observed at 0.015 g/mL (0.0925 ± 0.0040), suggesting the highest antimicrobial activity at this concentration these findings agree with recent research showing that L-asparaginase disrupts quorum sensing and biofilm formation in *P. aeruginosa* by depleting asparagine, a key metabolite in bacterial virulence pathways (Li and Lu, 2018). In contrast, higher concentrations such as

1.0 g/mL (0.16475 ± 0.00396) consistently showed elevated OD values, indicating reduced antimicrobial efficacy. Notably, the potent activity at lower concentrations suggests potential clinical utility against carbapenem-resistant *P. aeruginosa* strains, particularly when combined with existing antibiotics (Tirlangi et al., 2023).. These findings support the conclusion that L-asparaginase exerts a significant antimicrobial effect on *P. aeruginosa*, with maximum inhibition occurring at lower concentrations, particularly 0.015 g/mL

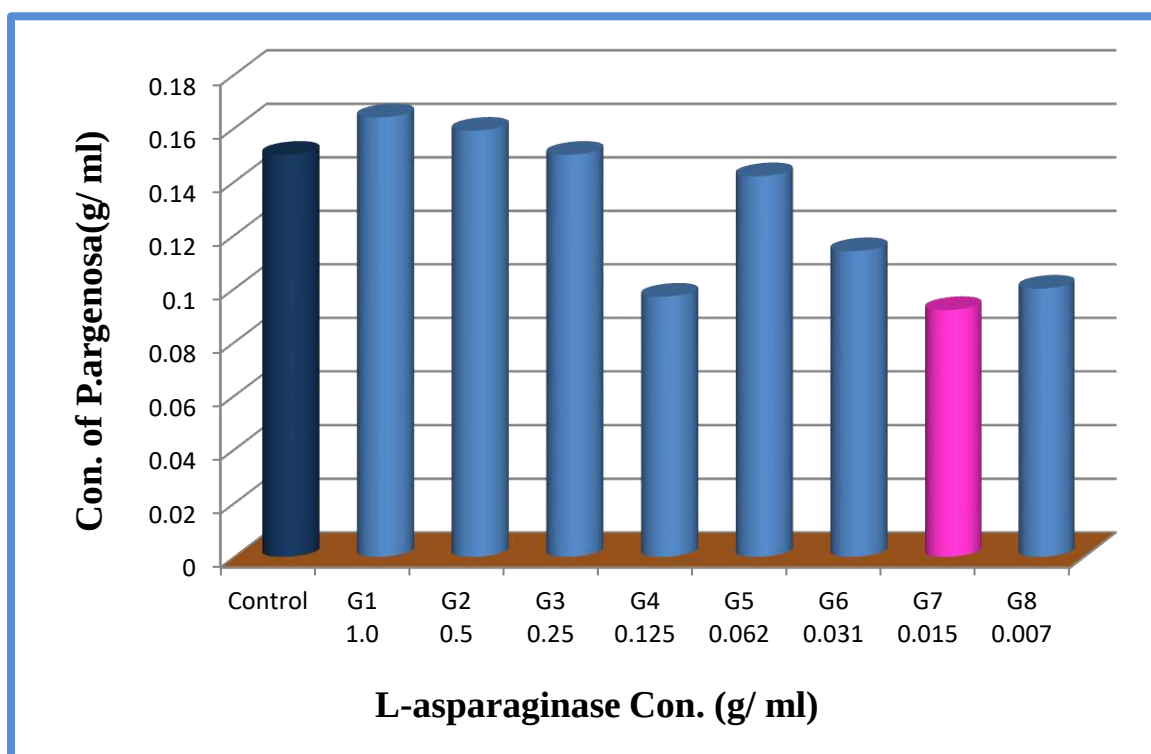
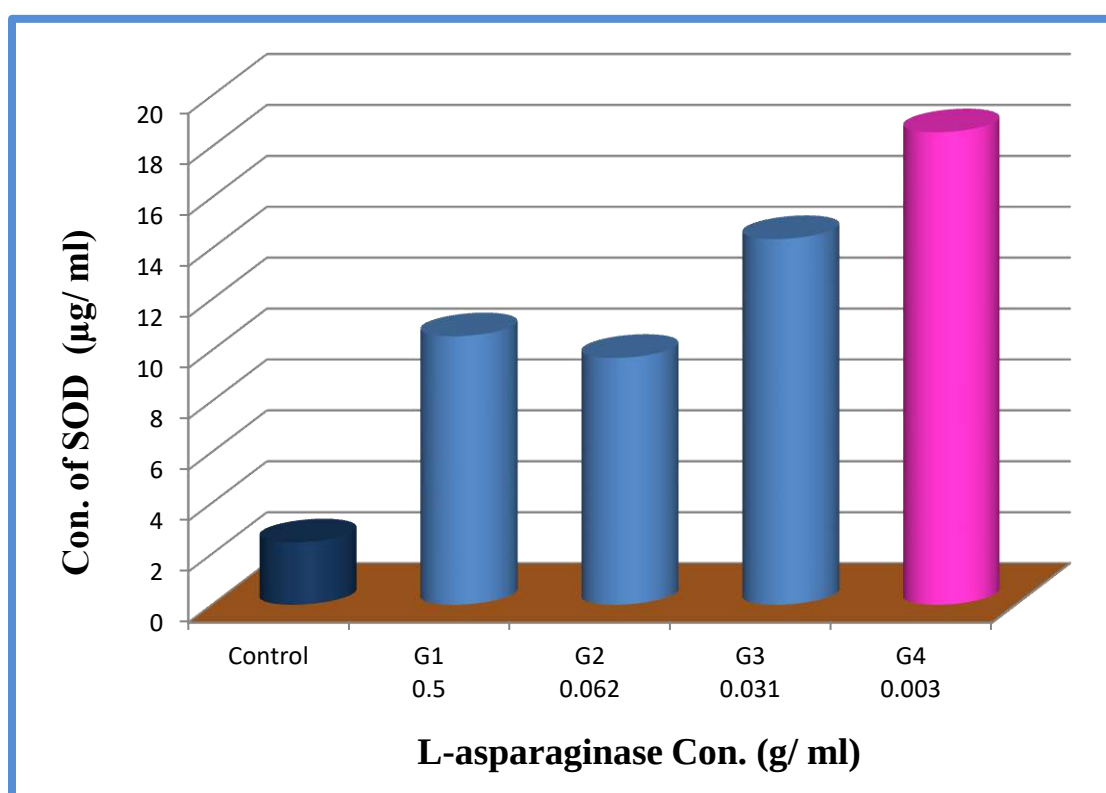


Figure (4-15): Effect L-asparaginase on *Pseudomonas aeruginosa*, (Control) It is the control group, (G1 to G8) they are groups at different L- asparaginase concentrations

4.5.4 Antioxidant activity

4.5.4.1 Antioxidant effect of L-Asparaginase combined with Superoxide Dismutase (SOD)

The synergistic action of L-asparaginase and superoxide dismutase (SOD) presents a promising therapeutic strategy, leveraging dual enzymatic mechanisms to target microbial pathogens and cancer cells. Recent studies indicate that L-asparaginase depletes asparagine, crippling protein synthesis in susceptible cells, while SOD mitigates oxidative stress by converting superoxide radicals into less harmful species, thereby enhancing cellular stress and apoptosis (Liu *et al.*, 2015). The analysis of the effect of L-asparaginase in combination with superoxide dismutase (SOD) on optical density (O.D.) revealed significant differences among the tested concentrations (Figure 4-16).

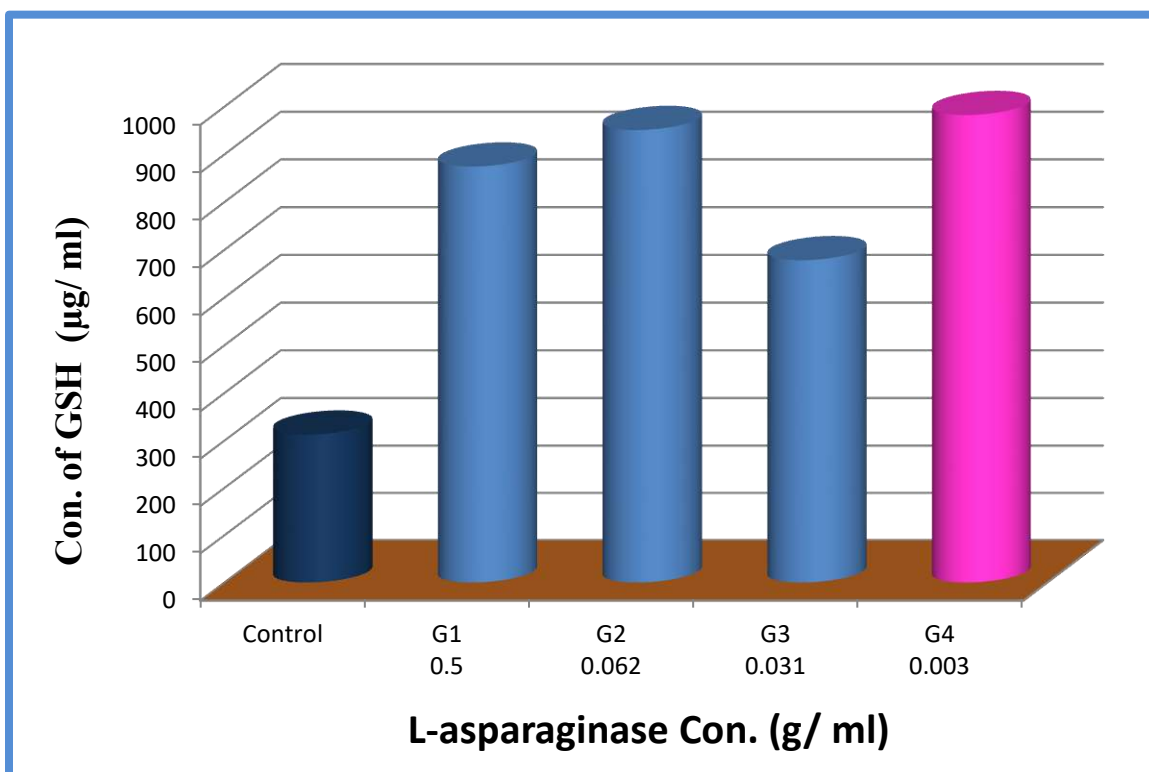


Figure(4-16): Effect L-asparaginase on anti-oxidant superoxide dismutase SOD, (Control) It is the control group, (G1 to G4) they are groups at different L- asparaginase concentrations

Descriptive statistics showed that the mean O.D. values varied considerably across concentrations, ranging from 2.4500 ± 0.68557 at the 0.5 concentration to 18.5600 ± 0.09381 at the 0.003 concentration, with a total mean of 11.1235 ± 5.50579 . The ANOVA test indicated highly significant differences between groups ($p < 0.0001$). Tukey's HSD test further confirmed that most pairwise differences were statistically significant ($p < 0.05$). Notably, the largest mean difference was observed between the 0.5 and 0.003 concentrations, while several other comparisons, such as between 0.5 and 0.031, also showed strong statistical significance. These findings suggest that varying concentrations of L-asparaginase combined with SOD significantly influence enzymatic activity, as reflected in the optical density measurements. Statistically, 0.003 concentration was significantly different from others, indicating a highly significant effect. These findings support recent research showing that the synergistic action of these enzymes creates a dual metabolic-oxidative stress mechanism, where L-asparaginase depletes asparagine while SOD disrupts redox homeostasis, leading to enhanced cellular apoptosis (Takahashi *et al.*, 2017) The inverse concentration-efficacy relationship, with maximal activity at the lowest tested concentration (0.003 g/mL), may reflect optimal enzyme-substrate ratios for synergistic action, as described in recent cancer therapy studies (Vardakas *et al.*, 2022).

4.5.4.2 The Antioxidant Effect of L-Asparaginase combined with Glutathione (GSH)

The analysis of the effect of L-asparaginase used in combination with glutathione peroxidase on optical density (O.D.) revealed significant differences among the tested concentrations (Figure 4-17).



Figure(4-17): Effect L-asparaginase on anti-oxidant glutathione GSH, (Control) It is the control group, (G1 to G4) they are groups at different L-asparaginase concentrations

The ANOVA test, conducted at a 0.05 significance level, indicated highly significant variation between groups ($p < 0.001$). Descriptive statistics showed that the mean O.D. values varied widely across concentrations, ranging from 312.75 ± 29.624 at concentration 0.5 to 982.50 ± 5.568 at concentration 0.003. Tukey's HSD test further confirmed that most pairwise differences were statistically significant,. The largest mean difference was observed between the

two 0.5 concentration groups, indicating a strong contrast in enzymatic activity. Several other comparisons also showed significant differences, such as between 0.5 and 0.062 (-638.250), and 0.5 and 0.031. The inverse dose-response relationship, where lower enzyme concentrations show enhanced efficacy, may reflect optimal substrate-enzyme ratios that prevent catalytic saturation while maintaining redox homeostasis (Mesnage *et al.*, 2017). These findings agree with recent research highlighting the potential of enzyme combinations to target oxidative stress in cancer and neurodegenerative diseases, where conventional antioxidants often fail due to poor specificity (Forman, 2021). However, not all comparisons reached statistical significance; These findings suggest that the concentration of L-asparaginase significantly influences its antioxidant activity when combined with glutathione peroxidase, with the lowest concentration (0.003) yielding the highest measured effect. This synergistic interaction likely occurs through complementary mechanisms: L-asparaginase depletes asparagine to disrupt metabolic pathways generating reactive oxygen species (ROS), while GPx directly reduces hydrogen peroxide and lipid peroxides using glutathione as a cofactor (Minetto, 2019).

Conclusions And Recommendations

Conclusions

1. The local isolates of *A. niger* could produce of L-asparaginase enzyme using submerged culture and *A. niger* SAP-1 isolated from the soil was tested, being the most abundant enzyme- producing.
2. Gel filtration chromatography utilizing Sephadex G-150 (Column chromatography) was active process for purification of enzyme.
- 3-The Study's L-asparaginase enzyme yield was 35.5%, which is regarded as a high percentage.
4. The purified fungal enzyme L-asparaginase showed cytotoxic activity against HepG2 hepatocellular, A375 melanoma cell line with less toxicity against normal cell
- 5- Fungal infection as the predominant etiology, followed by bacterial infections in this study
- 6-. The purified fungal enzyme L-asparaginase showed antimicrobial activity against fungal and bacterial isolates which were MDR.
7. Impact of L-asparaginase on glutathione peroxidase and superoxide dismutase (SOD), which were indicators of its antioxidant activity

Conclusion and Recommendation

.....

Recommendations

1-Possibility of depended on fungi as natural source of L-asparaginase especially *A. niger*

2-Gel filtration chromatography is considering to be an effective technique for enzyme purification.

3. Investigating the antitumor abilities of L-asparaginase and its treatment for tumors transplanted into mice in vivo.

4. Determine the cytotoxicity effect of purified asparaginase on other cell lines such as lung cancer or prostate cancer. etc.

5-tested fungal L-asparaginase activity against other bacteria and fungi, particularly those that cause systemic infections

6. Possible dependence on fungal L-asparaginase as an antioxidant

7- should be aware of fungal infections, especially systemic mycosis which have become more common in immunosuppressive conditions recently.

8-Further studies on other fungi that produce Asparaginase as other natural sources

References

References

References

A

- Ababneh**, Q., Aldaken, N. A., Jaradat, Z., Al Sbei, S., Alawneh, D. A., Al-zoubi, E. A., ... & Saadoun, I. (2021). Molecular epidemiology of carbapenem-resistant *Acinetobacter baumannii* isolated from three major hospitals in Jordan. *International Journal of Clinical Practice*, 75(12), e14998.
- Abhini**, K., Rajan, A. B., Zuhara, K. F., & Sebastian, D. (2022). Response surface methodological optimization of l-asparaginase production from the medicinal plant endophyte *Acinetobacter baumannii* ZAS1. *Journal of Genetic Engineering and Biotechnology*, 20(1), 22.
- Abou-Bakr**, H.A., El-Sahn, M.A. and El-Banna, A.A., (2013). Screening of tannase-producing fungi isolated from tannin-rich sources. *J. of Agri and Food Res*, 2(3), pp.1-12.
- Abu Zaid**, A., Yassien, M., Aboshanab, K., & Elissawy, A. (2021). *Streptomyces variabilis* Isolate MW091521: a new microbial source of heliomycin. *Applied Biochemistry and Microbiology*, 57, 564-570.
- Aggarwal**, S., & Kumari, A. (2024). Role of fungi in biotechnology Entrepreneurship with microorganisms (pp. 39-67): Elsevier.
- Agha**, A., Furnari, M., Chakik, R.M., Abdulhadi Ali, M.M., Alsaudi, D., Bazeed, M., Savarino, V. and Giannini, E.G., (2015). Hepatocellular Carcinoma Is the Most Frequent Final Diagnosis of Focal Liver Lesions Identified in a Cross-Sectional Evaluation of Patients with Chronic Liver Disease in Saudi Arabia. *Journal of Cancer Research*, Ahmad, S., Singh, S., Wasim, S., Anwar, M.,

References

- Parveen, H., & Shah, S. K. (2021). Mucormycosis and COVID-19: A Narrative Review. *Int. J. Curr. Microbiol. App. Sci*, 10(07), 598-609.
- Aisha**, A., Sajjad, A., Munawar, N., Azam, H. M. H., Ishtiaq, U., & Nawaz, S. (2025). Harnessing L-asparaginase from Solanaceae plants: Purification and molecular dynamics studies for cancer targeting. *Asian Journal of Natural Product Biochemistry*, 23(1).
- Ajayi**, O. A., & Olatunji, G. A. (2019). Comparative Study of Antimicrobial and Staining Activity of Selected Anthraquinone Dyes. *Pakistan Journal of Scientific & Industrial Research Series A: Physical Sciences*, 62(2), 92-97.
- Akram**, S., Ahmed, A., He, P., He, P., Liu, Y., Wu, Y., . . . He, Y. (2023). Uniting the role of endophytic fungi against plant pathogens and their interaction. *Journal of Fungi*, 9(1), 72.
- Al Yousef**, S. A. (2022). *Fusarium* sp. L-asparaginases: purification, characterization, and potential assessment as an antileukemic chemotherapeutic agent. *Environmental Science and Pollution Research*, 29(8), 11243-11254.
- Al-Dulimi**, A. G. H. (2021). Immobilization of L-asparaginase on gold nanoparticles for biomedical applications (Doctoral dissertation, Doctorate thesis, College of Biotechnology, Al-Nahrain University).
- Al-Hamdani**, A. H., Al-Dhalimi, M. A., and Alrufae, M. M. A. (2014). Epidemiologic study of dermatophytosis in Al-Najaf government. *Al- Kufa Journal for Biology* . Vol. 6(1).
- Ali**, S., Muzslay, M., Bruce, M., Jeanes, A., Moore, G., & Wilson, A. (2016). Efficacy of two hydrogen peroxide vapour aerial

References

- decontamination systems for enhanced disinfection of. *Journal of Hospital Infection*, 30, 1e8.
- Allocati**, N., Favalaro, B., Masulli, M., Alexeyev, M. F., & Di Ilio, C. (2003). *Proteus mirabilis* glutathione S-transferase B1-1 is involved in protective mechanisms against oxidative and chemical stresses. *Biochemical Journal*, 373(1), 305-311.
- Almohideb**, M. (2020). Epidemiological patterns of skin disease in Saudi Arabia: a systematic review and meta-analysis. *Dermatology Research and Practice*, 2020(1), 5281957.
- Alsharabasy**, A. (2015). Molecular biological studies to evaluate the treatment roles of irradiated scaffolds in ulcers and wounds of rat skin.
- Amaya-Chantaca**, D., Flores-Gallegos, A. C., Iliná, A., Aguilar, C. N., Sepúlveda-Torre, L., Ascacio-Vadlès, J. A., & Chávez-González, M. L. (2022). Comparative extraction study of grape pomace bioactive compounds by submerged and solid-state fermentation. *Journal of Chemical Technology & Biotechnology*, 97(6), 1494-1505.
- Amponsah-Dacosta**, E. (2021). Hepatitis B virus infection and hepatocellular carcinoma in sub-Saharan Africa: Implications for elimination of viral hepatitis by 2030? *World Journal of Gastroenterology*, 27(36), 6025.
- Ando**, M.; Sugimoto, K.; Kitoh, T.; Sasaki, M.; Mukai, K.; Ando, J.; Egashira, M.; Schuster, S.M. and Oshimi, K. (2005). Selective apoptosis of natural killer cell tumors by Lasparaginase. *British J. Haematol*, 130: 860–868.
- Ariaeenejad**, S., Kavousi, K., Zolfaghari, B., Roy, S., Koshiba, T., & Salekdeh, G. H. (2023). Efficient bioconversion of lignocellulosic

References

waste by a novel computationally screened hyperthermostable enzyme from a specialized microbiota. *Ecotoxicology and environmental safety*, 252, 114587.

Aristoy, M.-C., & Toldrá, F. (2024). Essential amino acids Handbook of seafood and seafood products analysis (pp. 287-306): CRC Press.

B

Bai, J., Tang, R., Zhou, K., Chang, J., Wang, H., Zhang, Q., ... & Sun, C. (2022). An asparagine metabolism-based classification reveals the metabolic and immune heterogeneity of hepatocellular carcinoma. *BMC medical genomics*, 15(1), 222.

Bailly, C., Thuru, X., & Quesnel, B. (2020). Combined cytotoxic chemotherapy and immunotherapy of cancer: modern times. *NAR cancer*, 2(1), zcaa002.

BALDEA, C. (2024). THE HIGHLIGHTING OF FLUFFY-TYPE COLONIES FOR MOLDS. *Analele Universitatii din Oradea, Fascicula Ecotoxicologie, Zootehnie si Tehnologii în Industria Alimentara*, 23.

Barry, J., Gadre, A., & Akuthota, P. (2020). Hypersensitivity pneumonitis, allergic bronchopulmonary aspergillosis and other eosinophilic lung diseases. *Current Opinion in Immunology*, 66, 129-135.

Barwant, M., & Lavhate, N. (2020). Isolation and maintenance of fungal pathogens *Aspergillus niger* and *Aspergillus flavus*. *Int. J. Appl. Nat. Sci*, 9(3), 47-52.

Batool, T., Makky, E.A., Jalal, M. and Yusoff, M.M., 2016. A comprehensive review on L-asparaginase and its applications. *Applied biochemistry and biotechnology*, 178, pp.900-923.

References

- Becker**, K., Both, A., Weißelberg, S., Heilmann, C., & Rohde, H. (2020). Emergence of coagulase-negative staphylococci. Expert review of anti-infective therapy, 18(4), 349-366.
- Bennett**, S.E.B.J.W., (2007). An overview of the genus *Aspergillus*. The *Aspergilli*, pp.23-34.
- Bhargavi**, P. L., & Prakasham, R. S. (2012). Proteolytic enzyme production by isolated *Serratia* sp RSPB11: role of environmental parameters. *Current Trends in Biotechnology and Pharmacy*, 6(1), 55-65.
- Bhosale**, H., Uzma, S., & Kadam, T. (2022). Isolation of L-asparaginase from marine bacterium *Bacillus subtilis* and its characterization. *Indian Journal of Geo-Marine Sciences (IJMS)*, 50(02), 97-106.
- Bhutada**, S. A., & Dahikar, S. B. (2017). L-asparaginase: an ultimate anti-neoplastic enzyme isolated from *Bacillus* species.
- Bizuayehu**, H. M., Dadi, A. F., Ahmed, K. Y., Tegegne, T. K., Hassen, T. A., Kibret, G. D., . . . Odo, D. B. (2024). Burden of 30 cancers among men: Global statistics in 2022 and projections for 2050 using population-based estimates. *Cancer*.
- Bongomin**, F., & Fayemiwo, S. A. (2021). Epidemiology of fungal diseases in Africa: a review of diagnostic drivers. *Current Medical Mycology*, 7(1), 63.
- Boudrant**, J., Woodley, J. M., & Fernandez-Lafuente, R. (2020). Parameters necessary to define an immobilized enzyme preparation. *Process Biochemistry*, 90, 66-80.

References

Brandenburg, G., Poppenborg, S., Radcke, C., Wittmann, J., Krähmer, R., Pasut, G., & Leenders, F. (2020). Challenges in the analytical characterization of PEGylated asparaginase. In *Polymer-Protein Conjugates* (pp. 205-231). Elsevier.

Brito-Lopez, C., van der Wielen, N., Barbosa, M., & Karlova, R. (2025). Plant growth-promoting microbes and microalgae-based biostimulants: Sustainable strategy for agriculture and abiotic stress resilience. *Philosophical Transactions B*, 380(1927), 20240251.

C

Costa, A. R., Batistão, D. W., Ribas, R. M., Sousa, A. M., Pereira, M. O., & Botelho, C. M. (2013). *Staphylococcus aureus* virulence factors and disease.

Calistri, L., Rastrelli, V., Nardi, C., Maraghelli, D., Vidali, S., Pietragalla, M., & Colagrande, S. (2021). Imaging of the chemotherapy-induced hepatic damage: Yellow liver, blue liver, and pseudocirrhosis. *World Journal of Gastroenterology*, 27(46), 7866.

Carta, G., & Jungbauer, A. (2020). *Protein chromatography: process development and scale-up*. John Wiley & Sons.

Casotti, M. C., Meira, D. D., Zetum, A. S. S., Campanharo, C. V., da Silva, D. R. C., Giacinti, G. M., . . . Altoé, L. S. C. (2024). Integrating frontiers: a holistic, quantum and evolutionary approach to conquering cancer through systems biology and multidisciplinary synergy. *Frontiers in Oncology*, 14, 1419599.

References

- Castro**, D., Marques, A. S. C., Almeida, M. R., de Paiva, G. B., Bento, H. B., Pedrolli, D. B., . . . Santos-Ebinuma, V. C. (2021). L-asparaginase production review: bioprocess design and biochemical characteristics. *Applied microbiology and biotechnology*, 105, 4515-4534.
- Chand**, S., Mahajan, R. V., Prasad, J. P., Sahoo, D. K., Mihooliya, K. N., Dhar, M. S., & Sharma, G. (2020). A comprehensive review on microbial l-asparaginase: Bioprocessing, characterization, and industrial applications. *Biotechnology and Applied Biochemistry*, 67(4), 619-647.
- Chauhan**, A., Jindal, T., Chauhan, A., & Jindal, T. (2020). Microbiological Methods for Water, Soil and Air Analysis. *Microbiological Methods for Environment, Food and Pharmaceutical Analysis*, 93-196.
- Chen**, C., & Zhang, J. (2024). Enhancing leukemia treatment: the role of combined therapies based on amino acid starvation. *Cancers*, 16(6), 1171.
- Chen**, D., Li, Y., Li, X., Hong, X., Fan, X., & Savidge, T. (2022). Key difference between transition state stabilization and ground state destabilization: increasing atomic charge densities before or during enzyme–substrate binding. *Chemical Science*, 13(27), 8193-8202.
- Cheng**, X., He, L., Xu, J., Fang, Q., Yang, L., Xue, Y., . . . Tang, R. (2020). Oxygen-producing catalase-based prodrug nanoparticles overcoming resistance in hypoxia-mediated chemo-photodynamic therapy. *Acta Biomaterialia*, 112, 234-249.

References

- Chowdhary, A., Sharma, C., Hagen, F., & Meis, J. F. (2014).** Exploring azole antifungal drug resistance in *Aspergillus fumigatus* with special reference to resistance mechanisms. *Future microbiology*, 9(5), 697-711.
- Christian, S. L. (2022).** Overview of the Maintenance of Authentic Cancer Cell Lines. *Cancer Cell Biology: Methods and Protocols*, 1-7.
- Chrysavgis, L., Giannakodimos, I., Diamantopoulou, P., & Cholongitas, E. (2022).** Non-alcoholic fatty liver disease and hepatocellular carcinoma: Clinical challenges of an intriguing link. *World Journal of Gastroenterology*, 28(3), 310.
- Cini, M., & Borragán, N. P.-S. (2022).** European union politics: oxford university press.
- Conrado, R., Gomes, T. C., Roque, G. S. C., & De Souza, A. O. (2022).** Overview of bioactive fungal secondary metabolites: Cytotoxic and antimicrobial compounds. *Antibiotics*, 11(11), 1604.
- Costa, A. R. Batistão, W. F. Ribas, R. M. Sousa, A. M. Pereira, M. O. and Botelho,**
- Cunha, J. R. (2021).** Effects of site-directed PEGylation on L-asparaginase thermostability. Universidade de São Paulo.
- Cuthbertson, L., Rogers, G. B., Walker, A. W., Oliver, A., Hafiz, T., Hoffman, L. R., ... & Van Der Gast, C. J. (2014).** Time between collection and storage significantly influences bacterial sequence composition in sputum samples from cystic fibrosis respiratory infections. *Journal of clinical microbiology*, 52(8), 3011-3016.

D

- Determann, H.** (2013). Gel Chromatography: Gel Filtration· Gel Permeation· Molecular Sieves: A Laboratory Handbook. Springer.
- Devi, R., Kaur, T., Guleria, G., Rana, K. L., Kour, D., Yadav, N., . Saxena, A. K.** (2020). Fungal secondary metabolites and their biotechnological applications for human health New and future developments in microbial biotechnology and bioengineering (pp. 147-161): Elsevier.
- Dhale, M. A., & Mohan-Kumari, H. P.** (2014). A comparative rapid and sensitive method to screen L-asparaginase producing fungi. *Journal of microbiological methods*, 102, 66-68.
- Dhankhar, R., Gupta, V., Kumar, S., Kapoor, R. K., & Gulati, P.** (2020). Microbial enzymes for deprivation of amino acid metabolism in malignant cells: biological strategy for cancer treatment. *Applied Microbiology and Biotechnology*, 104, 2857-2869.
- Djavid, A. R., Stonesifer, C., Fullerton, B. T., Wang, S. W., Tartaro, M. A., Kwinta, B. D., ... & Saenger, Y. M.** (2021). Etiologies of melanoma development and prevention measures: A review of the current evidence. *Cancers* , 13(19), 4914.
- Djavid, A. R., Stonesifer, C., Fullerton, B. T., Wang, S. W., Tartaro, M. A., Kwinta, B. D., . . . Saenger, Y. M.** (2021). Etiologies of melanoma development and prevention measures: a review of the current evidence. *Cancers*, 13(19), 4914.
- Du, W., Yao, Z., Li, J., Sun, C., Xia, J., Wang, B., . . . Ren, L.** (2020). Diversity and antimicrobial activity of endophytic fungi isolated

References

from *Securinega suffruticosa* in the Yellow River Delta. Plos one, 15(3), e0229589.

Durlach, V., Vergès, B., Al-Salameh, A., Bahougne, T., Benzerouk, F., Berlin, I., . . . Thomas, D. (2022). Smoking and diabetes interplay: a comprehensive review and joint statement. *Diabetes & metabolism*, 48(6), 101370.

E

El-Amier, Y. A., El-hayyany, L. Y., Ahmed, I. K., Al-Rubaye, A. A., Fahmy, A. A., Khattab, Y. Z., & Sultan, M. S. (2024). Evaluation of the VITEK® 2 Cards for Rapid Direct Identification and Antimicrobial Susceptibility Testing of Human Pathogenic Bacteria. *Egyptian Journal of Chemistry*, 67(2), 91-102.

El-Gendy, M. M. A. A., Awad, M. F., El-Shenawy, F. S., & El-Bondkly, A. M. A. (2021). Production, purification, characterization, antioxidant and antiproliferative activities of extracellular L-asparaginase produced by *Fusarium equiseti* AHMF4. *Saudi Journal of Biological Sciences*, 28(4), 2540-2548.

El-Gendy, M. M. A. A., Awad, M. F., El-Shenawy, F. S., & El-Bondkly, A. M. A. (2021). Production, purification, characterization, antioxidant and antiproliferative activities of extracellular L-asparaginase produced by *Fusarium equiseti* AHMF4. *Saudi Journal of Biological Sciences*, 28(4), 2540-2548.

El-Hadi, A. A., El-Refai, H. A., Shafei, M. S., Zaki, R., & Mostafa, H. (2017). Statistical optimization of L-asparaginase production by using. *Egyptian Pharmaceutical Journal*, 16(1), 16-23.

References

- Elkhateeb**, Y. A., & Fadel, M. (2022). Bioinformatic studies, experimental validation of phytase production and optimization of fermentation conditions for enhancing phytase enzyme production by different microorganisms under solid-state fermentation. *The Open Microbiology Journal*, 16(1).
- El-Naggar**, N. E. A., Shiha, A. M., Mahrous, H., & Mohammed, A. A. (2022). Green synthesis of chitosan nanoparticles, optimization, characterization and antibacterial efficacy against multi drug resistant biofilm-forming *Acinetobacter baumannii*. *Scientific Reports*, 12(1), 19869.
- El-Naggar**, N. E.-A., & El-Shweihy, N. M. (2020). Bioprocess development for L-asparaginase production by *Streptomyces rochei*, purification and in-vitro efficacy against various human carcinoma cell lines. *Scientific Reports*, 10(1), 7942.
- El-Naggar**, N. E.-A., & El-Shweihy, N. M. (2020). Bioprocess development for L-asparaginase production by *Streptomyces rochei*, purification and in-vitro efficacy against various human carcinoma cell lines. *Scientific Reports*, 10(1), 7942.
- Elshafei**, A., & El-Ghonemy, D. H. (2021). Extracellular glutaminase-free L-asparaginase from *Trichoderma viride* F2: purification, biochemical characterization and evaluation of its potential in mitigating acrylamide formation in starchy fried food. *Journal of microbiology, biotechnology and food sciences*, 11(2), e4336-e4336.
- Eryilmaz**, I. E., Bergel, C. C., Arıöz, B., & Egeli, Ü. (2025). Cytotoxic and Apoptotic Agent Encorafenib Controversially Alters Invasive

References

Properties of Castration-Resistant Prostate Cancer Cells with High and Moderate Metastatic Potential. *Osmangazi Tıp Dergisi*, 47(2), 263-270.

F

Fazeli, N., Alimadadi, N. and Nasr, S., 2021. Screening and optimization of process parameters for the production of L-asparaginase by indigenous fungal-type strains. *Iranian Journal of Science and Technology, Transactions A: Science*, 45(2), pp.409-416.

Fazili, A. B. A., Shah, A. M., Zan, X., Naz, T., Nosheen, S., Nazir, Y., . . . Song, Y. (2022). *Mucor circinelloides*: a model organism for oleaginous fungi and its potential applications in bioactive lipid production. *Microbial cell factories*, 21(1), 29.

Form, H. A. (2023). USA. CDC [16]. Diagnosis and Treatment of Fungal Infections, 280.

Forman, H. J., & Zhang, H. (2021). Targeting oxidative stress in disease: promise and limitations of antioxidant therapy. *Nature Reviews Drug Discovery*, 20(9), 689-709.

Frayman, K. B., Armstrong, D. S., Grimwood, K., & Ranganathan, S. C. (2017). The airway microbiota in early cystic fibrosis lung disease. *Pediatric pulmonology*, 52(11), 1384-1404.

Freitas, M., Souza, P., Cardoso, S., Cruvinel, K., Abrunhosa, L. S., Ferreira Filho, E. X., . . . O. Magalhães, P. (2021). Filamentous fungi producing L-asparaginase with low glutaminase activity isolated from Brazilian savanna soil. *Pharmaceutics*, 13(8), 1268.

References

Fu, X., Hou, R., Yang, P., Qian, S., Feng, Z., Chen, Z., . . . Zhou, B. (2022). Application of external carbon source in heterotrophic denitrification of domestic sewage: A review. *Science of the total environment*, 817, 153061.

G

Garcia, M. A. (2023). Human Papillomavirus (HPV) Vaccination in Pediatric, Adolescent, and Young Adult (PAYA) Cancer Survivors. State University of New York at Buffalo.

Garcia-Maset, R., Chu, V., Yuen, N., Blumgart, D., Yoon, J., Murray, B. O., . . . Rohn, J. L. (2025). Effect of host microenvironment and bacterial lifestyles on antimicrobial sensitivity and implications for susceptibility testing. *npj Antimicrobials and Resistance*, 3(1), 1-17.

Gefenas, E., Lekstutiene, J., Lukaseviciene, V., Hartlev, M., Mourby, M., & Cathaoir, K. (2022). Controversies between regulations of research ethics and protection of personal data: informed consent at a cross-road. *Medicine, Health Care and Philosophy*, 1-8.

Gulcin, İ., & Alwasel, S. H. (2022). Metal ions, metal chelators and metal chelating assay as antioxidant method. *Processes*, 10(1), 132.

H

Haji Mirghasemi, S. M., Pordeli, H. R., Fozouni, L., Komshikamar, M., Komshikamar, F., & Rubin, M. (2023). Exploring bacillus subtilis isolated from soil as a promising alternative for L-asparaginase enzyme production. *Health Biotechnology and Biopharma (HBB)*, 7(3), 45-61.

References

- Hallemeier**, C. L., Sharma, N., Anker, C., Selfridge, J. E., Lee, P., Jabbour, S., . . . Jethwa, K. R. (2023). American Radium Society Appropriate Use Criteria for the use of liver-directed therapies for nonsurgical management of liver metastases: Systematic review and guidelines. *Cancer*, 129(20), 3193-3212.
- Hameed**, A., Hussain, S. A., Ijaz, M. U., Ullah, S., Muhammad, Z., Suleria, H. A. R., & Song, Y. (2020). Antioxidant activity of polyphenolic extracts of filamentous fungus *Mucor circinelloides* (WJ11): Extraction, characterization and storage stability of food emulsions. *Food Bioscience*, 34, 100525.
- Haque**, M. A., Nath, N. D., Johnston, T. V., Haruna, S., Ahn, J., Ovissipour, R., & Ku, S. (2024). Harnessing biotechnology for penicillin production: Opportunities and environmental considerations. *Science of the Total Environment*, 174236.
- Haque**, M. A., Nath, N. D., Johnston, T. V., Haruna, S., Ahn, J., Ovissipour, R., & Ku, S. (2024). Harnessing biotechnology for penicillin production: Opportunities and environmental considerations. *Science of the total environment*, 174236.
- Hashem**, Z. S. (2025). Bacterial Metabolites in Defense: A Crucial Aspect of Microbial Interaction and Host Protection Metabolic Dynamics in Host-Microbe Interaction (pp. 101-120): Springer.
- Hassan**, T., & Hussein, S. (2022). Development of bioprocesses for production and purification of l-asparaginase from staphylococcus aureus, and in vitro effecacy against human breast cncer cell line. *Iraqi Journal of Agricultural Sciences*, 53(6), 1525-1538.

References

- Hiergeist, A.,** Reischl, U., & Gessner, A. (2016). Multicenter quality assessment of 16S ribosomal DNA-sequencing for microbiome analyses reveals high inter-center variability. *International Journal of Medical Microbiology*, 306(5), 334-342.
- Horváth, Z.,** Evelin, C.A., Oláh, P., Gyulai, R. and Lengyel, Z., 2021. Results of a Primary Skin-Cancer-Prevention Campaign in Early Childhood on Sun-Related Knowledge and Attitudes in Southern Hungary. *Cancers*, 13(15), p.3873.
- Hoyle, H.,** Stenger, C., & Przyborski, S. (2022). Design considerations of benchtop fluid flow bioreactors for bio-engineered tissue equivalents in vitro. *Biomaterials and Biosystems*, 8, 100063.
- Hussain, A. M.,** & Lafta, R. K. (2021). Cancer trends in Iraq 2000–2016. *Oman medical journal*, 36(1), e219.
- Hynds, R. E.,** Vladimirov, E., & Janes, S. M. (2018). The secret lives of cancer cell lines (Vol. 11, pp. dmm037366): The Company of Biologists Ltd.

I

- Ishak, N. H.,** Shaik, M. I., Yellapu, N. K., Howell, N. K., & Sarbon, N. M. (2021). Purification, characterization and molecular docking study of angiotensin-I converting enzyme (ACE) inhibitory peptide from shortfin scad (*Decapterus macrosoma*) protein hydrolysate. *Journal of food science and technology*, 1-11.

J

References

- Jacobsen, I. D.** (2023). The role of host and fungal factors in the commensal-to-pathogen transition of *Candida albicans*. *Current Clinical Microbiology Reports*, 10(2), 55-65.
- Jacobsen, S. M., & Shirtliff, M. E.** (2011). *Proteus mirabilis* biofilms and catheter-associated urinary tract infections. *Virulence*, 2(5), 460-465.
- Jan, S., Singh, R., Bhardwaj, R., Ahmad, P., & Kapoor, D.** (2020). Plant growth regulators: a sustainable approach to combat pesticide toxicity. *3 Biotech*, 10(11), 466.
- Jesudason, T.** (2024). WHO publishes updated list of bacterial priority pathogens. *The Lancet Microbe*, 5(9).
- Ji, X., Chen, J., Ye, J., Xu, S., Lin, B., & Hou, K.** (2023). Epidemiological analysis of global and regional lung cancer mortality: Based on 30-year data analysis of global burden disease database. Paper presented at the Healthcare.
- Job, N., Sruthy, K., Jose, D., Puthumana, J., Sarasan, M., Nevin, K., . . . Philip, R.** (2025). L-asparaginase from the mangrove endophyte *Penicillium citrinum* MEF 455: a focus on cancer surveillance gene expression in tumor cell lines HL-60 and NCI-H 460. *Toxicology Research*, 14(3), tfaf067.
- Joseph, J. A., Akkermans, S., & Van Impe, J. F.** (2022). Processing method for the quantification of methanol and ethanol from bioreactor samples using gas chromatography–flame ionization detection. *Acs Omega*, 7(28), 24121-24133.
- Jouhar, L., Jaafar, R. F., Nasreddine, R., Itani, O., Haddad, F., Rizk, N., & Hoballah, J. J.** (2020). Microbiological profile and

References

antimicrobial resistance among diabetic foot infections in Lebanon. *International Wound Journal*, 17(6), 1764-1773.

K

Kadry, S., Verdú, E., Damasevicius, R., Abualigah, L., Singh, V., & Rajinikanth, V. (2024). CNN segmentation of skin melanoma in pre-processed dermoscopy images. *Procedia Computer Science*, 235, 2775-2782.

Kaerger, K., Schwartz, V. U., Dolatabadi, S., Nyilasi, I., Kovács, S. A., Binder, U., ... & Voigt, K. (2015). Adaptation to thermotolerance in *Rhizopus* coincides with virulence as revealed by avian and invertebrate infection models, phylogeny, physiological and metabolic flexibility. *Virulence*, 6(4), 395-403.

Kamil Zaidan, H., Jasim Al-Khafaji, H. H., Al-dolaimy, F., Abed Hussein, S., Otbah Farqad, R., Thabit, D., . . . Alawadi, A. H. (2024). Exploring the therapeutic potential of Lawsone and nanoparticles in Cancer and Infectious Disease Management. *Chemistry & Biodiversity*, 21(4), e202301777.

Kandasamy, G. D., & Kathirvel, P. (2023). Insights into bacterial endophytic diversity and isolation with a focus on their potential applications—A review. *Microbiological Research*, 266, 127256.

Kango, N., Jana, U. K., & Choukade, R. (2019). Fungal enzymes: sources and biotechnological applications. *Advancing frontiers in mycology & mycotechnology: basic and applied aspects of fungi*, 515-538.

References

- Karlsson**, M., Atanasova, L., Jensen, D. F., & Zeilinger, S. (2017). Necrotrophic mycoparasites and their genomes. *Microbiology Spectrum*, 5(2), 10.1128/microbiolspec.funk-0016-2016.
- Khan**, M.A., Ali, R., Parveen, R., Najmi, A.K. and Ahmad, S., 2016. Pharmacological evidences for cytotoxic and antitumor properties of Boswellic acids from *Boswellia serrata*. *Journal of ethnopharmacology*, 191, pp.315-323.
- Kilikian**, B. V., Pessoa, A., Picó, G. A., & Braia, M. J. (2024). Purification Processes: Analytical Methods and Enzyme Stability Purification of Biotechnological Products (pp. 11-33): CRC Press.
- Killander**, D., Dohlwitz, A., Engstedt, L., Franzen, S., Gahrton, G., Gullbring, B., Holm, G., Holmgren, A., Höglund, S., Killander, A. and Lockner, D., 1976. Hypersensitive reactions and antibody formation during L-asparaginase treatment of children and adults with acute leukemia. *Cancer*, 37(1), pp.220-228.
- Klebe**, G. (2025). Inhibitors of hydrolases with an acyl–enzyme intermediate Drug Design: From Structure and Mode-of-Action to Rational Design Concepts (pp. 371-401): Springer.
- Koehler**, P., Bassetti, M., Chakrabarti, A., Chen, S. C., Colombo, A. L., Hoenigl, M., ... & Cornely, O. A. (2021). Defining and managing COVID-19-associated pulmonary aspergillosis: the 2020 ECMM/ISHAM consensus criteria for research and clinical guidance. *The Lancet Infectious Diseases*, 21(6), e149-e162.
- Kotzia**, G.A., Lappa, K. and Labrou, N.E., 2017. Tailoring structure–function properties of L-asparaginase: engineering resistance to trypsin cleavage. *Biochemical Journal*, 404(2), pp.337-343.

References

Kroll, S. (2020). Thermal stability Enzymes of psychrotrophs in raw food (pp. 121-152): CRC Press.

Kuplińska, A., & Rząd, K. (2021). Molecular targets for antifungals in amino acid and protein biosynthetic pathways. *Amino Acids*, 53(7), 961-991.

L

Lange, H., Rulli, F., & Crestini, C. (2016). Gel permeation chromatography in determining molecular weights of lignins: critical aspects revisited for improved utility in the development of novel materials. *ACS Sustainable Chemistry & Engineering*, 4(10), 5167-5180.

Larsson, P., Engqvist, H., Biermann, J., Werner Rönnerman, E., Forssell-Aronsson, E., Kovács, A., . . . Parris, T. Z. (2020). Optimization of cell viability assays to improve replicability and reproducibility of cancer drug sensitivity screens. *Scientific Reports*, 10(1), 5798.

Lei, T. Y., Liao, B. B., Yang, L. R., Wang, Y., & Chen, X. B. (2024). Hypervirulent and carbapenem-resistant *Klebsiella pneumoniae*: A global public health threat. *Microbiological Research*, 127839.

Lemij, A. A., Bastiaannet, E., De Glas, N. A., van den Bos, F., Portielje, J. E., Liefers, G.-J., & Derks, M. G. (2023). Breast cancer in the older population: a global challenge—an epidemiological perspective. *Annals of Breast Surgery*, 7.

Lenicek Krleza, J., Katusic Bojanac, A., & Jakovljevic, G. (2024). Determination of l-asparaginase activity and its therapeutic monitoring in children with hematological malignancies in a single croatian center. *Diagnostics*, 14(6), 623.

References

- Lewnard, J. A., & Hanage, W. P. (2019). Making sense of differences in pneumococcal serotype replacement. *The Lancet Infectious Diseases*, 19(6), e213-e220.
- Li, G., & Lu, C. D. (2018). Molecular characterization and regulation of operons for asparagine and aspartate uptake and utilization in *Pseudomonas aeruginosa*. *Microbiology*, 164(2), 205-216.
- Li, Y., Awasthi, M. K., Sindhu, R., Binod, P., Zhang, Z., & Taherzadeh, M. J. (2023). Biochar preparation and evaluation of its effect in composting mechanism: A review. *Bioresource Technology*, 384, 129329.
- Lin, M. F., & Lan, C. Y. (2014). Antimicrobial resistance in *Acinetobacter baumannii*: From bench to bedside. *World Journal of Clinical Cases: WJCC*, 2(12), 787.
- Liu, B., Zhou, H., Tan, L., Siu, K. T. H., & Guan, X.-Y. (2024). Exploring treatment options in cancer: tumor treatment strategies. *Signal transduction and targeted therapy*, 9(1), 175.
- Liu, Y., Li, J., & Lu, Y. (2015). Enzyme therapeutics for systemic detoxification. *Advanced drug delivery reviews*, 90, 24-39.
- Liu, Z., Zhou, Y., & Yuan, L. (2022). Hydrogen-bonded aromatic amide macrocycles: synthesis, properties and functions. *Organic & Biomolecular Chemistry*, 20(46), 9023-9051.
- Lu, C., Zou, K., Guo, B., Li, Q., Wang, Z., Xiao, W., & Zhao, L. (2023). Linker-peptide-mediated one-step purification and immobilization of α -L-rhamnosidase from *Bacteroides thetaiotaomicron* for direct biotransformation from epimedin C to icariin. *Enzyme and Microbial Technology*, 162, 110131.

References

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

Lukić, M., Pantelić, I., & Savić, S. D. (2021). Towards optimal pH of the skin and topical formulations: From the current state of the art to tailored products. *Cosmetics*, 8(3), 69.

M

Ma, X., Mi, Y., Zhao, C., & Wei, Q. (2022). A comprehensive review on carbon source effect of microalgae lipid accumulation for biofuel production. *Science of the total environment*, 806, 151387.

Maake, R. (2022). The effect of Mg^{2+} and Ni^{2+} on the kinetics and thermodynamics of ATP-binding, catalysis, and stability of *Klebsiella pneumoniae* nicotinate nucleotide adenylyltransferase. University of the Witwatersrand, Johannesburg (South Africa).

Maithani, D., Sharma, A., Gangola, S., Chaudhary, P., & Bhatt, P. (2022). Insights into applications and strategies for discovery of microbial bioactive metabolites. *Microbiological Research*, 261, 127053.

Maithani, D., Sharma, A., Gangola, S., Chaudhary, P., & Bhatt, P. (2022). Insights into applications and strategies for discovery of microbial bioactive metabolites. *Microbiological Research*, 261, 127053.

Marchetti, P., Fovez, Q., Germain, N., Khamari, R., & Kluza, J. (2020). Mitochondrial spare respiratory capacity: mechanisms, regulation, and significance in non-transformed and cancer cells. *The FASEB Journal*, 34(10), 13106-13124.

References

- Mason, A., & Raymond, J. (2022).** THE MICROSCOPE. Laboratory Exercises in Microbiology.
- McGlynn, K. A., Petrick, J. L., & El-Serag, H. B. (2021).** Epidemiology of hepatocellular carcinoma. *Hepatology*, 73(S1), 4-13.
- McGlynn, K. A., Petrick, J. L., & El-Serag, H. B. (2021).** Epidemiology of hepatocellular carcinoma. *Hepatology*, 73, 4-13.
- Meghwanshi, G. K., Kaur, N., Verma, S., Dabi, N. K., Vashishtha, A., Charan, P., . . . Kumar, R. (2020).** Enzymes for pharmaceutical and therapeutic applications. *Biotechnology and Applied Biochemistry*, 67(4), 586-601.
- Mesnage, R., Renney, G., Séralini, G. E., Ward, M., & Antoniou, M. N. (2017).** Multiomics reveal non-alcoholic fatty liver disease in rats following chronic exposure to an ultra-low dose of Roundup herbicide. *Scientific reports*, 7(1), 39328.
- Micu, G., Israel-Roming, F., & Cornea, C. P. (2020).** Methods for screening of novel L-asparaginase from microorganisms. *Scientific Bulletin Series F. Biotechnologies*, 24.
- Mihooliya, K. N., Nandal, J., Kumari, A., Nanda, S., Verma, H., & Sahoo, D. K. (2020).** Studies on efficient production of a novel l-asparaginase by a newly isolated *Pseudomonas resinovorans* IGS-131 and its heterologous expression in *Escherichia coli*. *3 Biotech*, 10, 1-11.
- Minetto, P. (2019).** Metabolic starvation triggered by l-asparaginase sensitizes multiple myeloma cells to proteasome inhibitors by inducing DNA damage accumulation.

References

- Moguel**, I. S., Yamakawa, C. K., Brumano, L. P., Pessoa Jr, A., & Mussatto, S. I. (2022). Selection and optimization of medium components for the efficient production of L-asparaginase by *Leucosporidium scottii* L115—A Psychrotolerant Yeast. *Fermentation*, 8(8), 398.
- Mohammadian**, M., Bakeshei, K. A., & Mohammadian-Hafshejani, A. (2020). International epidemiology of liver cancer: geographical distribution, secular trends and predicting the future. *Journal of Preventive Medicine and Hygiene*, 61(2), E259.
- Morellon-Sterling**, R., Tavano, O., Bolivar, J. M., Berenguer-Murcia, Á., Vela-Gutiérrez, G., Sabir, J. S., . . . Fernandez-Lafuente, R. (2022). A review on the immobilization of pepsin: A Lys-poor enzyme that is unstable at alkaline pH values. *International Journal of Biological Macromolecules*, 210, 682-702.
- Morgan**, R. N., Ali, A. A., Alshahrani, M. Y., & Aboshanab, K. M. (2023). New insights on biological activities, chemical compositions, and classifications of marine actinomycetes antifouling agents. *Microorganisms*, 11(10), 2444.
- Morris**, A. J., Kidd, S. E., Halliday, C. L., Chen, S. C., McKinney, W., Ryan, K., & Elvy, J. (2024). Update on methods used for mycological testing: wide diversity and opportunities for improvement persist. *Pathology*, 56(7), 1021-1027.
- Mudenda**, S. (2024). Global Burden of fungal infections and antifungal resistance from 1961 to 2024: Findings and future implications. *Pharmacology & Pharmacy*, 15(4), 81-112.

References

Mukhopadhyay, R., & Kumar, D. (2020). Trichoderma: a beneficial antifungal agent and insights into its mechanism of biocontrol potential. *Egyptian Journal of Biological Pest Control*, 30, 1-8.

Muneer, F., Siddique, M. H., Azeem, F., Rasul, I., Muzammil, S., Zubair, M., . . . Nadeem, H. (2020). Microbial L-asparaginase: purification, characterization and applications. *Archives of Microbiology*, 202, 967-981.

Muneer, F., Siddique, M.H., Azeem, F., Rasul, I., Muzammil, S., Zubair, M., Afzal, M. and Nadeem, H., 2020. Microbial L-asparaginase: purification, characterization and applications. *Archives of Microbiology*, 202, pp.967-981.

N

Nafiseh, D., & Mohammad, B. (2013). Over Production for Microbial Secondary Metabolites. *International Journal; of Life Sciences and Pharma Research*, 3, 23-37.

Nafiseh, D., & Mohammad, B. (2013). Overproduction of microbial secondary metabolites. *International Journal of Life Sciences and Pharma Research*, 3, 23–37.

Najem, M. H; Al-Salhi, M. H. and Hamim, S. S. (2016). Study of Dermatophytosis prevalence in Al-Nassiriyah city- Iraq. *World J Pharm Sci.* 4(4): 166-172.

Naranjo-Ortiz, M. A., & Gabaldón, T. (2020). Fungal evolution: cellular, genomic and metabolic complexity. *Biological Reviews*, 95(5), 1198-1232.

References

- Narayana**, K.J.P., Kumar, K.G. and Vijayalakshmi, M., 2008. L-asparaginase production by *Streptomyces albidoflavus*. Indian Journal of Microbiology, 48, pp.331-336
- Nischitha**, R., & Shivanna, M. (2021). Antimicrobial activity and metabolite profiling of endophytic fungi in *Digitaria bicornis* (Lam) Roem. and Schult. and *Paspalidium flavidum* (Retz.) A. Camus. 3 Biotech, 11(2), 53.
- Nouri**, H., Moghimi, H., & Khaleghian, A. (2020). Production and characterization of biochemical properties of L-Asparaginase by indigenous yeast isolated from soil of Iran. Koomesh, 22(1), 178-184.
- Nunes**, J. C., Cristóvão, R. O., Santos-Ebinuma, V. C., Faria, J. L., Silva, C. G., Neves, M. C., . . . Tavares, A. P. (2021). L-Asparaginase-based biosensors. Encyclopedia, 1(3), 848-858.
- Nüse**, B., Holland, T., Rauh, M., Gerlach, R. G., & Mattner, J. (2023). L-arginine metabolism as pivotal interface of mutual host–microbe interactions in the gut. Gut Microbes, 15(1), 2222961.

O

- Okafor**, U. C., & Mmaduabuchi, C. E. (2022). Assessment of the microbial quality of some cassava granules (garri) sold at Umuoji Major Markets, Anambra State Nigeria. Journal of Global Agriculture and Ecology, 14(1), 19-26.
- OKUYAN**, Ö. Ü. D. (2023). BÖLÜM 6. TEMEL TIP BİLİMLERİNDE GÜNCEL ÇALIŞMALAR 4, 99.

References

- Orfali**, R., Aboseada, M. A., Abdel-Wahab, N. M., Hassan, H. M., Perveen, S., Ameen, F., ... & Abdelmohsen, U. R. (2021). Recent updates on the bioactive compounds of the marine-derived genus *Aspergillus*. *RSC advances*, 11(28), 17116-17150.
- Osama**, S., El-Sherei, M., Al-Mahdy, D., Bishr, M., Salama, O., & Raafat, M. (2023). Optimization and characterization of antileukemic L-asparaginase produced by *Fusarium solani* endophyte. *AMB Express*, 13. doi: 10.1186/s13568-023-01602-2
- P
- Parmar**, F. A., Patel, J. N., & Upasani, V. N. (2021). Screening of microorganisms for production of therapeutic enzymes. *Screening*, 3(6), 39.
- Patel**, A. K., Dong, C.-D., Chen, C.-W., Pandey, A., & Singhania, R. R. (2023). Production, purification, and application of microbial enzymes *Biotechnology of microbial enzymes* (pp. 25-57): Elsevier.
- Patridge**, E., Gareiss, P., Kinch, M. S., & Hoyer, D. (2016). An analysis of FDA-approved drugs: natural products and their derivatives. *Drug discovery today*, 21(2), 204-207.
- Pattnaik**, S., Imchen, M., Kumavath, R., Prasad, R., & Busi, S. (2022). Bioactive microbial metabolites in cancer therapeutics: mining, repurposing, and their molecular targets. *Current microbiology*, 79(10), 300.
- Polouckova**, V. (2021). Correction in determination of specific activity of radionuclides by gamma spectrometry in building materials.

References

Paper presented at the IOP Conference Series: Materials Science and Engineering.

Premalatha, A., Vijayalakshmi, K., Shanmugavel, M., & Rajakumar, G. S. (2023). Optimization of culture conditions for enhanced production of extracellular α -amylase using solid-state and submerged fermentation from *Aspergillus tamaraii* MTCC5152. *Biotechnology and Applied Biochemistry*, 70(2), 835-845.

Ptak, A., & Szyc, M. (2024). Athlete's Foot: A Common Fungal Infection in Athletes and Beyond. The Use of Terbinafine in Treating Tinea Pedis and Onychomycosis. *Quality in Sport*, 34, 56195-56195.

Putriningsih, P., & Arjentina, I. (2017). Macroconidia of dermatophytes fungi on direct microscopic examinations. *Journal of Veterinary Medicine and Animal Science*, 1(1), 40-42.

R

Raj, J. E. E. A., Baskaran, A., Kumar, S. D., Sureka, I., Velmurugan, M., & Sathiyamurthy, K. (2016). Isolation and screening of L-asparaginase and L-glutaminase producing bacteria and their antimicrobial potential from environmental sources. *IOSR Journal of Pharmacy and Biological Sciences*, 11(3), 47-53.

ReFaey, K., Tripathi, S., Grewal, S. S., Bhargav, A. G., Quinones, D. J., Chaichana, K. L., . . . Dronca, R. S. (2021). Cancer mortality rates increasing vs cardiovascular disease mortality decreasing in the world: future implications. *Mayo Clinic Proceedings: Innovations, Quality & Outcomes*, 5(3), 645-653.

References

- Ren, X., & Palmer, L. D.** (2023). Acinetobacter metabolism in infection and antimicrobial resistance. *Infection and immunity*, 91(6), e00433-22.
- Rezaee, N., Hasanvand, P., Bagheri Lotfabad, T., Heydarinasab, A., Khodabandeh, M., & Yaghmaei, S.** (2023). Study on the use of bovine blood protein hydrolysate as a peptone in microbial culture media. *Preparative Biochemistry & Biotechnology*, 53(6), 622-633.
- Rinker, D. C., Sauters, T. J., Steffen, K., Gumilang, A., Raja, H. A., Rangel-Grimaldo, M., . . . Delbaje, E.** (2024). Strain heterogeneity in a non-pathogenic *Aspergillus* fungus highlights factors associated with virulence. *Communications Biology*, 7(1), 1082.
- Roberts, H., Ly, K. N., Yin, S., Hughes, E., Teshale, E., & Jiles, R.** (2021). Prevalence of HBV infection, vaccine-induced immunity, and susceptibility among at-risk populations: US households, 2013-2018. *Hepatology*, 74(5), 2353-2365.
- Rodríguez-Rodríguez, N., Martínez-Jiménez, I., García-Ojalvo, A., Mendoza-Mari, Y., Guillén-Nieto, G., Armstrong, D. G., & Berlanga-Acosta, J.** (2022). Wound chronicity, impaired immunity and infection in diabetic patients. *MEDICC review*, 24, 44-58.
- Roger, M., Fullard, N., Costello, L., Bradbury, S., Markiewicz, E., O'Reilly, S., . . . Karakesisoglou, I.** (2019). Bioengineering the microanatomy of human skin. *Journal of anatomy*, 234(4), 438-455.

References

Roky, A. H., Islam, M. M., Ahasan, A. M. F., Mostaq, M. S., Mahmud, M. Z., Amin, M. N., & Mahmud, M. A. (2025). Overview of skin cancer types and prevalence rates across continents. *Cancer pathogenesis and therapy*, 3(02), 89-100.

S

Sadiq, M. B., Fadl, S. E., Akin, M., Peng, M., Chaklader, M. R., Chaklader, M., . . . Abdel-Latif, H. (2024). OPEN ACCESS EDITED BY. *Nutrition and sustainable development goal 12: Responsible consumption*, 54.

Sajjad, H., Imtiaz, S., Noor, T., Siddiqui, Y. H., Sajjad, A., & Zia, M. (2021). Cancer models in preclinical research: A chronicle review of advancement in effective cancer research. *Animal models and experimental medicine*, 4(2), 87-103.

Salam, M. A., Al-Amin, M. Y., Salam, M. T., Pawar, J. S., Akhter, N., Rabaan, A. A., & Alqumber, M. A. (2023). Antimicrobial resistance: a growing serious threat for global public health. Paper presented at the Healthcare.

SALAMI, M. O. (2021). PRODUCTION, PURIFICATION AND CHARACTERISATION OF LASPARAGINASE FROM ACTINOMYCETES AND EVALUATION OF ITS ANTI-CANCER AND ACRYLAMIDE REDUCTION ACTIVITIES (Doctoral dissertation).

SALAMI, M. O. (2021). PRODUCTION, PURIFICATION AND CHARACTERISATION OF LASPARAGINASE FROM

References

ACTINOMYCETES AND EVALUATION OF ITS ANTI-CANCER AND ACRYLAMIDE REDUCTION ACTIVITIES.

- Salazar-Hamm, P., & Torres-Cruz, T. J.** (2024). The impact of climate change on human fungal pathogen distribution and disease incidence. *Current Clinical Microbiology Reports*, 11(3), 140–152.
- Salih, H. N.** (2022). Production, Purification and Characterization of Collagenase produced from locally isolated *Staphylococcus aureus* HN77. College of Science/University of Baghdad as a Partial Fulfillment of the
- Sánchez-Romero, M. I., Moya, J. M. G. L., López, J. J. G., & Mira, N. O.** (2019). Collection, transport and general processing of clinical specimens in Microbiology laboratory. *Enfermedades infecciosas y microbiología clinica* (English ed.), 37(2), 127-134.
- Sasaki, K., Jabbour, E., Montalban-Bravo, G., Darbaniyan, F., Do, K.-A., Class, C., . . . Borthakur, G.** (2022). Low-dose decitabine versus low-dose azacitidine in lower-risk MDS. *NEJM evidence*, 1(10), EVIDoa2200034.
- Saseedharan, S., Sahu, M., Chaddha, R., Pathrose, E., Bal, A., Bhalekar, P., ... & Krishnan, P.** (2018). Epidemiology of diabetic foot infections in a reference tertiary hospital in India. *brazilian journal of microbiology*, 49, 401-406.
- Sathyanarayan, S., Srinivasan, P. T., & Lakshmi, K. P.** (2021). Effect Of Organic (Neem Oil) And Inorganic Pesticides (Protrin And Phoskill) On Soil Microflora And Evaluation Of The Growth Characteristics Of *Agrobacterium* Sp. And *Pseudomonas* Sp. *Soil*

References

- Isolates. Journal Of Advanced Scientific Research, 2021(Icitnas), 156-168.
- Schmitt**, J., Razvi, A., & Grapentin, C. (2023). Predictive modeling of concentration-dependent viscosity behavior of monoclonal antibody solutions using artificial neural networks. Paper presented at the MABs.
- Selvaraj**, C., Rudhra, O., Alothaim, A. S., Alkhanani, M., & Singh, S. K. (2022). Structure and chemistry of enzymatic active sites that play a role in the switch and conformation mechanism. *Advances in Protein Chemistry and Structural Biology*, 130, 59-83.
- Shakambari**, G., Sameer Kumar, R., Ashokkumar, B., & Varalakshmi, P. (2017). Agro waste utilization for cost-effective production of L-asparaginase by *Pseudomonas plecoglossicida* RS1 with anticancer and acrylamide mitigation potential. *ACS omega*, 2(11), 8108-8117.
- Shannon**, A. H., Ruff, S. M., & Pawlik, T. M. (2022). Expert insights on current treatments for hepatocellular carcinoma: Clinical and molecular approaches and bottlenecks to progress. *Journal of Hepatocellular Carcinoma*, 1247-1261.
- Sharma**, K. M., Kumar, R., Panwar, S., & Kumar, A. (2017). Microbial alkaline proteases: Optimization of production parameters and their properties. *Journal of Genetic Engineering and Biotechnology*, 15(1), 115-126.
- Shi**, X., Wang, X., Yao, W., Shi, D., Shao, X., Lu, Z., . . . Wang, X. (2024). Mechanism insights and therapeutic intervention of tumor

References

metastasis: latest developments and perspectives. *Signal transduction and targeted therapy*, 9(1), 192.

Singh, R. S., Singhanian, R. R., Pandey, A., & Larroche, C. (Eds.). (2019). *Biomass, biofuels, biochemicals: advances in enzyme technology*. Elsevier.

Slak, J., Pomeroy, B., Kostyniuk, A., Grilc, M., & Likozar, B. (2022). A review of bio-refining process intensification in catalytic conversion reactions, separations and purifications of hydroxymethylfurfural (HMF) and furfural. *Chemical Engineering Journal*, 429, 132325.

Soliman, H. M., El-Naggar, N. E.-A., & El-Ewasy, S. M. (2020). Bioprocess optimization for enhanced production of L-asparaginase via two model-based experimental designs by alkaliphilic *Streptomyces fradiae* NEAE-82. *Current Biotechnology*, 9(1), 23-37.

Sousa, N. S. O. d., Almeida, J. D. R. d., Rocha, L. S. d., Moreira, I. d. M. B., Fernandes, F. d. S., Matsuura, A. B. J., . . . Frickmann, H. (2025). Antifungal Activity of Selected Naphthoquinones and Their Synergistic Combination with Amphotericin B Against *Cryptococcus neoformans* H99. *Antibiotics*, 14(6), 602.

T

Tafari, A. (2023). Exploring variability in free asparagine content to reduce the acrylamide-forming potential of wheat grain.

Takahashi, H., Inoue, J., Sakaguchi, K., Takagi, M., Mizutani, S., & Inazawa, J. (2017). Autophagy is required for cell survival under

References

- L-asparaginase-induced metabolic stress in acute lymphoblastic leukemia cells. *Oncogene*, 36(30), 4267-4276.
- Talukdar, D.**, Sharma, R., Jaglan, S., Vats, R., Kumar, R., Mahnashi, M. H., & Umar, A. (2020). Identification and characterization of cadmium resistant fungus isolated from contaminated site and its potential for bioremediation. *Environmental Technology & Innovation*, 17, 100604.
- Thoonen, K.**, Van Osch, L., De Vries, H., Jongen, S., & Schneider, F. (2020). Are environmental interventions targeting skin cancer prevention among children and adolescents effective? A systematic review. *International Journal of Environmental Research and Public Health*, 17(2), 529.
- Tirlangi, P. K.**, Wanve, B. S., Dubbudu, R. R., Yadav, B. S., Kumar, L. S., Gupta, A., ... & Reddy, P. N. (2023). Successful use of cefepime-zidebactam (WCK 5222) as a salvage therapy for the treatment of disseminated extensively drug-resistant New Delhi metallo- β -lactamase-producing pseudomonas aeruginosa infection in an adult patient with acute T-cell leukemia. *Antimicrobial Agents and Chemotherapy*, 67(8), e00500-23.
- Tripathi, N.**, & Sapra, A. (2020). Gram staining.
- Tosta Pérez, M.**, Herrera Belén, L., Letelier, P., Calle, Y., Pessoa, A., & Farías, J. G. (2023). L-Asparaginase as the gold standard in the treatment of acute lymphoblastic leukemia: A comprehensive review. *Medical Oncology*, 40(5), 150.

References

Trager, M. H., Geskin, L. J., Samie, F. H., & Liu, L. (2022). Biomarkers in melanoma and non-melanoma skin cancer prevention and risk stratification. *Experimental dermatology*, 31(1), 4-12.

Trager, M. H., Geskin, L. J., Samie, F. H., & Liu, L. (2022). Biomarkers in melanoma and non-melanoma skin cancer prevention and risk stratification. *Experimental dermatology*, 31(1), 4-12.

Tuttle, A. R., Trahan, N. D., & Son, M. S. (2021). Growth and maintenance of *Escherichia coli* laboratory strains. *Current protocols*, 1(1), e20.

V

Van Trimpont, M., Peeters, E., De Visser, Y., Schalk, A. M., Mondelaers, V., De Moerloose, B., ... & Van Vlierberghe, P. (2022). Novel insights on the use of L-asparaginase as an efficient and safe anti-cancer therapy. *Cancers*, 14(4), 902.

Vardakas, P., Veskoukis, A. S., Rossiou, D., Gournikis, C., Kapetanopoulou, T., Karzi, V., ... & Kouretas, D. (2022). A mixture of endocrine disruptors and the pesticide roundup® induce oxidative stress in rabbit liver when administered under the long-term low-dose regimen: Reinforcing the notion of real-life risk simulation. *Toxics*, 10(4), 190.

Vargas, M. I. P. (2021). Engineering Human Skin Equivalents to Prevent Infections in Skin Wounds.

Varghese, A. P., Neppolian, B., & Lakhera, S. K. (2023). Pitfalls of using nessler's reagent for ammonia detection in photocatalytic nitrogen fixation studies: Leveraging ¹H NMR for enhanced accuracy and

References

precision. *Industrial & Engineering Chemistry Research*, 62(32), 12530-12537.

Verma, N.; Kumar, K.; Kaur, G. and Anand, S. (2007). L Asparaginase: A promising chemotherapeutic agent. *Critical Rev. in Biotechnol. J.*, 27:45-62.

W

Wadhwa, K., Kapoor, N., Kaur, H., Abu-Seer, E. A., Tariq, M., Siddiqui, S., . . . Alghamdi, S. (2024). A Comprehensive Review of the Diversity of Fungal Secondary Metabolites and Their Emerging Applications in Healthcare and Environment. *Mycobiology*, 1-53.

Wakil, S. M., & Adelegan, A. A. (2015). Screening, production and optimization of L-asparaginase from soil bacteria isolated in Ibadan, South-western Nigeria. *Journal of Basic & Applied Sciences*, 11, 39-51.

Wang, A., Li, F., Qiao, M., Liu, J., Guo, X., Zhao, Y.-Y., . . . Shi, Y. (2025). Study on the effect of NH₃ on the solubility of KCl and NH₄Cl—from microscopic view. *Physical Chemistry Chemical Physics*.

Wang, C., Cao, Y., Yang, C., Bernards, R., & Qin, W. (2021). Exploring liver cancer biology through functional genetic screens. *Nature Reviews Gastroenterology & Hepatology*, 18(10), 690-704.

Wang, C., Liu, X., Mei, D., Deng, M., Zheng, Y., Zheludkevich, M. L., & Lamaka, S. V. (2022). Local pH and oxygen concentration at the interface of Zn alloys in Tris-HCl or HEPES buffered Hanks' balanced salt solution. *Corrosion Science*, 197, 110061.

References

- Wang**, C.-M., Chen, C.-Y., & Liao, W.-S. (2021). Enclosed paper-based analytical devices: Concept, variety, and outlook. *Analytica Chimica Acta*, 1144, 158-174.
- Wang**, J. S., Wang, H. J., & Qian, H. L. (2018). Biological effects of radiation on cancer cells. *Military medical research*, 5, 1-10.
- Wang**, S., Zhang, T., Bao, M., Su, H., & Xu, P. (2020). Microbial production of hydrogen by mixed culture technologies: a review. *Biotechnology journal*, 15(1), 1900297.
- Wang**, X., Xu, X., Liu, J., Liu, Y., Li, J., Du, G., . . . Liu, L. (2023). Metabolic engineering of *Saccharomyces cerevisiae* for efficient retinol synthesis. *Journal of Fungi*, 9(5), 512.
- Wang**, Y., Xu, W., Wu, H., Zhang, W., Guang, C., & Mu, W. (2021). Microbial production, molecular modification, and practical application of L-asparaginase: A review. *International Journal of Biological Macromolecules*, 186, 975-983.
- Workie**, M. S., Jalali, R., & Cheng, Z. (2023). Background: Cancer is a leading cause of death in the world, and the estimated new cancer cases were 19 million and the estimated cancer deaths were around 10 million worldwide in 2020. Proton therapy (PT) is a promising treatment for cancers, 17.
- World Health Organization**. (2022). Global antimicrobial resistance and use surveillance system (GLASS) report 2022. World Health Organization.

References

- Xu, S., Li, M., Hu, Z., Shao, Y., Ying, J., & Zhang, H. (2023).** The potential use of fungal co-culture strategy for discovery of new secondary metabolites. *Microorganisms*, 11(2), 464.
- Yadav, G. P., Dalbhat, C. G., & Mishra, H. N. (2022).** Effects of extrusion process parameters on cooking characteristics and physicochemical, textural, thermal, pasting, microstructure, and nutritional properties of millet-based extruded products: A review. *Journal of Food Process Engineering*, 45(9), e14106.

Y

- Yadav, H., Singh, S., & Sinha, R. (2024).** Fermentation Technology for Microbial Products and Their Process Optimization Industrial Microbiology and Biotechnology: A New Horizon of the Microbial World (pp. 35-64): Springer.
- Yang, Y., Liang, C., Wang, Y., Cheng, H., An, S., & Chang, S. X. (2020).** Soil extracellular enzyme stoichiometry reflects the shift from P-to N-limitation of microorganisms with grassland restoration. *Soil Biology and Biochemistry*, 149, 107928.
- Yang, Y., Liang, C., Wang, Y., Cheng, H., An, S., & Chang, S. X. (2020).** Soil extracellular enzyme stoichiometry reflects the shift from P-to N-limitation of microorganisms with grassland restoration. *Soil Biology and Biochemistry*, 149, 107928.
- Yang, Y., Liang, C., Wang, Y., Cheng, H., An, S., & Chang, S. X. (2020).** Soil extracellular enzyme stoichiometry reflects the shift from P-to N-limitation of microorganisms with grassland restoration. *Soil Biology and Biochemistry*, 149, 107928.

References

- Yayan, J., Franke, K.-J., Berger, M., Windisch, W., & Rasche, K. (2024).** Adhesion, metastasis, and inhibition of cancer cells: a comprehensive review. *Molecular biology reports*, 51(1), 165.
- Yuan, Q., Yin, L., He, J., Zeng, Q., Liang, Y., Shen, Y., & Zu, X. (2024).** Metabolism of asparagine in the physiological state and cancer. *Cell Communication and Signaling*, 22(1), 163.
- Yuvarajan, S., Hameed, A., Arun, A. B., Saptami, K., & Rekha, P. D. (2022).** Urease-negative uropathogen *Kalamielli piersonii* YU22 metabolizes urea by urea carboxylase and allophanate hydrolase enzyme system. *Microbiological Research*, 263, 127142.
- Z
- Zhang, X., Liu, Y., Gopalakrishnan, S., Castellanos-Garcia, L., Li, G., Malassiné, M., . . . Vachet, R. W. (2020).** Intracellular activation of bioorthogonal nanozymes through endosomal proteolysis of the protein corona. *ACS nano*, 14(4), 4767-4773.
- Zhgun, A. A. (2023).** Fungal BGCs for production of secondary metabolites: Main types, central roles in strain improvement, and regulation according to the piano principle. *International Journal of Molecular Sciences*, 24(13), 11184.
- Zhgun, A. A. (2023).** Fungal BGCs for production of secondary metabolites: main types, central roles in strain improvement, and regulation according to the piano principle. *International journal of molecular sciences*, 24(13), 11184.
- Zuarez-Easton, S., Zafran, N., Garmi, G., & Salim, R. (2017).** Postcesarean wound infection: prevalence, impact, prevention,

References

and management challenges. International journal of women's health, 81-88.

Zuñiga Martinez, M. d. L., López Mendoza, C. M., Tenorio Salazar, J., García Carrancá, A. M., Cerbón Cervantes, M. A., & Alcántara-Quintana, L. E. (2022). Establishment, authenticity, and characterization of cervical cancer cell lines. *Molecular & Cellular Oncology*, 9(1), 2078628.

Ковалевський, I. A. (2024). Біосинтез L-аспарагінази *Erwinia carotovora*.

Vi, V. C., T., Batool, E. A., Makky, N., Suhaity, and Mashitah M. Y. 2016. Production of L-asparaginase from natural substrates by *Escherichia coli*. ATCC 10536 and comparison of physiology parameters. *ARPN Journal of Engineering and Applied Sciences*. 11(16): 9680- 9684.

Kuplińska, A., & Rząd, K. (2021). Molecular targets for antifungals in amino acid and protein biosynthetic pathways. *Amino Acids*, 53(7), 961-991.

Starke, S. (2024). *Unraveling the role of the antifungal peptide AnAFP in Aspergillus niger through a systems biology perspective*. Technische Universität Berlin.

Dellièvre, S., & Aimanianda, V. (2023). Humoral immunity against *Aspergillus fumigatus*. *Mycopathologia*, 188(5), 603-621.

Castro, D., Marques, A. S. C., Almeida, M. R., de Paiva, G. B., Bento, H. B., Pedrolli, D. B., . . . Santos-Ebinuma, V. C. (2021). L-asparaginase production review: bioprocess design and

References

- biochemical characteristics. *Applied microbiology and biotechnology*, 105, 4515-4534.
- Patel**, A. K., Dong, C.-D., Chen, C.-W., Pandey, A., & Singhania, R. R. (2023). Production, purification, and application of microbial enzymes *Biotechnology of microbial enzymes* (pp. 25-57): Elsevier.
- Kassab**, M. M. (2023). Screening and production of fungal L-asparaginase enzymes as anticancer agents from high-contrast soil environments in Egypt. *Egyptian Academic Journal of Biological Sciences, G. Microbiology*, 15(1), 21-41.
- Hassan**, T., & Hussein, S. (2022). Development of bioprocesses for production and purification of l-asparaginase from *staphylococcus aureus*, and in vitro efficacy against human breast cancer cell line. *Iraqi Journal of Agricultural Sciences*, 53(6), 1525-1538.
- Breijyeh**, Z., Jubeh, B., & Karaman, R. (2020). Resistance of gram-negative bacteria to current antibacterial agents and approaches to resolve it. *Molecules*, 25(6), 1340.
- Georgiou**, G., & Kotzé, A. (2023). Eradication of antibiotic-resistant *E. coli*, *S. aureus*, *K. pneumoniae*, *S. pneumoniae*, *A. baumannii*, and *P. aeruginosa* with chlorine dioxide in vitro. *Medical Research Archives*, 11(7.2).
- Luo**, A.-G., Wang, Y.-Y., Xue, S.-S., Zhao, J., Hao, J.-W., Shi, S.-L., & Hu, B.-F. (2023). Screening, identification, and optimization of enzyme-producing conditions for cellulose-degrading bacteria in distillery lees. *Applied Sciences*, 13(13), 7693.

Appendix

Appendix

Vitek diagnosis

bioMérieux Customer:Microbiology Chart ReportPrinted January 20, 2024 8:08:30 PM

Patient Name: 135211Patient ID: 135211
Location:Physician:
Lab ID: 135211Isolate Number: 1

Organism Quantity:
Selected Organism : *Candida albicans*

Collected:

Comments:	

Identification Information		Analysis Time: 17.80 hours		Status: Final	
Selected Organism		98% Probability Bionumber: 4102546045327671			
ID Analysis Messages					
Susceptibility Information		Analysis Time: 15.32 hours			Status: Final
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Fluconazole	4	R	Amphotericin B	≥ 16	R
Caspofungin	≥ 8	R	Flucytosine	≥ 16	R

AES Findings	
Confidence:	Consistent

Appendix

bioMérieux Customer:

Microbiology Chart Report

Printed August 9, 2025 8:08:31 PM CDT

Patient Name: 136825

Patient ID: 136825

Location:

Physician:

Lab ID: 136825

Isolate Number: 1

Organism Quantity:

Selected Organism : *Candida albicans*

Collected:

Comments:	

Identification Information	Analysis Time: 17.77 hours	Status: Final
Selected Organism	95% Probability <i>Candida albicans</i>	
	Bionumber: 4102546045327771	
ID Analysis Messages		

Susceptibility Information	Analysis Time: 15.08 hours				Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation	
Fluconazole	>= 16	R	Amphotericin B	8	R	
Caspofungin	>= 8	R	Flucytosine	>= 16	R	
Micafungin	>= 16	R				

AES Findings	
Confidence:	Consistent

Appendix

bioMérieux Customer:Microbiology Chart ReportPrinted January 20, 2024 8:08:32 PM

Patient Name:Patient ID: 139775
Location:Physician:
Lab ID: 139775Isolate Number: 1

Organism Quantity:
Selected Organism : Candida albicans

Collected:

Comments:	

Identification Information	Analysis Time: 17.77 hours	Status: Final
Selected Organism	88% Probability Candida albicans	
ID Analysis Messages	Bionumber: 4112747165327371	

Susceptibility Information	Analysis Time: 13.60 hours	Status: Final
----------------------------	----------------------------	---------------

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Fluconazole	>= 16	R	Amphotericin B	4	R
Caspofungin	>= 8	R	Flucytosine	>= 16	R
Micafungin	4	R			

AES Findings	
Confidence:	Consistent

Appendix

bioMérieux Customer:

Microbiology Chart Report

Printed August 9, 2015 8:08:33 PM CDT

Patient Name: 143768

Patient ID: 143768

Location:

Physician:

Lab ID: 143768

Isolate Number: 1

Organism Quantity:

Selected Organism : *Candida albicans*

Collected:

Comments:	

Identification Information	Analysis Time: 17.80 hours	Status: Final
Selected Organism	99% Probability: <i>Candida albicans</i>	
	Bionumber: 4502546065327771	
ID Analysis Messages		

Susceptibility Information	Analysis Time: 15.60 hours	Status: Final
----------------------------	----------------------------	---------------

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Fluconazole	>= 16	R	Micafungin	>= 16	R
Voriconazole	>=16	R	Amphotericin B	8	R
Caspofungin	>= 8	R	Flucytosine	>= 16	R

AES Findings	
Confidence:	Consistent

Appendix

bioMérieux Customer:

Microbiology Chart Report

Printed January 9, 2024 7:58:14 PM CDT

Patient Name: 141265

Patient ID: 141265

Location:

Physician:

Lab ID: 141265

Isolate Number: 1

Organism Quantity:

Selected Organism : *Acinetobacter baumannii* complex

Collected:

Comments:	

Identification Information	Analysis Time: 4.83 hours	Status: Final
Selected Organism	99% Probability Bionumber: 0241010303500200	<i>Acinetobacter baumannii</i> complex
ID Analysis Messages		

Susceptibility Information		Analysis Time: 11.67 hours		Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
+Amoxicillin		R	Cefepime	≥ 32	R
+Amoxicillin/Clavulanic Acid		R	+Aztreonam		R
Ampicillin/Sulbactam	≥ 32	R	+Doripenem		
+Carbenicillin		R	Imipenem	≥ 16	R
+Ticarcillin		R	Meropenem	≥ 16	R
+Ticarcillin/Clavulanic Acid		R	Amikacin	≥ 64	R
+Piperacillin		R	Gentamicin	≥ 16	R
+Piperacillin/Sulbactam		R	+Nalidixic Acid		R
Piperacillin/Tazobactam	≥ 128	R	Ciprofloxacin	≥ 4	R
+Cefixime		R	+Norfloxacin		R
Cefotaxime	≥ 64	R	Tigecycline		
Ceftazidime	≥ 64	R	Colistin	≥ 16	R
Ceftazidime/Avibactam			+Trimethoprim		R
Ceftolozane/Tazobactam			Trimethoprim/ Sulfamethoxazole	160	R

AES Findings	
Confidence:	Consistent

Appendix

Appendix

bioMérieux Customer:

Microbiology Chart Report

Printed January 9, 2024 7:58:32 PM CDT

Patient Name: 145818

Patient ID: 145818

Location:

Physician:

Lab ID: 145818

Isolate Number: 1

Organism Quantity:

Selected Organism : *Acinetobacter baumannii* complex

Collected:

Comments:	

Identification Information	Analysis Time: 4.85 hours	Status: Final
Selected Organism	99% Probability Bionumber: 0241010203500310	<i>Acinetobacter baumannii</i> complex
ID Analysis Messages		

Susceptibility Information	Analysis Time: 13.15 hours	Status: Final
----------------------------	----------------------------	---------------

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
+Amoxicillin		R	Cefepime	≥ 32	R
+Amoxicillin/Clavulanic Acid		R	+Aztreonam		R
Ampicillin/Sulbactam	≥ 32	R	+Doripenem		R
+Ticarcillin			Imipenem	≥ 16	R
+Ticarcillin/Clavulanic Acid			Meropenem	≥ 16	R
+Piperacillin			Amikacin	≥ 64	R
+Piperacillin/Sulbactam			Gentamicin	≥ 16	R
Piperacillin/Tazobactam	≥ 128	R	Ciprofloxacin	≥ 16	R
+Cefixime		R	Tigecycline		
Cefotaxime	16	R	Colistin	≥ 1	R
Ceftazidime	≥ 64	R	+Trimethoprim		R
Ceftazidime/Avibactam			Trimethoprim/ Sulfamethoxazole	≥ 320	R
Ceftiozane/Tazobactam					

*= AES modified **= User modified

AES Findings	
Confidence:	Consistent

Appendix

bioMérieux Customer: Microbiology Chart Report Printed January 9, 2024 7:58:46 PM CDT

Patient Name: 147508 Patient ID: 147508
Location: Physician:
Lab ID: 147508 Isolate Number: 2

Organism Quantity:
Selected Organism : Acinetobacter baumannii complex

Collected:

Comments:	

Identification Information	Analysis Time: 4.83 hours	Status: Final
Selected Organism	99% Probability Bionumber: 0241010303500200	Acinetobacter baumannii complex
ID Analysis Messages		

Susceptibility Information	Analysis Time: 8.23 hours	Status: Final
----------------------------	---------------------------	---------------

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Amoxicillin/Clavulanic Acid			Imipenem	>= 16	R
Piperacillin/Tazobactam	>= 128	R	Meropenem	>= 16	R
Cefazolin	>= 64	R	Amikacin	>= 64	R
Cefuroxime			Gentamicin	>= 16	R
Cefuroxime Axetil			Ciprofloxacin	>= 4	R
Ceftazidime	>= 64	R	Fosfomycin		
Ceftriaxone	>= 64	R	Nitrofurantoin		
Cefepime	>= 32	R	Trimethoprim/Sulfamethoxazole	160	R
Ertapenem					

AES Findings	
Confidence:	Consistent

Appendix

bioMérieux Customer:

Microbiology Chart Report

Printed January 9, 2024 7:59:02 PM CDT

Patient Name: 152689

Location:

Lab ID: 152689

Patient ID: 152689

Physician:

Isolate Number: 1

Organism Quantity:

Selected Organism : *Acinetobacter baumannii* complex

Collected:

Comments:	

Identification Information	Analysis Time: 5.83 hours	Status: Final
Selected Organism	99% Probability Bionumber: 0241010103500210	<i>Acinetobacter baumannii</i> complex
ID Analysis Messages		

Susceptibility Information	Analysis Time: 10.72 hours	Status: Final
----------------------------	----------------------------	---------------

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
+Amoxicillin		R	Cefepime	≥ 32	R
+Amoxicillin/Clavulanic Acid		R	+Aztreonam		R
Ampicillin/Sulbactam	≥ 32	R	+Doripenem		
+Carbenicillin		R	Imipenem	≥ 16	R
+Ticarcillin		R	Meropenem	≥ 16	R
+Ticarcillin/Clavulanic Acid		R	Amikacin	≥ 64	R
+Piperacillin		R	Gentamicin	≥ 16	R
+Piperacillin/Sulbactam		R	+Nalidixic Acid		R
Piperacillin/Tazobactam	≥ 128	R	Ciprofloxacin	≥ 4	R
+Cefixime		R	+Norfloxacin		R
Cefotaxime	≥ 64	R	Tigecycline		
Ceftazidime	≥ 64	R	Colistin	≥ 16	R
Ceftazidime/Avibactam			+Trimethoprim		R
Ceftolozane/Tazobactam			Trimethoprim/ Sulfamethoxazole	≥ 320	R

AES Findings	
Confidence:	Consistent

Appendix

bioMérieux Customer:

Microbiology Chart Report

Printed August 9, 2024 7:59:52 PM CDT

Patient Name: 117377

Patient ID: 117377

Location:

Physician:

Lab ID: 117377

Isolate Number: 1

Organism Quantity:

Selected Organism : Proteus mirabilis

Source: d.f

Collected:

Comments:	

Identification Information	Analysis Time: 3.87 hours	Status: Final
Selected Organism	99% Probability	Proteus mirabilis
	Bionumber:	0017000340042200
ID Analysis Messages		

Susceptibility Information	Analysis Time: 9.00 hours			Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Ampicillin/Sulbactam	≥ 2	R	Meropenem	1	R
Piperacilin/Tazobactam	≥164	R	Amikacin	2	R
Cefotaxime	≥164	R	Gentamicin	≥16	R
Ceftazidime	≥164	R	Ciprofloxacin	≥8	R
Ceftazidime/Avibactam	≥164	R	Tigecycline		
Ceftolozane/Tazobactam	≥164	R	Colistin	≥ 16	R
Cefepime	≥164	R	Trimethoprim/ Sulfamethoxazole	≥230	R
Imipenem	1	R			

AES Findings	
Confidence:	Consistent

Appendix

bioMérieux Customer:

Microbiology Chart Report

Printed August 9, 2024 8:07:23 PM CDT

Patient Name:

Patient ID: 115301

Location:

Physician:

Lab ID: 115301

Isolate Number: 1

Organism Quantity:

Selected Organism : *Pseudomonas aeruginosa*

Source: d.f

Collected:

Comments:	

Identification Information	Analysis Time: 5.07 hours	Status: Final
Selected Organism	97% Probability Biounumber: 0043451203500040	<i>Pseudomonas aeruginosa</i>
ID Analysis Messages		

Susceptibility Information	Analysis Time: 15.33 hours	Status: Final
----------------------------	----------------------------	---------------

Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Ampicillin/Sulbactam			Meropenem	≥ 16	R
Cefotaxime			Gentamicin	≥ 16	R
Ceftazidime	≥ 64	R	Ciprofloxacin	≥ 4	R
Ceftazidime/Avibactam	≥ 16	R	Tigecycline		
Ceftolozane/Tazobactam	≥ 32	R	Colistin	2	S
Cefepime	≥ 32	R	Trimethoprim/ Sulfamethoxazole		
Imipenem	≥ 16	R			

AES Findings	
Confidence:	Consistent

Appendix

bioMérieux Customer:

Microbiology Chart Report

Printed August 9, 2024 7:55:32 PM CDT

Patient Name

Patient ID: 126980

Location:

Physician:

Lab ID: 126980

Isolate Number: 1

Organism Quantity:

Selected Organism : *Staphylococcus epidermidis*

Collected:

Comments:	

Identification Information	Analysis Time: 4.87 hours	Status: Final
Selected Organism	99% Probability <i>Staphylococcus epidermidis</i>	
ID Analysis Messages	Bionumber: 010000032620211	

Susceptibility Information		Analysis Time: 9.50 hours		Status: Final	
Antimicrobial	MIC	Interpretation	Antimicrobial	MIC	Interpretation
Cefoxitin Screen	POS	+	+Levofloxacin		R
Benzylpenicillin	≥ 0.5	R	Moxifloxacin		R
Ampicillin			Inducible Clindamycin Resistance	NEG	-
+Amoxicillin/Clavulanic Acid		R	Erythromycin	≥ 8	R
Oxacillin	≥ 4	R	Clindamycin	≥ 8	R
+Cefotaxime		R	Linezolid	≥ 12	R
+Ceftriaxone		R	Teicoplanin	≥ 12	R
+Cefepime		R	Vancomycin	≥ 64	R
Imipenem			+Doxycycline		
Gentamicin High Level (synergy)			Tetracycline		
Streptomycin High Level (synergy)			Tigecycline		
+Amikacin			Fosfomycin		
Gentamicin	≥ 16	R	Fusidic Acid	16	R
+Tobramycin			Rifampicin	≥ 16	R
Ciprofloxacin	≥ 8	R	Trimethoprim/ Sulfamethoxazole	≥ 320	R

AES Findings	
Confidence:	Consistent

الخلاصة :

درست هذه الدراسة إنتاج وتنقية وتطبيقات إنزيم الاسباراجينيز من الفطر *Aspergillus niger* المعزول من التربة. تم اختبار إنتاج الإنزيم خارج الخلية باستخدام طريقتين على وسط اكار الأسبارجين الصلب، وذلك كخطوة أولية لفحص قدرة العزلات على إنتاج الاسباراجينيز. تم اختيار ست سلالات من *A. niger* أظهرت أعلى قدرة على تكوين مناطق صفراء حول الآبار المزروعة (دلالة على تحلل الأسبرجين) لإجراء الفحص الثانوي. أظهرت هذه السلالات نشاطاً ملحوظاً لإنزيم الاسباراجينيز ، كما ظهر من خلال مناطق التحلل (٤.٥-٥ سم). وقد تم إجراء الفحص الثانوي باستخدام التخمر المغمر وتحليل نيسلر، وكشف عن أن إحدى العزلات (*A. niger* SAP-1) أظهرت أعلى نشاط إنزيمي بلغ ٢.٨١١ وحدة/ملغم.

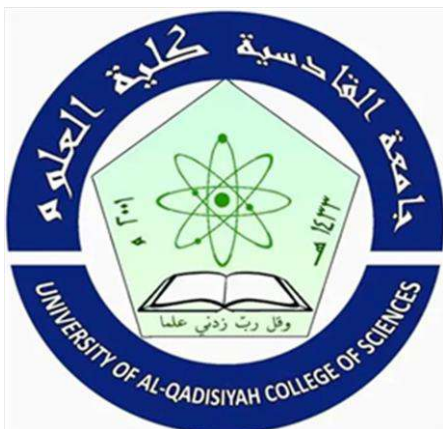
تم تنقية الإنزيم باستخدام كروماتوغرافيا الترشيح الهلامي، مما أدى إلى زيادة في النشاط النوعي بمقدار ٦.٣ ضعفاً ليصل إلى ٦٣٠ وحدة/ملغم، مع نسبة استرجاع بلغت ٣٥.٥%. أظهر تحليل HPLC لمستخلصات الفطر أن *A. niger* قد أنتج بالفعل إنزيم الاسباراجينيز ، حيث ظهرت قمم كروماتوغرافية بأزمنة احتجاز (٤.١٥ دقيقة) مماثلة لتلك الخاصة بمادة الاسباراجينيز التي تستخدم للمعايرة.

أظهرت نتائج اختبار النشاط المضاد للسرطان أن الإنزيم المنقى يمتلك فعالية قوية في اختبارات MTT، حيث أظهر سمية خلوية تعتمد على التركيز ضد خلايا سرطان الجلد الميلانيني (A375) بنسبة بقاء ٤٩.٣٨% عند تركيز ٤٠٠ ميكروغرام/مل، وضد خلايا سرطان الكبد (HepG2) بنسبة بقاء ٦٥.٠١% عند نفس التركيز، مع المحافظة على سلامة الخلايا الليفية الجلدية البشرية الطبيعية (HdFn).

أظهرت نتائج العزل والتشخيص الميكروبي أنه من بين ٣٠٠ عينة تم الحصول عليها من مرضى يعانون من إصابات مختلفة، تم تحديد العدوى الفطرية (٢٠٥ عينة ، ٦٨.٥%) كانت السائدة ، تليها العدوى البكتيرية (٧٧ عينة ، ٢٥.٥%)، ثم العدوى المختلطة (بكتيرية-فطرية) (١٨ عينة ، ٦.٠%). أظهرت البيانات الوبائية لتوزيع مسببات العدوى البكتيرية أن *Streptococcus pneumoniae* كانت السلالة السائدة بنسبة ٢٩.٨٩%، تلتها *Klebsiella pneumoniae* (23.39%)، *Staphylococcus* (15.58%)، و *Pseudomonas aeruginosa* (12.98%)، و *Acinetobacter baumannii* (11.68%).

أما من حيث الفطريات، فقد سيطرت (*Microsporum spp.* (43.6% و *Trichophyton spp.* (42.2%)) على الطيف الفطري، حيث شكّلتا معًا ما يقارب ٩٠% من الحالات، بينما ظهرت *Aspergillus niger* بنسبة ٥.٣% و *Coccidioides immitis* بنسبة ٤.٨%.

أظهرت نتائج اختبار النشاط المضاد للميكروبات للإنزيم الاسباراجينيز فاعلية واضحة ضد الفطريات (*Candida albicans, Rhizopus*) والبكتيريا (*Staphylococcus epidermidis, Acinetobacter*)، مع تثبيط ملحوظ عند تركيزات منخفضة جدًا (مثل ٠.٠١٥ ميكروغرام/مل ضد *P. aeruginosa*). كما لوحظت تأثيرات مضادة للأكسدة بشكل تآزري عند دمج إنزيم الاسباراجينيز مع إنزيمات مثل سوبر أوكسيد ديسميوتاز (SOD) أو غلوتاثيون بيروكسيداز، مما زاد من مستوى الإجهاد التأكسدي في الخلايا المستهدفة. تُبرز هذه النتائج إنزيم L-أسبرجيناز المستخلص من *A. niger* (العزلة SAP-1) كعامل علاجي واعد لعلاج السرطان والعدوى، لما يتمتع به من سُمية خلوية انتقائية، وخصائص مضادة للميكروبات، وقدرة تآزرية كمضاد للأكسدة. وتؤكد الدراسة على الأهمية السريرية للإنزيم في ظل تصاعد مقاومة الميكروبات وازدياد انتشار السرطان



وزارة التعليم العالي والبحث العلمي

جامعة القادسية

كلية العلوم

قسم علوم الحياة

الخصائص الصيدلانية المحتملة لأنزيم L-asparaginase الفطري المعزول من فطر *Aspergillus niger*

اطروحة مقدمة

رسالة

مقدمة إلى مجلس كلية العلوم/جامعة القادسية

كجزء من متطلبات نيل شهادة الدكتوراه فلسفة في علوم الحياة

تقدم بها

نور عبد الخضر صلال

بكالوريوس علم الاحياء / كلية العلوم / جامعة القادسية ٢٠١٦

ماجستير علم الاحياء / كلية العلوم / جامعة المستنصرية ٢٠٢٠

بإشراف

أ.د نيران عبيد جاسم