

STUDIES ON L-ASPARAGINASE FROM YEASTS ISOLATED FROM MANGROVE SEDIMENTS OF GOA

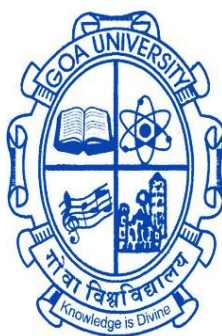
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GOA UNIVERSITY



BY

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DECLARATION

I, Aabha Arun Marathe, hereby declare that this thesis represents work which has been carried out by me and that it has not been submitted, either in part or full, to any other University or institution for the award of any research degree.

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CERTIFICATE

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“The morning will come again.
No darkness, no season is eternal.”
– Spring Day by BTS

DEDICATED WITH GRATITUDE

To my parents, brother and sister-in-law,
for believing in me beyond all doubt.

CONTENTS

Chapter I Introduction and Review of Literature	1-38
1.1 Introduction	1
1.2 Review of Literature	2
1.2.1 Mangrove ecosystem and its ecological importance	2
1.2.2 Mangroves: Source of yeast and bioactive compounds	12
1.2.3 The enzyme L-asparaginase	16
1.2.3.1 Classification of L-asparaginase	16
1.2.3.2 Structure of L-asparaginase	20
1.2.4 Sources of L-asparaginase	20
1.2.5 Applications of L-asparaginase as Chemotherapeutic agent	25
1.2.6 Applications of L-asparaginase in food Industry	27
1.2.7 Other applications of L-asparaginase	28
1.2.8 Optimization and characterization of L-asparaginase	28
1.2.9 From Bacteria to Eukaryotes: Advancing L-asparaginase Research	37
Chapter II Isolation of yeasts from mangrove sediments of Goa	40-63
2.1 Introduction	40
2.2 Methodology	43
2.2.1 Sampling	43
2.2.2 Isolation	43
2.2.3 Morphological Characterisation	44
2.2.3.1 Determining the colony characters of the isolates	44
2.2.3.2 Monochrome staining	44
2.2.3.3 Lacto-phenol cotton blue staining	44
2.3 Results	45
2.3.1 Sampling	45
2.3.2 Isolation	45
2.3.3 Morphological characterisation	45
2.4 Discussion	59

2.5 Conclusion	63
Chapter III Screening of the isolates for L-asparaginase activity	65-99
3.1 Introduction	65
3.2 Methodology	
3.2.1 Screening for L-asparaginase using rapid plate assay method	68
3.2.2 Confirmatory screening by paper chromatography method	68
3.2.3 Screening the positive isolates for L-glutaminase activity	69
3.2.4 Morphological and physiological analysis of L-asparaginase positive isolates	69
3.2.5 Quantitative analysis of L-asparaginase activity	69
3.2.6 Identification of the isolates	70
3.3 Results	70
3.3.1 Qualitative estimation of L-asparaginase production by yeast isolates	70
3.3.2 Glutaminase activity of the positive isolates	88
3.3.3 Morphology and physiological studies of the L-asparaginase positive isolates	88
3.3.4 Quantitative estimation of L-asparaginase production by yeast isolates	89
3.3.5 Identification of CMS4 and MMS8	94
3.4 Discussion	98
3.5 Conclusion	99
Chapter IV Optimization and characterization of L-asparaginase from selected isolate	101-135
4.1 Introduction	101
4.2 Methodology	103
4.2.1 Effect of physical parameters, carbon and nitrogen sources on L-asparaginase production by selected isolates	103
4.2.2 Time course analysis of L-asparaginase production by selected isolates	103
4.2.3 Effect of Glucose, L-asparagine and Sodium chloride	104

concentration on enzyme production by MMS8	
4.2.4 Optimization of medium composition by RSM	104
4.2.5 Partial purification of enzyme L-asparaginase	105
4.2.5.1 Ammonium sulphate precipitation	
4.2.5.2 Size Exclusion Chromatography	
4.2.6 Estimation of molecular weight using SDS PAGE technique	106
4.2.7 Characterization of the partially purified L-asparaginase	106
4.2.7.1 Effect on physical and reaction parameters on enzyme activity	106
4.2.7.1.1 Effect of incubation time on L-asparaginase activity	
4.2.7.1.2 Effect of incubation temperature on L-asparaginase activity	
4.2.7.1.3 Effect of pH on L-asparaginase activity	
4.2.7.1.4 Effect of substrate concentration on L-asparaginase activity	
4.2.7.2 Enzyme stability	107
4.2.7.2.1 Effect of physicochemical parameters	
4.2.7.2.2 Effect of metal ions	
4.2.7.2.3 Effect of inhibitors	
4.3 Results	108
4.3.1 Effect of physical parameters on enzyme production	108
4.3.2 Effect of carbon and nitrogen sources on enzyme production	111
4.3.3 Time course analysis	114
4.3.4 Effect of Glucose, L-asparagine and Sodium chloride concentration on enzyme production by MMS8	117
4.3.5 Optimization of medium composition by RSM	122
4.3.6 Partial purification of enzyme L-asparaginase	124
4.3.7 Characterization of partially purified enzyme	124
4.3.7.1 Effect of physical and reaction parameters on enzyme activity	

4.3.7.1.1	Effect of incubation time on L-asparaginase activity	
4.3.7.1.2	Effect of pH on L-asparaginase activity	
4.3.7.1.3	Effect of incubation temperature on L-asparaginase activity	
4.3.7.1.4	Effect of substrate concentration on L-asparaginase activity	
4.3.7.2	Enzyme stability	127
4.3.7.2.1	Effect of physicochemical parameters	
4.3.7.2.2	Effect of metal ions and inhibitors	
4.4	Discussion	129
4.5	Conclusion	135
Chapter V	Application of bioactivity of L-asparaginase as anticancer compound	137-146
5.1	Introduction	137
5.2	Methodology	140
5.2.1	Quantitative estimation of cytotoxic potential of L-asparaginase enzyme by MTT assay method	140
5.2.2	Qualitative estimation of cytotoxic potential of L-asparaginase enzyme by DAPI staining method	141
5.3	Results	145
5.4	Discussion	145
5.5	Conclusion	146
	SUMMARY AND CONCLUSION	148-150
	APPENDICES	152-167
	BIBLIOGRAPHY	169-189
	PUBLICATIONS	191-200

List of tables

Chapter I

Table 1.1: Microbial diversity of mangrove ecosystem

Table 1.2: L-asparaginase producing microbes obtained from multiple sources

Table 1.3: Optimization strategies for L-asparaginase enzyme production

Table 1.4: Purification strategies, yields and biochemical properties of L-asparaginase obtained from microbial sources

Table 1.5: Studies on recombinant expression of L-asparaginase enzyme

Chapter II

Table 2.1: Diverse media used for yeast isolation

Table 2.2: The Sampling locations

Table 2.3: Physical Parameters of the samples

Table 2.4: Yeast isolates from the nine sampling locations

Table 2.5: The yeast isolated from mangrove sediments. A- isolates grown on YMA, B- monochrome microscopy images at 100x, C- lactophenol cotton blue staining images at 40x

Table 2.6 Colony Characteristics of the isolates grown on YMA

Table 2.7 The microbial diversity of mangroves from Goa

Chapter III

Table 3.1: Methods of determination of L-asparaginase activity

Table 3.2: Screening of isolates AMS1 to AMS9 for L-asparaginase production over 120h using PR dye

Table 3.3: Screening of isolates AMS1 to AMS9 for L-asparaginase production over 120h using BTB dye

Table 3.4: Screening of isolates AMS10, BMS1 to BMS3 and DMS1 to DMS4 for L-asparaginase production over 120h using PR dye

Table 3.5: Screening of isolates AMS10, BMS1 to BMS3 and DMS1 to DMS4 for L-asparaginase production over 120h using BTB dye

Table 3.6: Screening of isolates CMS1-5 and RiMS1 and 2 for L-asparaginase production over 120h using PR dye

Table 3.7: Screening of isolates CMS1-5 and RiMS1 and 2 for L-asparaginase production over 120h using BTB dye

Table 3.8: Screening of isolates MMS1 to MMS6, MMS9, MMS13 and MMS14 for L-asparaginase production over 120h using PR dye

Table 3.9: Screening of isolates MMS1 to MMS6, MMS9, MMS13 and MMS14 for L-asparaginase production over 120h using BTB dye

Table 3.10: Screening of isolates MMS7, MMS8, MMS10, MMS11 and MMS12 for L-asparaginase production over 120h using PR dye

Table 3.11: Screening of isolates MMS7, MMS8, MMS10, MMS11 and MMS12 for L-asparaginase production over 120h using BTB dye

Table 3.12: Screening of isolates RMS1 to RMS6 for L-asparaginase production over 120h using PR dye

Table 3.13: Screening of isolates RMS1 to RMS6 for L-asparaginase production over 120h using BTB dye

Table 3.14: Screening of isolates SMS1 to SMS6 and RcMS1 to RcMS3 for L-asparaginase production over 120h using PR dye

Table 3.15: Screening of isolates SMS1 to SMS6 and RcMS1 to RcMS3 for L-asparaginase production over 120h using BTB dye

Table 3.16: Yeast isolates producing L-asparaginase screened using PR dye

Table 3.17: Yeast isolates producing L-asparaginase screened using BTB dye

Table 3.18: Chromatograms of the L-asparaginase-producing isolates. a. CFS collected after 24h of incubation, b. CFS collected after 96h of incubation. In the Chromatogram, the spots are named ‘A’- L-aspartate, ‘B’- L-asparagine and ‘C’, ‘D’ and ‘E’ – CFS.

Table 3.19 Preliminary identification of isolates grown on Hichrome *Candida* differential medium

Table 3.20: Glutaminase assay of the positive isolates

Table 3.21: Morphological analysis of the selected isolates CMS4 and MMS8.

Table 3.22: Biochemical characterization of selected isolates CMS4 and MMS8

Chapter IV

Table 4.1 The actual and coded variables for CCD to optimize L-asparaginase production by isolate MMS8

Table 4.2: The experiment design and observed L-asparaginase activity

Table 4.3: Analysis of variance for the response surface model of L-asparaginase production by isolate MMS8.

Table 4.4: Model adequacy and statistical summary of the RSM model

Table 4.5 Purification profile of enzyme L-asparaginase obtained from isolate MMS8

Table 4.6: L-asparaginase production by different yeasts

Table 4.7: The optimized medium conditions for L-asparaginase production obtained from microbes

Table 4.8: The characteristics of purified L-asparaginase obtained from microbes

Table 4.9: The characteristics of recombinant L-asparaginase

Chapter V

Table 5.1: Methods implemented to estimate cytotoxicity of L-asparaginase enzyme on multiple cell lines

List of Figures

Chapter I

Figure 1.1: Action of enzyme L-asparaginase. a. Reaction catalysed by L-asparaginase enzyme. b. Illustration of the catalytic mechanism of L-asparaginase, depicting the hydrolysis of L-asparagine into L-aspartate and ammonia. The illustration was created using BioRender.com and adapted from Batool et al. (2016) and Cachumba et al. (2016).

Figure 1.2: Schematic representation of Type I and Type II L-asparaginase in yeast cells. Type I L-asparaginase is localized intracellularly and functions primarily in cellular nitrogen metabolism. Type II L-asparaginase is periplasmic or secreted and is capable of extracellular L-asparagine depletion.

Figure 1.3: Selective cytotoxic effect of L-asparaginase based on cellular L-asparagine availability. Normal cells synthesize L-asparagine via asparagine synthetase, whereas tumour lymphoblasts depend on extracellular L-asparagine; enzymatic depletion inhibits protein synthesis and induces cell death. Created using BioRender.com and adapted from Batool et al. (2016) and Cachumba et al. (2016).

Chapter II

Figure 2.1: Collection of mangrove sediment samples from different sites. Adpai, b. Divar island, c. Madkai and d. Ribandar a. Borim, b. Camorlim, c. Raia, d. Shiroda and e. Rachol

Chapter III

Figure 3.1: Positive isolates on Hichrome candida differential agar incubated for a. 24h, b. 48h and c. 72h

Figure 3.2: Scanning electron microscopy images of L-asparaginase positive isolates. a. SMS1, b. RMS3, c. CMS4, d. MMS7, e. MMS8, f. MMS10, g. MMS11 and h. MMS12.

Figure 3.3: Enzyme activity of all the L-asparaginase positive isolates. a. Over 120 h of incubation period and b. at 96 h of incubation. The symbol ‘#’ for CMS4 and ‘\$’ for MMS8 is used to represent a comparatively statistically significant enzyme activity value.

Figure 3.4: Phylogenetic tree of isolate CMS4. The tree was constructed by the Neighbour Joining Method based on the rDNA sequences that included ITS1, 5.8s rRNA and ITS2

regions. *Penicillium chrysogenum* was used as an outgroup. The numbers at the nodes indicate the bootstrap values(percentage) resulting from 10000 replicates.

Figure 3.5: Phylogenetic position of isolate MMS8. The tree was constructed by the Neighbour Joining Method based on the rDNA sequences that included ITS1, 5.8s rRNA and ITS2 regions. *Penicillium chrysogenum* was used as an outgroup. The numbers at the nodes indicate the bootstrap values(percentage) resulting from 10000 replicates.

Chapter IV

Figure 4.1: Study of effect of physical factors on enzyme production by isolate CMS4 (1) and MMS8 (2): a) Incubation time, b) Incubation temperature. The symbols ‘*’ for enzyme activity and ‘#’ for biomass are used to represent a comparatively statistically significant result.

Figure 4.2: Study of effect of physical factors on enzyme production by isolate CMS4 (1) and MMS8 (2): a) pH of Medium, b) Inoculum size. The symbols ‘*’ for enzyme activity and ‘#’ for biomass are used to represent a comparatively statistically significant result.

Figure 4.3: Study of effect of nutrition factors on enzyme production by isolate CMS4 (1) and MMS8 (2): a) Carbon source, b) Combination of Nitrogen source (10 g/L), c) Nitrogen source (5 g/L) with L-asparagine (5 g/L). The symbols ‘*’ for enzyme activity and ‘#’ for biomass are used to represent a comparatively statistically significant result.

Figure 4.4 Time course study of enzyme production by isolates grown in MCDB optimized by OFAT method. a) Isolate CMS4 and b) Isolate MMS8

Figure 4.5: Effect of change in concentration of factors on L-asparaginase production by MMS8: a) Glucose concentration, b) Sodium chloride (Salt) and c) L-asparagine. The symbols ‘*’ for enzyme activity and ‘#’ for biomass are used to represent a comparatively statistically significant result.

Figure 4.6: Three-dimensional response surface analysis depicting the influence of process variables on L-asparaginase production. a) Normal residual plot indicating the suitability of the model. b) Interactive influence of L-asparagine and glucose; c) Interaction between Sodium chloride and L-asparagine and d) Interaction between glucose and sodium chloride.

Figure 4.7: Partial purification of L-asparaginase produced by isolate MMS8: a. Size exclusion chromatogram; b. SDS PAGE analysis (left to right): Lane 1 Protein marker,

Lane 2 Partially purified protein, Lane 3 Cell free supernatant and Lane 4 Dialyzed ammonium sulphate precipitate of protein; c. Purification profile

Figure 4.8: Characterization of the partially purified enzyme by studying the effect of factors on enzyme activity: a) Incubation time, b) pH of the reaction mixture, c) Incubation temperature, d) Substrate concentration and e) Lineweaver-Burke plot for determination K_m and V_{max} .

Figure 4.9: Determination of stability of the partially purified enzyme by studying the effect of factors on enzyme activity: a) Incubation time, b) pH of the reaction mixture, c) Metal ions and d) Inhibitors

Chapter V

Figure 5.1: Effect of varying concentrations of L-asparaginase on percent cell viability. Control 1 consists of HeLa cells grown in DMEM and Control 2 consists of HeLa cells grown in DMEM with the solvent. a. Crude extract containing enzyme in MCDB. The symbol ‘*’ for Control 1 and ‘#’ for Control 2 is used to represent a comparatively statistically significant result; and b. partially purified enzyme in PBS. The symbol ‘*’ for Control 1 and ‘#’ for Control 2 at 24h; ‘\$’ for Control 1 and ‘@’ for Control 2 at 48h, is used to represent a comparatively statistically significant result.

Figure 5.2: DAPI staining images of effect of crude enzyme (CFS) on viability of HeLa cells. Controls are C1 (DMEM medium+ HeLa cells) and C2 (DMEM +HeLa cells+MCDB). The tests include HeLa cells grown in DMEM medium with CFS ranging from 2U/mL to 4 U/mL.

Figure 5.3: DAPI staining images of effect of partially purified enzyme on HeLa cells on incubating for 48h. Controls are C1 (DMEM medium+ HeLa cells) and C2 (DMEM +HeLa cells+PBS). The tests include HeLa cells grown in DMEM medium with partially purified L-asparaginase enzyme ranging from 2U/mL to 10 U/mL.

Abbreviations

CDB	Czapek Dox Broth
SDB	Sabouraud's Dextrose Broth
PDB	Potato Dextrose Broth
YMB	Yeast malt broth
MCDM	Modified Czapek Dox Media
CCD	Central composite design
RSM	Response Surface Methodology
SDS	Sodium dodecyl sulphate
PAGE	Polyacrylamide gel electrophoresis
CFS	Cell free supernatant
ALL	Acute Lymphoblastic Leukemia
ITS	Internal transcribing sequence
BTB	Bromothymol blue
PR	Phenol red
EDTA	Ethylene diamine tetra acetic acid

CHAPTER I

INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction

Tropical and subtropical forests with flora that have undergone adaptation to survive in saline conditions with changing tides are called mangroves. Mangrove forests are observed in area along the coast where waves are absent which leads to build up of sediment with no oxygen in the surrounding soil (Smith, 2012). They are found in intermediate regions of land, sea and rivers (Ghizelini *et al.*, 2012). Mangroves have ample carbon resources that assist microbial growth and promote adaptation to saline environment, supporting diverse microbial communities. The primary productivity of this ecosystem is influenced by physiological mechanisms and interactions between soil nutrients, microbes and vegetation (Thatoi *et al.*, 2013). Mangrove, an important tropical ecosystem rich in diversity needs to be studied before these valuable resources deplete, as it is most susceptible to damage by the current climate changes and human activities (Sarkar *et al.*, 2019; Huxham *et al.*, 2017; Ghizelini *et al.*, 2012).

The mangroves, a marine ecosystem is known to have a rich abundance of microorganisms that are a source of multiple bioactive compounds and enzymes of commercial importance (Gunde-Cimerman *et al.*, 2014; Bevilacqua, 2016; Chrapusta *et al.*, 2017; Halder *et al.*, 2019). These bioactive compounds include antioxidants, cell growth inhibitors, pigments, anti-inflammatory compounds, vitamins and photo-protective compounds (Galasso *et al.*, 2018; Chrapusta *et al.*, 2017). The marine ecosystem is a source of these novel and unexplored bioactive compounds that need recognition (Galasso *et al.*, 2018; Chrapusta *et al.*, 2017; Wang *et al.*, 2014).

L-asparagine amidohydrolase catalyzes conversion of L-asparagine to aspartic acid and ammonia. Normal cells can synthesize non-essential amino acid L-asparagine while the cancerous cells lack the ability to do so and depend on circulating L-asparagine. L-asparaginase as a chemotherapeutic agent causes depletion of available L-asparagine to cancerous cells, leading to death of these cells as process of protein synthesis is obstructed. Commercially L-asparaginase is obtained from bacterial sources (Cachumba *et al.*, 2016). It is observed that L-asparaginase from bacterial origin may cause unfavorable effects such as hypersensitivity and immune inactivation (Cachumba *et al.*, 2016). Research is now focused on enzyme of eukaryotic origin with less adverse effects (Ammulu *et al.*, 2017; Batool *et al.*, 2016; Cachumba *et al.*, 2016).

Yeasts are single-celled eukaryotic microorganisms having membrane bound nucleus (Bevilacqua, 2016). They are prominent part of fermentation industry. Yeasts are preferred due to their ability to produce recombinant products and show fast cell growth. Protein production in yeasts include post-translation modifications resulting in synthesis of proteins that resemble those of mammalian origin (Deak, 2009). Bioactive agents of eukaryotic origin like yeast may be more acceptable source for commercial application as therapeutic agent (Cachumba *et al.*, 2016). Utilizing yeasts to obtain these bioactive compounds and enzymes is a requirement of the modern world (Cachumba *et al.*, 2016; Imhoff, 2016).

The yeast diversity present in the marine ecosystem is yet to be explored. The state of Goa is situated on the south-west coast of India and is flanked by Arabian Sea. The coast of Goa consists of multiple locations with mangroves that represent marine ecosystems. The organisms residing in these regions are exposed to changing salinity, harsh temperature and continuous exposure to sunlight. Organisms under stressful conditions produce bioactive compounds for protection from these intense conditions. This research aims at investigating the potential L-asparaginase producing yeasts obtained from mangrove sediments for their anti-neoplastic applications.

1.2 Review of Literature

1.2.1 Mangrove ecosystem and its ecological importance

Tropical and subtropical forests with flora that have undergone adaptation to survive in saline conditions with changing tides are called mangroves. Mangrove forests barricade the coastlines and protect it from the ocean's currents. These forests flourish in regions with heavy rainfall as they require freshwater physiologically while surviving in saline conditions and are hence called facultative halophytes. These forests are adapted to the existing environmental conditions of waterlogging, salinity, tides and storms through morphological, anatomical and physiological changes. Mangroves are ecologically beneficial for the coastal areas as they play a significant role in protection of the coastline by preventing acidification, eutrophication including filtering and deposition of micro plastics. Mangroves facilitate survival of diverse flora and fauna as it is rich in multiple resources. They sequester carbon and aid fisheries supporting livelihood of coastal communities/population (Thatoi *et al.*, 2013a; Mukherji *et al.*, 2019).

The global forest resource assessment revealed that 14.72Mha of mangrove forests span 113 countries though the distribution is varying with South and South East Asia having the

largest share. Mangrove forest cover across the world including Asia showed a decline from 1990 to 2020, indicating a need for aiming towards conservation. Aquaculture and agriculture including coastal erosion due to extreme weather conditions and rising sea levels are the cause of decline in mangrove forest covers (Mukherji et al., 2019; Forest Survey of India, 2023).

The mangrove ecosystem is regarded as a highly productive and dynamic habitat that supports diverse life forms belonging to microbial communities including bacteria, algae, plankton and fungi. Primary detritus in mangrove forests is provided by the plant materials from mangrove vegetation while further colonization by microorganisms aids the carbon sync formation (Mukherji et al., 2019).

Mangroves are a dynamic ecosystem with complex interactions, making them highly productive. The mutualistic interactions between plants, animals and microbes result in nutrient cycling that leads to organic richness of these ecosystems. Microbe-rich mangrove sediments have contributed to the productivity and habitat restoration (Holguin et al., 2001; Mendes and Tsai, 2014). A threat to the existing mangrove ecosystem worldwide includes pollution, deforestation and human intrusions. In a polluted ecosystem, the microbial diversity is determined by a shift towards the microbial communities capable of sustaining in such unfavourable conditions (Grativola et al., 2017). The detritus-rich sediments are abundant in yeast diversity and thus aid in studying yeast with biotechnological potential (Hagler et al., 2006).

The sediments possess abundant organic carbon and mineral nutrients that support the sustenance of varied types of microorganisms. Indian mangrove sediments exhibit pH values ranging from 6 to 8, with varying salinity influenced by changing seasons and tides, that range between 5 and 30 psu. Nazareth and Gonsalves (2022) reported that the mangrove sediments of Goan mangroves have near neutral pH values throughout the year, with temperatures varying from 26 °C to 30 °C. These physical parameters of the sediments are conducive to the growth and maintenance of a wide range of microbes. Mangrove sediments are a repository of multiple groups of microorganisms, including yeasts.

India ranks third globally in mangrove diversity and mangrove forests are found along the East and West coasts including the Andaman, Nicobar having highest species richness and Lakshadweep islands hosting few representative species. Mangrove species face threat of extinction due to poor regeneration and two such species *Heritiera fomes* and *Sonneratia*

griffithi are present in Indian mangrove forests (Mukherji et al., 2019; Forest Survey of India, 2023).

India harbours 46 true mangrove species and about 4100 associated species. The successful conservation and restoration of these habitats across the country was done by implementing advanced methods that include canal bank plantations, raised bed method and community-based management (Mukherji et al., 2019; Forest Survey of India, 2023). States also carried out notable conservative efforts by launching programs such as Mangrove Cell (Maharashtra) and Kannuar Mission (Kerala).

India has launched the MISHTI program for mangrove restoration, aiming towards mangrove cover expansion by about 540 km² across 11 states and 2 union territories, focusing mainly on awareness, community participation, including plantation efforts. The estimated mangrove forest cover across India is 4991.68 km² having varying density of the plants ranging from very dense to open. States of Maharashtra and Andhra Pradesh reported increase in the mangrove area by plantation and natural regeneration (Forest Survey of India, 2023).

The tidal range along the Indian coast is varied and contributes towards shaping the structure and function of mangroves. The tidal amplitude differs from minimal (Kerala), 1.5 to 2 m in Goa to peak at 8-9 m in Gulf of Khambhat. Seasonal freshwater specifically during monsoon influences the tides and salinity. The waterlogged conditions deplete available oxygen, promoting root adaptations for effective respiration. Mangrove sediments or soil have distinct physicochemical characteristics. The coastal erosion is a source of the clay rich sediments that support the mangroves. The sediment displaying varying salinity and rich in organic matter contains 4% coarse sand, 38% fine sand, 30-33% silt and 4% clay (Dhargalkar et al., 2014; Forest Survey of India, 2023).

The availability of nutrients regulates physiological processes like photosynthesis in the mangrove forest, affecting the productivity and species diversity. Mangroves sequester carbon in their root system, that develops into sync as decomposition is hindered by waterlogging and anaerobic conditions. The nutrient cycling essential for sustaining the mangrove ecosystem is facilitated by tidal flushing and microbial activity. Variations in hydrology and topography result in the formation of mangrove ecotypes, including basin (stagnant water), estuarine (brackish water), fringe (border) and scrub (stress conditions) forests (Dhargalkar et al., 2014).

The ecologically significant adaptations are displayed by mangroves to sustain the dynamic conditions of fluctuating tides, salinity gradients, lack of oxygen and sediment

properties. The prominent adaptations observed are pneumatophores, stilt roots, thick cuticles and vivipary, mainly intended to exclude excessive salt. Modified leaves with thick cuticles and sunken stomata diminish water loss and specialized aerial roots called pneumatophores aid aeration. The stilt roots act as anchors in the swampy sediment. The salt tolerance strategies employed by the plants in the mangrove ecosystem include salt exclusion by leaves and selective ion uptake by roots. The mechanism of reproduction in mangroves called vivipary involves the development of a seedling on the parent plant, subsequent dispersal of the buoyant propagule and establishment of a sapling in a suitable habitat (Dhargalkar et al., 2014).

Mangroves generate abundant detritus, an essential component of estuarine food webs sufficient to fuel juvenile fish, crabs and shrimps central to coastal fisheries and support a diverse community of marine organisms. The tidal movements redistribute nutrients to adjacent ecosystems, facilitate regeneration and mineralization processes and aid ecological balance. The role of mangroves as natural filters contributes to maintaining the ecosystem by enhancing coastal water quality. Local communities obtain food, medicine, fuel wood and construction materials from mangroves that support cultural practices and sustain livelihoods. Fisheries are dependent on mangroves, as they offer breeding and nursery habitats for multiple commercially valuable species. Fishery yields are adversely affected due to reductions in mangrove cover. Additionally, mangroves function as powerful carbon sinks, storing carbon in their biomass and sediments as well as contributing meaningfully to climate change mitigation (Thatoi et al., 2013; Dhargalkar et al., 2014; Mukherji et al., 2019).

Mangrove ecosystems are exceptionally complex and productive habitats that harbour highly diverse microbial communities (Kathiresan & Qasim, 2005). The intertidal nature of mangroves, which creates strong physicochemical gradients in salinity, oxygen availability, and organic matter content, supports the coexistence of terrestrial, marine, and transitional microorganisms (Alongi et al., 1998; Holguin et al., 2001). The major microbial groups present include bacteria, fungi, actinomycetes, and algae (Hyde et al., 1998; Hong et al., 2009).

Bacteria represent the major driving force behind biogeochemical cycling in mangrove ecosystems due to their ability to perform key processes such as nitrogen fixation, phosphate solubilization, sulphate reduction, sulphur oxidation, cellulose degradation, and methanogenesis (Nedwell et al., 1994; Holguin et al., 2001; Dar et al., 2008). Another core component of this ecosystem is fungi. Mangrove fungi are broadly categorized based on

their position within the ecosystem, with fungi inhabiting non-submerged regions classified as terrestrial, while those occupying submerged and periodically inundated zones at the land–sea interface are considered marine fungi (Sarma & Hyde, 2001).

Mangrove-associated fungi are taxonomically dominated by Ascomycetes, with fewer representatives of Basidiomycetes and anamorphic fungi, and their diversity varies according to geographical region and local physicochemical conditions (Hyde et al., 1998; Shearer et al., 2007). Fungi from mangrove forests along the Indian subcontinent predominantly exhibit saprophytic lifestyles with strong enzymatic capabilities for organic matter degradation, as well as endophytic associations that enhance nutrient absorption, stress tolerance, and survival under saline conditions (Suryanarayanan et al., 1998; Ravikumar & Vittal, 1996).

Diverse actinomycetes also inhabit mangrove ecosystems, particularly members of *Streptomyces* and related genera, which are well known for their capacity to produce bioactive secondary metabolites (Sivakumar, 2001; Hong et al., 2009). Collectively, the extensive microbial diversity of mangrove forests highlights these ecosystems as promising reservoirs for the discovery of novel antibiotics, industrial enzymes, and anticancer compounds, underscoring their significant biotechnological potential (Sivakumar et al., 2007; Gupta et al., 2009a, b).

Microorganisms are central to mangrove ecosystem functioning, linking detrital inputs to nutrient regeneration and maintaining the resilience and productivity of these ecologically and economically vital forests. Mangrove ecosystems provide organic matter to adjacent estuarine and nearshore waters in the form of detritus that forms the base of complex food webs (Odum & Heald, 1975a). This carbon-rich matter supports diverse and metabolically active microbial communities that transform dead mangrove biomass into bioavailable forms of carbon, nitrogen, phosphorus, and sulfur (D’Croz et al., 1989). A tightly coupled interaction exists between mangrove vegetation and microorganisms: plant root exudates stimulate microbial growth and activity, while microbial mineralization replenishes nutrients essential for plant productivity (Rivera-Monroy et al., 1995a, b).

Bacteria are responsible for most carbon fluxes in mangrove sediments (Kathiresan & Qasim, 2005). Labile organic compounds leached from mangrove litter, including sugars and tannins, are rapidly metabolized, whereas complex lignocellulosic fractions undergo slower degradation (Cundell et al., 1979). Nitrogen cycling is mediated by processes including ammonification, denitrification, and biological nitrogen fixation, contributing to nutrient conservation within sediments, although anthropogenic nutrient inputs may

enhance nitrogen losses (Rivera-Monroy et al., 1995b; Sengupta & Choudhury, 1991). In the predominantly anaerobic sediments, sulphate-reducing bacteria play a key role in organic matter mineralization, sulphur cycling, and the release of bioavailable iron and phosphorus (Nedwell et al., 1994; Alongi et al., 1998; Holguin et al., 2001).

Fungi act as primary decomposers of mangrove litter and are essential for the degradation of lignocellulosic substrates, enabling subsequent bacterial decomposition (Nakagiri et al., 1996; Matondkar et al., 1981). Actinomycetes support ecosystem functioning through the production of extracellular enzymes involved in organic matter degradation and phosphate solubilization, while their secondary metabolites enhance the growth of higher trophic levels such as juvenile fish and crustaceans (Sivakumar et al., 2007; Sahu et al., 2007). Phytoplankton, benthic microalgae, and cyanobacteria contribute significantly to primary production by fixing carbon and nitrogen, thereby enhancing estuarine productivity and sediment stability (Nedumaran et al., 2008).

Sediment microbes are a significant part of the mangrove ecosystem. Interactions between the sediment microbes, nutrients and plants are interdependent as microbes release nutrients from dead plant parts which in turn aids growth of living plants. Yeasts are mainly saprophytes that derive nutrition from decaying matter and are sustained by detritus rich mangrove sediments.

Mangroves are distributed mainly in tropical and subtropical regions across the globe except for the poles. Asia, North and South America and Europe are the biggest contributors towards studies on yeast diversity while Africa and Australia are less explored. Ascomycetes yeasts are commonly found in mangroves across the world. Seven per cent of the world's total mangroves are present in India constituting the fourth largest mangrove forest area. The Indian sub-continent has distinct seasonal and ecological variations that support the vast species diversity in mangroves and provide opportunity for future study of sediment microbe composition and their metabolic properties, including yeast.

Situated on the west coast of India, Goa is a state with an extensive mangrove forest. The Forest Department of Goa implemented actions to elevate the mangrove cover across the state of Goa from 22 km² (Dhargalkar et al., 2014) to 29 km² (Forest Survey of India, 2023) to emphasize the role and contribution of the mangrove ecosystem. The lack of available information regarding the Goan mangrove yeast and their potential as sources of bioactive compounds demonstrates the need for further exploration.

Table 1.1: Microbial diversity of mangrove ecosystem

Taxonomic group	Location and Characteristics
Algae (Thatoi et al., 2013a; Mukherji et al., 2019)	
<i>Bostrychia</i> spp., <i>Cologlossa</i> spp., <i>Catenella</i> spp.	on the roots and trunks
<i>Rhizoclonium</i> spp., <i>Enteromorpha</i> spp., <i>Cladophora</i> spp.	sediments
<i>Chlorophyta</i> spp., <i>Chrysophyta</i> spp., <i>Phaeophyta</i> spp., <i>Rhodophyta</i> spp., <i>Cyanophyta</i> spp.	Sundarbans mangroves of West Bengal
<i>Gloeocapsa</i> spp., <i>Chlorella</i> spp., <i>Ulva</i> spp., <i>Anabaena</i> spp., <i>Spirogyra</i> spp., <i>Oscillatoria</i> spp., <i>Phormidium</i> spp.	Bhitarakanika, Orissa
Cyanobacteria (Thatoi et al., 2013a; Mukherji et al., 2019)	
<i>Lyngbya</i> sp., <i>Anacystis</i> sp.	sediments
<i>Aphanocapsa</i> spp., <i>Nodularia</i> spp., <i>Trichodesmium</i> spp.	Pichavaram mangroves, Nitrogen fixation
<i>Leptolyngbya</i> spp., <i>Oscillatoria</i> spp., <i>Synechocystis</i> spp.	Sundarbans
Fungi (Thatoi et al., 2013a; Mukherji et al., 2019)	
<i>Aspergillus</i> spp., <i>Penicillium</i> spp., <i>Fusarium</i> spp., <i>Trichoderma</i> spp.	Mangalvan mangrove ecosystem, ligninolytic, cellulolytic, pectinolytic, amylolytic, proteolytic
<i>Aspergillus niger</i> , <i>Gongronella</i> sp., <i>Halosarpheia ratnagiriensis</i>	Mangrove detritus, sediment, wood, Chorao Island, Goa
<i>Alternaria</i> spp., <i>Aspergillus</i> spp., <i>Cladosporium</i> spp., <i>Colletotrichum</i>	Endophytic fungi

spp., <i>Fusarium</i> spp., <i>Paecilomyces</i> spp., <i>Penicillium</i> spp., <i>Pestalotiopsis</i> spp., <i>Phoma</i> spp., <i>Phomopsis</i> spp., <i>Phyllosticta</i> spp., <i>Trichodema</i> spp.	
<i>Aspergillus niger</i>	the rhizosphere of black mangrove <i>Avicennia germinans</i> , Marine phosphate-solubilizing fungus
Actinomycetes (Thatoi et al., 2013a; Mukherji et al., 2019)	
<i>Actinomadura</i> spp., <i>Microbispora</i> spp., <i>Nonomuraea</i> spp., <i>Actinoplanes</i> spp., <i>Micromonospora</i> spp., <i>Verrucosipora</i> spp., <i>Arthrobacter</i> spp., <i>Isophtericola</i> spp., <i>Micrococcus</i> spp., <i>Microbacterium</i> spp., <i>Nocardia</i> spp., <i>Rhodococcus</i> spp., <i>Streptomyces</i> spp.	mangrove soils and plants in China
<i>Brevibacterium</i> spp., <i>Dermabacter</i> spp., <i>Kocuria</i> spp., <i>Kytococcus</i> spp., <i>Microbacterium</i> spp., <i>Nesterenkonia</i> spp., <i>Rothia</i> spp.	mangrove sediment in Brazil
<i>Nonomuraea maheshkhaliensis</i>	Mangrove rhizosphere mud, Bangladesh.
<i>Streptomyces albidoflavus</i>	Pichavaram mangrove, antitumor properties
<i>Streptomyces</i> spp.	Bhitarkanika mangrove forest, Orissa, Phosphate solubilisation
Bacteria (Thatoi et al., 2013a; Mukherji et al., 2019)	
<i>Azospirillum</i> spp., <i>Azotobacter</i> spp., <i>Rhizobium</i> spp., <i>Clostridium</i> spp., <i>Klebsiella</i> spp.	sediments, rhizosphere and root surfaces, Nitrogen fixation
<i>Azotobacter</i> spp.	Sediments, Pichavaram mangroves, Nitrogen fixation

<i>Vibrio campbellii</i> , <i>Listonella anguillarum</i> , <i>Vibrio aestuarianus</i> , <i>Phyllobacterium</i> spp.	rhizosphere of mangroves in Mexico, Nitrogen fixation
<i>Bacillus amyloliquefaciens</i> , <i>B. atrophaeus</i> , <i>Paenibacillus macerans</i> , <i>Xanthobacter agilis</i> , <i>Vibrio proteolyticus</i> , <i>Enterobacter aerogenes</i> , <i>E. taylorae</i> , <i>E. asburiae</i> , <i>Kluyvera cryocrescens</i>	black mangrove (<i>Avicennia germinans</i>) roots, Mexico, Phosphate solubilisation
<i>Pseudomonas aeruginosa</i>	mangrove soils, Great Nicobar, Phosphate solubilisation
<i>Pseudomonas</i> spp., <i>Bacillus</i> spp., <i>Corynebacterium</i> spp., <i>Vibrio</i> spp., <i>Micrococcus</i> spp., <i>Alcaligenes</i> spp.	Water, sediment, Porto Novo, Chennai, Phosphate solubilisation
<i>Chromatiales</i> , <i>Desulfobacterales</i>	Sediment, Brazil, sulfur-oxidizing
<i>Desulfovibrio desulfuricans</i> , <i>Desulfovibrio desulfuricans aestuarii</i> , <i>Desulfovibrio salexigens</i> , <i>Desulfovibrio sapovorans</i> , <i>Desulfotomaculum orientis</i> , <i>Desulfotomaculum acetoxidans</i> , <i>Desulfosarcina variabilis</i> , <i>Desulfococcus multivorans</i>	Sediment, Mangroves at Agasaim, Daboli, Mirabaug, along Zuari estuary, Goa, Sulphate reducing bacteria
<i>Bacillus cereus</i> , <i>Bacillus licheniformis</i> , <i>Bacillus pumilus</i> , <i>Bacillus</i> sp.	mangroves in the Philippines, Cellulose degrading
<i>Chloronema</i> spp., <i>Chromatium</i> spp., <i>Beggiatoa</i> spp., <i>Thiopedia</i> spp., <i>Leucothiobacteria</i> spp.	Mangrove ecosystem of Cochin (India), Purple non-sulfur bacteria
<i>Methanococcoides methylutens</i>	Sediment, Pichavaram, Methanogenic bacteria
<i>Methanococcoides methylutens</i> , <i>Methanosarcina semesiae</i>	Sediment, Tanzanian mangrove, Methanogenic bacteria
<i>Methanopyrus kandleri</i> , <i>Methanothermococcus thermolithotrophicus</i>	Sediment, BrazilMethanogenic bacteria

Protozoa (Thatoi et al., 2013a; Mukherji et al., 2019)	
<i>Crassostrea cucullata</i>	Bay of Bengal
<i>Crassostrea gryphoides</i> ,	Frasergunj, Digha, and Kaikhali , West Bengal
<i>Chromulina</i> spp, <i>Astasia</i> spp, <i>Heteronema</i> spp., <i>Spumella</i> spp., <i>Euglena</i> <i>acus</i> , <i>Paranema</i> spp., <i>Prorodon</i> spp., <i>Cyclidium</i> spp., <i>Zoothamnium</i> spp.	degraded leaf litter of <i>Avicennia</i> sp. and <i>Excoecaria</i> sp. from Coringa mangrove forest at Kakinada

1.2.2 Mangroves: Source of yeast and bioactive compounds

The term manglicolous fungi is used to describe fungi obtained from the mangrove ecosystem. Here they are present on surfaces of the mangrove plant parts both aerial and submerged in water. Manglicolous yeast have immense application as bioactive agent/compound producers including novel compounds as these compounds are tolerant and stable even under harsh conditions. They enjoy a reputation for producing better yield and compounds with use in fermentation for production of biofuel, feed additives, probiotics in aquaculture and biocontrol agents. The available knowledge regarding the diversity and economic potential of manglicolous yeast is limited, hence there is a requirement for more focused research to explore the existing mangrove ecosystem.

Mangroves sustain and shelter a number of organisms like snails, molluscs, oysters, clams, mussels, shrimp, birds, insects including diverse microbial communities. The mangrove mycobiota includes yeast as an integral part discovered in the entire ecosystem, the vegetation, sediment, water and invertebrates. The yeast community of the mangrove ecosystem contributes towards the bioproductivity by nutrient cycling.

Microorganisms develop the metabolic ability to detoxify/degrade toxic mangrove secondary metabolites to sustain. This competition to survive promotes the production of novel bioactive compounds (Solntsev et al., 2019). These compounds find industrial applications due to their properties, such as salt tolerance, thermal stability, and substrate flexibility (Sengupta et al., 2015). Bioprocess technology has been used to produce yeast enzymes with applications in the food and medical industries. Characteristics of yeast, including ease of generation both environmentally and after genetic modification, cost-effective downstream processing, stability, safety, and extracellular products, are the reasons for preference in large-scale production of enzymes and other bioactive compounds (Elsanhoty et al., 2017).

Yeasts are recognized as extracellular enzyme producers aided by the intracellular biochemical interactions. In a mangrove ecosystem, nutrient cycling and biodegradation of substrates, including organic material and hydrocarbons, are facilitated by these enzymes. (Kutty et al., 2012; Kutty et al., 2014; Vidya and Sebastian, 2020). Microorganisms develop the metabolic ability to detoxify toxic secondary metabolites produced by mangroves, enabling them to sustain themselves. This competition to survive promotes the production of novel bioactive compounds (Solntsev et al., 2019). These compounds find

industrial applications based on their properties, such as salt tolerance, thermal stability and substrate flexibility (Sengupta et al., 2015). Industries based on healthcare and nutrition utilize fermentation technology to obtain enzymes of yeast origin. Yeast is preferred in large-scale production of bioactive compounds, including enzymes. This is attributed to the characteristic of yeasts comprising vigorous growth in both native and modified form, producing stable, safe and extracellular compounds resulting in cost-effective downstream processing (Elsanhoty et al., 2017). Yeasts can assimilate multiple nutrient sources; industrial byproducts and wastes in culture media can be used for large scale production (Cheng and Yang, 2016). Extracellular enzyme production by yeasts is dependent on multiple physical and chemical factors that include pH, temperature, salinity, composition and substrate concentration. Enhanced enzyme production can be achieved through optimization of factors and medium components responsible for growth and enzyme production. Moreover, this knowledge can support production of enzyme at a much larger scale using fermentation technology.

Asia, North and South America and Europe are the biggest contributors towards studies on yeast diversity with maximum studies reported from Asian countries like India, Thailand, Taiwan, Philippines, Pakistan, China, and Saudi Arabia. Multiple studies from Asian regions have reported isolation of a total 43 yeast genera. Ascomycetes yeasts are commonly found in mangroves across the world that predominantly comprises of genera *Candida*, *Cryptococcus*, *Debaryomyces*, *Geotrichum*, *Kluyveromyces*, *Rhodotorula*, *Saccharomyces*, and *Pichia*.

Studies on South Asian mangroves suggest the presence of diverse fungal communities, with most work on mangrove yeast is reported from mangroves from Thailand. Novel strains of yeast were isolated from Thailand and Taiwan. These include novel strains of *Kluyveromyces* species and *Heterocephalacria mucosa* from Ranong (Thailand) mangroves and *Vishniacozyma taiwanica* and *V. changhuana* from Taiwan. A total of 191 yeasts were obtained from eight estuarine mangroves in Andaman Sea and Gulf of Thailand.

The Indian subcontinent has east and west coasts and the southernmost Lakshadweep, Andaman and Nicobar Islands rich in mangrove forests. The studies on mangrove forests of Muthupet, Pichavaram, Sundarbans and Mahanadi delta from the east coast of India included the isolation of the yeasts belonging to genera *Kuraishia*, *Barnettozyma*, *Saccharomyces*, *Debaryomyces*, *Aureobasidium*, *Dioszegia*, *Leucosporidium*, *Rhodotorula*, *Cytobasidium*, *Geotrichum* and *Candida* (Manivannan and Kathiresan, 2009; Kathiresan et

al. 2011; Thatoi et al., 2013b; Saravanakumar et al. 2013; Senthilraja and Kathiresan 2015; Jayalakshmi and Umamaheshwari, 2016; Sarkar & Bhaskara Rao, 2023).

The reported studies carried out on manglicolous yeast isolated from the west coast of India across the states of Gujarat, Goa and Kerala included the yeast genera *Hortaea*, *Candida*, *Kluyveromyces*, *Debaryomyces*, *Torulaspora*, *Trichosporon*, *Rhodotorula*, *Cyberlindnera*, *Wickerhamiella*, *Pichia* and *Starmerella* (Prabhakaran and Gupta, 1990; Nazareth et al., 2015; Kachiprath et al., 2019, Patel and Patel, 2020; Nimsi et al., 2023). In a comprehensive approach, Prasannakumar et al. (2020) created a DNA barcode library of marine yeast isolates obtained from mangrove sediments and the continental shelves of the Indian coastline. The study reported fifty-five genera of yeast and for the first time, thirteen species were barcoded.

Existing literature contains reports on the contribution of manglicolous yeasts as single cell proteins; producers of biofuel, immunostimulants and biosurfactants; biocontrol agents; bioremediators, including application in nanoparticle synthesis (Subramanian et al., 2010; Senthilraja et al., 2011; Subramanian et al., 2014; Shen et al., 2019; Hodhod et al., 2020; He et al., 2022)

The saprophytic nature of mangrove yeast makes them a potential source of hydrolytic enzyme namely cellulase, xylanase, pectinase, chitinase, inulinase, glutaminase, lipase, ligninase, urease, DNase, pectinase, and amylase (Da Costa and D'Souza, 1979; Chi et al., 2009; Jiang et al., 2016; Pothayi et al., 2022; Kachiprath et al., 2019). Mangrove yeasts with the ability to produce decomposing enzymes can be implemented to support bioremediation. Mangrove yeasts belonging to the genus *Candida* can degrade oil and hydrocarbons (Da Costa and D'Souza, 1979; Kachiprath et al., 2019). In a study by He et al (2022) isolated *Geotrichum* sp. CS-67 from Futian mangrove reserve that was reportedly involved in heavy metal sequestration. Biosurfactant production by mangrove yeast is a topic of great interest, with studies reporting yeast strains from mangroves comprising of *S. cerevisiae*, *C. tropicalis*, *S. bombicola*, *Yarrowia lipolytica*, and *Rhodotorula paludigena* VA 242 for their biosurfactant production potential (Thenmozhi et al., 2018; Patel and Patel, 2020; He et al., 2022; Nimsi et al., 2022). Mangrove yeast isolates have agricultural application including their role as phosphate solubilizing plant growth promoters (Nurhariyati et al., 2020; Nimsi et al., 2023) and biocontrol agents, as observed in a study reporting action of *Sporidiobolus pararoseus* ZMY-1 against *Botrytis cinerea* fungi that affects the strawberry fruit with grey mold disease (Shen et al., 2019). Rekha et

al (2022) reported the enhancement in pigment of koi fish with a diet that included *Rhodotorula paludigena* VA242 isolated from mangrove sediment.

Mangrove yeast, inhabiting the fluctuating environmental conditions, is a good source of defensive compounds that include antibiotics and natural killer toxins (Buzdar et al., 2011; Zhao et al., 2019). Hodhod et al (2020) in their study on *H. werneckii* yeast strains isolated from intertidal decayed wood and leaves of *A. marina*, discovered the production of antimicrobial agents effective against multiple pathogens, namely methicillin-resistant *Staphylococcus aureus* (MRSA), *Campylobacter jejuni*, and *Salmonella typhimurium*. Studies also show antitumor activity of mangrove yeasts, as observed with *S. cerevisiae* extract, which has a therapeutic index of 16 against MCF-7 cancer cell lines (Senthilraja and Kathiresan, 2015).

Xylitol is implemented as a dietary alternative in foods for diabetic patients and can be produced from xylose and corn cob hydrolysate by mangrove yeast, as reported in a study conducted on *Cyberlindnera* isolate obtained from mangrove wetlands on Indian west coast (Kamat et al., 2013). A study on *Kuraishia capsulate* (formerly *Pichia capsulate*) isolated from Pichavaram resulted in the successful synthesis of silver nanoparticles in culture (Subramanian et al., 2010).

The ability of mangrove yeasts to produce bioactive compounds such as enzymes, xylitol, biofuels, single-cell oil, anticancer agents, antimicrobials and biosurfactants including their application in nanoparticles, has attracted the attention of researchers at global level, in India as well as Goa. The promising potential of mangrove-derived yeasts as a source of L-asparaginase remains insufficiently explored in several aspects. Current research is limited in the development of economical bioprocesses for enzyme production, efficient purification strategies and biochemical characterization of enzyme. Additionally, extensive evaluation of the enzyme under physiological conditions is necessary to establish its therapeutic efficacy, specificity and compatibility for clinical applications. Information about the immunogenicity, serum stability, pharmacokinetic properties and biocompatibility of yeast-derived L-asparaginase remains limited. There is a need for research focusing on the development of recombinant expression systems capable of achieving high-yield enzyme production facilitating commercial scale manufacturing.

1.2.3 The enzyme L-asparaginase

L-asparaginase amidohydrolase (EC 3.5.1.1) facilitates the conversion of L-asparagine to L-aspartic acid and ammonia. L-asparaginase enzyme contributes in the biosynthesis of amino acids that are derived from aspartate including lysine, threonine, methionine and isoleucine. The precursor molecule of these pathways is aspartate, formed mainly through asparagine hydrolysis by L-asparaginase and the tricarboxylic acid cycle (Mesas et al., 1990; Azevedo et al., 1997; Batool et al., 2016).

The hydrolysis of L-asparagine proceeds through a two-step mechanism. The amide carbon of L-asparagine is attacked by the nucleophilic residue within the active site on activation by a strong basic residue, resulting in β -acyl-enzyme intermediate formation. Nucleophilic attack on the ester carbon by an activated water molecule subsequently results in hydrolysis of the molecule, releasing the reaction products ammonia and L-aspartate. The mechanism exhibited by L-asparaginase shares similarities with that of serine proteases that have an active site consisting of a serine (nucleophile), a histidine (base) and an aspartate residue (stabilizing acid) (Cachumba et al., 2016).

Lubkowski et al. (2020) reported that the reaction catalysed by *E. coli* type II L-asparaginase (EcAII) proceeds through a double-displacement (ping-pong) mechanism involving the formation of a covalent intermediate. According to their findings, threonine functions as a weak nucleophile and interacts with the substrate in the presence of a distant general base, which contrasts with the direct general-base participation typical of many hydrolytic enzymes. The tetrahedral intermediates formed during catalysis are stabilized by a structural motif not previously described in other enzyme systems. Although this mechanistic model was established using EcAII and its engineered variants, the authors suggest that it may be broadly applicable to many and possibly all, L-asparaginases.

1.2.3.1 Classification of L-asparaginase

The enzyme is classified into three major classes including bacterial-type, plant-type, and rhizobial-type L-asparaginases, based on their amino acid sequences and biochemical characteristics. The bacterial-derived L-asparaginases is further divided into two types: Type 1, cytoplasmic, constitutive, and Type 2, periplasmic, nitrogen-dependent expression. The type 1 enzyme, coded by an A gene having a homodimer structure, exhibits low affinity for L-asparagine and substantial L-glutaminase activity. Type 2 enzyme coded by an Z gene is reportedly a tetramer that displays a high L-asparagine specificity and affinity. *E. coli* produces both type 1 and type 2 enzymes. The type 2 enzymes are

preferred in pharmaceutical applications for their specificity, affinity and anti tumor activity (Batoool et al., 2016; Cachumba et al., 2016; Izadpanah Qeshmi et al., 2018; Loch and Jaskolski, 2021). Yeast produces two types of L-asparaginase, the intracellular type I encoded by ASP1 and the extracellular type II encoded by ASP3 gene (Wriston and Yellin., 1973; Coral-Medina et al., 2022).

Coral-Medina et al. (2022) reported that ASP3 is regulated through nitrogen catabolite repression (NCR), its transcription is upregulated under limited availability of preferred nitrogen sources, enabling more effective utilization of extracellular asparagine. ASP3 gene is found in select yeast strains, mainly those used in bioethanol production, brewing and cider fermentation including laboratory research strains. ASP3 improves yeast growth in the presence of L-asparagine as sole nitrogen source. This growth advantage was not observed during the fermentation of apple juice, even though asparagine is its main nitrogen source, establishing that the benefit of L-asparaginase expression is limited to specific conditions and nitrogen assimilation in yeast is influenced by complex regulatory mechanisms.

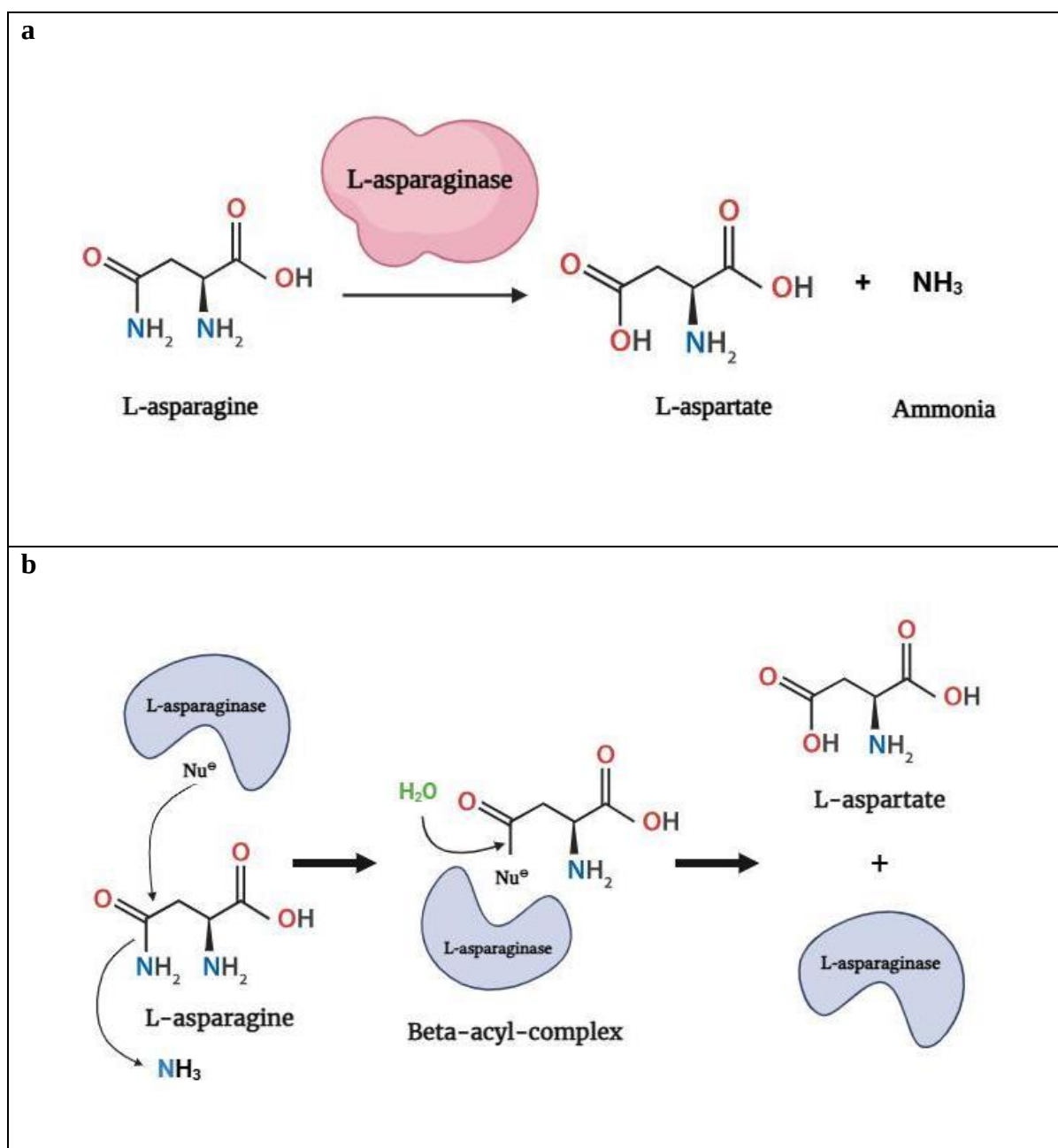


Figure 1.1: Action of enzyme L-asparaginase. a. Reaction catalysed by L-asparaginase enzyme. b. Illustration of the catalytic mechanism of L-asparaginase, depicting the hydrolysis of L-asparagine into L-aspartate and ammonia. The illustration was created using BioRender.com and adapted from Batool et al. (2016) and Cachumba et al. (2016).

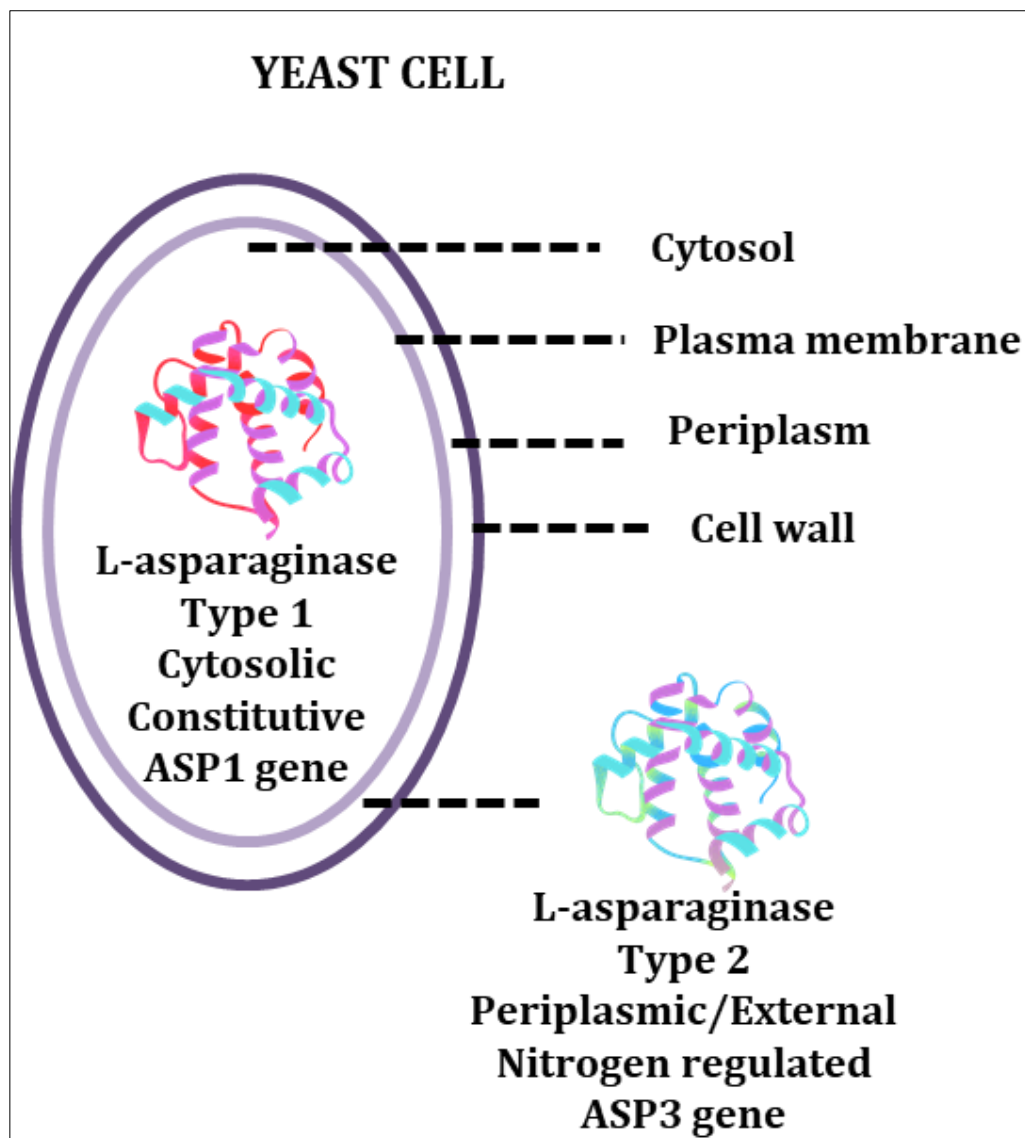


Figure 1.2: Schematic representation of Type I and Type II L-asparaginase in yeast cells. Type I L-asparaginase is localized intracellularly and functions primarily in cellular nitrogen metabolism. Type II L-asparaginase is periplasmic or secreted and is capable of extracellular L-asparagine depletion. The illustration was created using Canva based on published literature (Wriston and Yellin., 1973; Coral-Medina et al., 2022)

1.2.3.2 Structure of L-asparaginase

Elucidation of the structure of an enzyme aids in understanding its functional properties and enzymatic efficiency. L-asparaginase enzyme is reported to have a varied structural configuration comprising monomers, dimers, tetramers and hexamers depending on the source of the enzyme. The bacterial L-asparaginases including *E. coli* type II enzyme have common biochemical features, including the active site composition, quaternary structure and an active form as a homotetramer. The monomer unit contains 330 amino acid residues, a large N-terminal domain, a connecting chain of 20 amino acids and a smaller C-terminal domain. The active site lies between adjacent monomer domains consisting of ligand binding and flexible pocket with a conserved region containing nucleophilic Thr 15 residue (Loch and Jaskolski, 2021). Five key residues are essential for catalysis Thr12, Tyr25, Thr89, Asp90, and Lys162. These amino acids form the core catalytic machinery, while several additional residues contribute to substrate recognition and specificity (Lubkowski et al., 2020). The high degree of conservation of these catalytic residues across bacterial L-asparaginases underscores their fundamental role in enzyme function. L-asparaginase from *E. carotovora* consists of eight monomers arranged into two tetramers (ABCD and EFGH), each composed of four identical subunits that form a dimer-of-dimers structure. Each monomer is made up of 327 amino acids and contains 14 α -helices, eight β -strands, and the characteristic N- and C-terminal domains. The active site is located between adjacent monomers (A and C; B and D), where one monomer contributes key residues such as Thr15 and Thr95, while the neighboring monomer provides Ser254 (Batoool et al., 2016; Cachumba et al., 2016; Izadpanah Qeshmi et al., 2018)

1.2.4 Sources of L-asparaginase

Sources of obtaining L-asparaginase include plants, animals and microorganisms. Fast growing microorganisms, with their ability to utilize multiple substrates and form different products in bulk, make a desirable source of enzyme L-asparaginase. Known microbial producers of L-asparaginase bacteria, yeast and fungi are capable of synthesizing multiple forms of L-asparaginase both intracellular and extracellular with distinct biochemical properties. Fungal L-asparaginases are mainly secreted extracellularly, simplifying extraction and downstream purification. The presence of toxic substances having an impact on human health makes the use of certain fungal strains challenging. The unicellular microbes, bacteria and yeast are preferred as they are amenable during optimization of culture conditions to enhance enzyme production, introduction of genetic

modification, the downstream processing and product recovery (Batool et al., 2016; Ammulu et al., 2017; Brumano et al., 2019)

Promising L-asparaginase producers belonging to diverse microbial classes have been reported, including bacterial source *Pseudomonas fluorescens*, *Serratia marcescens*, *Escherichia coli*, *Erwinia carotovora*, *Proteus vulgaris*, actinobacterial source *Streptomyces karnatakensis*, *Streptomyces venezuelae* and yeast *Saccharomyces cerevisiae*, *Pichia polymorpha*, *Candida utilis*, *Issatchenkia orientalis* and *Rhodotorula glutinis* along with fungal genera such as *Aspergillus*, *Penicillium*, *Fusarium*, *Trichoderma*, *Mucor* *Rhizomucor* and *Cladosporium* (Cachumba et al., 2016; da Cunha et al., 2019; Chand et al., 2020; Muneer et al., 2020). Microbes isolated from extreme and unique environments can produce multiple bioactive compounds and enzymes, as these molecules provide protection and aid survival under challenging conditions. Isolates obtained from marine and endophytic environments produced L-asparaginase, including bacteria *Bacillus cereus* MAB5, *Pseudomonas aeruginosa* AVP17, *Enterobacter hormaeche*; actinobacteria *Nocardiopsis alba* NIOT-VKMA08, *Streptomyces albidoflavus* and fungi belonging to genera *Colletotrichum*, *Eupenicillium*, *Talaromyces*, *Alternaria*, *Chaetomium*, *Cladosporium*, *Colletotrichum*, *Curvularia*, *Nigrospora*, *Paecilomyces*, *Phaeotrichoconis*, *Phoma* and *Pithomyces* (Cachumba et al., 2016; Izadpanah Qeshmi et al., 2018)

The L-asparaginase approved for use in treatment of ALL was obtained from *E. coli* and *E. carotovora*. Commercially, the bacterial sources of enzyme are utilized for clinical purposes. Limitations of the enzyme of bacterial origin include the hypersensitivity reactions that result from prolonged use, associated immunological complications, antibody-mediated enzyme inactivation, allergic reactions and related disorders pertaining to the substantial glutaminase activity. Additional characteristics, including low stability and restricted pH tolerance, exhibited by the enzyme present constraints in application. An alternative enzyme of eukaryotic origin with fewer adverse effects is the focus of current research (Batool et al., 2016; Cachumba et al., 2016; Ammulu et al., 2017; Teixeira et al., 2017; Izadpanah Qeshmi et al., 2018; Brumano et al., 2019; da Cunha et al., 2019; Shakambari et al., 2019; Chand et al., 2020; Muneer et al., 2020; Fonseca et al., 2021)

Fungal L-asparaginase is widely employed in the food industry to prevent acrylamide formation during baking and frying of carbohydrate rich foods. L-asparaginases obtained from fungi belonging to genus *Aspergillus* are most commonly utilized for their Generally Recognized as Safe (GRAS) status. Commercial enzyme preparations include

PreventASe® (DSM) obtained from *A. niger* having optimal activity at acidic pH and temperature of 50 °C and Acrylaway® (Novozymes), based on a cloned enzyme of *A. oryzae* origin, exhibiting optimum at near neutral pH and temperatures of 60 °C, that are extensively applied in industrial food processing. The catalytic performance of the enzyme varies with the composition of the food. An ideal L-asparaginase for application in the food industry requires thermal stability, proteolytic resistance and is safe for consumption (da Cunha et al., 2019).

Table 1.2: L-asparaginase producing microbes obtained from multiple sources

Reference	Location	Sample Type	Isolate	Screening Method (Qualitative and quantitative)
(Kiranmayi et al., 2014)	Nizampatnam, Andhra Pradesh, India	Mangrove sediments	<i>Pseudonocardia endophytica</i> VUK-10	Rapid plate assay with PR and Nesslerization method
(Damare & Kajawadekar, 2020)	Goa	Mangrove detritus	<i>Peroneutypa</i> sp. GU-S, <i>Lecanicillium</i> sp. GU-G	Rapid plate assay with PR and Nesslerization method
(Shakambari et al., 2015)	Gulf of Mannar, Rameswaram, India	Marine water	<i>Pseudomonas plecoglossicida</i> RS-1	Rapid plate assay with PR and Nesslerization method
(Freire et al., 2021)	Antarctica	Marine sediment	<i>Leucosporidium muscorum</i>	Rapid plate assay with PR and L-aspartic acid β -hydroxamate (AHA) colorimetric method
(Moguel et al., 2022)	Antarctica	Marine sediments	<i>Leucosporidium scotti</i> L115	Rapid plate assay with PR and L-aspartic acid β -hydroxamate (AHA) colorimetric method
(Ashok et al., 2019)	Antarctic	Soil and Moss	<i>Trichosporon asahii</i> IBBLA1	Rapid plate assay with PR and BTB, and the Nesslerization method
(Ratuchne et al., 2023)	-	Oil mill and slaughterhouse fridge effluents	<i>Meyerozyma guilliermondii</i>	Rapid plate assay with PR and Nesslerization method
(Alrumman et al., 2019)	Red Sea of Saudi Arabia	Water samples	<i>Bacillus licheniformis</i>	Rapid plate assay with PR and Nesslerization method

(Darvishi et al., 2019)	German collection of microorganisms and cell cultures (DSMZ), Germany	-	<i>Yarrowia lipolytica</i>	Rapid plate assay with PR and Nesslerization method
(Thenmozhi et al., 2011)	Vellar estuary, Parangipettai, India	Mangrove rhizosphere sediment	<i>Bacillus cereus</i> MAB5	Rapid plate assay with PR and Nesslerization method
(Meena et al., 2015b)	Phoenix Bay in Port Blair, India	Marine sediments	<i>Streptomyces griseus</i> NIOT-VKMA29	Rapid plate assay with PR and Nesslerization method
(Doriya & Kumar, 2016).	Vizag, Kanyakumari and Kerala	Soil, agricultural residue	<i>Aspergillus sp.</i>	Rapid plate assay with PR and BTB, and the Nesslerization method
(Sisay et al., 2024)	Republic of Kenya	Soil	<i>Candida palmioleophila</i> JK12	Rapid plate assay with PR and Nesslerization method
(Meghavarnam & Janakiraman, 2017).	Karnataka, India	Soil	<i>Fusarium culmorum</i> (ASP-87)	Rapid plate assay with PR and BCP, and Nesslerization method
(Vala et al., 2018a)	Gulf of Khambhat, Gujarat, India	Marine	<i>Aspergillus niger</i> AKV-MKBU (<i>Avicennia marina</i> associated endophyte)	Rapid plate assay with PR and Nesslerization method
(Prihanto et al., 2019)	Aeng Sareh Beach, Madura Island, Indonesia	Mangrove stem	<i>Lysinibacillus fusiformis</i> B27 (<i>Rhizophora mucronata</i> associated endophyte)	Rapid plate assay with BTB and Nesslerization method
(Freitas et al., 2021)	Brazil	Savanna (Cerrado) soil	<i>Penicillium sizovae</i>	Rapid plate assay with PR and L-aspartic acid β -hydroxamate (AHA) colorimetric method

1.2.5 Applications of L-asparaginase as a chemotherapeutic agent

Amino acid depletion therapy is an effective way to treat cancers that obtain specific amino acids extracellularly. The tumour cells unable to synthesize required amino acids are susceptible to fluctuations in the available amino acids while the normal cells with functional biosynthetic pathways remain unharmed. L-asparagine is a non-essential amino acid with a notable role in protein synthesis, glycoprotein formation and ammonia detoxification. L-asparagine synthesis from aspartate in a normal cell is catalysed by L-asparagine synthetase and requires ATP. Some leukemic cells with insufficient L-asparagine synthetase enzyme depend on extracellular L-asparagine and L-asparagine depletion selectively impacts these cells while the normal cells remain unaffected.

Normal cells are able to synthesize L-asparagine, while the cancer cells lack this ability and depend on the circulating L-asparagine. For cancer treatment, L-asparaginase is introduced into the bloodstream, wherein it depletes the available L-asparagine levels in the serum leading to cancer cell death as the process of protein synthesis is hampered (Cachumba et al. 2016; Fonseca et al., 2021). This has led to L-asparaginase being applied in treatment of cancer, mainly in types of leukemia, lymphomas and sarcomas.

Leukemia malignancy of white blood cells is a frequently diagnosed cancer among children aged 2 to 4 and comparatively less observed in adolescents, including young adults. Acute lymphoblastic leukemia (ALL) is characterized by uncontrolled proliferation of lymphoid precursors with displacement of healthy hematopoietic cells that infiltrate organs such as the spleen, liver, and lymph nodes. Clinically, on the basis of prognostic indicators, ALL can be grouped into low, intermediate, and high-risk categories. Advanced therapies have aided in survival up to 5 years in 90% of the patients, though ALL is a major cause of childhood mortality. These treatments involve chemotherapy the most favoured, corticosteroids, radiation therapy and in selective cases, transplantation of stem cells or bone marrow. Implementation of multidrug therapies improved patient outcomes and research efforts were directed towards optimization of drug combinations to obtain therapeutic efficacy with negligible toxicity (Teixeira et al., 2017; Fonseca et al., 2021)

In a study conducted by Kidd (1953), it was observed that administration of guinea pig serum inhibited the growth of subcutaneous lymphosarcoma cells. Subsequently, Broome (1963) identified the serum factor responsible for this antileukemic activity as enzyme L-asparaginase. Consequent research efforts were directed toward isolating L-asparaginase

from microbial sources due to its ease in production, genetic manipulation and purification compared to plant and animal sources. The ease of medium optimization to enhance enzyme production, along with the ability of microbes to utilize diverse substrates, including various waste materials, further highlights the effectiveness of microbial sources. This led to clinical approval and utilization of bacterial L-asparaginase derived from *Escherichia coli* and *Erwinia chrysanthemi* in the therapeutic management of acute lymphoblastic leukemia (ALL).

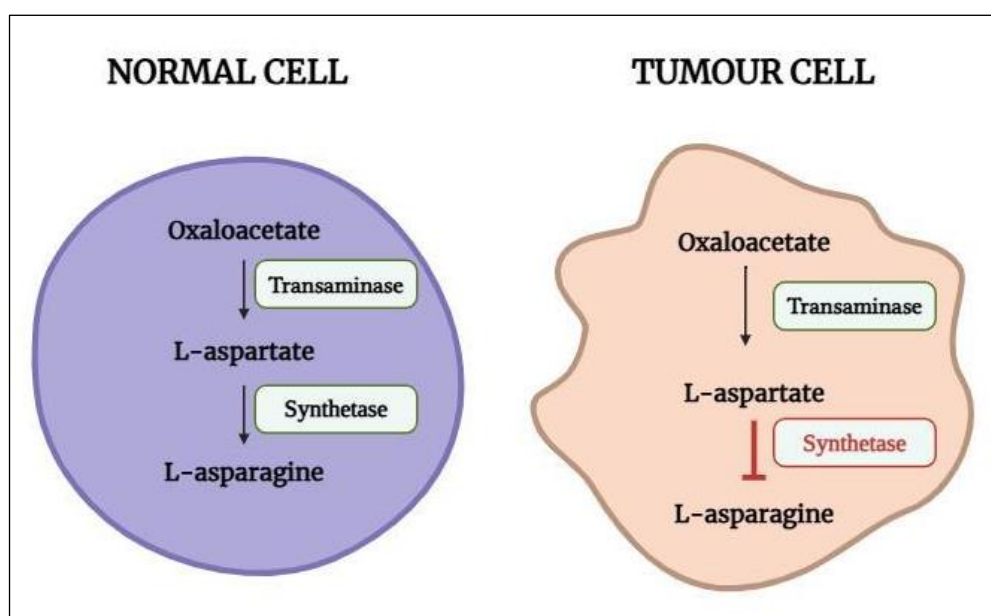


Figure 1.3: Selective cytotoxic effect of L-asparaginase based on cellular L-asparagine availability. Normal cells synthesize L-asparagine via asparagine synthetase, whereas tumour lymphoblasts depend on extracellular L-asparagine; enzymatic depletion inhibits protein synthesis and induces cell death. Created using BioRender.com and adapted from Batool et al. (2016) and Cachumba et al. (2016).

The antineoplastic activity of the enzyme L-asparaginase is the basis for its chemotherapeutic use. The enzyme is administered along with other drugs to enhance treatment effectiveness for cancers, including ALL, Hodgkin's lymphoma, and other types of leukaemia (Batool et al., 2016). The remission induction treatment of Acute Lymphoblastic Leukemia (ALL) and lymphoblastic lymphoma largely uses L-asparaginase as a chemotherapeutic agent. This treatment aims at reducing the cancer cells in the bloodstream using effective agents. L-asparaginase of prokaryotic origin, mainly *Escherichia coli* and *Erwinia chrysanthemi* are used in commercial treatment methods.

The microbial L-asparaginase preparations exist under several proprietary names, including *E. coli* L-asparaginase named Elspar®, Kidrolase®, Oncaspar® (PEGylated variant) and *E. chrysanthemi* L-asparaginase named Crisantaspase®, and Erwinase®/Erwinaze®. The enzyme preparation containing l-asparaginase obtained from *E. coli* has been used in treatment of ALL, leukemic meningitis and other lymphomas. The enzyme of *E. chrysanthemi* origin is used in combination with other agents or as an alternative to *E. coli* formulations upon hypersensitivity.

Resistance to L-asparaginase therapy has been shown to follow two distinct mechanisms. The first mechanism involves the elevated activity of the enzyme asparagine synthetase, supported by an observed increase in levels of the enzyme mRNA and protein. Silent antibodies produced by the patients showing no allergic symptoms acts as neutralizing antibodies, enhancing the drug clearance, affecting the L-asparaginase therapy. Other resistance mechanisms include the secretion of L-asparagine produced by bone-marrow mesenchymal cells in the leukemic microenvironment and enzyme degradation caused by proteases from leukemic cells (Cachumba et al. 2016; Teixeira et al., 2017).

L-asparaginase enzyme is a therapeutic enzyme with clinical success and prolonged use for treatment is limited by the harmful effects. The harmful effects exhibited include hepatotoxicity, pancreatitis, neurotoxicity, edema, fever, dermatological reactions, diabetes, leukopenia, bleeding disorders, and hypersensitivity ranging from mild allergy to anaphylaxis (Batool et al., 2016). The L-asparaginase, having glutaminase activity, resulted in multiple side effects due to low substrate specificity. Additional limitations include short half-life and formation of silent antibodies, resulting in rapid clearance of the enzyme and repeated injections of the formulations can lead to aggravated toxicity. The constraints associated with L-asparaginases emphasizes the need for a potential L-asparaginase with improved immunological compatibility and enhanced stability (Batool et al., 2016; Teixeira et al., 2017; Fonseca et al., 2021)

1.2.6 Applications of L-asparaginase In Food Industry

Processing of carbohydrate rich foods, including potatoes and cereals under high temperature conditions results in the formation of the potential carcinogen acrylamide by the Maillard reaction between reducing sugars and free asparagine. Detection of acrylamide is recurrent in thermally processed foods that include coffee, baked and fried products. Formation of acrylamide is dependent on multiple factors including pH, temperature, processing duration, moisture content, and sugar composition (da Cunha et al., 2019).

An effective methodology implemented in the depletion of asparagine levels is using L-asparaginase enzyme to obstruct acrylamide formation while maintaining the flavour profile of the food. L-asparaginase obtained from fungi (*A. niger*, *A. oryzae* and *Cladosporium* sp.) and bacteria (*B. licheniformis*, *B. subtilis*, *Paenibacillus barengoltzii* and *Thermococcus zilligii*) have shown reduction in acrylamide levels ranging from 34 to 97 % in multiple baked and fried products (Shi et al., 2017). Fungal L-Asparaginases (particularly from *Aspergillus* spp.) find application in the food industry. Their limited pH and thermal tolerance make their use challenging as food processing requires high temperature conditions (Cachumba et al. 2016; da Cunha et al., 2019).

1.2.7 Other applications of L-asparaginase

Additional applications of the enzyme are in detection of the asparagine levels in biological fluids and food products. The conventional methods used include chromatography and XPS-based spectroscopy, although accurate are costly, laborious and require expertise (Batoool et al., 2016; Cachumba et al. 2016). An advanced approach using biosensors containing immobilized L-asparaginase provides a rapid and cost-effective method. It detects the ammonium production during asparagine hydrolysis as fluorescence signals or pH-dependent colorimetric shifts (Batoool et al., 2016; da Cunha et al., 2019).

1.2.8 Optimization and characterization of L-asparaginase

Optimization strategies are aimed at process improvement, supported by the development of dedicated algorithms and software that further enhance their applicability and practicality. The purpose of any bioprocess is to implement working conditions that yield the maximum product concentration. Environmental and nutritional conditions are altered during the optimization studies on microbial systems to establish the major influential factors and experimentally validate their role in the enhancement of the output (Ashok & Kumar, 2017).

Multiple optimization methodologies can be utilized, from a relatively simple One factor at a time (OFAT) approach to complex multivariate statistical designs, including Central composite design (CCD) and Plackett-Burman design (PBD) each having a distinct purpose and system. The OFAT approach, though simple, is laborious, expensive, time intensive and incomplete to an extent, as it is unable to account for interactions among the variables.

The methods based on statistical analysis aid in defining experimental objectives, identifying the key variables, designing an appropriate optimization strategy, minimizing experimental error and saving time and cost. Optimization results in improvement of

productivity and product quality by efficiently identifying the suitable operating conditions that should be robust, reliable, practical and streamlined (Ashok & Kumar, 2017). Medium and process optimization is essential to enhance enzyme production to a level substantial enough for industrial use. The factors that influence microbial metabolism and the associated pathways for enzyme production and release include carbon and nitrogen source concentration, pH, temperature, incubation period, inoculum size and salinity. Optimization of these parameters aids in the enhancement of L-asparaginase production. Enzyme production by a microorganism can be improved by systematically evaluating different nutritional components, their concentration in the medium and the pertinent physicochemical parameters, subsequently implementing the conditions having a significant and positive impact.

An affordable enzyme production process, including the utilization of inexpensive and nutrient-rich substrates, is beneficial for scaling up the process (Freire et al. 2021; Moguel et al. 2022). Enzyme production in yeast is influenced by the type and concentration of carbon and nitrogen sources incorporated in the fermentation medium.

In yeast, the pathways involved in carbohydrate utilization follow a similar pattern, with notable biochemical and genetic variations among species. Catabolite repression leads to the selective expression of genes responsible for sugar assimilation, making choice of carbon source a regulatory factor affecting enzyme production. Enzymes involved in amino acid assimilation and conversion including proteases, amidases and ureases are modulated by nitrogen catabolite repression, characterized by the preferential utilization of readily assimilable nitrogen sources among those available (Moguel et al. 2022).

The critical medium components influencing enzyme synthesis during fermentation are carbon and nitrogen sources (Cachumba et al., 2016). Glucose is the most common carbon source (Doriya & Kumar, 2016) and excessive carbon concentration may lead to suppression of L-asparaginase due to catabolite repression. Nitrogen sources such as L-asparagine and L-proline are potent inducers (Sarquis et al., 2004), while urea, yeast extract, and peptone can also enhance yields (Baskar & Renganathan, 2010).

The biochemical and kinetic properties of enzyme are dependent on the source of the enzyme. Extracellular L-asparaginase is advantageous because it accumulates directly in the culture broth, facilitating simplified recovery, enabling efficient and cost-effective downstream processing (Meena et al., 2015; de Castro Girao et al. 2016; El-Naggar et al., 2016).

Protein purification is an essential step following the enhanced production of an enzyme. Protein purification aids the determination of optimal parameters to ensure maximum enzyme action including the enzyme stability and specificity. Protein free from contaminants is more effective and safer as a chemotherapeutic agent. Enzyme free from L-glutaminase activity is preferred to reduce the deleterious effects.

Purification strategies predominantly involve salting out by ammonium sulphate precipitation followed by multiple chromatography techniques including size exclusion (Sephadex) and ion exchange (DEAE). Ammonium sulfate precipitation concentrates the protein and removes impurities, followed by dialysis to eliminate excess salts. Another approach involves precipitation of proteins using solvents including ethanol, methanol and acetone. Further purification by chromatographic separation using Gel-filtration matrices such as Sephadex G-100 to isolate fractions based on molecular size followed by enzymatic assays to verify the protein of interest is obtained. These are then pooled and subjected to ion-exchange chromatography using CM-Sephadex C-50 to achieve charge based protein separation on elution with a sodium chloride gradient. These most commonly implemented classical approaches for protein purification can be time-consuming and may result in moderate yields (Muneer et al., 2020; Chand et al., 2020).

Modern purification strategies, including thermo-separating aqueous two-phase systems, have improved the extraction of intracellular L-asparaginase, producing higher purity, activity and in combination with high-pressure homogenization, these systems further enhance enzyme recovery. Recombinant expression technologies have also contributed to more efficient purification. Periplasmic L-asparaginase expressed in *Pichia pastoris* can be released by freeze-thaw treatment, ethanol permeabilization or alkaline extraction with or without cysteine supplementation (Muneer et al., 2020; Chand et al., 2020).

Purified enzymes are routinely evaluated by native PAGE for homogeneity and by SDS-PAGE to confirm subunit composition and molecular weight. L-asparaginases vary noticeably in molecular mass and quaternary structure. *Serratia* species produce pentameric or hexameric enzymes (170–180 kDa), while *E. coli* L-asparaginase is a homotetramer of four subunits of approximately 38 kDa. In *Pseudomonas* species, both monomeric forms (~160 kDa) and smaller subunits (33–34 kDa) have been reported (Muneer et al., 2020; Chand et al., 2020).

There is notable variation in the biochemical properties of microbial L-asparaginases. Optimal pH values range from slightly acidic to strongly alkaline (pH 5–10), depending on the organism. While *E. coli* L-asparaginase exhibits maximal activity near pH 6, many

bacterial and fungal enzymes prefer alkaline conditions; for example, *Aspergillus niger* AK10 reaches optimum activity at pH 9, and alkaliphilic *Streptomyces fradiae* NEAE-82 at pH 8.5. Temperature optima usually fall between 25 and 45 °C, although thermophilic archaea such as *T. kodakarensis* and *T. gammatolerans* produce highly thermostable enzymes with optimum activity around 85 °C. Metal ions and inhibitors strongly influence catalytic behavior, with divalent ions often acting as inhibitors, while monovalent ions or reducing agents may enhance activity. Kinetic parameters including Michaelis constant (K_m) and maximum velocity (V_{max}) vary widely among species, reflecting differences in substrate affinity and catalytic efficiency (Muneer et al., 2020; Chand et al., 2020).

Table 1.3: Optimization strategies for L-asparaginase enzyme production

Organism	Methodology	Optimized conditions (pH, temperature, time, agitation, medium composition)	Initial yield	Final yield
<i>Bacillus licheniformis</i> (Alrumman et al., 2019)	OFAT	pH 6.5, 37 °C, 72 h, 250 rpm, glucose and (NH ₄) ₂ SO ₄	8.1 U/mL	7.78 U/mg
<i>Bacillus cereus</i> MAb5 (Thenmozhi et al., 2011)	RSM CCD	soyabean meal (6.282 8 g/L), L-asparagine (5.5 g/L), wood chips (1.383 8 g/L) and NaCl (4.535 4 g/L).		51.54 U/mL
<i>Erwinia carotovora</i> EC-113 (Maladkar et al., 1993)	OFAT	Lactose, monosodium glutamate, corn steep liquor, tryptone and yeast extract, L-asparagine (0.2%)		
<i>Escherichia coli</i> ATCC 11303 (Kenari et al., 2011)	RSM	1.08% lactose, 1.79% tryptone, 1.6% yeast extract, 2% KH ₂ PO ₄ and 0.19% l-asparagine.	0.1 U/mL	1.03 U/mL
<i>Pseudomonas plecoglossicida</i> RS1 (Shakambari et al., 2015)	OFAT, PBD, CCD	pH 6.5, 37°C, M-9 medium containing KH ₂ PO ₄ (0.2 g % w/v), effluent (0.8 mL% v/v)	0.87 IU/mL	3.25 IU/mL
<i>Pseudomonas oryzae</i> habitans (Bhagat et al., 2016)	OFAT	pH 7.0, 30 °C, M-10 medium with 0.75% peptone, glucose and asparagine	2.1U/mL	4.3 U/mL
<i>Nocardiosis alba</i> NIOT-VKMA08 (Meena et al., 2015a)	OFAT	NIOT-ASP medium- 1.5% maltose, 2 % yeast extract, 1.5 % l-asparagine	6.73 IU/mL	18.47 IU/mL
<i>Streptomyces brodiae</i> NEAE-115 (El-Naggar et al., 2019)	PBD, FCCD	pH 7, 30 °C, 7 days, 150 rpm, Dextrose 2 g, starch 20 g, L-asparagine 10 g, KNO ₃ 1 g, K ₂ HPO ₄ 1 g, MgSO ₄ ·7H ₂ O 0.5 g, NaCl 0.1 g	6.60 U/mL	162.11 U/mL
<i>Streptomyces koyangensis</i> SK4 (Saleena et al., 2023)	PBD	pH 7.5, 20°C, 7day, 125 rpm, asparagine dextrose broth containing L-asparagine 7.5 g/L.	23.12 IU/ml	136 IU/ml
<i>Streptomyces ginsengisoli</i> (Deshpande et al., 2014)	OFAT	pH 8, 30 °C, 5 days, ADS broth containing glucose, peptone, 0.05 g% asparagine	2.52 μ mol/mL	3.23 μ mol/mL
<i>Streptomyces griseus</i> NIOT-VKMA29 (Meena et al., 2015b)	OFAT, BBD	pH 8.04, 35 °C, 144 h, yeast extract (2.04%), starch (1.48%), L-asparagine (1.5%)	5.36 IU/mL	56.78 IU/mL
<i>Streptomyces rochei</i> (El-	PBD and CCD	3 g/L yeast extract, 2.6 g/L dextrose and pH 6.7	44.821	175.96

Naggar & El-Shweihy, 2020)	RSM		U/gds	U/gds
<i>Pseudonocardia endophytica</i> VUK-10 (Kiranmayi et al., 2014)	OFAT	Asparagine glucose salts broth containing 1% maltose and 1% L-asparagine, pH 8.0, 30°C, 4 days, 120 rpm.	3.96 IU/ml	7.42 IU/ml
<i>Penicillium cyclopium</i> (El-Refai et al., 2014)	OFAT, PBD, RSM-CCD	composition (g/l) sucrose, 1; Lasparagine, 0.5; KH ₂ PO ₄ .7H ₂ O, 0.75 and MgSO ₄ . 7H ₂ O, 0.72; KCl, 0.72; (NH ₄) ₂ SO ₄ , 11.7 and time 3 days , 40°C and pH 8	68 U/mL	160 IU m/L
<i>Aspergillus terreus</i> MTCC 1782 (Baskar & Renganathan, 2009; Baskar & Renganathan, 2012)	ANN-GA	L-proline (1.7%), NaNO ₃ (1.99%), L-asparagine (1.38%), glucose (0.65%)	34.98 IU/mL	40.86 IU/mL
<i>Aspergillus oryzae</i> CCT 3940 (Dias & Sato., 2016)	PBD, RSM-CCD	Modified Czapeck medium: proline (2%), glucose (0.5%), L-asparagine inducer (0.2%), yeast extract (0.5%), pH8, 72h 150rpm, 30 °C.	19.1 U/mL	67.49 U/mL
<i>Aspergillus niger</i> AKV-MKBU (Vala et al., 2018a)	PBD, RSM-CCD	L-asparagine (14g/L), CuNO ₃ .3H ₂ O (0.015 g/L), ZnSO ₄ .7H ₂ O (0.012 g/L), FeSO ₄ .7H ₂ O (0.007 g/L)	7.5642 U/mL	13.1252 U/mL
<i>Fusarium culmorum</i> (ASP-87) (Meghavarnam & Janakiraman, 2017).	OFAT	pH 7.0, 30 °C, 6 days, glucose (0.1%), ammonium chloride (0.1%), 3 mm of particle size of the substrate Soybean meal and wheat bran (1:1)	7.21 U/gds	18.91 U/gds
<i>Candida palmioleophila</i> JK12 (Sisay et al., 2024)	OFAT	25°C, 96 h, 100rpm, Fructose, yeast extract	34.04 U/mL	67.6 U/mL
<i>Meyerozyma guilliermondii</i> (Ratuchne et al., 2023)	CCRD	pH 4.0, 28°C, 75 h, 150 rpm, 1.0% L-asparagine, 0.5% glucose	2.32 U/mL	24.75 U/mL
<i>Lysinibacillus fusiformis</i> B27 (Prihanto et al., 2019)	L16 (Taguchi's model)	pH 6, 35°C, 24 h, M9 medium with 2% L-asparagine	3.18 U/ml	8.51 U/ml

OFAT- One Factor at a Time, PBD- Plackett–Burman Design, RSM- Response Surface Methodology, CCD- Central Composite Design, CCRD- Central Composite Rotatable Design, BBD- Box-Behnken Design, FCCD- Face-centred Central Composite Design, ANN- Artificial Neural Network, GA- Genetic Algorithm

Table 1.4: Purification strategies, yields and biochemical properties of L-asparaginase obtained from microbial sources

Organism	Methodology	Characteristics of the enzyme (pH, Temperature, Km, Vmax, Molecular weight, Purification fold, Specific activity)
<i>Pseudomonas oryzae</i> (Bhagat et al., 2016)	Ultrafiltration, desalting with Milli Q, SP Sephadex (Amicon) column	8.0, 37–40 °C, 10.0 mM, $0.92 \times 10^2 \mu\text{mol}^{-1} \text{mg protein}^{-1}$, 54 and 50 kDa, $4239 \mu\text{mol min}^{-1} \text{mg protein}^{-1}$
<i>Erwinia carotovora</i> , EC-113 (Maladkar et al., 1993)	DEAE cellulose chromatography, sephadex G-200 gel filtration, hydroxylapatite ion-exchange and affinity chromatography on sepharose CL-6B.	8.0, 50°C, $1.8 \times 10^{-5} \text{ M}$, 30
<i>Escherichia coli</i> (Borah et al., 2012)	Ammonium sulphate precipitation (60%), DEAE-Sepharose column	6.0, 55°C, 153kDa, 0.039 U/ml/min
<i>Bacillus licheniformis</i> (Alrumman et al., 2019)	Ammonium sulphate precipitation (70%), Sephadex G-100 column	7.5, 37°C, 0.049995 M, 45.45 $\mu\text{mol/ml/min}$, 37 kDa, 4.63, 36.08 U/mg
<i>Pseudomonas plecoglossicida</i> RS1 (Shakambari et al., 2015)	Ammonium sulfate precipitation (80%), DEAE Sepharose ion exchange chromatography	8.5, 55°C, $2.25 \pm 0.61 \text{ mM}$, $8.9 \pm 0.81 \text{ IU/mL/min}$, 46 kDa, 4.36, $9.43 \pm 0.40 \text{ IU/mg}$
Recombinant <i>Escherichia coli</i> (Jiao et al., 2020)	Affinity chromatography Ni-NTA column	8.0, 40°C, 3.22 mmol L^{-1} , $1.55 \text{ IU } \mu\text{g}^{-1}$, 35 kDa, 11.4, 400 IU mg^{-1}
Recombinant <i>Escherichia coli</i> (Sudhir et al., 2020)	Affinity chromatography Ni-NTA column	37 °C, 0.42 mM, 2778.9 $\mu\text{mol/min}$, ~37 kDa, 597.7 IU/mg
<i>Streptomyces brodiae</i> NEAE-115 (El-Naggar et al., 2018)	ammonium sulphate precipitation, ion-exchange chromatography DEAE-Sepharose CL-6B	8.5, 37 °C, $2.139 \times 10^{-3} \text{ M}$, $152.6 \text{ U mL}^{-1} \text{ min}^{-1}$, 67 kDa, 7.835, 76.671 U/mg
<i>Nocardiopsis alba</i> NIOT-VKMA08 (Meena et al., 2015)	Ammonium sulphate precipitation (45%), ion exchange chromatography Q-Sepharose	8.0, 37 °C, 0.127 mM, 5.50 $\text{U } \mu\text{g}^{-1}$, 58.44, 93.28 IU/mg

	FPLC	
<i>Streptomyces rochei</i> (El-Naggar & El-Shweihy, 2020)	ammonium sulphate precipitation, ion-exchange chromatography DEAE-Sepharose CL-6B	64 kD, 16.18, 13.57 U/mg
<i>Aspergillus fumigatus</i> WL002 (Dutta et al., 2015)	Ultrafiltration (30 kDa), Ammonium sulfate precipitation (60%), DEAE-Sepharose column, Sephadex G-100	9.5, 50 °C, 7.02×10^{-3} M, 355.3 μ mol/ mg. min 35 kDa, 355.03IU/mg, 232.04.
<i>Cladosporium</i> sp. (Kumar & Manonmani, 2013)	Methanol precipitation, DEAE-cellulose column, Sepharose 6B	pH 6.3, 30 °C, 0.1M, 4.44 μ mol/ mL.min, 121 kDa, 83.3 IU/mL, 867.7.
<i>Aspergillus oryzae</i> CCT 3940 (Dias et al., 2016)	Ammonium sulphate precipitation (80%), dialysis, Q Sepharose Fast Flow, SP Sepharose Fast Flow, CM Sepharose Fast Flow	pH 8, 50 °C, 0.66×10^{-3} M, 313 IU/mL, 115 kDa , 282 IU/mg, 28.6
<i>Aspergillus niger</i> AKV-MKBU (Vala et al., 2018b)	Ethanol precipitation, DEAE cellulose	pH 7, 30 °C, 0.8141mM, 6.228 μ mol/ mg, 90 kDa ,46.75 IU/mg, 10.36
<i>Trichoderma viride</i> (Lincoln et al., 2015)	Acetone precipitation, DEAE-cellulose column	pH 7, 37 °C, 2.56 μ M, 279.27 U/mL, 99 ± 1 kDa ,78.2 IU/mg, 13.0.
<i>Rhodosporidium toruloides</i> CBS14 (Ramakrishnan & Joseph, 1996).	concentrated by keeping dialysis bag with crude enzyme buried in dry Sephadex G-200 over night, loaded onto a PBE94 ion exchanger (20 x 0.8 cm)	6.35, 37°C, 1.43×10^{-3} M, 133 μ mol/min/ mg protein, 87 kDa,205, 10.508 U/mg protein
<i>Saccharomyces cerevisiae</i> X2180-1A (L-asparaginase type 2) (Dunlop et al., 1978)	heat and pH treatment, ethanol fractionation, ammonium sulphate fractionation, Sephadex G-25 chromatography and DEAE-cellulose chromatography	7, 35 °C, 35×10^{-4} M, 57.8 μ mol/min/ml, 250, 30.5 U/mg
<i>Saccharomyces cerevisiae</i> X2180-1A (L-asparaginase type 1) (Dunlop et al., 1978)	Protamine sulphate treatment, ammonium sulphate fractionation, gel permeation chromatography, and DEAE-cellulose chromatography.	8, 35 °C, 7.4×10^{-4} M, 0.6 μ mol/min/ml, 81, 5.4 U/mg

Table 1.5: Studies on recombinant expression of L-asparaginase enzyme

Reference	Gene of interest	Gene source	Expression host	Enzyme activity
(de Castro Girão et al., 2016)	ASP3	<i>Saccharomyces cerevisiae</i>	<i>Pichia pastoris</i>	204.4 IU/mg
(Lopes et al., 2019)	ASP3	<i>Saccharomyces cerevisiae</i>	<i>Escherichia coli</i>	225.6 IU/g cells
(Santos et al., 2017)	ASP1	<i>Saccharomyces cerevisiae</i>	<i>Escherichia coli</i>	110 IU/mg
(Aktar et al., 2023)	ASP1	<i>Lachancea thermotolerans</i>	<i>Escherichia coli</i>	313.82 U/mg
(Biasoto et al., 2023)	pJAG-PHO-ASP3	<i>Saccharomyces cerevisiae</i>	<i>Pichia pastoris</i> Glycoswitch pep4- sub2-	218.2 IU/mg
(Jiao et al., 2020)	AsAsnase	<i>Acinetobacter soli</i>	<i>Escherichia coli</i> BL21 (DE3)	400 IU/mg
(Sudhir et al., 2020)	BliA (ansA3)	<i>Bacillus licheniformis</i> (MTCC 429)	<i>Escherichia coli</i> BL21 (DE3)	597.7 IU/mg
(Freitas et al., 2022)	pPsASNase	<i>Penicillium sizovae</i>	<i>Komagataella phaffii</i> X-33	3.05 U/g _{cell}
(Eisele et al., 2011)	FvNase	<i>Flammulina velutipes</i>	<i>E. coli</i> BL21 (DE3)	16 U/mL

1.2.9 From Bacteria to Eukaryotes: Advancing L-asparaginase Research

A limitation associated with the therapeutic proteins is the immunogenicity exhibited by induction anti-drug antibodies (ADA). The clinical efficacy of these proteins is negatively impacted by the neutralizing antibodies that bind the functional sites and result in a need for dosage increase. Non-neutralizing antibodies cause rapid drug clearance by forming an immune complex. Commercial *E. coli* L-asparaginase used in the treatment of ALL induces immunogenicity because of its microbial origin and relatively large size. These antibodies cause silent inactivation by diminishing the enzymatic efficiency while no outward allergic symptoms appear. L-asparaginase applied during treatment can undergo cleavage by lysosomal proteases in both normal and tumor cells. This results in enhanced antigen processing and immune activation.

The hypersensitivity reactions leading to treatment termination include urticaria to anaphylaxis, bronchospasm and serum sickness. Multiple strategies have been explored to minimize immunogenicity. One of the approaches explored included PEGylation by covalently attaching polyethylene glycol (PEG) to the enzyme reducing epitope exposure. PEGylated enzyme has reportedly resulted in resistance, hepatotoxicity and occasional silent inactivation, particularly in older patients. The other solution implemented was incorporating L-asparaginase obtained from *Erwinia chrysanthemi* in treatment. Other methods to overcome this constraint include co-administration of corticosteroids that can reduce immune reaction and using protein engineering tools to deimmunize through epitope mapping and targeted mutagenesis (Batoool et al. 2016; Cachumba et al. 2016; Fonseca et al., 2021).

The secondary L-glutaminase activity of commercial L-asparaginase is a significant functional limitation of the enzyme. Its lack of specificity causes biochemical changes that have both negative and positive effects. Leukemic cells lacking the enzyme asparagine synthetase are mainly affected by the depletion of L-asparagine due to L-asparaginase treatment, ultimately leading to cell death. Conversely, in leukemic cells that express asparagine synthetase, the depletion of L-glutamine caused by the enzyme's co-activity enhances cytotoxicity by disrupting protein synthesis and limiting L-asparagine production mediated by asparagine synthetase. Normal cells need amino acid L-glutamine because it plays a crucial role in various biochemical pathways, such as nitrogen transport and protein biosynthesis.

The reduction of L-glutamine levels caused by L-asparaginase's L-glutaminase activity has been linked to side effects including hepatotoxicity, pancreatitis, hyperammonemia,

neurotoxicity, hyperglycemia, leukopenia, and coagulation disorders like thrombosis and hemorrhage. It has been reported that reducing the L-glutaminase activity of L-asparaginase retains its anticancer potential and improves therapeutic application with less toxicity (Batool et al. 2016; Cachumba et al. 2016; Fonseca et al., 2021).

The adverse effects of the enzyme of bacterial origin are acute allergic reactions, hypersensitivity, and immune inactivation. The efficiency of L-asparaginase as a chemotherapeutic agent diminishes with prolonged exposure during treatment, as circulating antibodies cause the clearance of the enzyme, resulting in the development of silent immunity (Cachumba et al., 2016; Teixeira et al., 2017). L-asparaginase of eukaryotic origin can help to avoid the undesirable effects of a prokaryotic enzyme as an anti-neoplastic agent.

The primary focus of current research is to explore an alternative, preferably eukaryotic, source of enzyme L-asparaginase that exhibits anti-tumor activity with the least detrimental effects (Batool et al. 2016; Cachumba et al. 2016).

CHAPTER II

**ISOLATION OF YEASTS FROM MANGROVE
SEDIMENTS OF GOA**

2.1 Introduction

Tropical mangrove forests are active and transforming. The presence of ever-changing environmental conditions and organic richness aids to the survival of diverse microbial communities. The sediments, in particular, support the sustenance of varied types of microorganisms due to their abundance in organic carbon and mineral nutrients. Mangrove sediments from India exhibit pH values ranging from 6 to 8, with varying salinity influenced by changing seasons and tides, that range between 5 and 30 psu. Nazareth and Gonsalves (2022) reported that the mangrove sediments of Goan mangroves have near neutral pH values throughout the year, with temperatures varying from 26 °C to 30 °C. These physical parameters of the sediments are conducive to the growth and maintenance of a wide range of microbes. Mangrove sediments are a repository of multiple groups of microorganisms, including yeasts. The nutrient cycling fuelled by these microbes results in detritus generation from plant wastes and, in turn, helps in maintaining healthy vegetation cover. The metabolic interactions between microorganisms present in the sediments contribute to the nutrient availability and species richness.

Aquatic, marine, atmospheric and terrestrial are the diverse ecosystem that yeasts inhabit, as they are largely ubiquitous and a few yeast species are restricted to distinct environments. In an ecosystem, yeasts often coexist with other types of microbes, comprising bacteria and fungi. This results in a need for use of selective techniques consisting of media that aid yeast proliferation and obstruct fungi and bacteria. Yeasts can sustain at conditions of pH and water activity that bacteria cannot and the media specific for yeasts utilizes this ability of yeasts. Supplementing media with antimicrobial agents aids in preventing fungal or bacterial contamination (Kurtzman et al., 2011).

Yeasts, being mesophiles, can grow at an incubation temperature in the range of 20 to 25°C. The incubation temperatures vary in association with the environments the yeasts are obtained. The dependency of parameters, including incubation temperature and pH of the medium, on the source and related factors during the isolation assists selective isolation of yeasts. The selectivity of the medium is enhanced by incorporating antimicrobial agents to prevent the growth of microbes other than yeast. Antibiotics are used individually or in combination to suppress bacterial growth, including tetracycline (50 mg/L), chloramphenicol (100-300 mg/L) and penicillin G, along with streptomycin sulphate (150-

500 mg/L). Antifungal agents are used cautiously to avoid deleterious effects on growth of yeast. Cyclosporin A (4-10 mg/L), cycloheximide (100-500 mg/L) and pimaricin (5-100 mg/L) are used to inhibit filamentous fungi (Kurtzman et al., 2011).

Constituents of the mangrove ecosystem, including the plants and their parts, sediments, water and invertebrates, are a reservoir of yeast. The isolation of yeast from mangrove ecosystems comprises the utilization of both regular and advanced methods. Advanced methods are necessary to obtain yeasts from mangrove forests, including rare species and those that tend to grow slowly. Multiple sample collection and processing methods are available in the literature that are based on the techniques used in isolation of marine yeast.

The isolation of marine yeasts is influenced by two factors: the preferred traits of the isolates and the anticipated yeast cell density in the collected sample. To resolve these limitations, the samples are enriched before isolation, ensuring yeast isolation with preferred traits. In the enrichment step, a known amount of the sample is suspended in a liquid medium for several days and then serially diluted before plating. Regular fungal growth medium and modified medium supplemented with an appropriate antimicrobial agent have been used in multiple studies reported (Zaky et al., 2014). These media composition and other parameters are summarized in Table 2.1. Distinct colonies from the media plates are initially collected and purified using streaking methods. Morphological characterization following isolation helps in confirming and distinguishing the yeast isolates. It includes microscopy imaging of stained cells and recording the colony morphology of isolates grown on agar. The obtained yeasts are maintained on a medium similar to that used for isolation, lacking antimicrobial agents (Zaky et al., 2014).

Table 2.1 Diverse media used for yeast isolation

Medium	Composition (w/v) and other parameters	References
Sabouraud's dextrose agar	4% dextrose, 1% peptone, 1.5% agar, 50% seawater pH 5.6, 35 °C, 48 h.	(Kumar et al., 2011)
Yeast extract Peptone, Dextrose Agar	glucose 2%, peptone 2%, yeast extract 1%, agar 2.5%, seawater, chloramphenicol (0.05%), 25 °C, 96 h	(Buzdar et al., 2011)
Czapek Dox basal medium	3% sucrose, 0.3% NaNO ₃ , 0.1% K ₂ HPO ₄ , 0.05% MgSO ₄ ·7H ₂ O, 0.05% KCl, 1.5% NaCl, traces of FeSO ₄ , ZnSO ₄ ·7H ₂ O, Co(NO ₃) ₂ ·6H ₂ O, Penicillin (400 U/mL) and streptomycin (1mg/L), 28 °C, 72 h	(Khot et al., 2012)
Wickerham's medium	1.0% glucose, 0.3% yeast extract, 0.3% malt extract, 0.5% peptone, and 2.0% agar, seawater, chloramphenicol (200 mg/L), pH 7, 28 °C, 120 h	(Kachiprath et al., 2019)
Nitrogen depleted medium	2.0% glucose, 0.085% KH ₂ PO ₄ , 0.015% K ₂ HPO ₄ ·3H ₂ O, 0.05% MgSO ₄ ·7H ₂ O, 0.01% NaCl, 0.01% CaCl ₂ ·6H ₂ O, traces of H ₃ BO ₃ , CuSO ₄ ·5H ₂ O, KI, FeCl ₃ ·3H ₂ O, MnSO ₄ ·H ₂ O, Na ₂ MoO ₄ ·2H ₂ O, ZnSO ₄ ·7H ₂ O, chloramphenicol (100mg/L), pH 5.5, 30°C, 48 h	(Hoonde et al., 2019)

2.2 Methodology

Isolation of yeasts from sediment samples collected from nine different mangrove forests across the state of Goa was carried out.

2.2.1 Sampling

The samples were collected from nine different mangroves across the state of Goa as listed in Table 2.2. Sampling was done in two sets in February 2020 and February 2021. Mangrove sediment samples were collected in sterile bags and were promptly processed in the laboratory. The physical parameters of the samples as seen in Table No 2.3, including pH, temperature and salinity, were determined after appropriate treatment. 10 g of the sediment was dissolved in 90 mL of water for 2 h at 120 rpm under room temperature conditions. This mixture was used to determine the pH using a pH meter and salinity using a salinometer of the sample. The temperature of the sediment sample was recorded during sampling using a portable thermometer. The collected sediment sample was added to culture broth for enrichment (Khot et al., 2012; Nazareth and Gonsalves, 2022).

2.2.2 Isolation

Mangrove sediment samples collected were subjected to enrichment. 1 g of sediment sample was inoculated in 150 mL culture broth containing chloramphenicol (200 mg/L) and incubated at 28°C 120 rpm for 96 h. Regular fungal media including Czapek Dox Broth (CDB), Sabouraud's Dextrose Broth (SDB), Potato Dextrose Broth (PDB) and Yeast malt broth (YMB) were used in yeast isolation. On completion of the incubation period the enriched sample was serially diluted using physiological saline (0.85%) up to 10^{-8} . 0.1 mL of the diluted broth was plated using a spread plate method on respective fungal media containing chloramphenicol (200 mg/L) and incubated at 28°C for 48 h. From the colonies obtained, distinct colonies were selected and purified using streaking methods. They were maintained on YMA at 4 °C. The pure cultures were then named based on sample code indicating the location, sampling site (mangroves) and sample type (sediment) (Kurtzman et al., 2011; Khot et al., 2012; Chang et al., 2018; Hoonde et al., 2019).

2.2.3 Morphological Characterisation

The yeast isolates grown on agar plate were observed and images were compiled to study colony morphology. The cells of the yeast isolates were visualized using microscopy techniques aided by staining.

2.2.3.1 Determining the colony characters of the isolates

The yeast isolates were grown on YMA at 28°C for 48 h. The colony morphology of the isolates was described in the terms of texture, colour, surface, shape, margin and elevation. (Kurtzman et al., 2011)

2.2.3.2 Monochrome staining

Monochrome staining of the isolates using crystal violet was carried out. Crystal violet, a basic dye with positive charge binds to the negatively charged components on yeast cell surface and enhances the visualization of cell morphology. Yeast cells were suspended in sterile saline and this suspension was used to prepare a smear on a clean and dry glass slide. The smear was dried and heat fixed prior to staining. The smear was stained using crystal violet followed by washing of the slide with distilled water to remove excess stain. Stained cells were observed under an oil emulsion (100×) objective. Microscopy images of the isolates were recorded.

2.2.3.3 Lacto-phenol cotton blue staining

The isolates were stained with Lacto phenol cotton blue. The phenol component of the stain acts as a fungicide while the lactic acid maintains cell structure and the cotton blue dye stains the chitin present in the fungal cell wall. A clear visual of the fungal cell morphology is obtained using this method. A cell suspension of the yeast isolate was prepared in sterile saline. Lacto phenol cotton blue stain was added to the drop of the suspension placed on a clear dry glass slide. The wet mount was covered with coverslip and observed under a microscope at 40× power. Microscopy images of the isolates were compiled.

2.3 Results

2.3.1 Sampling

A total of nine mangroves were chosen for sampling from across the state of Goa. The details of the sampling locations are included in Table 2.2 and Figure 2.1. Sampling sites were mainly associated with Zuari river (six sites), while two mangroves sampled were from regions with Mandovi river and Madakai mangroves interacting with waters from both the rivers. The selected locations belonged to regions with dynamic physicochemical conditions and anthropogenic activities, indicating possibility of inhabitation by robust microbes able to sustain such conditions. As seen in Table 2.3 the temperature of the sediment measured during sampling ranged from 29°C to 32°C while the salinity remained constant at 2 psu. The pH of the sediment showed pH near neutral with lowest being 5.56 from Divar island site and highest being 8.28 from Madkai sampling site.

2.3.2 Sampling

Sediments were collected from nine mangroves across the state of Goa. The enrichment culture technique was implemented to isolate yeasts from nine sites and 53 yeasts were successfully obtained. The names of each isolated yeast, along with the location they were obtained from are listed in Table 2.4. The highest number of yeasts were obtained from Madkai followed by Adpai mangrove sediments and the least number from Raia sediments.

2.3.3 Morphological characterisation

The visual records of morphological properties of all the isolates are shown in Table 2.5 and the colony characters are described in Table 2.6. The isolated yeast showed a typical oval shaped cell when observed at 40x and 100x magnification after staining using monochrome and lactophenol cotton blue method. The majority of the yeasts displayed circular colonies with smooth surfaces and mucoid textures. Isolates MMS4 and MMS9 were pigmented and others were white and crème coloured. All isolates showed raised colonies with entire margins.

Table 2.2: The Sampling locations

Sr. No	Sampling Site Name	Latitude	Longitude	River
1	Adpai	15° 22' 13" N	73° 57' 59" E	Zuari
2	Divar Island	15° 30' 32" N	73° 54' 44" E	Mandovi
3	Madkai	15° 25' 14" N	73° 55' 35" E	Mandovi & Zuari
4	Ribandar	15° 29' 59" N	73° 53' 24" E	Mandovi
5	Borim	15° 20' 46" N	74° 00' 16" E	Zuari
6	Camorlim	15° 21' 15" N	74° 00' 09" E	Zuari
7	Rachol	15° 18' 32" N	74° 00' 36" E	Zuari
8	Raia	15° 19' 21" N	73° 59' 24" E	Zuari
9	Shiroda	15° 18' 44" N	74° 00' 45" E	Zuari

Table 2.3: Physical Parameters of the samples

Location	Temperature (°C)	Salinity(psu)	pH
Adpai	32	2	7.43
Divar Island	30	2	5.56
Madkai	30	2	8.28
Ribandar	29	2	7.84
Borim	30	2	7.43
Camorlim	30	2	7.43
Rachol	30	2	7.48
Raia	30	2	7.84
Shiroda	30	2	7.48

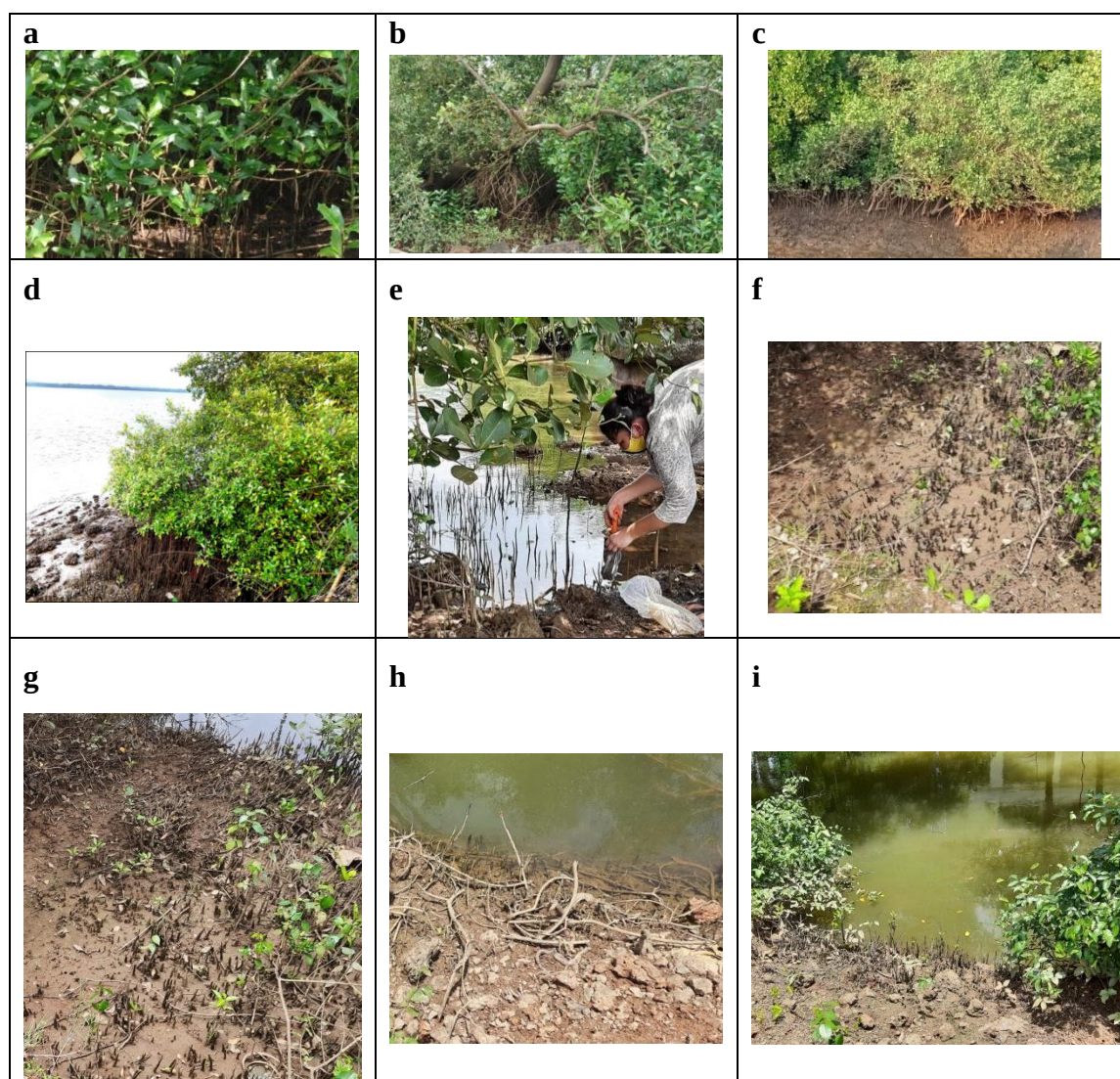
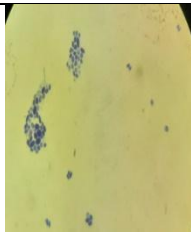
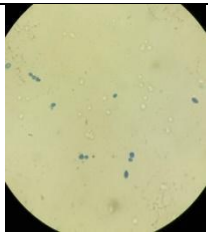
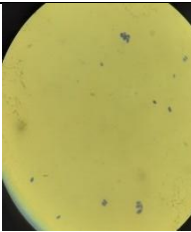
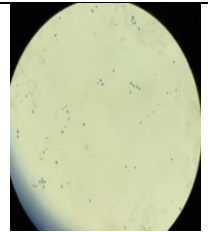
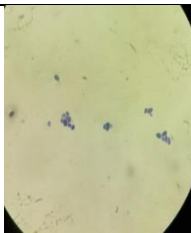
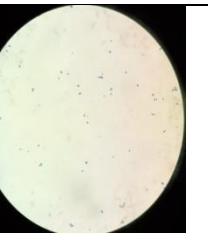
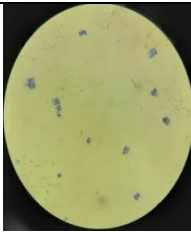
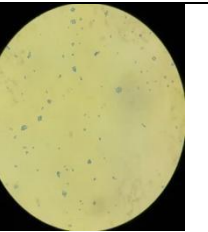
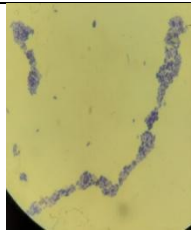
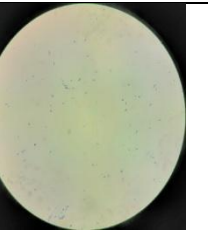
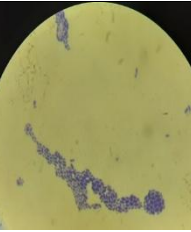
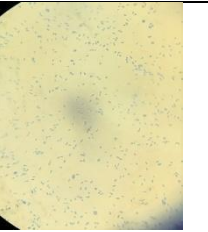


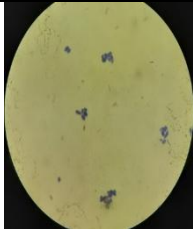
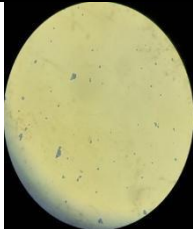
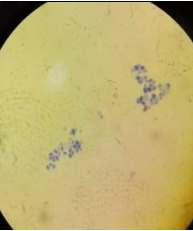
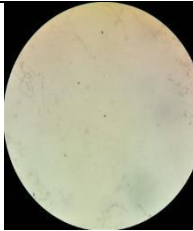
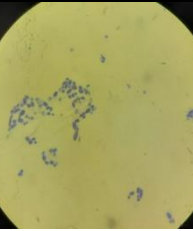
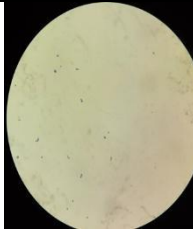
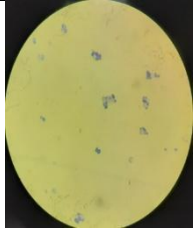
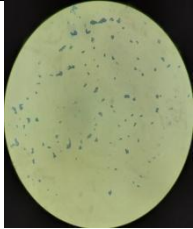
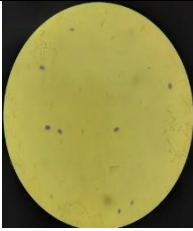
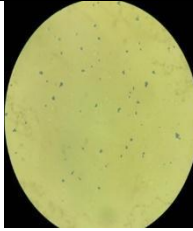
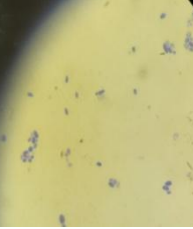
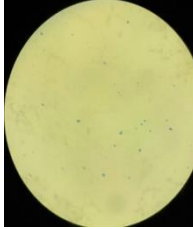
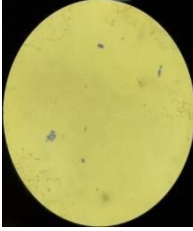
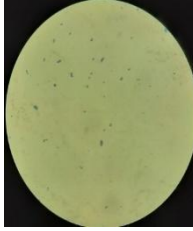
Figure 2.1: Collection of mangrove sediment samples from different sites. a) Adpai, b) Divar island, c) Madkai, d) Ribandar e) Borim, f) Camorlim, g) Raia, h) Shiroda and i) Rachol.

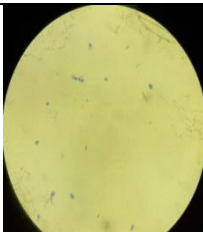
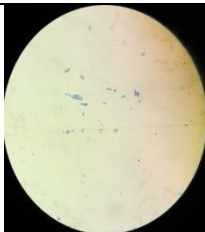
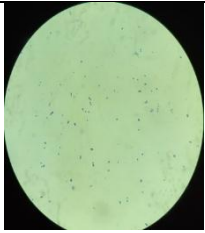
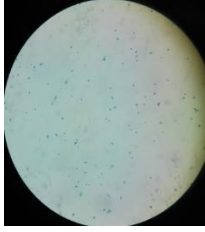
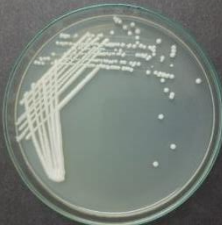
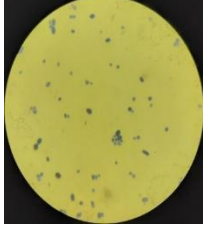
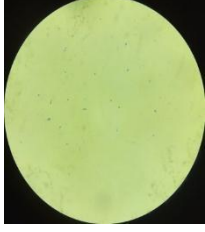
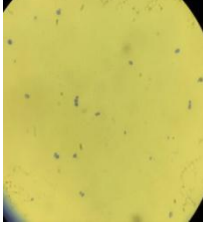
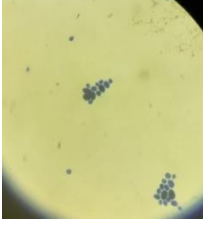
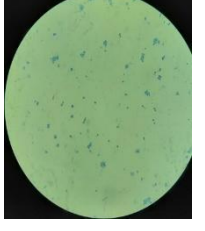
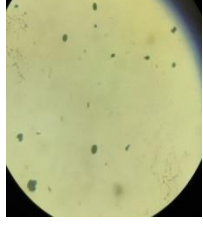
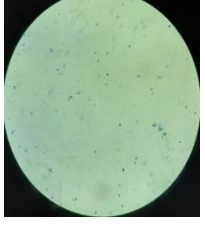
Table 2.4: Yeast isolates from the nine sampling locations

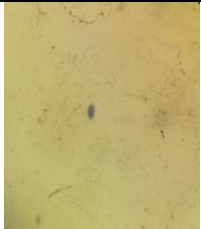
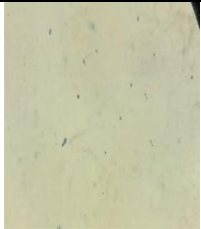
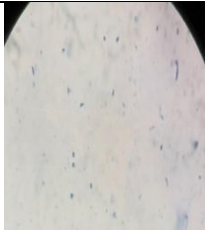
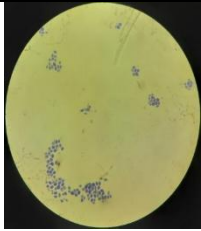
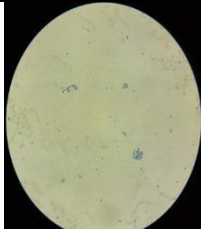
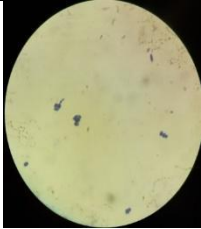
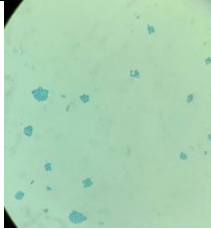
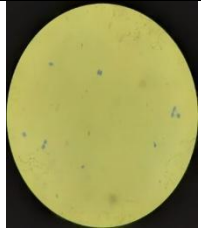
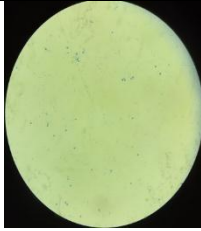
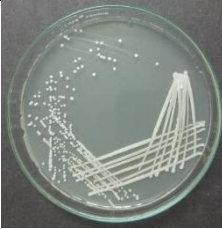
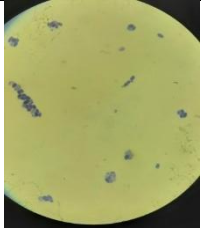
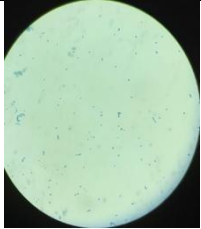
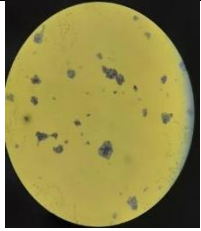
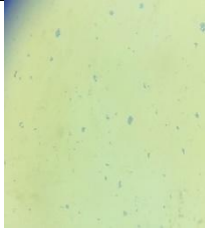
Sampling site	Number of Isolates	Isolates
Adpai	10	AMS1, AMS2, AMS3, AMS4, AMS5, AMS6, AMS7, AMS8, AMS9, AMS10.
Divar Island	4	DMS1, DMS2, DMS3, DMS4
Madkai	14	MMS1, MMS2, MMS3, MMS4, MMS5, MMS6, MMS7, MMS8, MMS9, MMS10, MMS11, MMS12, MMS13, MMS14
Ribandar	6	RMS1, RMS2, RMS3, RMS4, RMS5, RMS6
Borim	3	BMS1, BMS2, BMS3
Camorlim	5	CMS1, CMS2, CMS3, CMS4, CMS5
Rachol	3	RcMS1, RcMS2, RcMS3
Raia	2	RiMS1, RiMS2
Shiroda	6	SMS1, SMS2, SMS3, SMS4, SMS5, SMS6
Total	53	

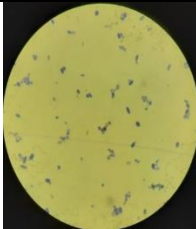
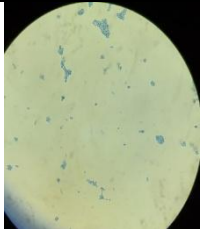
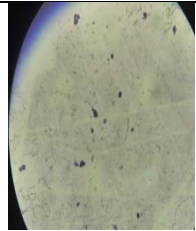
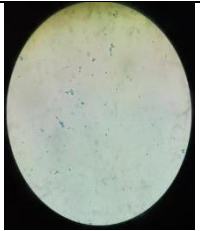
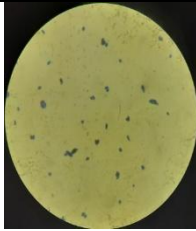
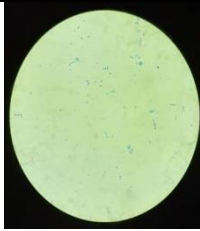
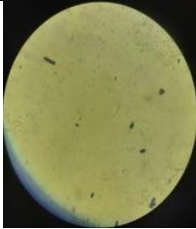
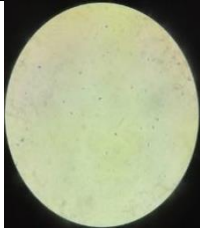
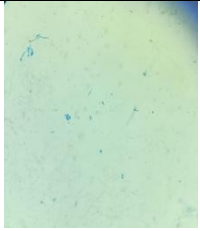
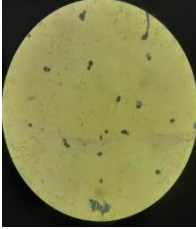
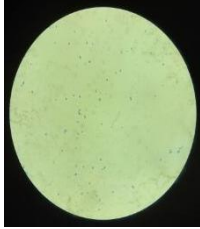
Table 2.5: The yeast isolated from mangrove sediments. A- Isolates grown on YMA, B- monochrome microscopy images at 100x, C- lacto phenol cotton blue staining images at 40x

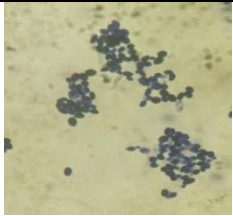
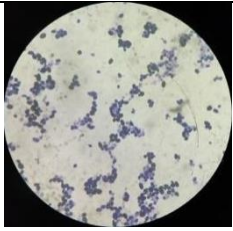
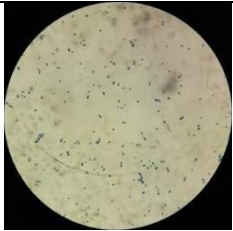
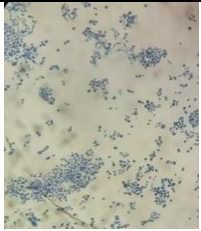
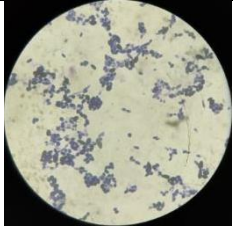
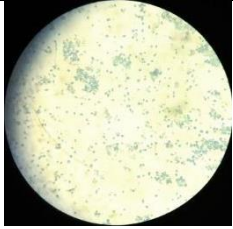
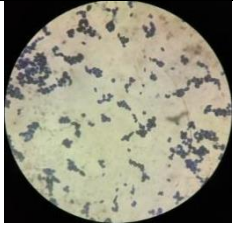
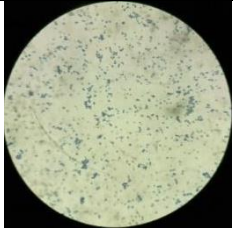
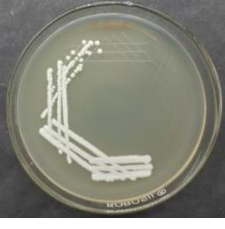
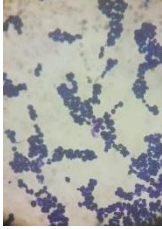
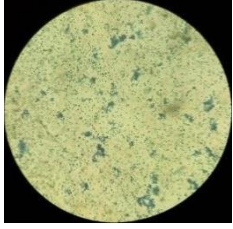
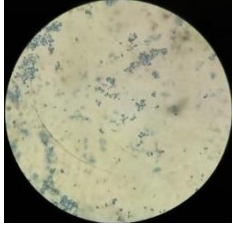
Sr. No.	Isolate	A	B	C
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2	AMS2			
3	AMS3			
4	AMS4			
5	AMS5			
6	AMS6			

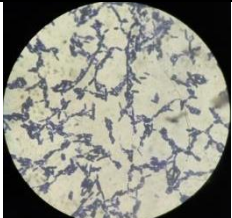
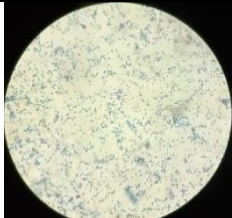
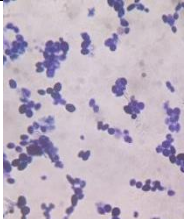
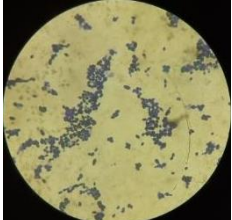
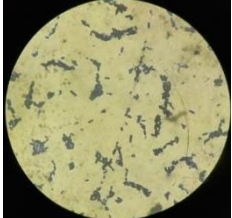
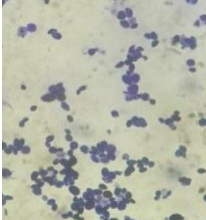
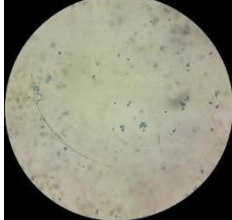
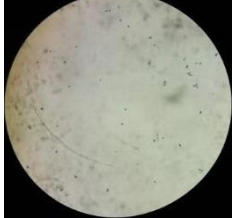
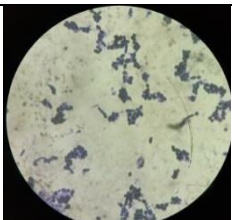
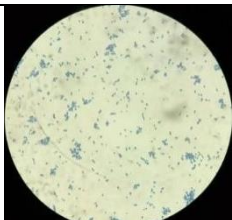
7	AMS7			
8	AMS8			
9	AMS9			
10	AMS10			
11	DMS1			
12	DMS2			
13	DMS3			

14	DMS4			
15	MMS1			
16	MMS2			
17	MMS3			
18	MMS4			
19	MMS5			
20	MMS6			

21	MMS7			
22	MMS8			
23	MMS9			
24	MMS10			
25	MMS11			
26	MMS12			
27	MMS13			

28	MMS14			
29	RMS1			
30	RMS2			
31	RMS3			
32	RMS4			
33	RMS5			
34	RMS6			

35	BMS 1			
36	BMS2			
37	BMS3			
38	CMS1			
39	CMS2			
40	CMS3			
41	CMS4			

42	CMS5			
43	RcMS1			
44	RcMS2			
45	RcMS3			
46	RiMS1			
47	RiMS2			
48	SMS1			

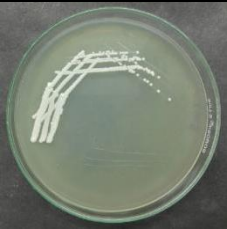
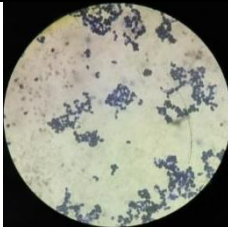
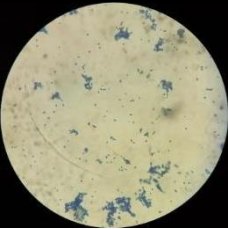
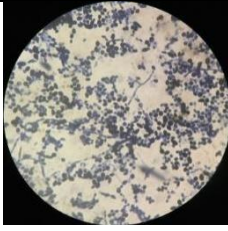
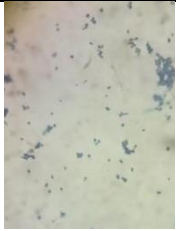
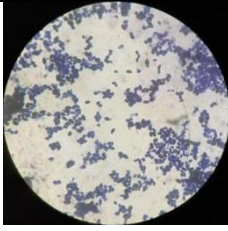
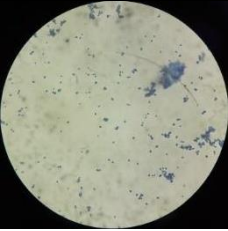
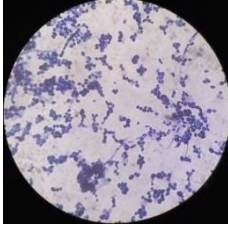
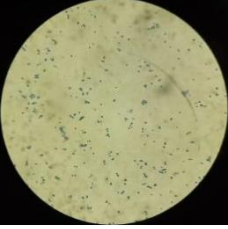
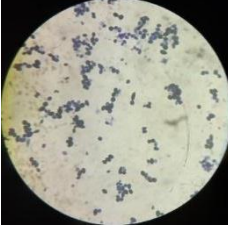
49	SMS2			
50	SMS3			
51	SMS4			
52	SMS5			
53	SMS6			

Table 2.6 Colony Characteristics of the isolates grown on YMA

Sr. No.	Isolate	Colour	Shape	Texture	Surface	Elevation	Margine
1	AMS1	White	Circular	Mucoid	Smooth	Raised	Entire
2	AMS2	White	Circular	Mucoid	Smooth	Raised	Entire
3	AMS3	White	Circular	Mucoid	Smooth	Raised	Entire
4	AMS4	White	Circular	Mucoid	Smooth	Raised	Entire
5	AMS5	White	Circular	Mucoid	Smooth	Raised	Entire
6	AMS6	White	Circular	Mucoid	Smooth	Raised	Entire
7	AMS7	White	Circular	Mucoid	Smooth	Raised	Entire
8	AMS8	White	Circular	Mucoid	Smooth	Raised	Entire
9	AMS9	White	Circular	Friable	Wrinkled	Raised	Entire
10	AMS10	White	Circular	Mucoid	Smooth	Raised	Entire
11	DMS1	White	Circular	Mucoid	Smooth	Raised	Entire
12	DMS2	White	Circular	Mucoid	Smooth	Raised	Entire
13	DMS3	White	Circular	Mucoid	Smooth	Raised	Entire
14	DMS4	White	Circular	Mucoid	Smooth	Raised	Entire
15	MMS1	White	Circular	Mucoid	Smooth	Raised	Entire
16	MMS2	White	Circular	Friable	Smooth	Raised	Entire
17	MMS3	White	Circular	Friable	Wrinkled	Raised	Entire
18	MMS4	Maroon	Circular	Mucoid	Smooth	Raised	Entire
19	MMS5	White	Circular	Friable	Smooth	Raised	Entire
20	MMS6	White	Circular	Friable	Smooth	Raised	Entire
21	MMS7	White	Circular	Mucoid	Smooth	Raised	Entire
22	MMS8	White	Circular	Mucoid	Smooth	Raised	Entire
23	MMS9	Orange	Circular	Mucoid	Smooth	Raised	Entire
24	MMS10	White	Circular	Friable	Smooth	Raised	Entire
25	MMS11	White	Circular	Mucoid	Smooth	Raised	Entire

26	MMS12	White	Circular	Mucoid	Smooth	Raised	Entire
27	MMS13	White	Circular	Mucoid	Smooth	Raised	Entire
28	MMS14	White	Circular	Friable	Wrinkled	Raised	Entire
29	RMS1	White	Circular	Mucoid	Smooth	Raised	Entire
30	RMS2	White	Circular	Mucoid	Wrinkled	Raised	Entire
31	RMS3	White	Circular	Mucoid	Smooth	Raised	Entire
32	RMS4	White	Circular	Friable	Wrinkled	Raised	Entire
33	RMS5	White	Circular	Mucoid	Wrinkled	Raised	Entire
34	RMS6	White	Circular	Mucoid	Smooth	Raised	Entire
35	BMS1	White	Circular	Mucoid	Smooth	Raised	Entire
36	BMS2	White	Circular	Mucoid	Smooth	Raised	Entire
37	BMS3	White	Circular	Mucoid	Smooth	Raised	Entire
38	CMS1	White	Circular	Mucoid	Smooth	Raised	Entire
39	CMS2	White	Circular	Mucoid	Smooth	Raised	Entire
40	CMS3	White	Circular	Mucoid	Smooth	Raised	Entire
41	CMS4	White	Circular	Mucoid	Smooth	Raised	Entire
42	CMS5	White	Circular	Mucoid	Smooth	Raised	Entire
43	RcMS1	White	Circular	Mucoid	Smooth	Raised	Entire
44	RcMS2	White	Circular	Mucoid	Smooth	Raised	Entire
45	RcMS3	White	Circular	Mucoid	Smooth	Raised	Entire
46	RiMS1	White	Circular	Mucoid	Smooth	Raised	Entire
47	RiMS2	White	Circular	Mucoid	Smooth	Raised	Entire
48	SMS1	White	Circular	Mucoid	Smooth	Raised	Entire
49	SMS2	White	Circular	Mucoid	Smooth	Raised	Entire
50	SMS3	White	Circular	Mucoid	Smooth	Raised	Entire
51	SMS4	White	Circular	Mucoid	Smooth	Raised	Entire
52	SMS5	White	Circular	Mucoid	Smooth	Raised	Entire
53	SMS6	White	Circular	Mucoid	Smooth	Raised	Entire

2.4 Discussion

Mangrove forests are ecologically rich and dynamic habitats. The mangrove sediments contain organic matter and decaying plant materials, making them rich in nutrients and increasing their habitability. The fluctuating conditions, including temperature and salinity variations, allow sustenance of only a few adaptive microorganisms. They adapt by producing several bioactive compounds that enhance the uniqueness of the organisms. This robust nature is exhibited by yeasts.

The Indian subcontinent has east and west coasts, rich in mangrove forests flanking its sides. The studies on mangrove forests of Muthupet, Pichavaram, Sundarbans and Mahanadi delta from the east coast of India included the isolation of the yeasts belonging to genera *Kuraishia*, *Barnettozyma*, *Saccharomyces*, *Debaryomyces*, *Aureobasidium*, *Dioszegia*, *Leucosporidium*, *Rhodotorula*, *Cytobasidium*, *Geotrichum* and *Candida* (Manivannan and Kathiresan, 2009; Kathiresan et al. 2011; Thatoi et al., 2013b; Saravanakumar et al. 2013; Senthilraja and Kathiresan 2015; Jayalakshmi and Umamaheshwari, 2016; Sarkar & Bhaskara Rao, 2023).

The reported studies carried out on manglicolous yeast isolated from the west coast of India across the states of Gujarat, Goa and Kerala included the yeast genera *Hortaea*, *Candida*, *Kluyveromyces*, *Debaryomyces*, *Torulaspora*, *Trichosporon*, *Rhodotorula*, *Cyberlindnera*, *Wickerhamiella*, *Pichia* and *Starmerella* (Prabhakaran and Gupta, 1990; Nazareth et al., 2015; Kachiprath et al., 2019, Patel and Patel, 2020; Nimsi et al., 2023). In a study by Prasannakumar et al. (2020) from mangrove sediments and the continental shelves of the Indian coastline, fifty-five genera of yeast, including thirteen species, were barcoded, and a DNA barcode library of marine yeasts was created.

The nutrient-packed sediments favour the growth and survival of multiple microorganisms. In the present study, sediment samples as a source of yeasts were collected from the nine locations across the state of Goa. The physicochemical characters across these sites remained similar. A difference in the pH value of sediment sample at Madkai (8.28) and Divar island (5.56) was observed. The Madkai sampling site is in fishing landing zone region subjected to fishing related activities and could be the reason for the pH value 8.3 of the sediment sample observed. The Divar sampling location being an island is pristine and less affected by anthropological interferences. This sampling site is not affected by extensive fishing and related activities resulting in having a pH value of 5.56.

Isolation of yeast was carried out by the enrichment method, instrumental in the selective recovery of yeast over other classes of microbes. Regular fungal media having an acidic pH and containing the antibiotic chloramphenicol were chosen for the isolation of yeast. This choice of media suppressed bacterial growth while providing conducive conditions for yeast growth. In a study by Khot et al. (2012) using the enrichment culture technique, three yeast isolates were obtained from mangrove sediments. An enrichment method was implemented in the successful isolation of 178 yeasts from eight mangrove forests in Thailand (Hoondie et al., 2019).

Studies conducted on the microbial diversity of mangroves from Goa are included in Table 2.7. The table indicates that while the bacterial and filamentous fungal diversity has been studied extensively, there remains a considerable scope for exploring diversity of other microbial classes. The present study includes nine distinct locations two from North Goa district and remaining from South Goa district. The highest number of isolates (14) including two pigmented yeasts were isolated from the Madkai mangroves whereas two yeasts were recovered from Raia mangroves.

Table 2.7 The microbial diversity of mangroves from Goa

Taxonomic identification	Sample type	Physical parameters	Location	Reference
Yeast				
<i>Hortaea werneckii</i>	water	32°C, 7.03, 2.5 psu	Santacruz, Mandovi	(Nayak et al., 2012)
<i>Saccharomycetaceae</i> spp., <i>Saccharomyces cerevisiae</i> , <i>Pichia kudriavzevii</i>	Sediment	-	Ribander, Mandovi and Cortalim, Zuari	(Haldar & Nazareth, 2019)
<i>Candida</i> spp , <i>Debaryomyces</i> spp , <i>Geotrichum</i> spp <i>Cryptococcus</i> spp , <i>Rhodospiridium</i> spp, <i>Kloeckera</i> spp, <i>Saccharomyces</i> spp , <i>Trichosporon</i> spp	water	-	Chapora, Mandovi, Zuari estuaries of Goa	(Haldar & Nazareth, 2019)
Fungi				
<i>Agaricomycetes</i> , <i>Sordariomycetes</i> , <i>Dothideomycetes</i> , <i>Eurotiomycetes</i>	Sediment	-	Ribander, Mandovi and Cortalim, Zuari	(Haldar & Nazareth, 2019)
<i>Aspergillus</i> spp, <i>Penicillium</i> spp	Sediment and water	-	Ribander	(Bicholkar & Nazareth, 2015).
<i>Aspergillus niveus</i>	sediment		Sinquerim, Goa, India	(Devi et al., 2006)
<i>Aspergillus</i> spp, <i>Penicillium</i> spp, <i>Eurotium</i> spp	Sediment and water	32°C, 7.03, 2.5 psu	Santacruz and Ribander, Mandovi	(Nayak et al., 2012)
<i>Aspergillus sydowi</i> , <i>Aspergillus terreus</i> , <i>Purpureocillium lilacinum</i>	sediments	29 and 40 °C, 6–6.5, 32- 35 PSU	Divar mangroves	(Lotlikar et al., 2018)
<i>Aigialus grandis</i> , <i>Trichocladium achrasporum</i> , <i>Morosphaeria ramunculicola</i> , <i>Halorosellinia oceanica</i> , <i>Rimora mangrovei</i> , <i>Dactylospora haliotrepha</i> and <i>Rhizophila marina</i> .	Degrading plant (Rhizophora mucronata and Avicennia marina)	-	Chorao mangroves, Mandovi river, Goa	(Sarma & Raghukumar, 2013)
<i>Acaulospora</i> , <i>Claroideoglossum</i> , <i>Entrophospora</i> , <i>Funnelformis</i> , <i>Gigaspora</i> , <i>Glomus</i> , <i>Rhizophagus</i> , <i>Sclerocystis</i> , and <i>Scutellospora</i> .	Soil and root samples	-	Chorao mangroves, Mandovi river, Goa	(Gaonkar & Rodrigues, 2020).

Bacteria				
<i>Gammaproteobacteria</i>	sediment	28 °C, 6.29, 1 psu	Divar island, Mandovi estuary	(Fernandes et al., 2014)
<i>Desulfobacterales, Actinobacteria</i>	sediment	27.7 °C, 6.65, 0 psu	Tuvem, Chapora	(Fernandes et al., 2014)
<i>Bacillus, Corynebacterium, Micrococcus, Actinomyces, Flavobacterium</i>	sediment and water	-	Ribandar, Banastari, St. Cruz, Panjim and Mercas	(Desai et al., 2004)
<i>Proteobacteria, Firmicutes, Actinobacteria, Fusobacteria</i>	Sediments and water	28–30°C	Vagalim, Cortalim, Siolim, Chandor, Sarmanas (Piligao), Navelim, Marcel, Assolna, Diwar, Talpona, Chorao, Galgibag, and 4-Pillar (Panjim).	(Parab et al., 2020)
<i>E. coli, Listeria spp, Salmonella spp, Vibrio spp.</i>	Sediments, Water, Oyster, prawns, Crabs, fish	29°C -30.6°C, 5.5-7.2, 28 to 35 psu	Ribander, Tiswadi, Madkai, Betim, Divar, Old Goa, Cortalim, Durbhat, Racchol, Curtorim	(Poharkar et al., 2016)
<i>Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes</i>	Sediments	6.5, 35 °C	Ribander, Mandovi	(Haldar & Nazareth, 2018)
<i>Gemmatimonadetes, Proteobacteria, Firmicutes, Actinobacteria</i>	Sediments	6.7, 35 °C	Cortalim, Zuari	(Haldar & Nazareth, 2018)

2.5 Conclusion

In this study, 53 yeasts were successfully isolated from sediment samples collected from nine mangrove sites across the state of Goa, two from North Goa district and remaining from South Goa district. The highest number of isolates (14), including two pigmented yeasts, were isolated from the Madkai mangroves, whereas two yeasts were recovered from Raia mangroves. The morphology of the isolated yeasts was studied on the basis of colony characters and microscopic features. The colony characters of the isolates, when grown on YMA, ranged from smooth creamy textures to wrinkled, while the microscopy studies revealed typical oval shaped cells.

CHAPTER III

SCREENING OF THE ISOLATES FOR

L-ASPARAGINASE ACTIVITY

3.1 Introduction

The L-asparaginase amidohydrolase catalyzes the hydrolysis of L-asparagine to form aspartate and ammonia. A healthy cell is capable of synthesizing L-asparagine using the enzyme asparagine synthetase to fulfill its metabolic requirement. L-asparagine is an essential amino acid in tumor cells, necessary for the normal functioning of metabolic processes, including protein synthesis and cell growth. The action of enzyme L-asparaginase depletes the level of available L-asparagine to tumor cells targeting these crucial processes, ultimately resulting in cell death. This has led to L-asparaginase being applied in treatment of cancer, mainly in types of leukemia, lymphomas and sarcomas. Sources of obtaining L-asparaginase include plants, animals and microorganisms. Fast growing microorganisms, with their ability to utilize multiple substrates and form different products in bulk, make a desirable source of enzyme L-asparaginase. Known microbial producers of L-asparaginase are bacteria, yeast and fungi. The unicellular microbes, bacteria and yeast are preferred as they are amenable during optimization of culture conditions to enhance enzyme production, introduction of genetic modification and the downstream processing and product recovery. Commercially the bacterial sources of enzyme are utilized as chemotherapeutic agent. The L-asparaginase approved for use in treatment of ALL was obtained from *E. coli* and *E. carotovora*. Limitations of the enzyme of bacterial origin include the hypersensitivity reactions due to prolonged use, allergic reactions. An alternative enzyme of eukaryotic origin with cytotoxic potential is focus of current research. (Batoool et al., 2016; Ammulu et al., 2017; Brumano et al., 2019)

Detection of L-asparaginase production by microbes led to developing interest in implementing these fast-growing organisms to produce L-asparaginase in bulk. The earliest primary method to detect L-asparaginase production was based on the quantification of ammonia released by Nessler's test. The screening method being laborious led to the search for an easy and simple alternative technique. The semi quantitative method based on the principle of change in pH values of culture filtrate due to ammonia released on action of L-asparaginase was formulated. Detection of microbes producing L-asparaginase is a crucial step and was facilitated by the plate assay method. The rapid plate assay technique contains modified media containing L-asparagine as sole nitrogen source and an indicator dye to detect a change in pH. A colored zone around the growing colony indicates L-asparaginase production. Appropriate controls are used to avoid false detection of L-asparaginase producers (Gulati et al. 1997; Mahajan et al. 2013).

The enzyme activity of L-asparaginase can be estimated on the basis of formation of ammonia and aspartate and the L-asparagine consumed. The methods applied in determination of L-asparaginase activity are listed in Table 3.1.

The enzyme substrate specificity and suitability as a chemotherapeutic agent is dependent on its glutaminase activity. Determination of glutaminase activity aids in reinforcing the enzyme specificity as well as application in cancer treatment. A significant glutaminase activity indicates reduced selectivity and undesirable toxicity due to glutamine depletion in normal cells. Glutaminase free L-asparaginase with enhanced efficiency and clinical safety is preferred (Batoool et al. 2016; Cachumba et al. 2016; Fonseca et al., 2021).

Microbial identification helps in understanding the taxonomic identity, functional potential, beneficial roles and pathogenicity of the microorganisms. It enables determination of their growth requirements, facilitates the exploration of their biotechnological potential and ensures implementation of appropriate biosafety measures (Agarwal et al., 2011; Nimsi et al., 2023). Preliminary identification consists of morphological and biochemical analysis. Morphology studies involve the use of microscopic techniques to understand the shape, size, structure and other physical features. Biochemical characterisation, including carbohydrate assimilation, fermentation tests, and enzyme activity assays, aids in distinguishing physiologically similar species (Agarwal et al., 2011; Nimsi et al., 2023). Molecular identification is confirmatory due to its high precision and reliability. Fungal molecular identification involves isolation of genomic DNA followed by sequencing conserved regions that include the ITS or D1/D2 domains of rDNA and construction of a phylogenetic tree (White et al., 1990; Nimsi et al., 2023).

Table 3.1: Methods of determination of L-asparaginase activity

Sr. No.	Method	Principle
a) Qualitative methods (Batool et al., 2016)		
1	Rapid plate assay	Ammonia released causes a change in pH of the medium, detected by the indicator dye present in it.
2	Paper /Thin Layer Chromatography	Separation and asparagine and aspartate produce a brown and purple color on interaction with 2% Ninhydrin reagent.
b) Quantitative methods (Batool et al., 2016)		
1	Nesslerization method	Ammonia formed reacts with Nessler's reagent to produce brown coloration
2	Hydroxylamine method	L-aspartate formed reacts with hydroxylamine to form aspartyl β -hydroxamate
3	Berthelot Method	Ammonia reacts with hypochlorite and phenol under alkaline conditions to form indophenol blue
4	Coupled enzyme assay	Ammonia produced is utilized by glutamate dehydrogenase and NADH consumption is estimated.

3.2 Methodology

The comprehensive screening of 53 isolates obtained from mangrove sediments of Goa for L-asparaginase production was conducted. Identification of the selected potent isolates was carried out.

3.2.1 Screening for L-asparaginase using rapid plate assay method

The Isolates were primarily screened for L-asparaginase production using a rapid plate assay technique with modified Czapek Dox Media (MCDA) containing phenol red (PR) dye (Gulati et al. 1997) and confirmatory screening using bromothymol blue (BTB) indicator (Mahajan et al. 2013). A zone with a change in color around the colony was considered a positive test. The initial color of plates containing PR and BTB was yellow. A zone around the colony with a change in color from yellow to red for PR and yellow to green for BTB was considered positive. Control consisted of plain MCDA with L-asparagine as sole nitrogen source (without indicator) and Czapek Dox Agar (CDA) (HiMedia) containing the respective indicator dye. Isolates were monitored for 120 h and those showing prominent zones were used for secondary analysis.

3.2.2 Confirmatory screening by paper chromatography method

Secondary screening of the selected isolates was carried out using paper chromatography. Modified Czapek Dox Broth (MCDB) containing L-asparagine as the sole Nitrogen source (Gulati *et al.*,1997) was inoculated with 1% inoculum of the respective isolate having an absorbance of 0.500 at 600nm. This experiment was performed in triplicate. The flasks were incubated at 28°C, 120 rpm for 96 hours. 2mL of culture broth was collected aseptically at 48 and 96 h, centrifuged at 10000 rpm for 10 minutes at 4°C and the supernatant was used as a sample to perform paper chromatography. Paper chromatography was conducted using Whatman filter paper no. 1. The cell free supernatant, standards L-asparagine (1%) and L-aspartate (1%) were spot inoculated. The spots were allowed to dry completely. The spot inoculated paper was then placed in a saturated chromatographic chamber containing a mobile phase consisting of n-Butanol: Acetic Acid: Water in a ratio 5:4:1. The chromatogram was allowed to run and later air dried. It was developed by spraying with 2% Ninhydrin solution, air drying and later on oven drying at 80°C (Mu and Plummer, 2001).

3.2.3 Screening the positive isolates for L-glutaminase activity

Modified Czapek Dox Agar containing L-glutamine as the sole Nitrogen source and BTB (0.007% w/v) as indicator dye was used for screening of the isolates for L-glutaminase activity (Ashok et al., 2019). Control consisted of plain Modified Czapek Dox media (without indicator) and Czapek Dox Media (HiMedia) containing BTB (0.007% w/v). A zone with a change in color around the colony was considered a positive test.

3.2.4 Morphological and physiological analysis of L-asparaginase positive isolates

The purified isolates were inoculated on HiCrome™ Candida Differential Agar (Himedia) and incubated at 28°C for 72 hours. Isolates were observed for growth characteristics and color development. The distinction between *Candida* species is based on the color produced during growth of the isolates by the chromogenic reactions. Scanning Electron Microscopy provides detailed surface morphology of yeast cells, including three-dimensional images that aid in visualizing cell shape, size, surface structures, and morphological variations among isolates. L-asparaginase positive isolates were each grown in YMB at 28°C, 120 rpm, overnight. 1mL of this culture broth was collected and centrifuged at 4°C, 10000 rpm. The pellet was resuspended in minimal volume of saline and fixed on glass stubs using a 0.5% glutaraldehyde solution. The glass stubs containing the cells were placed undisturbed for 16 hours at 25°C, followed by sequential dehydration for 10 minutes each in acetone solutions with increasing concentration (20% to 100%) (Bang & Swanson, 2008). These dehydrated stubs were sputter coated and imaging was conducted using Scanning electron microscope Zeiss. The magnification of 2000X was maintained for all isolates.

3.2.5 Quantitative analysis of L-asparaginase activity

Quantitative estimation was performed following the method described by Imada et al. (1973). The method is based on quantifying ammonia released on hydrolysis of L-asparagine by Nessler's method. The reaction mixture consisted of 1.0 mL 0.5 M Tris buffer pH 7.2, 0.5 mL 0.04 M L-asparagine solution as substrate, 0.5 mL cell free supernatant as crude enzyme and was incubated at 37 °C for 30 min. The reaction was inhibited by adding 0.5 mL 1.5 M Trichloroacetic acid. Finally, 0.2 mL of Nessler's reagent was added to 0.1 mL reaction mixture in 3.7 mL distilled water and absorbance of

this was measured at 450 nm. Isolates with the highest L-asparaginase activity were selected for the following studies.

Statistical analysis

The experiments were performed in triplicate. The findings were expressed as mean with their standard deviation. The statistical analysis of the enzyme activity data of the L-asparaginase positive isolates was performed by a one-way analysis of variance (ANOVA) ($p \leq 0.05$), followed by a T-test and a Bonferroni post-hoc test. The statistical tests were performed using Microsoft Excel 2021.

3.2.6 Identification of the isolates

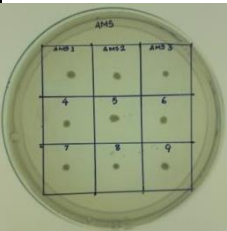
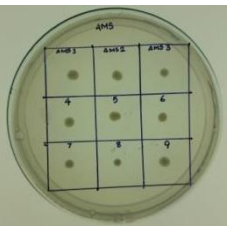
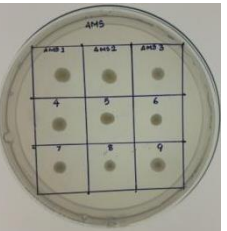
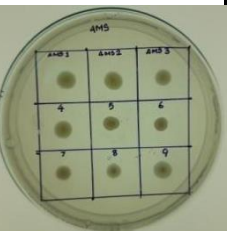
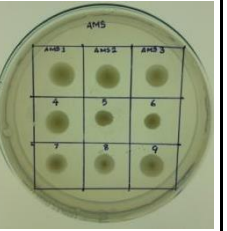
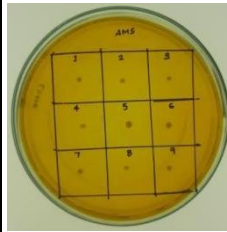
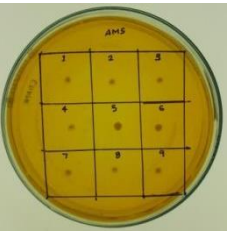
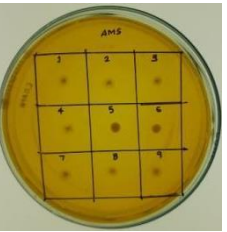
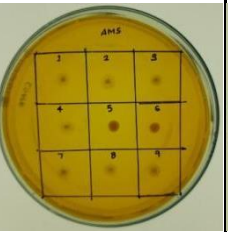
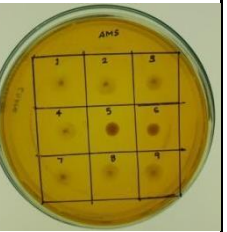
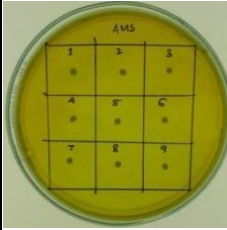
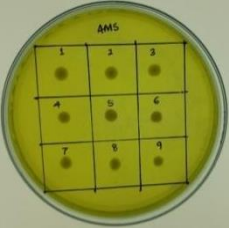
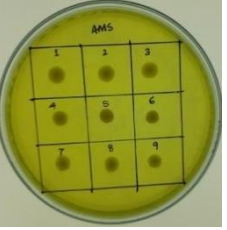
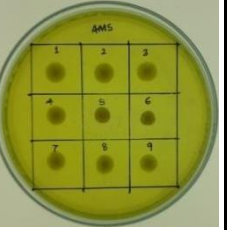
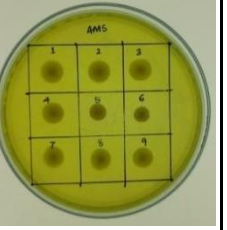
The above selected isolates were identified using morphological, biochemical and molecular techniques. Morphological identification was conducted by noting colony characters and performing microscopic studies. Biochemical characterization was performed using carbohydrate assimilation tests following the method reported by Kurtzman et al. (2011). For molecular identification, yeast genomic DNA was extracted following the method as reported by Green and Sambrooke (2018). The fungal Internal Transcribed Spacer (ITS) sequences of the extracted genomic DNA of the selected isolates were amplified using the universal primer ITS1 5'-TCCGTAGGTGAACCTGCGGG-3' and ITS4 5'-TCCTCCGCTTATTGATATG'. The amplified sequence was analysed to establish sequence similarity using BLAST database and to construct a phylogenetic tree using MEGA11 software.

3.3 Results

3.3.1 Qualitative estimation of L-asparaginase production by yeast isolates

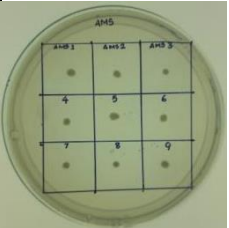
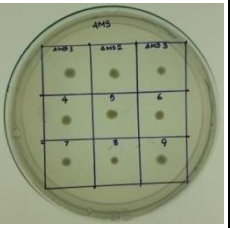
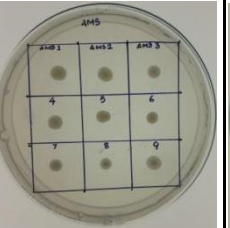
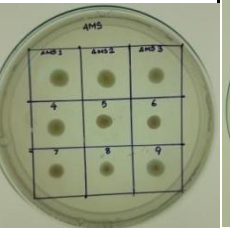
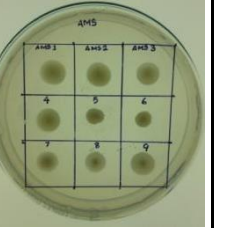
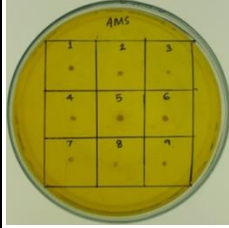
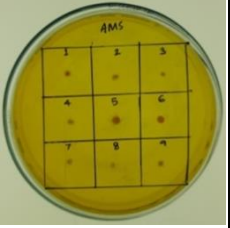
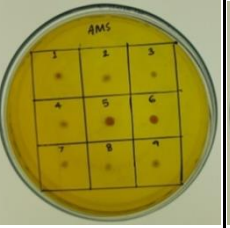
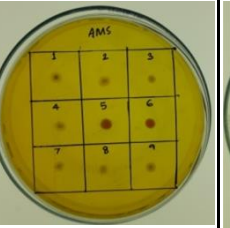
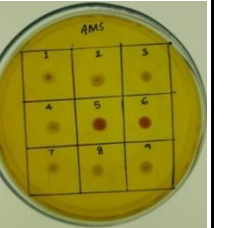
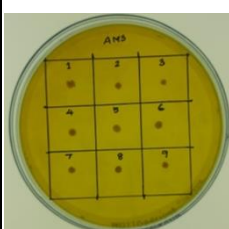
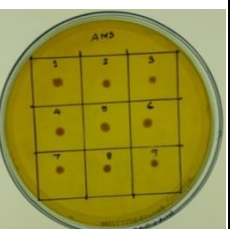
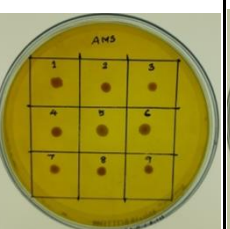
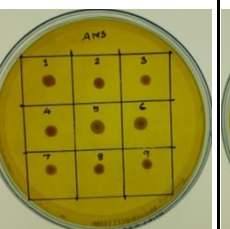
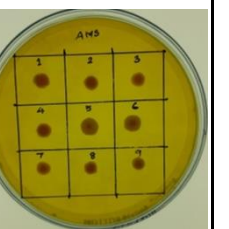
In this study, the screening of 53 yeast isolates from the mangrove forest was conducted using indicator dyes PR and BTB for L-asparaginase production (Tables 3.2 to 3.15). It resulted in eight isolates displaying a positive test, as depicted in Table 3.16 and 3.17. L-asparaginase activity of the positive isolates was confirmed using paper chromatography technique. The L-asparagine and L-aspartate with cell free supernatant was used to perform paper chromatography. The positive isolates as depicted in Table 3.18 showed spots on the chromatogram with a changing intensity of brown coloration compared with CFS collected at 24h and 96h of incubation. The position of the spots was observed more towards that of L-aspartate, indicating conversion of L-asparagine to L-aspartate.

Table 3.2: Screening of isolates AMS1 to AMS9 for L-asparaginase production over 120h using PR dye

Time	24h	48h	72h	96h	120h
C1					
C2a					
Ta					

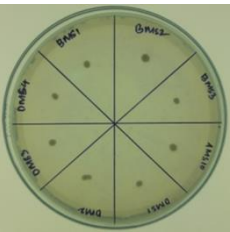
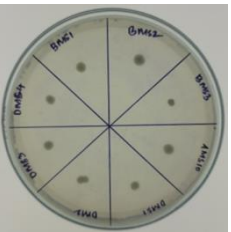
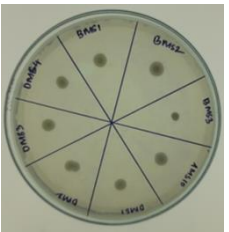
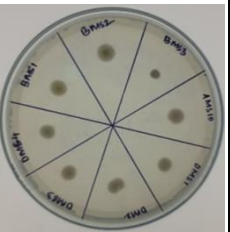
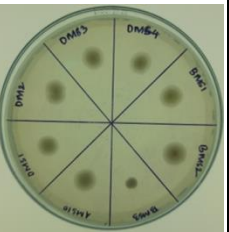
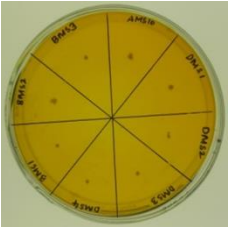
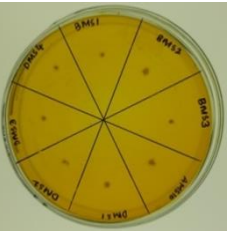
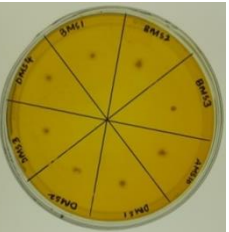
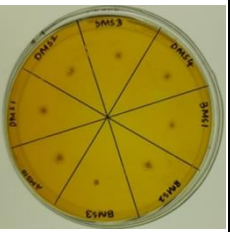
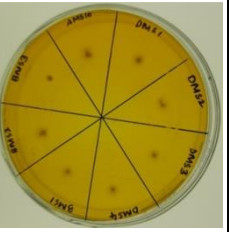
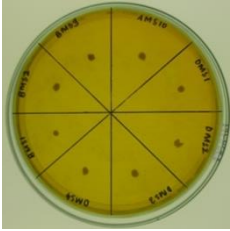
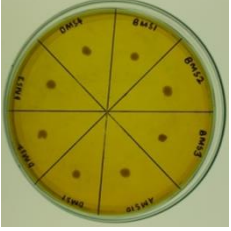
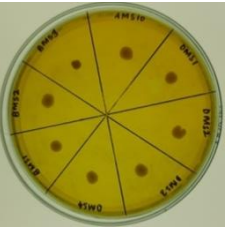
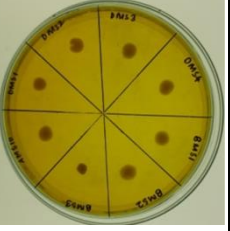
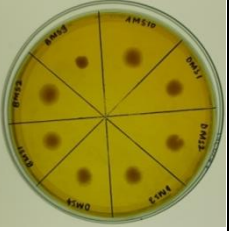
Controls are C1(MCDA) and C2a (CDA with PR). Test is Ta (MCDA with PR).

Table 3.3: Screening of isolates AMS1 to AMS9 for L-asparaginase production over 120h using BTB dye

Time	24h	48h	72h	96h	120h
C1					
C2b					
Tb					

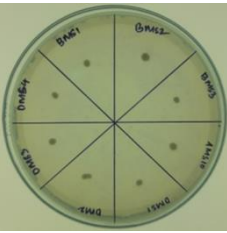
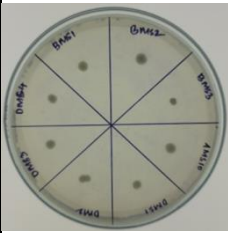
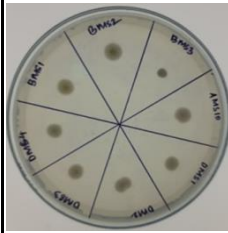
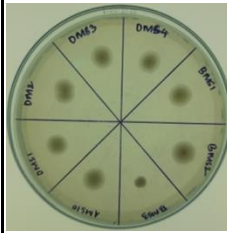
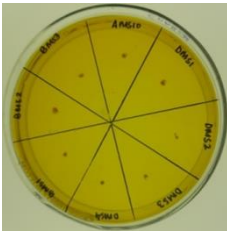
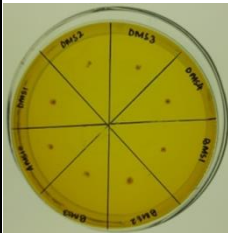
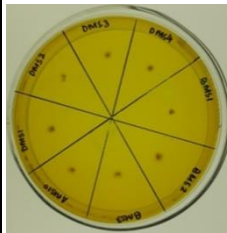
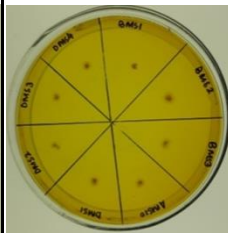
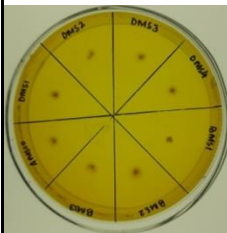
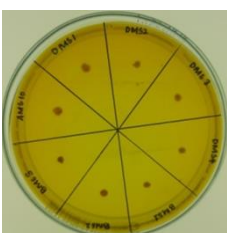
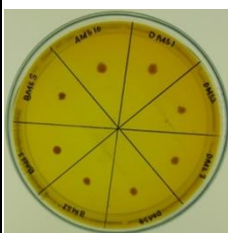
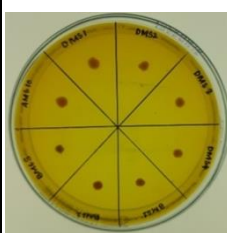
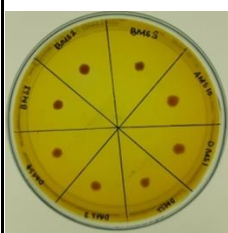
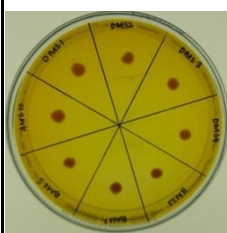
Controls are C1(MCDA) and C2b (CDA with BTB). Test is Tb (MCDA with BTB).

Table 3.4: Screening of isolates AMS10, BMS1 to BMS3 and DMS1 to DMS4 for L-asparaginase production over 120h using PR dye

Time	24h	48h	72h	96h	120h
C1					
C2a					
Ta					

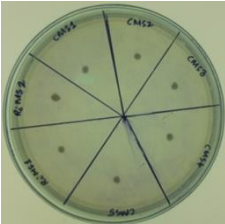
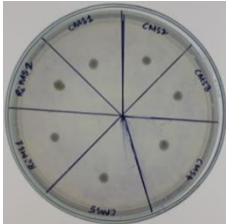
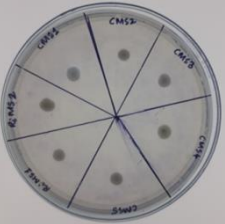
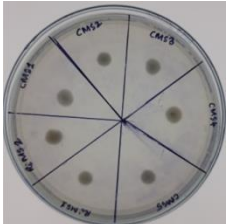
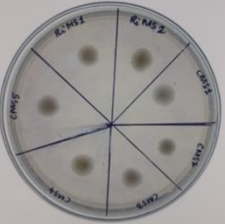
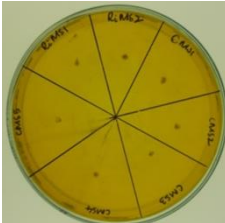
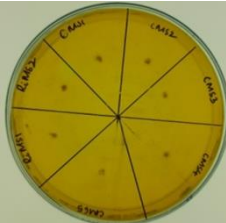
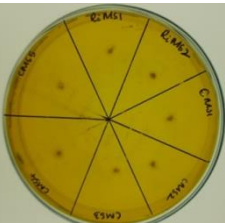
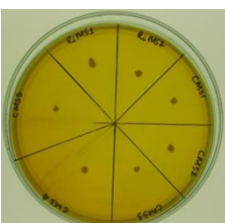
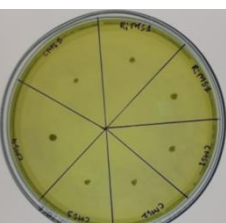
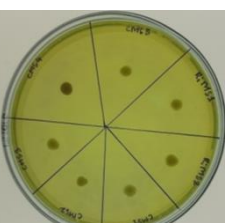
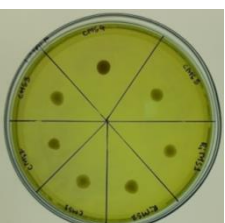
Controls are C1(MCDA) and C2a (CDA with PR). Test is Ta (MCDA with PR).

Table 3.5: Screening of isolates AMS10, BMS1 to BMS3 and DMS1 to DMS4 for L-asparaginase production over 120h using BTB dye

Time	24h	48h	72h	96h	120h
C1					
C2b					
Tb					

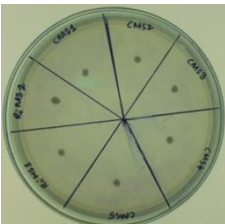
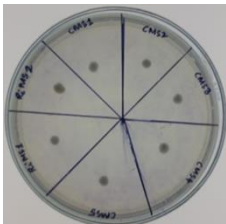
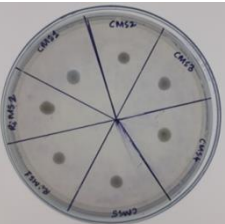
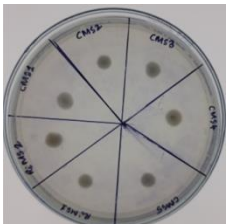
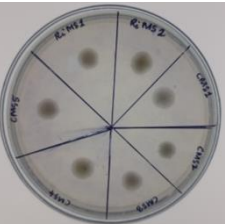
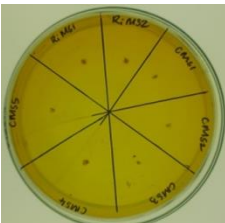
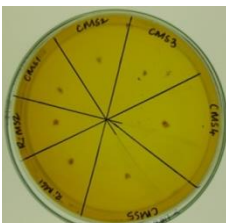
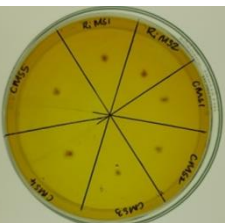
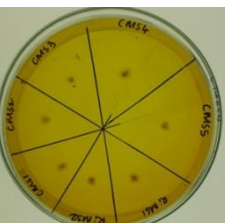
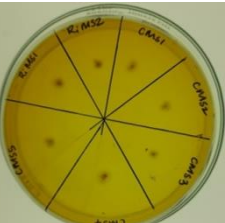
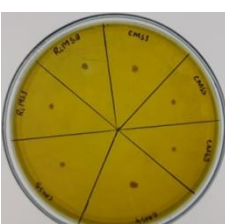
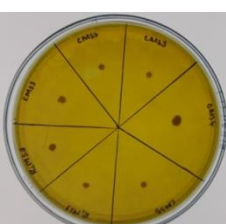
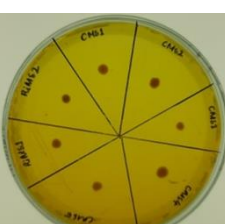
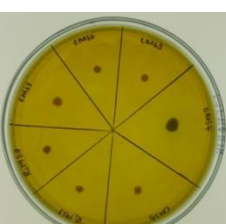
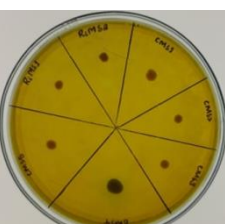
Controls are C1(MCDA) and C2b (CDA with BTB). Test is Tb (MCDA with BTB).

Table 3.6: Screening of isolates CMS1-5 and RiMS1 and 2 for L-asparaginase production over 120h using PR dye

Time	24h	48h	72h	96h	120h
C1					
C2a					
Ta					

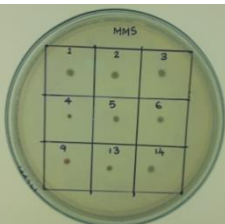
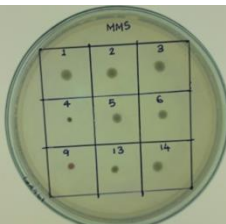
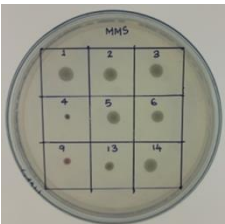
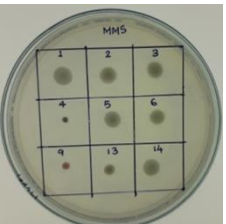
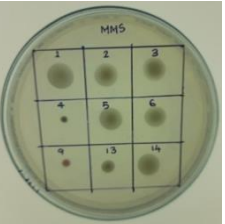
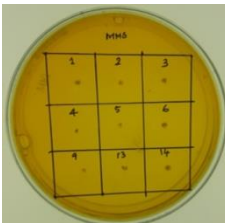
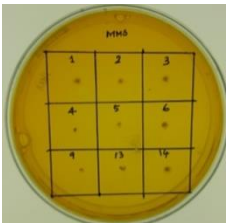
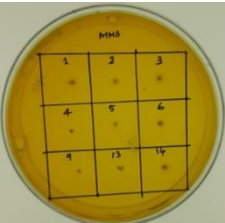
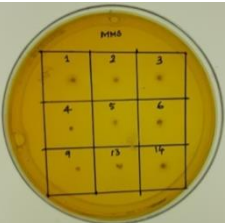
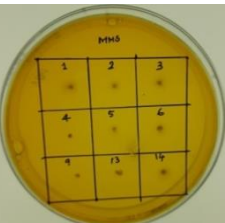
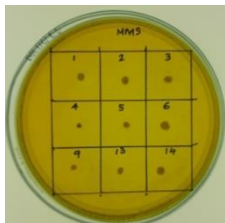
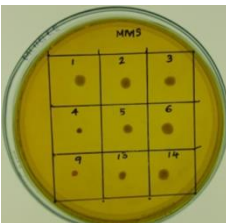
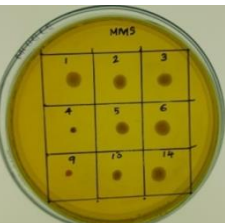
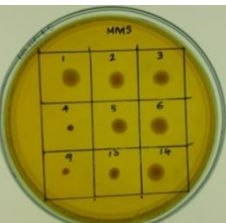
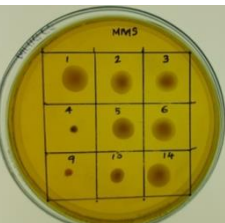
Controls are C1(MCDA) and C2a (CDA with PR). Test is Ta (MCDA with PR).

Table 3.7: Screening of isolates CMS1-5 and RiMS1 and 2 for L-asparaginase production over 120h using BTB dye

Time	24h	48h	72h	96h	120h
C1					
C2b					
Tb					

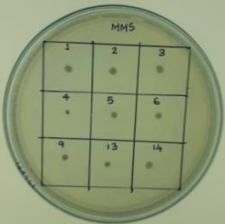
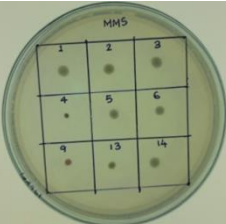
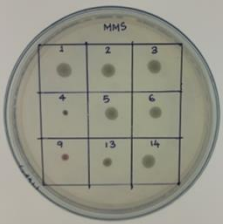
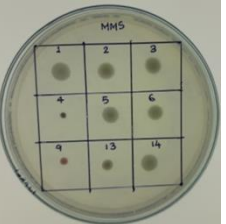
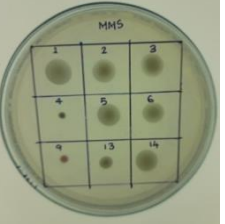
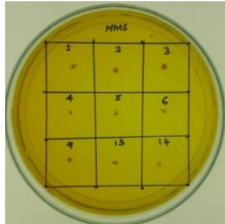
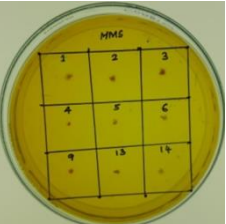
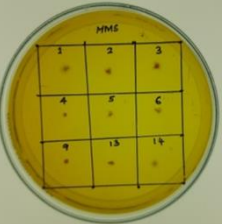
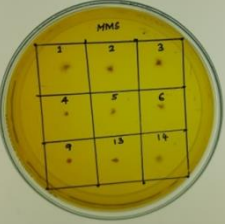
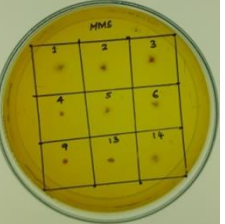
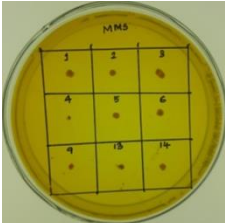
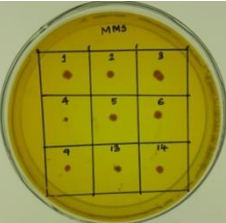
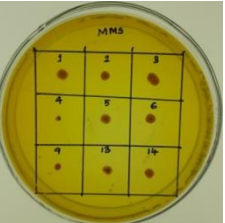
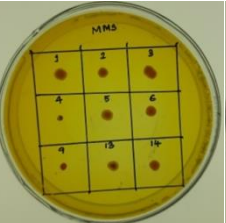
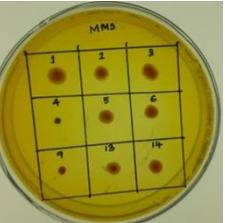
Controls are C1(MCDA) and C2b (CDA with BTB). Test is Tb (MCDA with BTB).

Table 3.8: Screening of isolates MMS1 to MMS6, MMS9, MMS13 and MMS14 for L-asparaginase production over 120h using PR dye

Time	24h	48h	72h	96h	120h
C1					
C2a					
Ta					

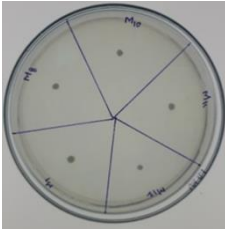
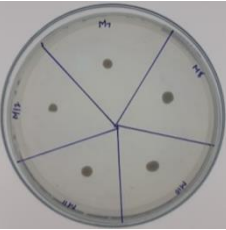
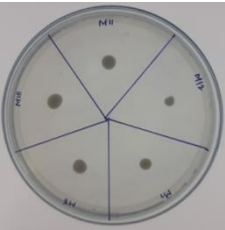
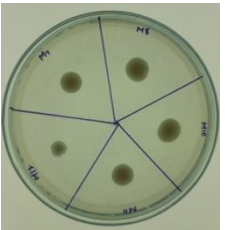
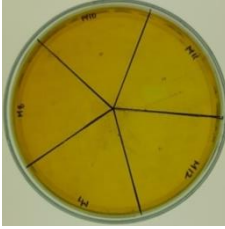
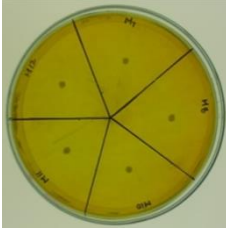
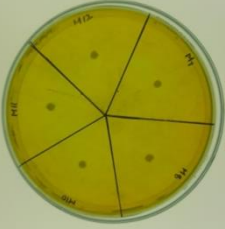
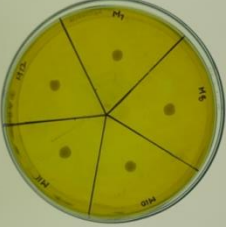
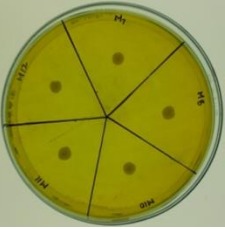
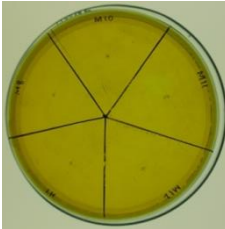
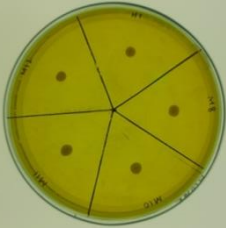
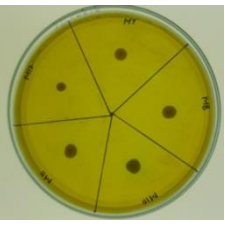
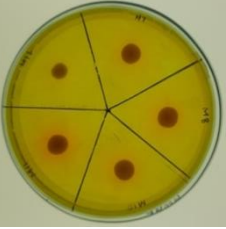
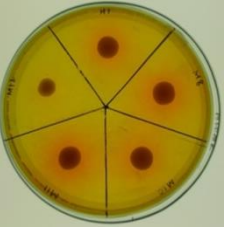
Controls are C1(MCDA) and C2a (CDA with PR). Test is Ta (MCDA with PR).

Table 3.9: Screening of isolates MMS1 to MMS6, MMS9, MMS13 and MMS14 for L-asparaginase production over 120h using BTB dye

Time	24h	48h	72h	96h	120h
C1					
C2b					
Tb					

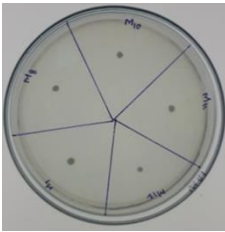
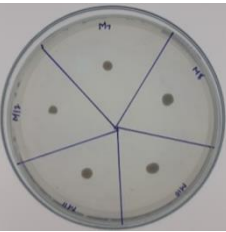
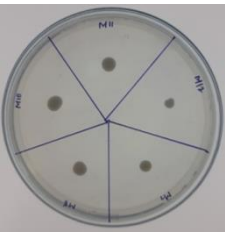
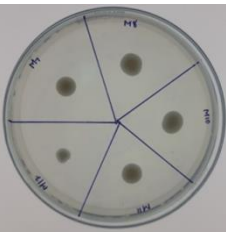
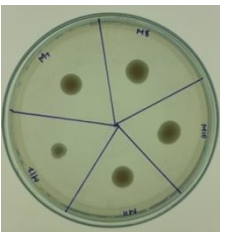
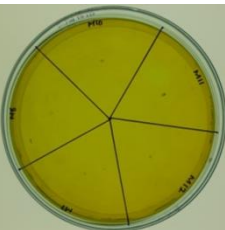
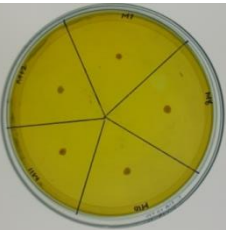
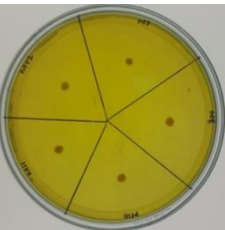
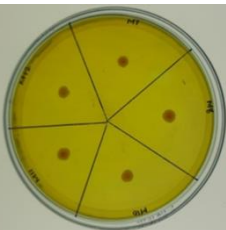
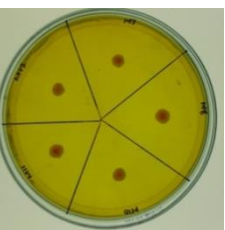
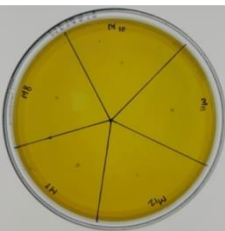
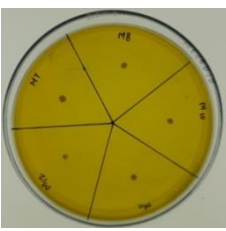
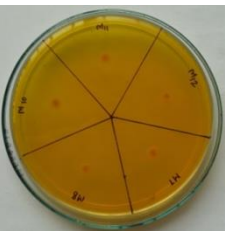
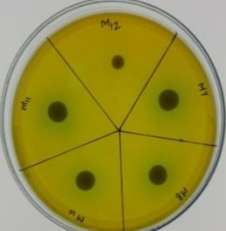
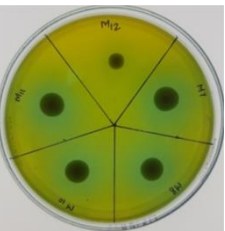
Controls are C1(MCDA) and C2b (CDA with BTB). Test is Tb (MCDA with BTB).

Table 3.10: Screening of isolates MMS7, MMS8, MMS10, MMS11 and MMS12 for L-asparaginase production over 120h using PR dye

Time	24h	48h	72h	96h	120h
C1					
C2a					
Ta					

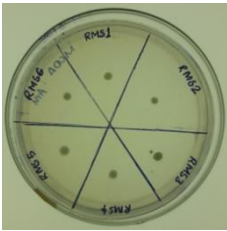
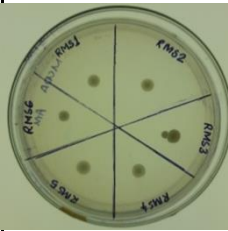
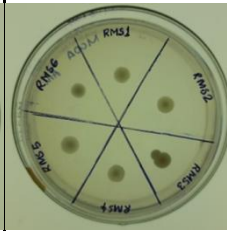
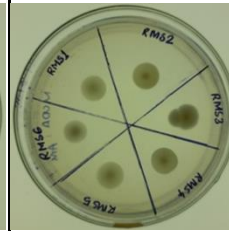
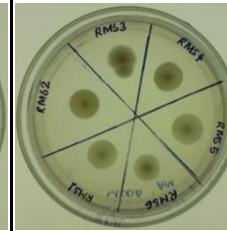
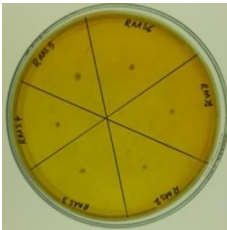
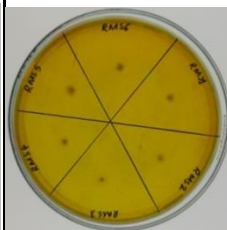
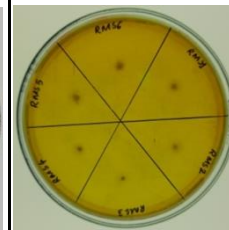
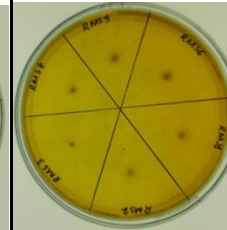
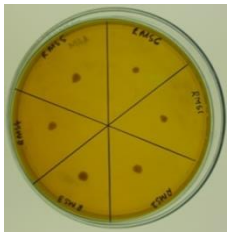
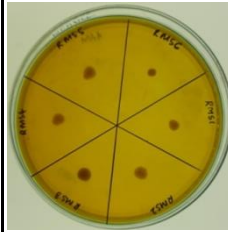
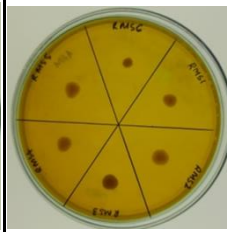
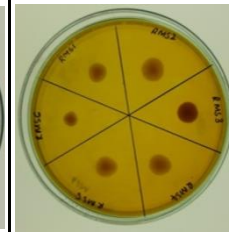
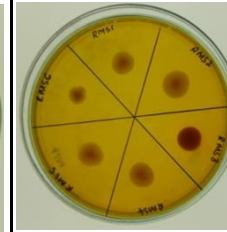
Controls are C1(MCDA) and C2a (CDA with PR). Test is Ta (MCDA with PR).

Table 3.11: Screening of isolates MMS7, MMS8, MMS10, MMS11 and MMS12 for L-asparaginase production over 120h using BTB dye

Time	24h	48h	72h	96h	120h
C1					
C2b					
Tb					

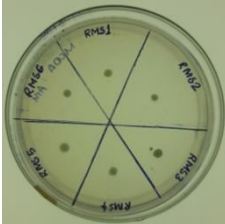
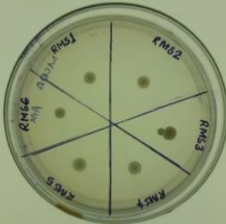
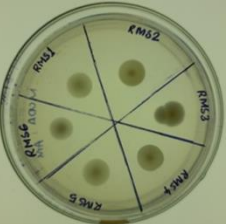
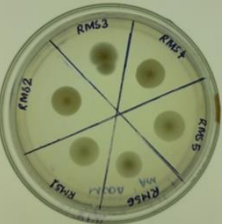
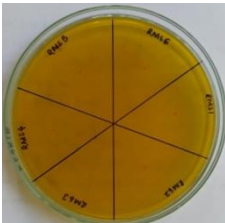
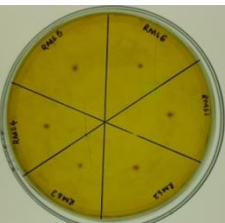
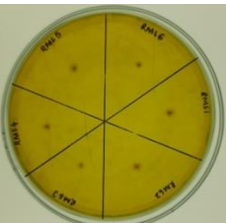
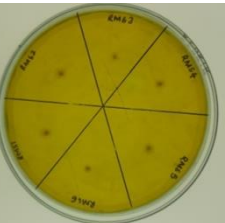
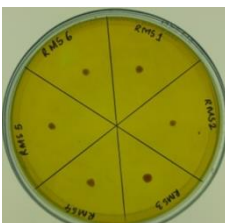
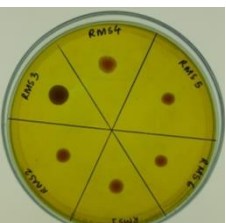
Controls are C1(MCDA) and C2b (CDA with BTB). Test is Tb (MCDA with BTB).

Table 3.12: Screening of isolates RMS1 to RMS6 for L-asparaginase production over 120h using PR dye

Time	24h	48h	72h	96h	120h
C1					
C2a					
Ta					

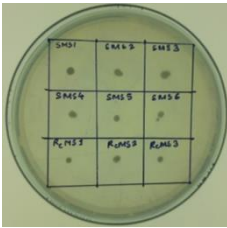
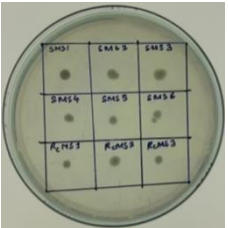
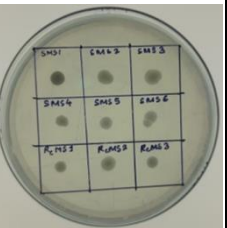
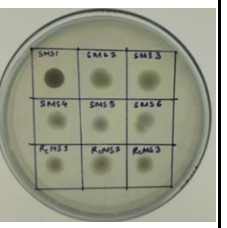
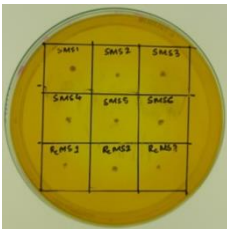
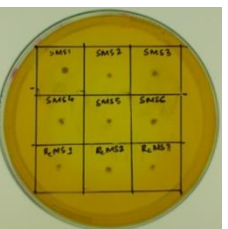
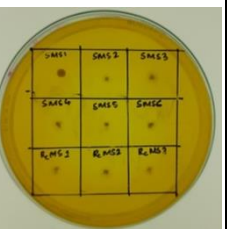
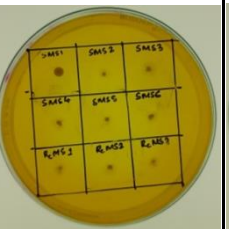
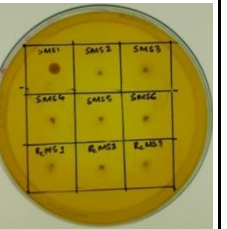
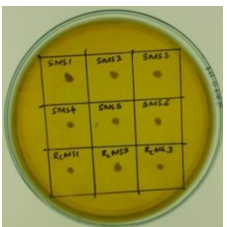
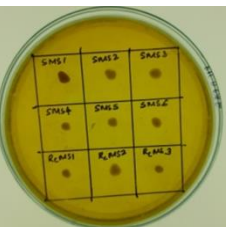
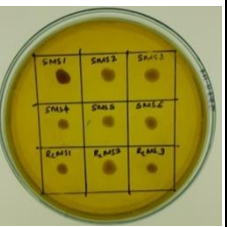
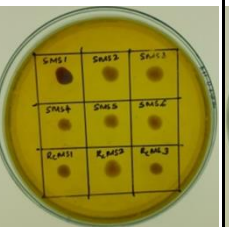
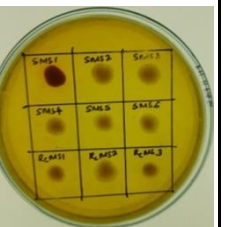
Controls are C1(MCDA) and C2a (CDA with PR). Test is Ta (MCDA with PR).

Table 3.13: Screening of isolates RMS1 to RMS6 for L-asparaginase production over 120h using BTB dye

Time	24h	48h	72h	96h	120h
C1					
C2b					
Tb					

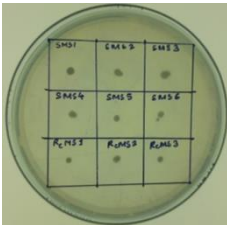
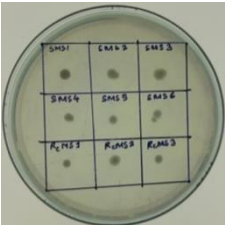
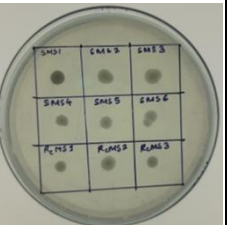
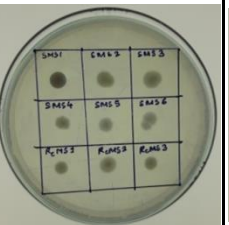
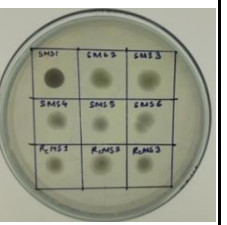
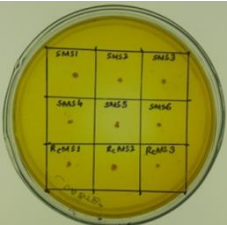
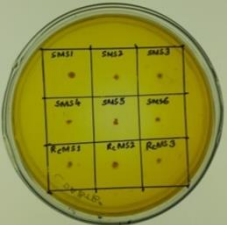
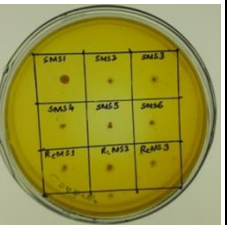
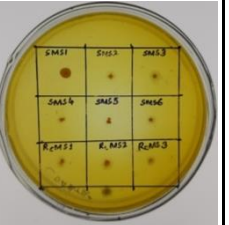
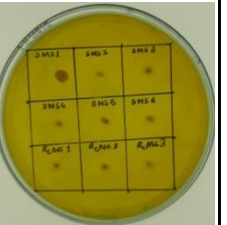
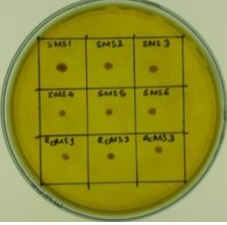
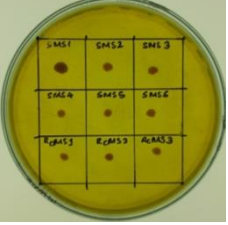
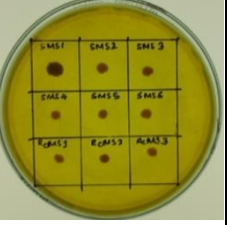
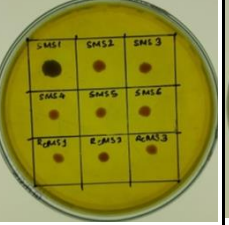
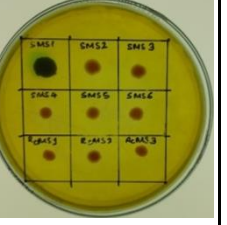
Controls are C1(MCDA) and C2b (CDA with BTB). Test is Tb (MCDA with BTB).

Table 3.14: Screening of isolates SMS1 to SMS6 and RcMS1 to RcMS3 for L-asparaginase production over 120h using PR dye

Time	24h	48h	72h	96h	120h
C1					
C2a					
Ta					

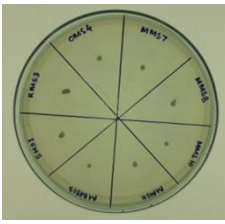
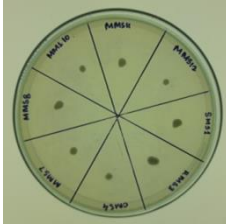
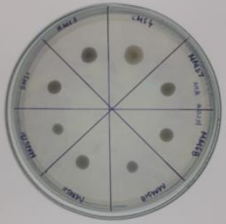
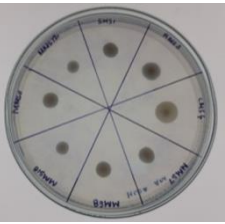
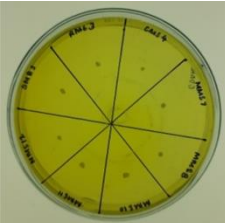
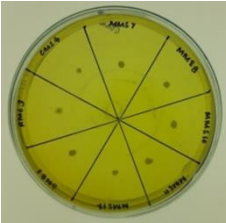
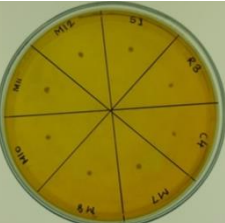
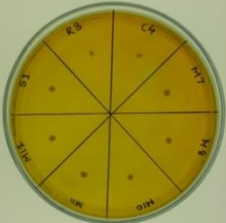
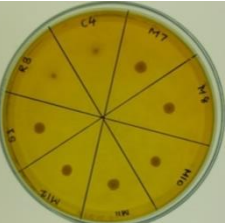
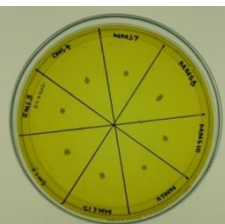
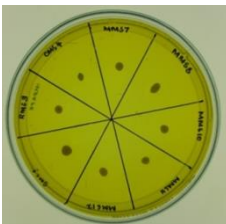
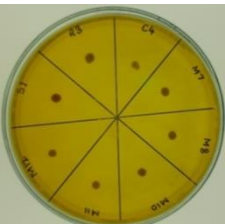
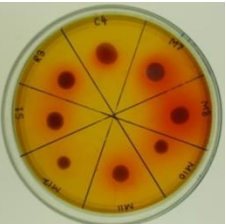
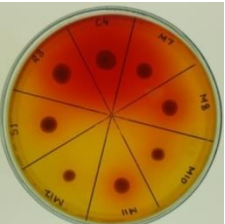
Controls are C1(MCDA) and C2a (CDA with PR). Test is Ta (MCDA with PR).

Table 3.15: Screening of isolates SMS1 to SMS6 and RcMS1 to RcMS3 for L-asparaginase production over 120h using BTB dye

Time	24h	48h	72h	96h	120h
C1					
C2b					
Tb					

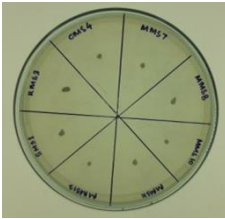
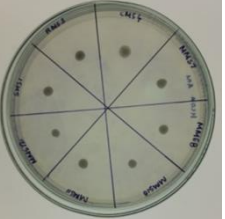
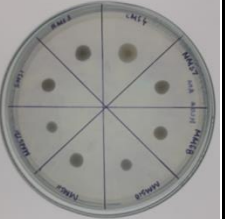
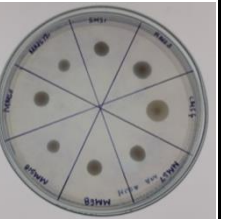
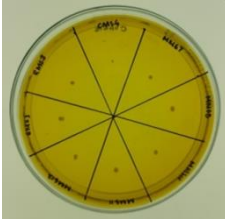
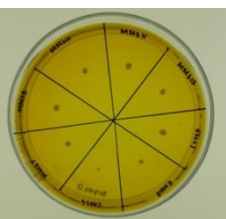
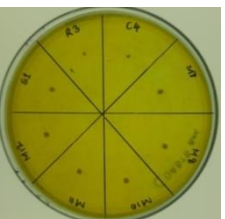
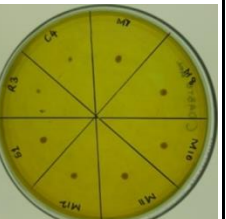
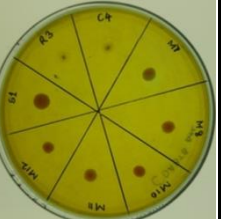
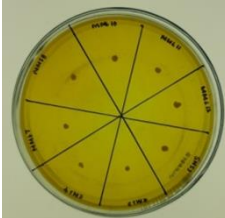
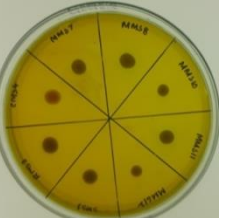
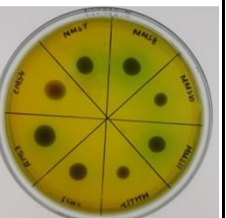
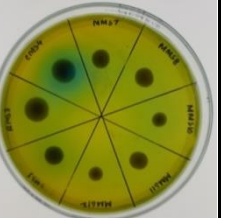
Controls are C1(MCDA) and C2b (CDA with BTB). Test is Tb (MCDA with BTB).

Table 3.16: Yeast isolates producing L-asparaginase screened using PR dye

Time	24h	48h	72h	96h	120h
C1					
C2a					
Ta					

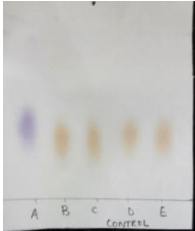
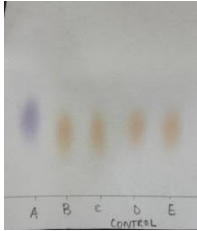
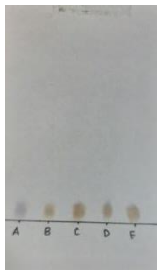
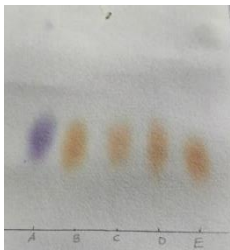
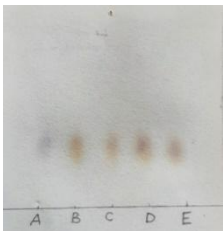
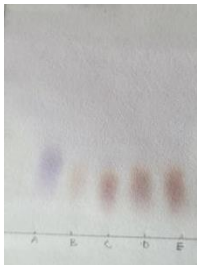
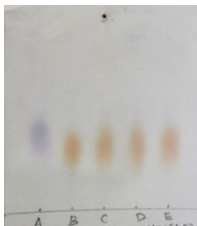
Controls are C1(MCDA) and C2a (CDA with PR). Test is Ta (MCDA with PR).

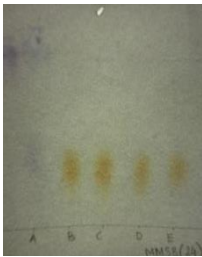
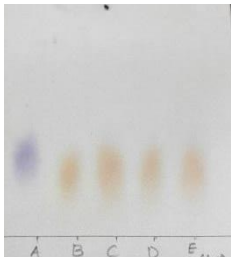
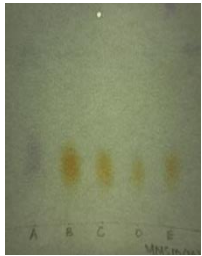
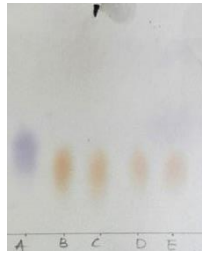
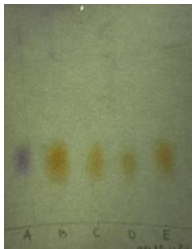
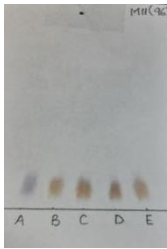
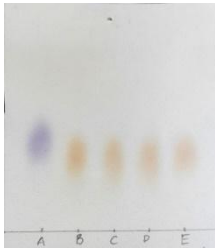
Table 3.17: Yeast isolates producing L-asparaginase screened using BTB dye

Time	24h	48h	72h	96h	120h
C1					
C2b					
Tb					

Controls are C1(MCDA) and C2b (CDA with BTB). Test is Tb (MCDA with BTB).

Table 3.18: Chromatograms of the L-asparaginase positive isolates. a. CFS collected after 24h of incubation, b. CFS collected after 96h of incubation. In the Chromatogram, the spots are named ‘A’- L-aspartate, ‘B’- L-asparagine and ‘C’, ‘D’ and ‘E’ – CFS.

Isolate	a. 24h	b. 96h
Control		
SMS1		
RMS3		
CMS4		
MMS7		

MMS8		
MMS10		
MMS11		
MMS12		

3.3.2 Glutaminase activity of the positive isolates

The isolates positive for L-asparaginase production were then tested using a plate assay method for L-glutaminase activity. The isolates showed growth and no zone around the colony, as presented in Table 3.20.

3.3.3 Morphology and physiological studies of the L-asparaginase positive isolates

The preliminary identification of the positive isolates using Hichrome *Candida* differential agar aided in distinguishing the isolates belonging to the genus *Candida* on the basis of the colony color. *C. albicans* typically produces light green colonies, *C. tropicalis* metallic blue to purple colonies, *C. krusei* pink, fuzzy, spreading colonies, and *C. glabrata* mauve to cream colonies. Images of the positive isolates grown on chromogenic agar displaying

colour are compiled in Figure 3.1 and Table 3.19. Isolate CMS4, produced a blue colour, indicating *Candida tropicalis*. Isolate RMS3 produced pink colour while isolates MMS7, MMS8, MMS10 and MMS11 displayed off-white to pale pink colour. Isolates SMS1 and MMS12 showed off-white coloured growth. The structural details including size, morphology and budding phenomenon of the L-asparaginase positive isolates were elucidated using the Scanning Electron Microscopy technique. The images obtained are presented in Figure 3.2. The positive isolates were displayed as oval shaped cells with some cells showing budding in yeast.

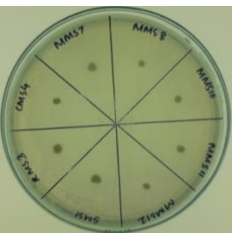
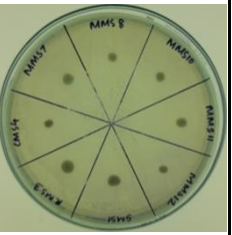
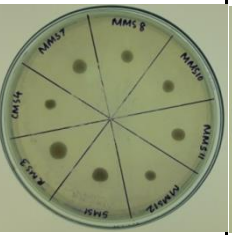
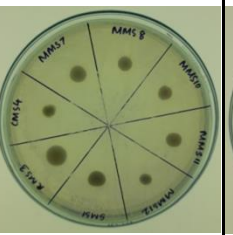
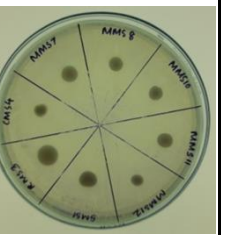
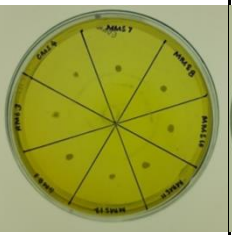
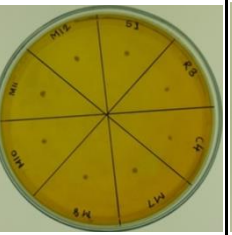
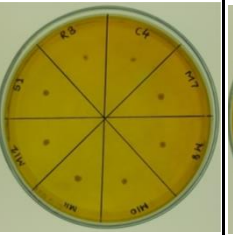
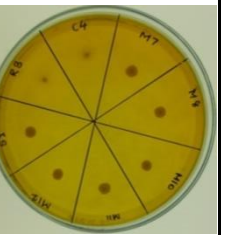
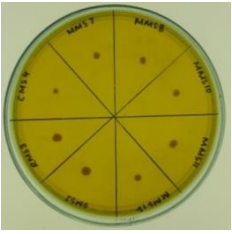
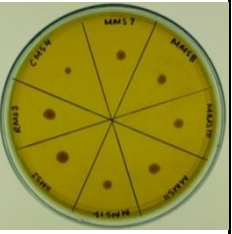
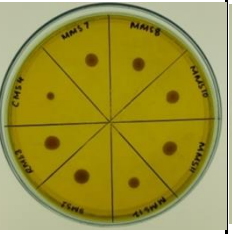
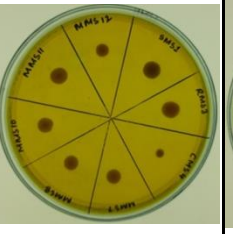
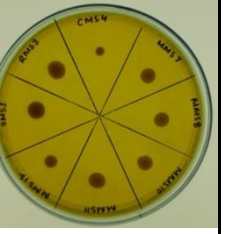
Table 3.19 Preliminary identification of isolates grown on Hichrome *Candida* differential medium

Isolate	Colour of the colony	Preliminary identification
SMS1	Off white	<i>Candida glabarata</i>
RMS3	Pink	-
CMS4	Blue	<i>Candida tropicalis</i>
MMS7	Off white to pale pink	-
MMS8	Off white to pale pink	-
MMS10	Off white to pale pink	-
MMS11	Off white to pale pink	-
MMS12	Off white	<i>Candida glabarata</i>

3.3.4 Quantitative estimation of L-asparaginase production by yeast isolates

The quantitative analysis of the eight positive isolates for enzyme production for a period of 24 to 120 h was conducted by Nessler's method as depicted in Figure 3.3a. The positive isolates all showed enzyme activity at 96 h of incubation, as seen in Figure 3.3b. Isolate CMS4 has the highest enzyme activity (35.03 U/mL), followed by MMS8 (31.52 U/mL). Enzyme activity of isolates CMS4 ([#]) and MMS8 (^{\$}) showed a significant difference ($p < 0.05$), in comparison with other positive isolates as presented in Figure 3.3b. Hence, isolates CMS4 and MMS8 were selected for further studies.

Table 3.20: Glutaminase assay of the L-asparaginase positive isolates

Time	24h	48h	72h	96h	120h
C					
C'					
T					

The controls are C(MCDA), C' (CDA with BTB). Test is T (MCDA with BTB)

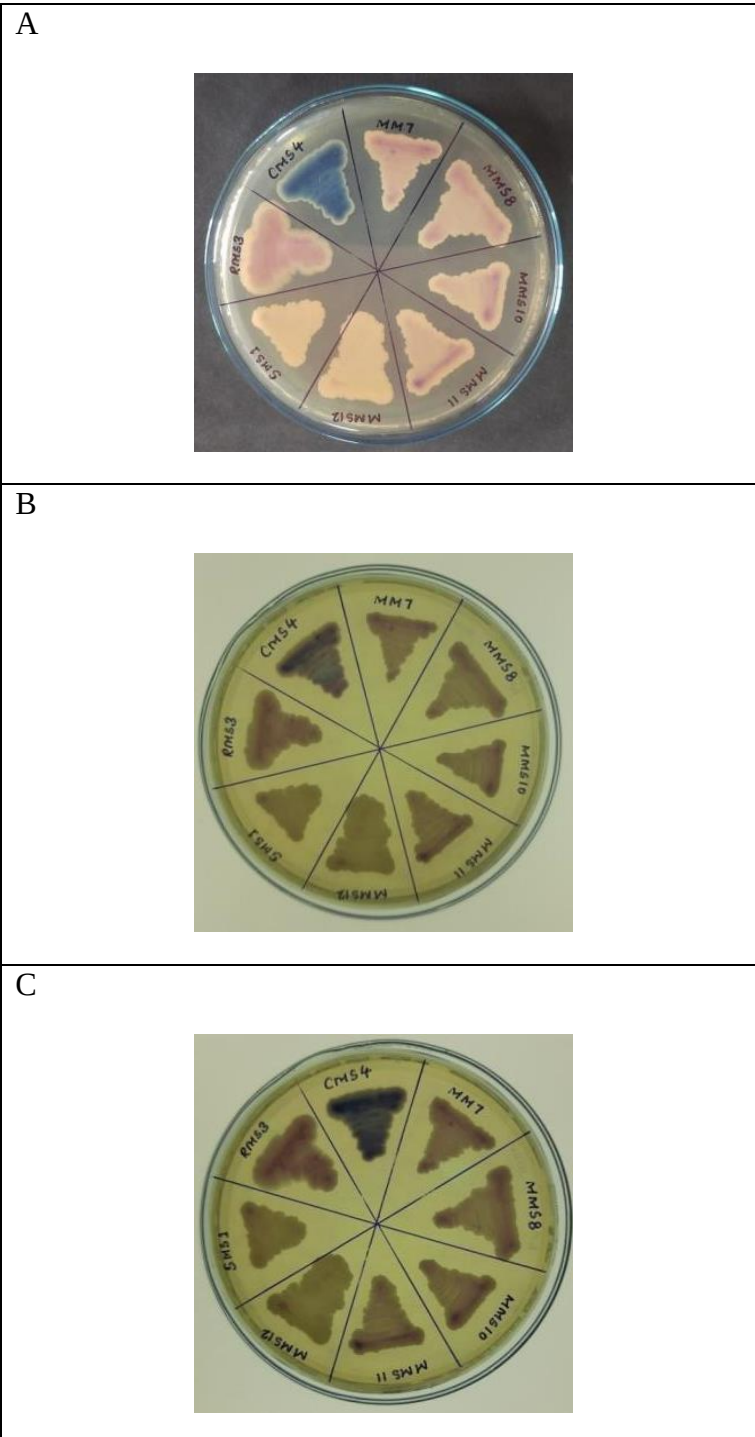


Figure 3.1: Positive isolates on Hichrome candida differential agar incubated for a. 24 h, b. 48h and c. 72h

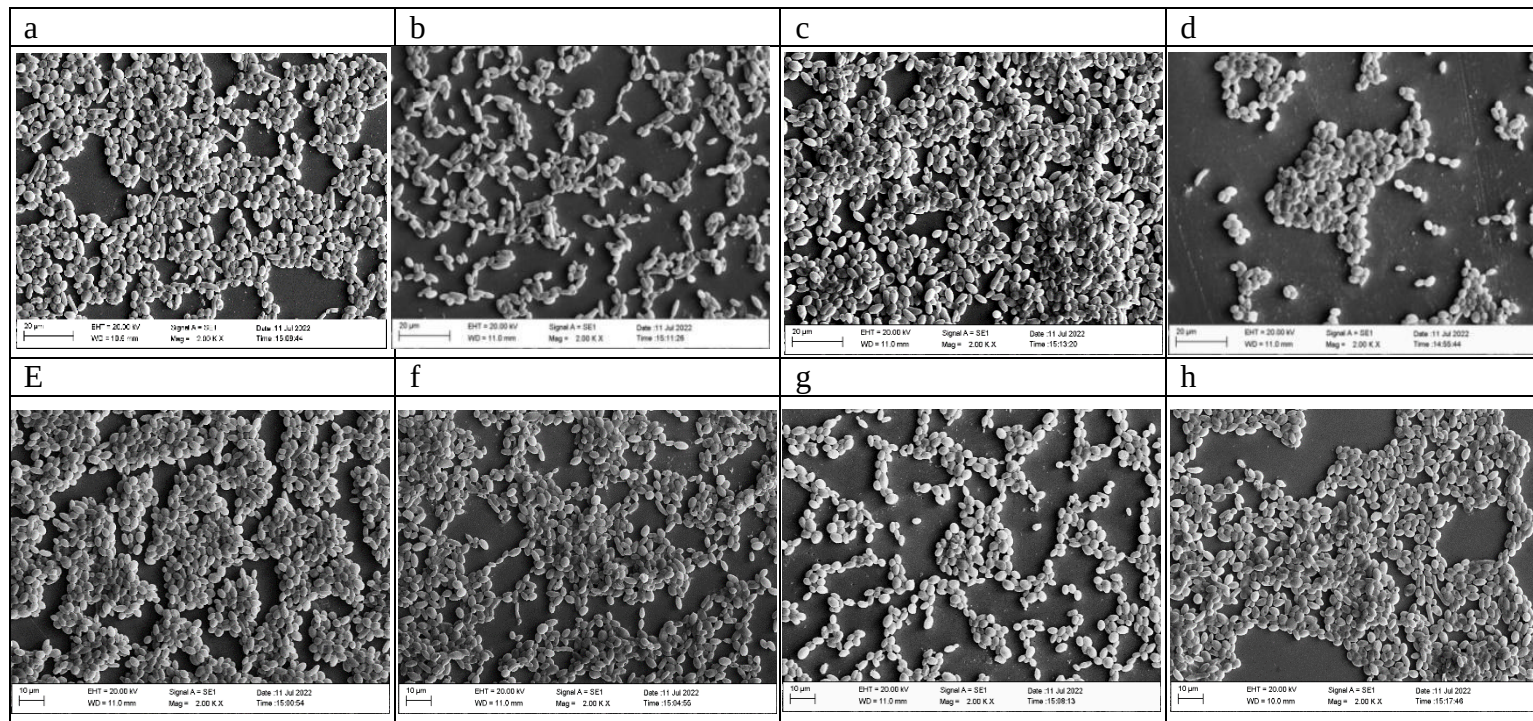


Figure 3.2: Scanning electron microscopy images of L-asparaginase positive isolates. a. SMS1, b. RMS3, c. CMS4, d. MMS7, e. MMS8, f. MMS10, g. MMS11 and h. MMS12

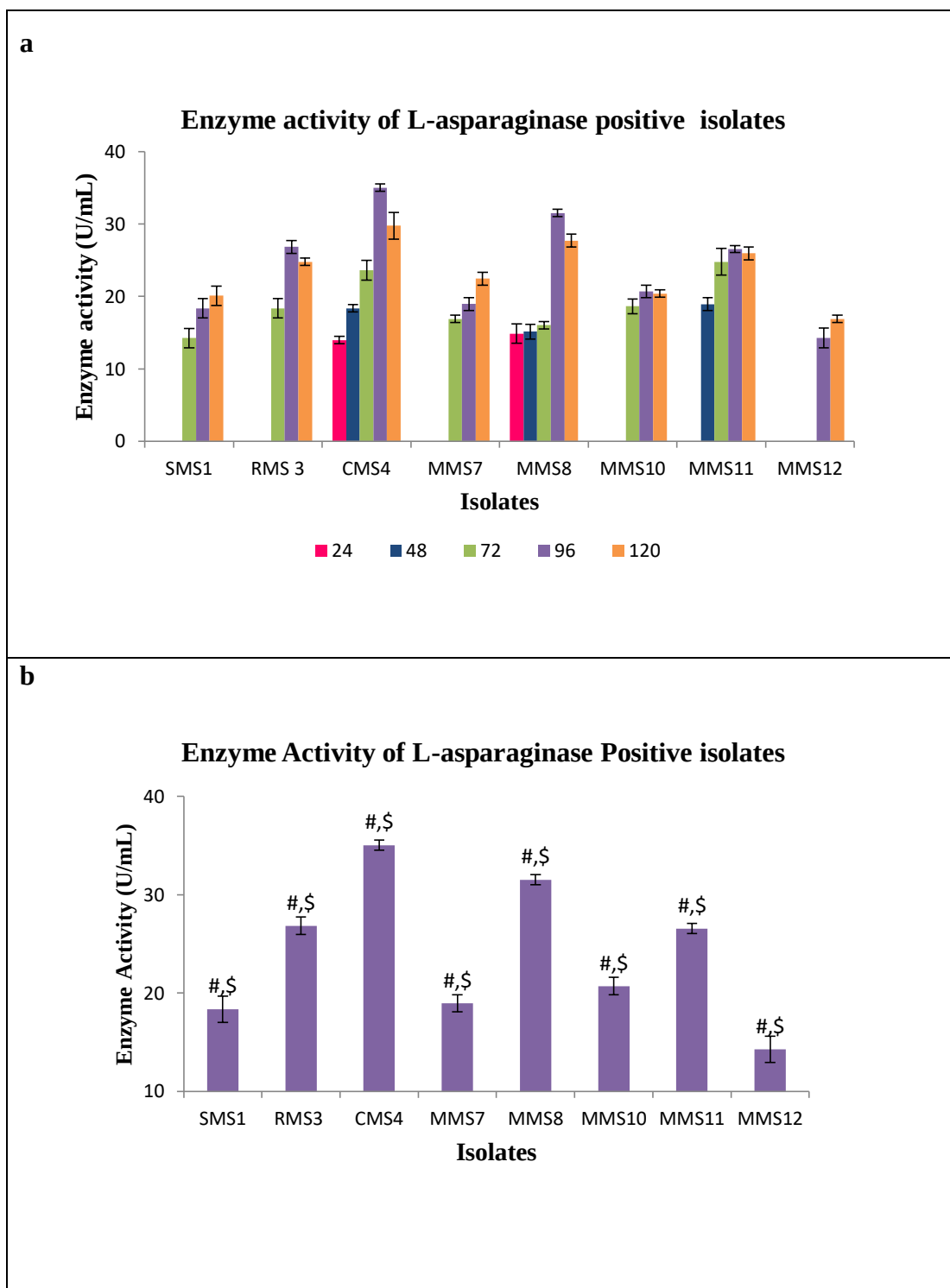


Figure 3.3: Enzyme activity of the 8 L-asparaginase positive isolates. a. Over 120 h of incubation period and b. at 96 h of incubation. The symbol ‘#’ for CMS4 and ‘\$’ for MMS8 is used to represent a comparatively statistically significant enzyme activity value.

3.3.5 Identification of CMS4 and MMS8

The selected isolates MMS8 and CMS4 were identified using morphological, biochemical, followed by molecular methods. The morphological identification included colony characters as seen in Table 3.21 CMS4 displays a white-colored, circular, smooth, mucoid and elevated colony with an entire margin. Isolate MMS8 exhibits a white-colored, circular, smooth, mucoid and raised colony with an entire margin. Structural details of the cells obtained by SEM, showing a characteristic oval shape having a 3µm length are displayed in Table 3.21. The biochemical characteristics of the isolate are summarized in Table 3.22. Isolates CMS4 and MMS8 were able to assimilate simple carbohydrates including glucose, fructose and sucrose. Complex carbohydrates and sugar alcohols were not utilized by the selected isolates.

The molecular identification was based on the sequencing of the ITS region consisting of ITS1, 5.8S rRNA and ITS2. The consensus sequence was submitted to GenBank and the nucleotide sequence accession numbers OR113692 (CMS4) and OR095858 (MMS8) were obtained. The phylogenetic relationship of the isolates with that of previously cultured species was established by the neighbor joining method using MEGA 11 software to construct a phylogenetic tree, illustrated in Figure 3.4 and Figure 3.5 respectively. BLAST analysis of isolate CMS4 showed >98% similarity with species belonging to Genus *Candida*, with closest identity to *Candida tropicalis*. BLAST analysis of isolate MMS8 showed >98% identity with species belonging to Genus *Cyberlindnera*, with closest identity to *Cyberlindnera subsufficiens* (99.23%) followed by *Cyberlindnera saturnus* (98.65%).

Table 3.21: Morphological analysis of the selected isolates CMS4 and MMS8.

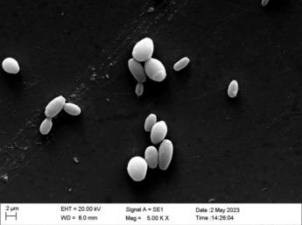
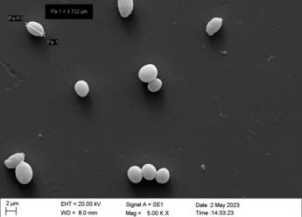
Isolate	A. Grown on YMA	B. SEM images	
CMS4			
MMS8			

Table 3.22: Biochemical characterization of selected isolates CMS4 and MMS8

Biochemical Tests	Results	
	MMS8	CMS4
Carbohydrate assimilation		
Lactose	-	-
Xylose	-	-
Maltose	-	+
Fructose	+	+
Dextrose	+	+
Galactose	-	+
Sucrose	+	+
L-Arabinose	-	-
Mannose	+	+
Inulin	+	+
Raffinose	-	-
Trehalose	-	+
Melibiose	-	-
Sodium gluconate	-	-
Glycerol	-	-
Salicin	-	-
Adonitol	-	-
Dulcitol	-	-
Inositol	-	-
Sorbitol	-	-

Mannitol	-	-
Arabitol	-	-
Erythritol	-	-
Xylitol	-	-
D-Arabinose	-	-
Sorbose	-	-
Cellulose	-	-
Starch	-	-
Temperature		
25 °C	+	+
28 °C	+	+
30 °C	+	+
37 °C	-	+
Growth on media with high osmotic pressure		
1% Sodium Chloride	+	+
10% Sodium Chloride	-	-
5% Glucose	+	+
10% Glucose	+	+

‘+’= positive and ‘-’= negative

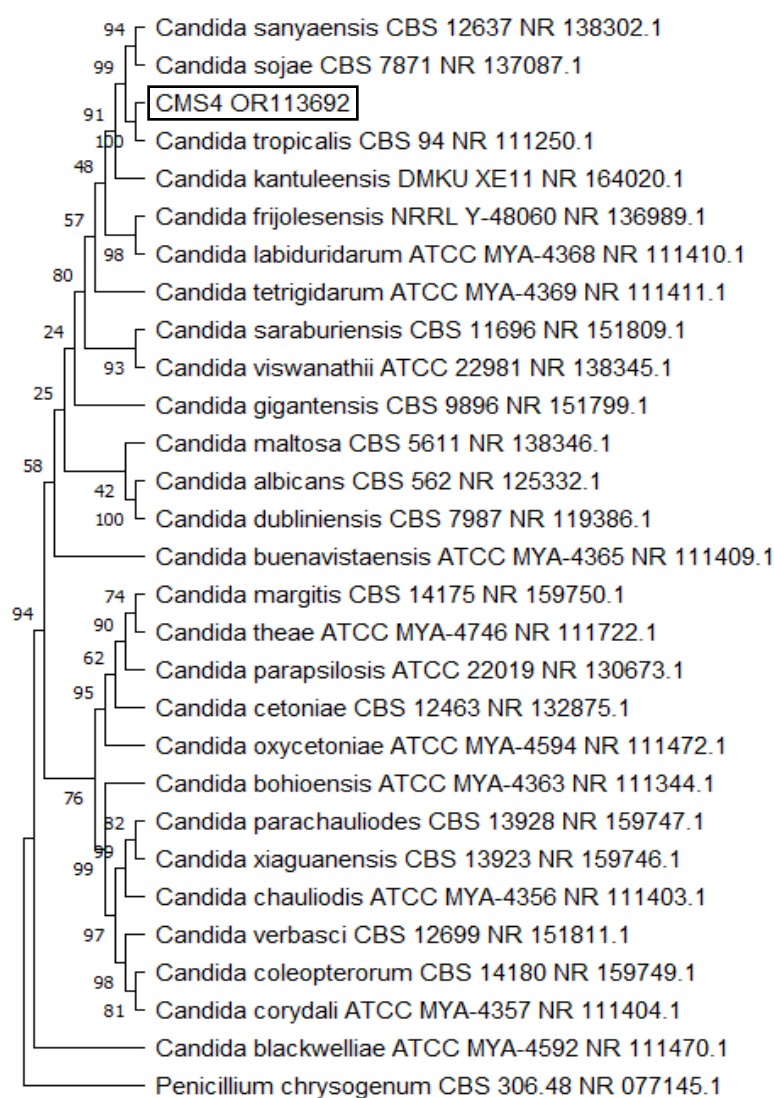


Figure 3.4: Phylogenetic tree of isolate CMS4. The tree was constructed by the Neighbour Joining Method based on the rDNA sequences that included ITS1,5.8s rRNA and ITS2 regions. *Penicillium chrysogenum* was used as an outgroup. The numbers at the nodes indicate the bootstrap values (percentage) resulting from 10000 replicates.

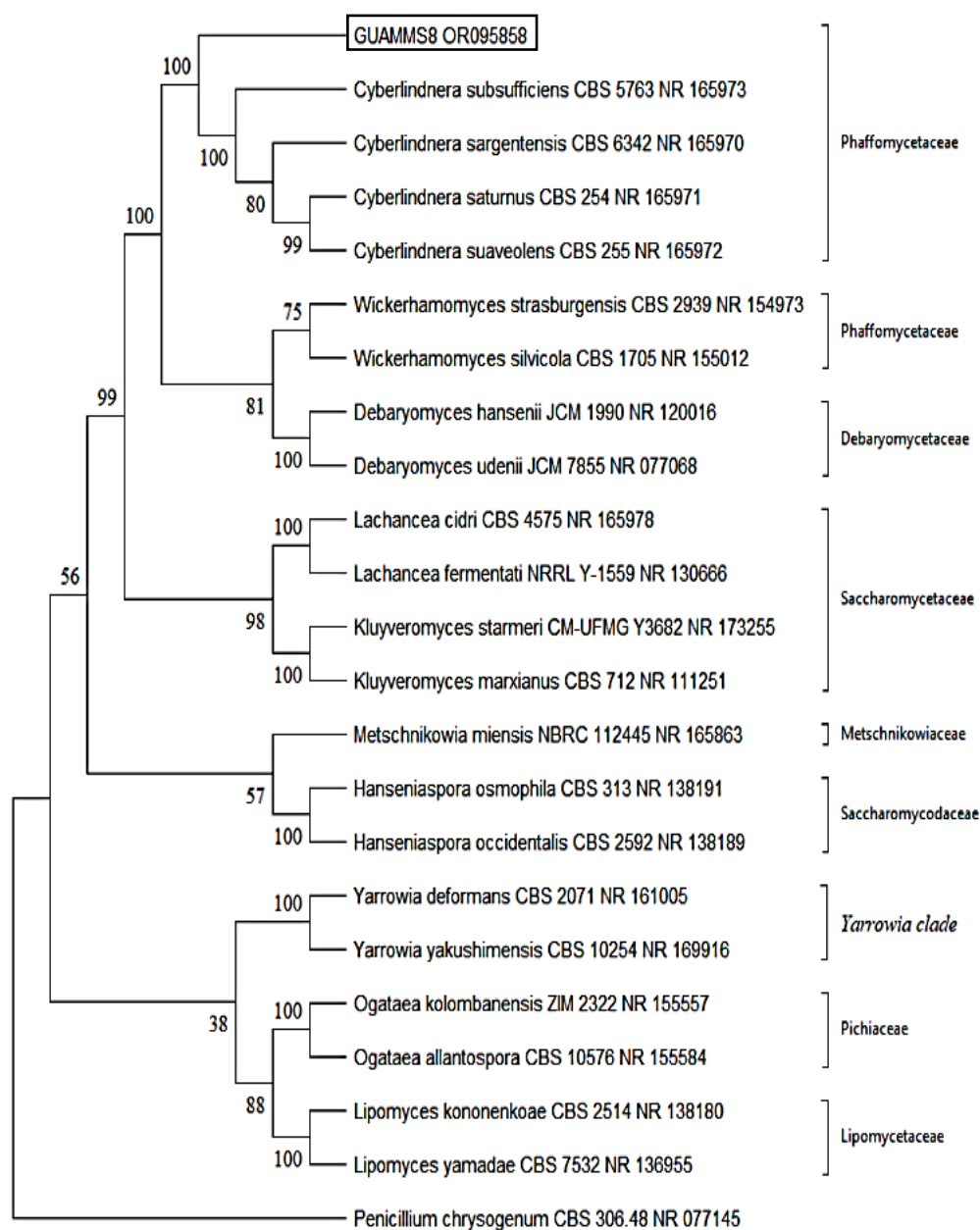


Figure 3.5: Phylogenetic position of isolate MMS8. The tree was constructed by the Neighbour Joining Method based on the rDNA sequences that included ITS1, 5.8s rRNA and ITS2 regions. *Penicillium chrysogenum* was used as an outgroup. The numbers at the nodes indicate the bootstrap values (percentage) resulting from 10000 replicates.

3.4 Discussion

This study investigated yeast obtained from mangrove sediments as a potential source of antineoplastic enzyme L-asparaginase. Eight isolates tested positive for L-asparaginase activity on screening using two different indicator dyes. The L-asparaginase positive isolates displayed no glutaminase activity. El-Naggar et al. (2016) reported successful screening of actinomycetes for L-asparaginase production using a plate assay containing PR. In a study on fungal species isolated from Antarctic moss and soil by Ashok et al. (2019), effective screening of 30 glutaminase free L-asparaginase producing fungal isolates was reported using the rapid plate assay technique with PR and BTB. Alrumman et al. (2019) isolated 40 marine bacteria from three coastal sites of Red Sea of Saudi Arabia and screened them for L-asparaginase production using the plate assay method with PR to obtain 28 L-asparaginase producing bacteria.

L-asparaginase activity of the positive isolates was determined using the Nesslerization method. From these, isolates CMS4 and MMS8, having the highest enzyme activity, were selected for further study to examine the influence of medium composition and parameters on enzyme production. In a study on actinomycetes isolated from soil sample by El-Naggar et al. (2016), L-asparaginase activity of *Streptomyces fradiae* NEAE-82 estimated using Nessler's method was reportedly 29507.688 U. Bhagat et al, (2016) reported 27 endophytic bacteria exhibiting L-asparaginase activity ranging from 0.6 to 2.3 U/mL with chosen isolate *Pseudomonas oryzihabitans* having an activity of 2.1 U/mL. Ashok et al. (2019) reported isolates *Trichosporon asahii* IBBLA1 and *Coprinopsis cinerea* IBBLA4 having enzyme activity of 15 U mL⁻¹ and 13.5 U mL⁻¹, respectively, determined using the same method. The L-asparaginase activities exhibited by filamentous fungi and actinomycetes are generally higher, while the activities of the selected isolates, CMS4 (35.03 U/mL) and MMS8 (31.52 U/mL), are comparable to those previously reported from bacteria and yeast.

The potent selected isolates were identified as belonging to genus *Candida* (CMS4) and genus *Cyberlindnera* (MMS8) based on morphological, biochemical and molecular tests. This is the first report on L-asparaginase producing tropical mangrove yeast belonging to the genus *Cyberlindnera*.

Yeasts from the genus *Candida* are ubiquitous and prior reports indicate their abundance in harsh and challenging conditions of the marine environment, including the mangrove forests. *Candida etchellsii* (MY1), *Candida parapsilosis* (MY6) (Kachiprath & Gopi, 2019)

and *C. tropicalis* (Pothayi et al., 2022), obtained from mangrove ecosystem, have been reported as producers of hydrolytic enzymes, including lipase and protease. Manglicolous yeasts belonging to the genus *Candida* have been reported for their use as food supplements (Kachiprath & Gopi, 2019), phosphate solubilizers (Nurhariyati et al., 2020) and as producers of biosurfactants (Nimsi et al., 2022).

The production of L-asparaginase by isolate CMS4, identified as member of genus *Candida*, further highlights the ability of yeasts within this genus to produce bioactive compounds with biotechnological. *Candida tropicalis* is a recognized opportunistic pathogenic yeast and the study of enzyme L-asparaginase from isolate CMS4 may help in broadening the understanding of this enzyme of eukaryotic origin, including the development of safer variants through recombinant expression or purification approaches.

In the food and pharmaceutical industries, the most preferred microorganisms are those categorized as generally recognized as safe (GRAS). Yeasts from the genus *Cyberlindnera* find application in the fermentation and cheese-making industry (Kamat et al. 2013). This indicates that the enzyme produced by the selected isolate MMS8 has probable potential for the development of future medical formulations.

3.5 Conclusion

This is a primary study of yeast isolated from sediments of the mangrove forest of Goa, India for their potential as a source of enzyme L-asparaginase. Screening by the rapid plate assay method with two indicator dyes followed by confirmation by paper chromatography, resulted in obtaining eight glutaminase free L-asparaginase positive isolates from a total of 53. The selected potent isolates CMS4 and MMS8, were identified by morphological, biochemical and molecular methods as belonging to genus *Candida* and genus *Cyberlindnera*, respectively. This is the first report on L-asparaginase producing tropical mangrove yeast belonging to the genus *Cyberlindnera*.

CHAPTER IV

**OPTIMIZATION AND CHARACTERIZATION OF
L-ASPARAGINASE FROM SELECTED ISOLATE**

4.1 Introduction

L-asparaginase is an enzyme of clinical and industrial importance. It is widely used for the treatment of Acute Lymphoblastic Leukemia (ALL) as well as in the food industry to curb acrylamide formation during high-temperature food manufacturing. The broad application of this enzyme has led to extensive research interest in achieving high-yield and stable production (Batoool et al. 2016; Cachumba et al. 2016). Yeast are an advantageous microbial source for enzyme production due to their rapid growth rates, generally regarded as safe (GRAS) status, and ability to carry out post-translational modifications in the synthesized protein (Kamat et al. 2013; Darvishi et al. 2018).

Medium and process optimization is essential to enhance enzyme production to a level substantial enough for industrial use. The factors that influence microbial metabolism and the associated pathways for enzyme production and release include carbon and nitrogen source concentration, pH, temperature, incubation period, inoculum size, and salinity. Optimization of these parameters aids in the enhancement of L-asparaginase production. Enzyme production by a microorganism can be improved by systematically evaluating different nutritional components, their concentration in the medium and the pertinent physicochemical parameters, subsequently implementing the conditions having a significant and positive impact. An affordable enzyme production process, including the utilization of inexpensive and nutrient-rich substrates, is beneficial for scaling up the process (Freire et al. 2021; Moguel et al. 2022).

Enzyme production in yeast is influenced by the type and concentration of carbon and nitrogen sources incorporated in the fermentation medium. In yeast, the pathways involved in carbohydrate utilization follow a similar pattern, with notable biochemical and genetic variations among species. Catabolite repression leads to the selective expression of genes responsible for sugar assimilation, making choice of carbon source a regulatory factor affecting enzyme production. Enzymes involved in amino acid assimilation and conversion including proteases, amidases and ureases are modulated by nitrogen catabolite repression, characterized by the preferential utilization of readily assimilable nitrogen sources among those available (Moguel et al. 2022).

The preliminary optimization method used is One Factor at a Time (OFAT). To understand the influence of an individual factor, a single factor is altered while keeping the others

constant. This approach results in the identification of key influential parameters and offers a limited understanding of the interactive effects among the factors examined. OFAT optimization yields conditions that support the process rather than true optimum conditions and it requires relatively exhaustive experimentation. OFAT method is used as an initial screening tool to establish the important factors and process parameters (Priya et al. 2011; Ashok et al. 2019; Freire et al. 2021).

RSM is a statistical optimization approach that helps in overcoming the limitations presented by the OFAT method. In this approach, the simultaneous evaluation of multiple interacting factors and parameters enables the determination of optimal conditions. RSM designs including Central Composite Design (CCD) and Box–Behnken Design allow determination of optimal conditions with fewer experimental trials (Meena et al., 2015; Friere et al. 2021; Moguel et al. 2022).

CCD is a highly efficient design incorporating factorial, axial and central points to estimate both linear and second-order effects. The development of a predictive polynomial model by CCD describes the interactions between the factors and the enzyme produced, enabling the prediction and optimization of the response within the experimental range. It generates three-dimensional response surfaces and contour plots that illustrate the effect of factors on enzyme yield. Thus, CCD enables the statistical validation of optimized conditions with comparatively fewer experiments, providing an in-depth understanding of factor interactions on enzyme production (Darvishi et al., 2018; Moguel et al., 2022; Ratuchne et al., 2023).

Protein purification is an essential step following the enhanced production of an enzyme. Protein purification aids the determination of optimal parameters to ensure maximum enzyme action including the enzyme stability and specificity. Protein free from contaminants is more effective and safer as a chemotherapeutic agent. Enzyme free from L-glutaminase activity is preferred to reduce the deleterious effects. Reports suggest the implementation of multiple methodologies to obtain purified L-asparaginase having potential application as exhibited in Table 1.4. The biochemical and kinetic properties of enzymes are dependent on the source of the enzyme. Yeast produces two types of L-asparaginase, the intracellular type I and the extracellular type II. Extracellular L-asparaginase is advantageous because it accumulates directly in the culture broth,

facilitating simplified recovery, enabling efficient and cost-effective downstream processing (Meena et al., 2015; de Castro Girao et al. 2016; El-Naggar et al., 2016).

L-asparaginase enzyme has extensive applications in the health and food industries. Current knowledge about L-asparaginase of yeast origin is limited and needs further exploration. Comprehensive characterization of the enzyme, including the determination of its kinetic parameters and thermal stability, is essential for assessing its suitability, enhancing its functional performance and confirming its potential for industrial application (Dias et al., 2019).

4.2 Methodology

4.2.1 Effect of physical parameters, carbon and nitrogen sources on L-asparaginase production by selected isolates

A study on the effect of medium composition and physical parameters on enzyme production by the identified potent isolates was carried out using one factor at a time approach (Priya et al. 2011; Ashok et al. 2019; Freire et al. 2021). MCDB medium was used and the influence of physical parameters consisting of incubation time from 12 to 120 h, temperature ranging from 25 °C to 37 °C and pH of medium in the range from 5.8 to 7.6 on enzyme production was analysed (Moguel et al. 2022). The study on the effect of carbon sources on enzyme production included glucose, fructose and sucrose (Freire et al. 2021). Nitrogen sources namely peptone, yeast extract, ammonium chloride, sodium nitrate and L-asparagine were studied to understand their influence on enzyme production (Freire et al. 2021; Moguel et al. 2022). Effect of the inoculum size ranging from 1% to 5% on the enzyme production was estimated (Thenmozhi et al., 2011; Freire et al. 2021). Optimized medium composition and physical parameters were determined based on the factors and their variable levels that support maximum enzyme production.

4.2.2 Time course analysis of L-asparaginase production by selected isolates

This study used the respective optimized modified Czapek Dox media with 1% L-asparagine having pH 7.0. The medium was inoculated with 1 % of a 24 h grown culture of isolate MMS8 in Yeast Malt Broth (YMB) after a wash using sterile 0.85% saline solution, having absorbance of 0.5 at 600 nm and incubated at 28 °C. Aliquots of culture broth were collected every 12 h up to 120 h. The collected aliquots were then centrifuged at 10,000 rpm for 10 min at 4 °C to obtain cell free supernatant (Meena et al. 2015). The cell free supernatant was used to estimate L-asparaginase activity (Imada et al. 1973) and

extracellular protein content (Lowry et al. 1951). The pellet obtained was used to determine growth in terms of biomass (mg/mL). The time course analysis indicates the effect of the optimized medium on enzyme production by selected isolates.

4.2.3 Effect of Glucose, L-asparagine and Sodium chloride concentration on enzyme production by MMS8

A study to determine the effect of glucose, L-asparagine and sodium chloride on L-asparaginase production by MMS8 was conducted. MCDB was used for these experiments supplemented with glucose ranging from 1 g/L to 40 g/L, L-asparagine having different concentrations (2.5 g/L to 25 g/L) and sodium chloride in varying concentrations (0.5 to 2.5 %), respectively. The medium was inoculated with 1% inoculum of isolate MMS8 having an absorbance of 0.500 at 600nm. This experiment was performed in triplicate. The flasks were incubated at 28°C, 120 rpm for 5 days. Culture broth was collected aseptically every 24 hours, centrifuged at 10000 rpm for 10 minutes at 4°C. The Cell Free Supernatant (CFS) was used for enzyme assay. Enzyme assay was conducted by Nesslerization method (El-naggar et al., 2016; Imada et al.,1973; Vala et al.,2018). Growth was measured in terms of biomass.

Statistical analysis

The experiments were performed in triplicates. The findings were expressed as mean with their standard deviation. Assessment of the statistically significant impact of varying levels of a factor on enzyme production, measured through enzyme activity and biomass was performed by a one-way analysis of variance (ANOVA) ($p \leq 0.05$) followed by a T-test and Bonferroni post-hoc test. The statistical tests were performed using Microsoft Excel 2021.

4.2.4 Optimization of medium composition by RSM

The Design Expert software (Statease) was used to determine the interactions among the variables and their impact on enzyme production using the Central composite design (CCD) of Response Surface Methodology (RSM). The variables L-asparagine, glucose, and sodium chloride were examined in detail using a 2^3 Central Composite Design (CCD). A total of 20 experimental runs were conducted and the three selected factors were evaluated at five levels ($-\alpha$, -1,0,1, α ; with $\alpha= 1.6817$). Experimental

error was assessed by including six replicates at the central point (0,0,0; runs 9 to 14) (Darvishi et al., 2018; Moguel et al., 2022; Ratuchne et al., 2023).

The variations in the response relative to the evaluated factors were represented by a quadratic model, shown in Equation (4.1). In this model, Y denotes the predicted response; β_0 is the intercept; β_i represents the linear effect coefficients; β_{ii} corresponds to the quadratic effect coefficients; β_{ij} indicates the interaction (cross-product) coefficients; X refers to the independent variables; and ε represents the experimental error.

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum \beta_{ij} X_i X_j + \varepsilon \quad (4.1)$$

A validation experiment was carried out under the selected optimal conditions in triplicate to confirm the effectiveness of the optimized medium formulation. The enzyme production was carried out in 100 mL optimized media in a 250 mL flask, incubated at 28 °C and 120 rpm for 96 hours.

4.2.5 Partial purification of enzyme L-asparaginase

The partial purification of enzyme was carried out in multiple phases involving ammonium sulphate precipitation followed by dialysis and size exclusion chromatography. Protein content and enzyme activity were determined at each step.

4.2.5.1. Ammonium sulphate precipitation

Protein was extracted from the crude extract by ammonium sulphate precipitation method (Meena et al., 2015, El-Naggar et al., 2016). Precipitate was obtained at 25% saturation which was later separated by centrifugation at 10,000 rpm at 4°C. Further this precipitate was dialysed against 0.05M Tris buffer pH 7.2 at 4°C for 4 hrs with continuous replacement of the buffer.

4.2.5.2 Size Exclusion Chromatography

The dialysed fraction was purified further by size exclusion chromatography. The column consisted of Sephadex G100 in 0.05 M Tris Buffer pH 7.2. The inner diameter of column was 2.5 cm having a bed height of 18 cm and void volume 15 mL with 2.5 mL/min flow rate. 1 mL of the sample was placed on the column and eluted using the same buffer. Fractions of 2 mL were collected and analysed for enzyme activity by Nessler's method and protein content using Folin's Phenol reagent. In total 100 fractions were collected and

fractions showing highest enzyme activity were pooled together and used for enzyme characterization.

4.2.6 Estimation of molecular weight using SDS PAGE technique

The molecular weight of the extracted enzyme was determined by performing SDS-PAGE (sodium dodecyl sulfate–polyacrylamide gel electrophoresis) according to the method described by Laemmli, with a separating acrylamide gel of 8% and a stacking gel 5% containing 0.1% SDS. The protein gels were stained with a silver staining method. The molecular mass of the protein bands were compared to the bands obtained using Protein Molecular Weight Marker (Genei) ranging from 29 to 205 kDa

4.2.7 Characterization of the partially purified L-asparaginase

4.2.7.1 Effect on physical and reaction parameters on enzyme activity

L-asparaginase activity of partially purified enzyme over a wide range of pH, incubation time temperature and substrate concentration was evaluated. The reaction mixture conditions were the same as described in section 3.2.5 with appropriate changes according to the parameter evaluated.

4.2.7.1.1 Effect of incubation time on L-asparaginase activity

The stability of partially purified enzyme was determined by incubating it for 3 hr at 37 °C with reaction mixture containing 0.04 M L-asparagine in 0.5 M tris buffer pH 7.2 (Meena et al., 2015, El-Naggar et al., 2016)

4.2.7.1.2 Effect of incubation temperature on L-asparaginase activity

The optimal temperature for partially purified enzyme was determined by incubating enzyme-substrate mixtures for 30 min in the temperature range of 25 to 45 °C at pH 7.2, and enzyme activity was determined by Nessler's method (Meena et al., 2015, El-Naggar et al., 2016)

4.2.7.1.3 Effect of pH on L-asparaginase activity

The impact of pH on partially purified enzyme was studied within the range of 4.0 to 10.5. This was accomplished using a 15 mM sodium acetate buffer for pH 4.0 to 6.0, potassium phosphate buffer for pH 6.5 to 8.0 and carbonate-bicarbonate buffer for pH 8.5 to 10.5 (Meena et al., 2015, El-Naggar et al., 2016)

4.2.7.1.4 Effect of substrate concentration on L-asparaginase activity

The effect of substrate concentration on partially purified enzyme was determined by incubating it for 30 min at 37 °C with reaction mixture containing L-asparagine ranging from 0.01 M to 0.1M in 0.5 M tris buffer pH 7.2 (Meena et al., 2015, El-Naggar et al., 2016).

4.2.7.2 Enzyme stability

4.2.7.2.1 Effect of physio-chemical parameters

The enzyme stability was determined under the optimal conditions over a time period of 5 h. In order to determine the pH stability of the enzyme under different pH the purified enzyme was suspended in buffers with varying pH in a 1:1 ratio and stored at 25 °C for 24 h. The treated enzyme was then used to estimate L-asparaginase activity (Ratuchne et al., 2023).

4.2.7.2.2 Effect of metal ions

The effect of metal ions on the enzyme activity of purified enzyme was studied. The enzyme was incubated with 1mM solution of the metal salts in ratio 1:1 for 1 h at 25°C. L-asparaginase activity of the enzyme after treatment was measured (Meena et al., 2015).

4.2.7.2.3 Effect of inhibitors

The impact of inhibitors on L-asparaginase activity was assessed by incubating enzyme with the inhibitors in 1:1 ratio for 30 min at 25°C and later determining the activity of the treated enzyme. The inhibitors tested included 2.5 mM Urea, 2mM EDTA, 2% SDS, 1% Tween 20 and 1% Triton (Meena et al., 2015).

Statistical analysis

All experiments were performed in triplicate; the data are presented as mean $\pm$ standard deviation (SD). Statistical analyses were carried out using SPSS (version 21). The significance of differences among groups was assessed by one-way ANOVA followed by Tukey's multiple comparison test, with $p < 0.05$ considered statistically significant. Kinetic parameters (K_m and V_{max}) were determined by non-linear regression analysis using the Michaelis–Menten equation.

4.3 Results

4.3.1 Effect of physical parameters on enzyme production

The monitoring of enzyme activity (U/mL) and growth in terms of biomass (mg/mL) for an interval of 12 h till 120 h as displayed in Figure 4.1 a1 and 4.1 a2 indicates that both enzyme activity and biomass increase steadily, with a distinct increase at 96 h of incubation. On analysing data, it was observed that in comparison with findings at the incubation period of 96 h, the enzyme activity was significantly different over the period from 12 to 96 h ($p < 0.05$), with no significant change ($p > 0.05$) observed further. A significant change in biomass as depicted in Figure 4.1 a2 was observed over 36 h of incubation ($p < 0.05$) followed by similar values ($p > 0.05$) for rest of the incubation period.

The study of the effect of incubation temperature on enzyme production as depicted in Figure 4.1 b1 and 4.1 b2 shows that the enzyme activity and biomass rise with increasing temperature up to 28 °C, having maximum enzyme activity and biomass, declining with further increase in temperature. No growth was observed at 37 °C incubation temperature in case of isolate MMS8 as shown in Figure 4.1 b2. The T-test results indicated notable differences in enzyme activity and biomass over the range of tested temperatures compared to 28 °C ($p < 0.05$). This implies that the optimum temperature for maximum enzyme production for selected isolates is 28 °C.

The effect of the pH of medium on enzyme production by isolates CMS4 and MMS8 as illustrated in Figure 4.2 a1 and 4.2 a2 revealed that the enzyme activity and biomass increase with increasing pH from 5.8 to 6.5 and 7.0, showing highest enzyme activity and biomass at pH 6.5 and 7.0 respectively. However, there is a decline in enzyme activity and biomass at pH 7.6. Among the tested pH of medium, significant distinction in enzyme activity and biomass was observed in comparison with pH 6.5 and 7.0 ($p < 0.05$), indicating that the optimum pH of medium is 6.5 and 7.0 respectively.

The influence of inoculum size on enzyme production by isolates CMS4 and MMS4 as depicted in the Figure 4.2 b1 and 4.2 b2 respectively, indicated a minor increase in enzyme activity and biomass with increase in the inoculum size from 1% to 5%. It showed highest activity and biomass at 2% inoculation of the isolates. The statistical analysis determined no significant ($p > 0.05$) change in enzyme production amongst the tested inoculum sizes.

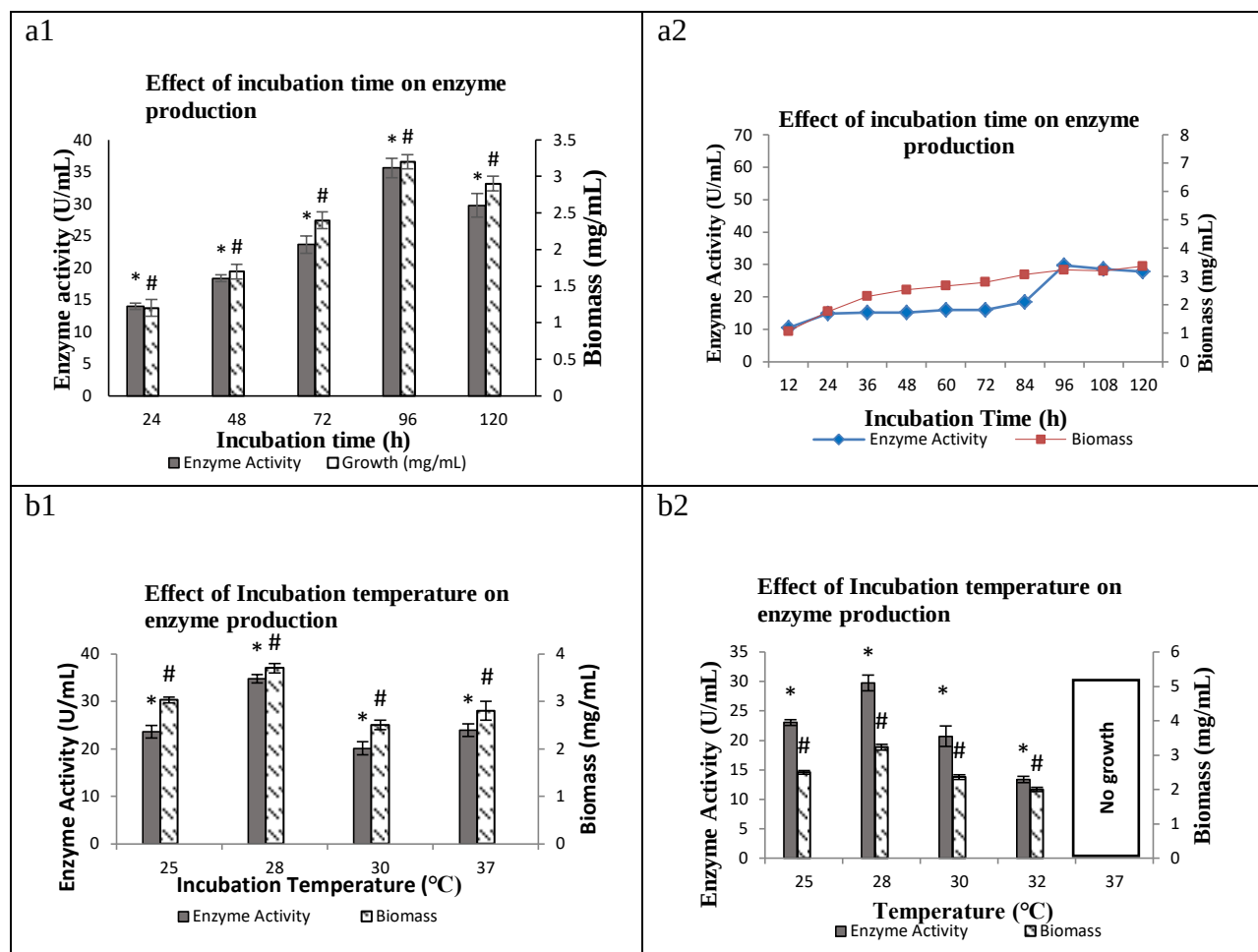


Figure 4.1: Study of effect of physical factors on enzyme production by isolate CMS4 (1) and MMS8 (2): a) Incubation time, b) Incubation temperature. The symbols “*” for enzyme activity and “#” for biomass are used to represent a statistically significant result on comparison with conditions showing maximum enzyme production.

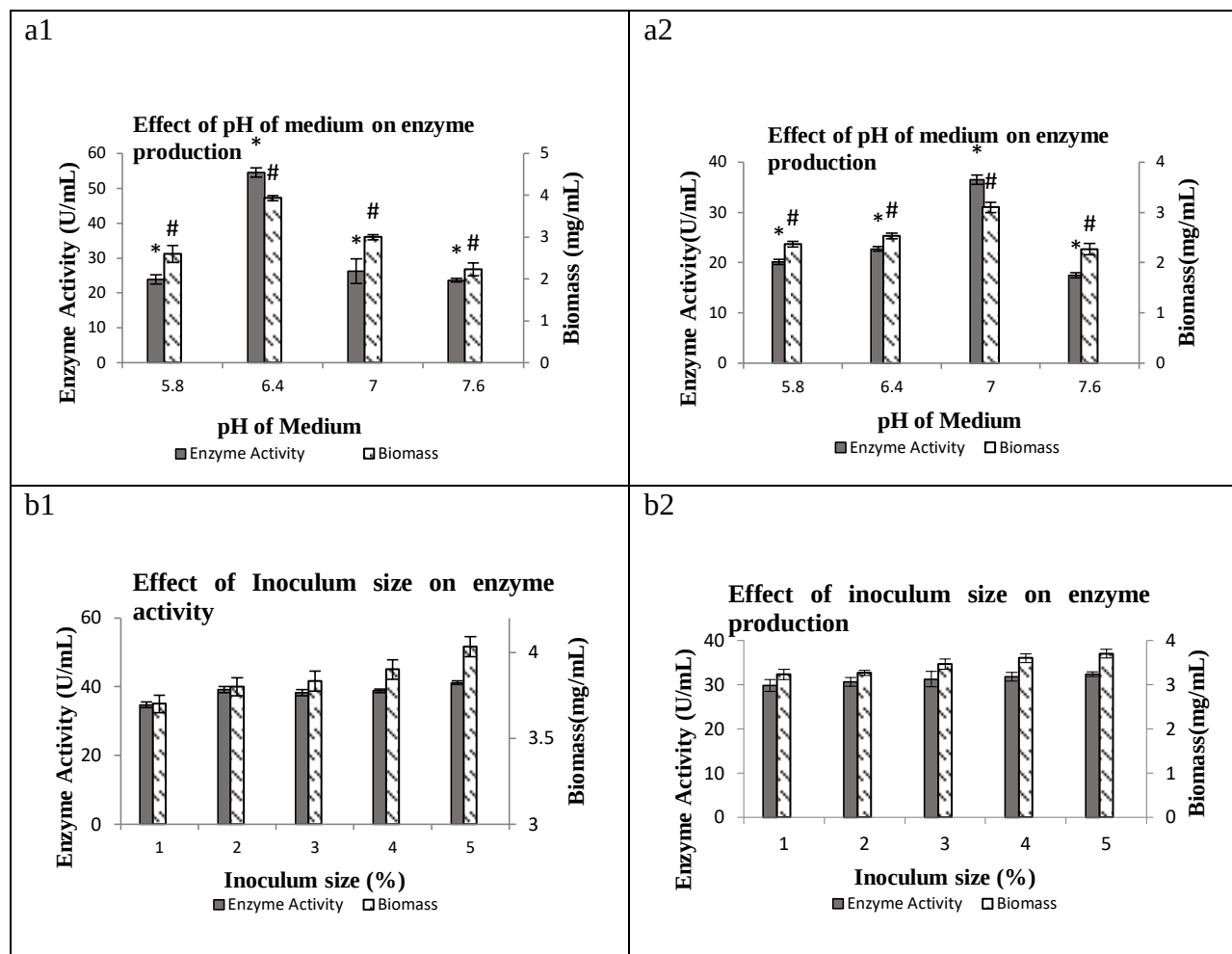


Figure 4.2: Study of effect of physical factors on enzyme production by isolate CMS4 (1) and MMS8 (2): a) pH of Medium, b) Inoculum size. The symbols ‘*’ for enzyme activity and ‘#’ for biomass are used to represent a statistically significant result on comparison with conditions showing maximum enzyme production

4.3.2 Effect of carbon and nitrogen sources on enzyme production

A study of media incorporated with different carbohydrates as carbon sources chosen based on the results of assimilation test (Table 3.22) namely glucose, fructose, maltose, galactose, trehalose and sucrose was conducted. Enzyme production by CMS4 and MMS8 as shown in Figure 4.3 a1 and 4.3 a 2 revealed highest biomass and L-asparaginase activity in the case of glucose as a carbon source followed by fructose and sucrose. Glucose as carbon source showed a statistically significant enzyme activity ($p < 0.05$) and biomass ($p < 0.05$) when compared with fructose and sucrose.

Evaluation of the effect of nitrogen source on enzyme production by CMS4 and MMS8 was conducted using MCDB supplemented with L-asparagine, ammonium chloride, sodium nitrate, peptone and yeast extract as sole nitrogen sources and in combination with L-asparagine. It is seen in Figure 4.3 b1 and 4.3 b2 that maximum growth was recorded when media contained yeast extract followed by peptone and sodium nitrate while the lowest growth was observed in medium containing ammonium chloride.

An enzyme production of 33.57 ± 1.67 U/mL, 29.77 ± 1.34 U/mL was observed when L-asparagine (10g/L) was the sole nitrogen source in the medium respectively. A similar pattern was observed in the study of medium incorporated with nitrogen source (5g/L) in combination with L-asparagine(5g/L) as presented in Figure 4.3 c1 and 4.3 c2. L-asparaginase production was observed when L-asparagine (5g/L) was the sole source of nitrogen. Analysis of biomass production using Bonferroni test for different nitrogen sources implied yeast extract ($p < 0.05$) as an effective nitrogen source.

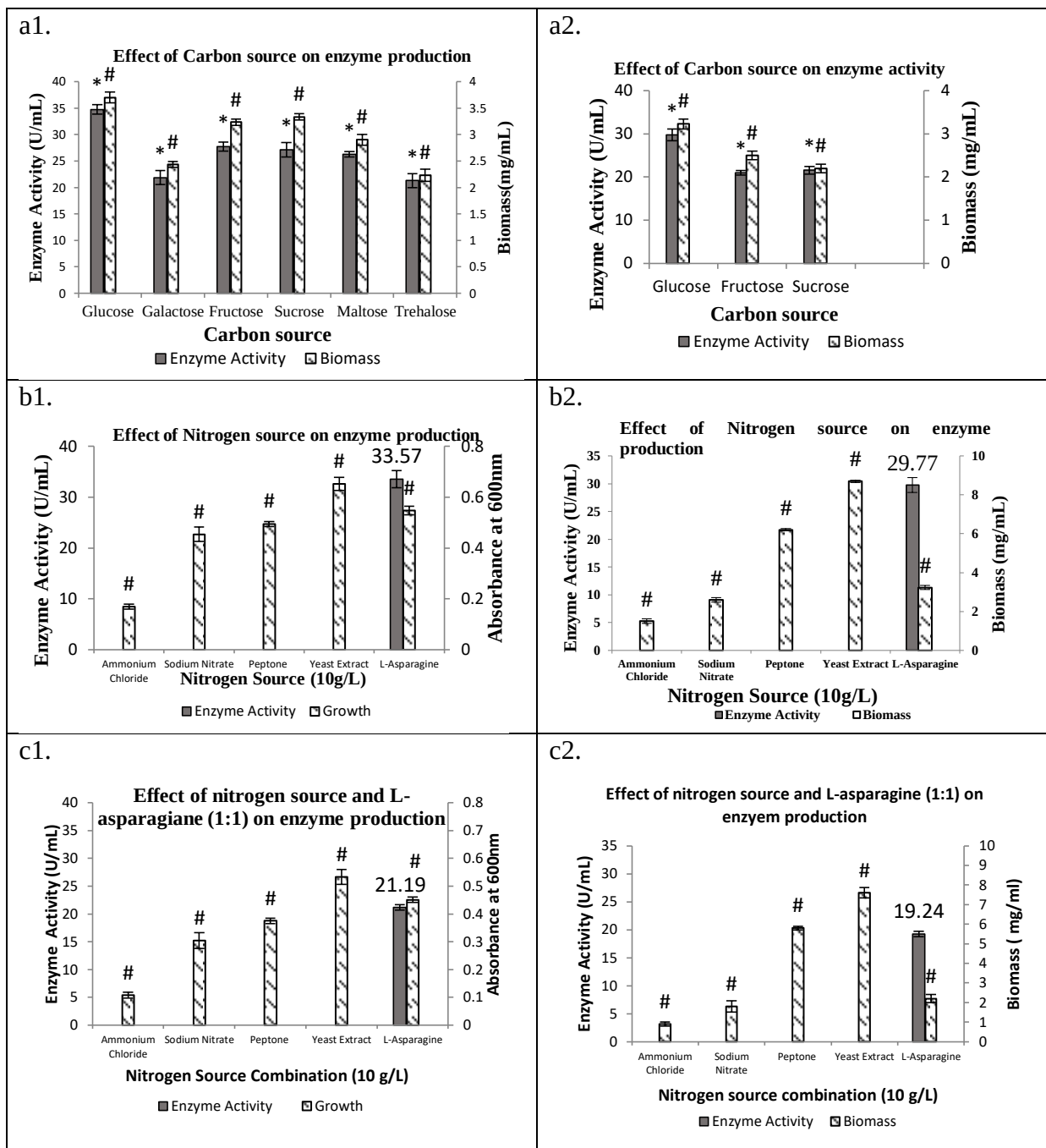


Figure 4.3: Study of effect of nutrition factors on enzyme production by isolate CMS4 (1) and MMS8 (2): a) Carbon source, b) Nitrogen source (10 g/L), c) Combination of Nitrogen source (5 g/L) with L-asparagiane (5 g/L). The symbols ‘*’ for enzyme activity and ‘#’ for biomass are used to represent a statistically significant result on comparison with conditions showing maximum enzyme production

4.3.3 Time course analysis

The L-asparaginase enzyme activity of isolate CMS4 remained constant for a period of 36 h to 78 h as shown in the Figure 4.4 a. A steep increase in enzyme activity and absorbance at 600nm was noted at an incubation time of 96 h followed by a gradual decrease up to 120 h. On performing the Bonferroni post hoc test for overall time course, the results revealed a significant difference in enzyme activity in comparison with 96 h ($p < 0.05$) of incubation period. The test indicated a distinct change in biomass over the incubation period of 120 h ($p < 0.05$).

The L-asparaginase enzyme activity of isolate MMS8 remained constant for a period of 24 h as shown in the Figure 4.4 b. A steep increase in enzyme activity and biomass was noted at an incubation time of 48 h followed by a gradual decrease up to 120 h. The extracellular protein content as shown in Figure 4.4 b, increased sharply during a 48 h incubation period and declined after 96 h. On performing the Bonferroni post hoc test for overall time course, the results revealed a significant difference in enzyme activity in comparison with 48 h ($p < 0.05$) of incubation period. The test indicated a distinct change in biomass over the incubation period of 120 h ($p < 0.05$) except at 60 h and 72 h ($p > 0.05$) of incubation. This analysis implied a notable difference in protein content during the 120 h incubation period compared to findings at 48 h ($p < 0.05$) with similar values at 36 h and 60 h ($p > 0.05$) of incubation.

On using modified Czapek Dox medium, the enzyme activity of the isolates CMS4 and MMS8 was determined to be 33.57 ± 1.67 U/mL and 29.77 ± 1.34 U/mL, respectively, after an incubation period of 96 h. It was observed that under optimal medium composition and parameter conditions that include glucose as carbon source, L-asparagine as the sole nitrogen source and an initial pH of 6.5 for CMS4 and 7.0 for MMS8 with an incubation temperature of 28 °C, resulted in enhanced enzyme production. The enzyme activity of the isolate CMS4 increased to 52.57 ± 1.34 U/mL after 96 h, while isolate MMS8 showed a two-fold increase reaching 57.54 ± 0.88 U/mL after 48 h of incubation.

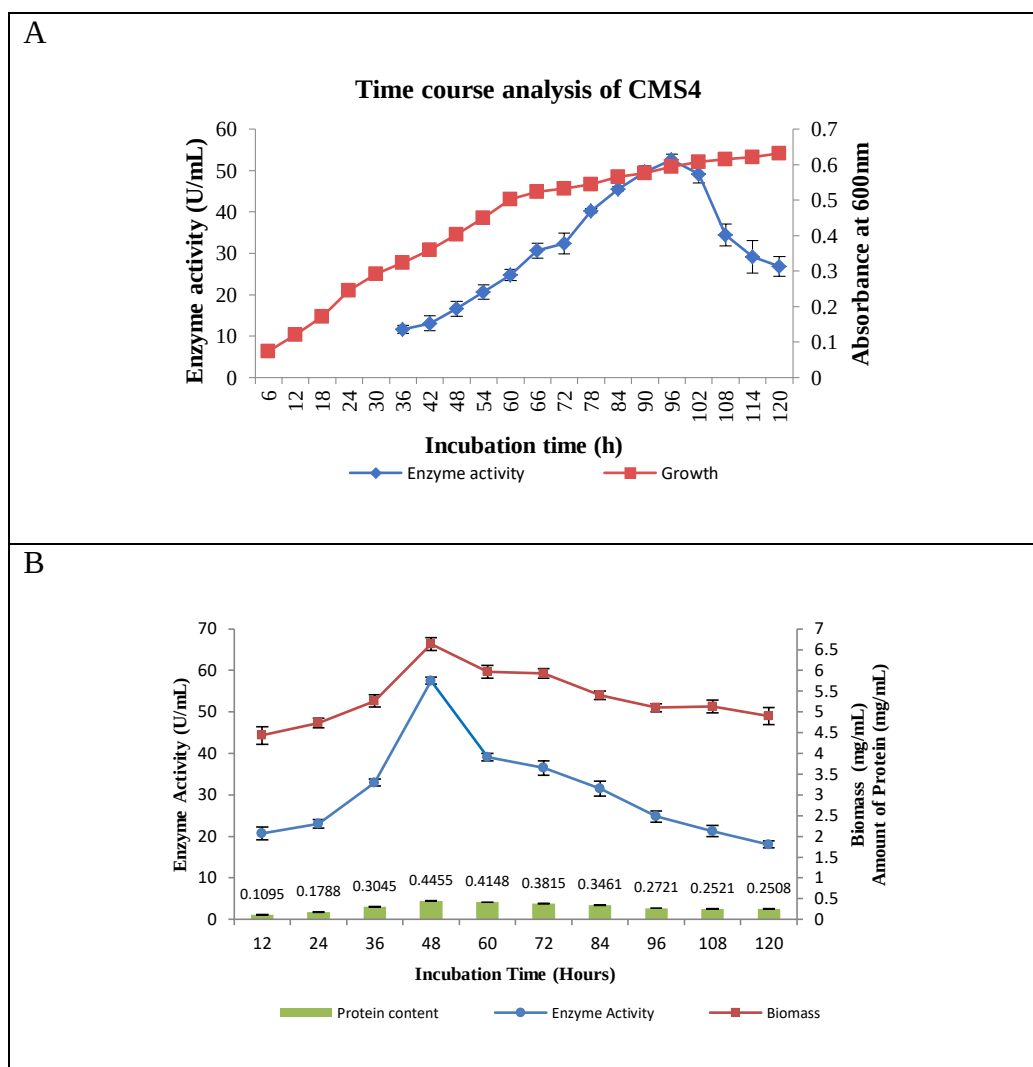


Figure 4.4: Time course study of enzyme production by isolates grown in MCDB optimized by OFAT method. a) Isolate CMS4 and b) Isolate MMS8

4.3.4 Effect of Glucose, L-asparagine and Sodium chloride concentration on enzyme production by MMS8

A study on the effect of Glucose, L-asparagine and Sodium chloride concentration on enzyme production by MMS8. Highest L-asparaginase activity recorded at a glucose concentration of 5g/L as shown in Figure 4.5 a1. Enzyme activity increased progressively up to 5g/L glucose; with increase in glucose concentration from 10 g/L onwards a decline was observed. In contrast the biomass increased steadily with the increasing glucose concentration as depicted in Figure 4.5 a2 having highest biomass production at 40g/L glucose concentration. Isolate MMS8 produced a statistically significant enzyme activity

($p < 0.05$) and biomass ($p < 0.05$) in media consisting of 5g/L of glucose when compared with other concentrations.

Evaluation of the effect of salt concentration on enzyme production by MMS8 was conducted using a medium supplemented with Sodium chloride ranging from 0.5 % to 2.5 %. The enzyme activity and biomass exhibited a progressive decline with increasing salt concentrations as seen in Figure 4.5 b. Maximum enzyme activity and growth was recorded when media contained 0.5 g/L salt. Statistical analysis indicated a significant difference in enzyme activity and biomass ($p < 0.05$) at 0.5 g/L sodium chloride compared to other tested salt concentrations.

The effect of L-asparagine concentration (2.5 to 20 g/L) on enzyme production by isolate MMS8 as illustrated in Figure 4.5 c revealed that the enzyme activity and biomass increase with increasing L-asparagine concentration from 2.5 g/L to 10 g/L, showing highest enzyme activity and biomass at 10 g/L. The enzyme production as observed remained steady with further increase in the L-asparagine concentration. Statistical analysis revealed a significant distinction in enzyme activity and biomass of other tested L-asparagine concentrations, in comparison with 10 g/L L-asparagine ($p < 0.05$). Significant variation was not observed among concentrations ranging from 15g/L to 20g/L ($p > 0.05$).

The study on the effect of glucose, L-asparagine and salt concentration on enzyme production by MMS8 revealed that enzyme production increased gradually and decreased with further increase in glucose and L-asparagine concentration, while it decreased with increasing salt concentration. The isolate MMS8 was obtained from an environment having low salinity (2 psu) and showed growth and enzyme production in media containing up to 2.5% sodium chloride concentrations, implying the halotolerance of the selected isolate.

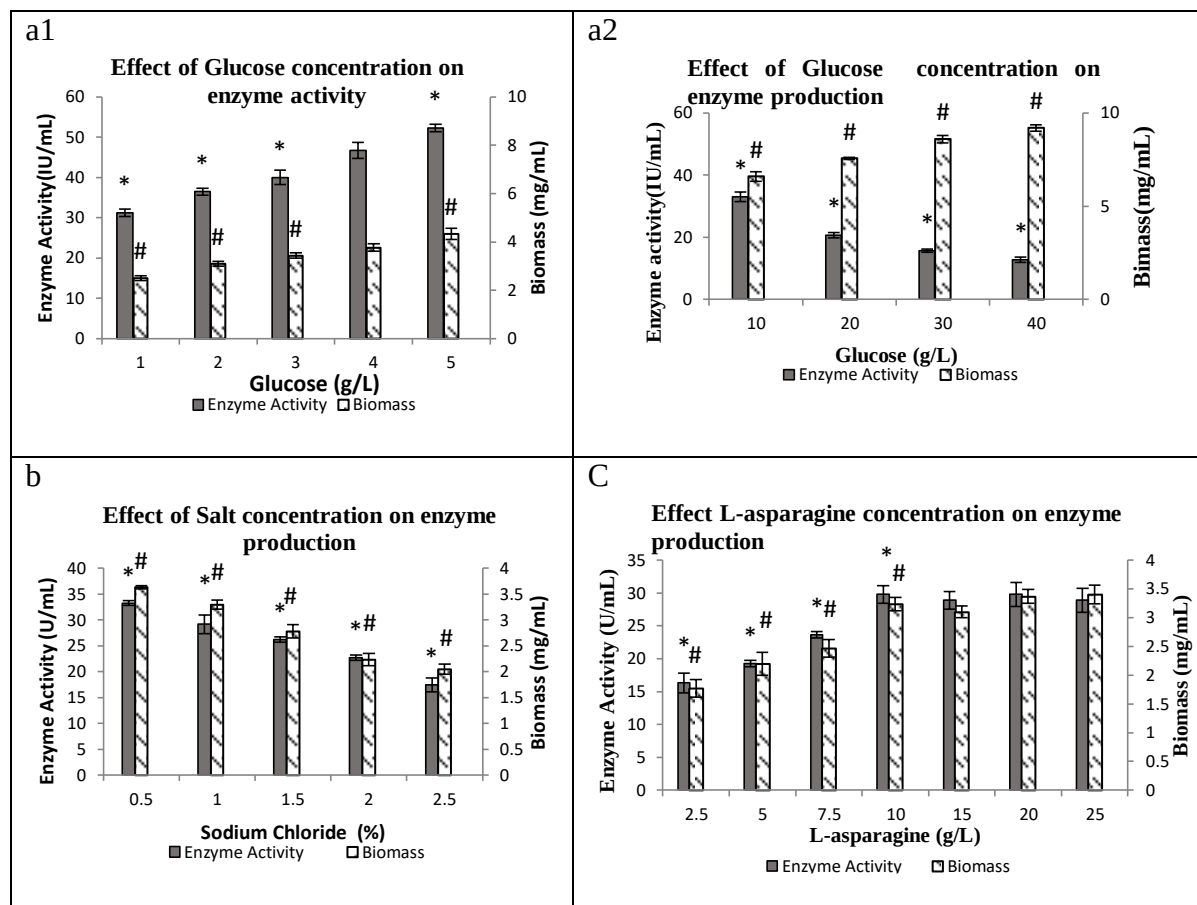


Figure 4.5: Effect of change in concentration of factors on L-asparaginase production by MMS8: a) Glucose concentration, b) Sodium chloride (Salt) and c) L-asparagine. The symbols “*” for enzyme activity and “#” for biomass are used to represent a comparatively statistically significant result.

4.3.5 Optimization of medium composition by RSM

Glucose, L-asparagine and sodium chloride were the nutrient sources examined in greater detail to determine their optimal concentrations for formulating a medium that maximizes L-asparaginase production by MMS8. These three factors were evaluated at various concentration levels and arranged according to a 2^3 Central Composite Design (CCD). The experimental conditions and corresponding L-asparaginase yields are summarized in Table 4.2.

Statistical analysis of the data represented in Table 4.3 demonstrates that the model was significant. Analysis of variance for the model indicated that the linear terms for Glucose and L-asparagine and the quadratic terms for all three variables were significant. The interaction terms exhibited no significant influence on the response. The coefficient of determination (R-square) having a value closer to 1 is indicative of a reasonable adjustment of the model to the data obtained. In the current study the model has a R square value of 0.9740, as depicted in the Table 4.4, implying a reliable model adjustment.

A regression analysis was subsequently conducted and after removing the non-significant terms, the remaining data were fitted to a second-order polynomial model (Equation 4.2), in which the concentrations of glucose, L-asparagine and sodium chloride were represented as A, B and C, respectively.

$$\text{Activity (U/mL)} = 72.12 + 4.61A + 17.86B + .01671C - 0.5943AB - 0.2390AC + 0.0022BC - 15.07A^2 - 2.70B^2 - 6.17C^2 \quad (4.2)$$

To identify the variable ranges responsible for enhanced enzyme production, response surface methodology was applied. Interactions between levels of each variable can be seen in the three-dimensional plots. The response surfaces shown in Figure 4.6 indicate a distinct region associated with the highest enzyme production (dark red area). The model showed a curvature in the response surface at the optimum. The variation in colour and intensity across the surface plot corresponds to differences in enzyme yield, with dark red indicating the highest production levels, green/yellow intermediate and blue representing the lowest. In this model, the surface plot shows that the highest enzyme activity (red region) occurs near the edge of the design space. Considering the limitations associated with the model, the optimal conditions were selected from the central region of the plot to minimize potential experimental and extrapolation errors. The normal probability plot of the residuals is presented in Figure 4.6 a. The close alignment of the residual points with

the diagonal reference line indicates that the residuals follow the normal distribution, that validates the model.

Based on this model, the statistical software predicted an optimal combination of 4.37 g/L glucose, 11.28 g/L L-asparagine and 1.28% sodium chloride for achieving an enzyme production of 75 U/mL. The accuracy of the model was determined by verifying the response levels under optimal conditions suggested by the CCD. The observed L-asparaginase activity value of 74.21 U/mL was closer to the targeted enzyme activity of 75 U/mL. The percent prediction accuracy of the model, having a value > 95%, demonstrated the validity of the model under the conditions tested.

Table 4.1: The actual and coded variables for CCD to optimize L-asparaginase production by isolate MMS8

Factor	Name	Units	- α	- 1	0	+1	+ α
A	Glucose	g/L	0.6364	2	4	6	7.36
B	L-Asparagine	g/L	1.59	5	10	15	18.41
C	Sodium chloride	%	0.1591	0.5	1.0	1.5	1.84

Table 4.2: The experiment design and observed L-asparaginase activity

Run	A: Glucose (g/L)	B: L-asparagine (g/L)	C: Sodium chloride (%)	Enzyme activity (U/mL)
1	2	5	0.5	24.37
2	6	5	0.5	36.42
3	2	15	0.5	61.98
4	6	15	0.5	70.39
5	2	5	1.5	28.44
6	6	5	1.5	38.26
7	2	15	1.5	64.79
8	6	15	1.5	73.51
9	4	10	1	70.89
10	4	10	1	72.14
11	4	10	1	69.39
12	4	10	1	71.10
13	4	10	1	75.84
14	4	10	1	74.10
15	7.36359	10	1	34.33
16	0.63641	10	1	20.13
17	4	18.40896	1	92.16
18	4	1.59104	1	32.26
19	4	10	1.840895	49.56
20	4	10	0.1591	55.25

Table 4.3: Analysis of variance for the response surface model of L-asparaginase production by isolate MMS8.

Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	8249.77	9	916.64	80.10	< 0.0001*
A	289.66	1	289.66	25.31	0.0005*
B	4356.39	1	4356.39	380.69	< 0.0001*
C	0.3815	1	0.3815	0.0333	0.8588
AB	2.83	1	2.83	0.2469	0.6300
AC	0.4570	1	0.4570	0.0399	0.8456
BC	0.0000	1	0.0000	3.422×10^{-6}	0.9986
A ²	3273.09	1	3273.09	286.03	< 0.0001*
B ²	105.34	1	105.34	9.21	0.0126*
C ²	548.63	1	548.63	47.94	< 0.0001*
Residual	114.43	10	11.44		
Lack of Fit	86.74	5	17.35	3.13	0.1179
Pure Error	27.69	5	5.54		
Cor Total	8364.20	19			

The symbol “*” represents a statistically significant result. A: Glucose, B: L-asparagine, C: Sodium chloride, df: Degrees of freedom.

Table 4.4: Model adequacy and statistical summary of the RSM model

Statistical parameters	Enzyme Activity
Standard Deviation	3.38
Mean	55.77
C.V. %	6.07
R ²	0.9863
Adjusted R ²	0.9740
Predicted R ²	0.9166
Adeq Precision	30.4206
Lack of Fit (p-values)	0.1178

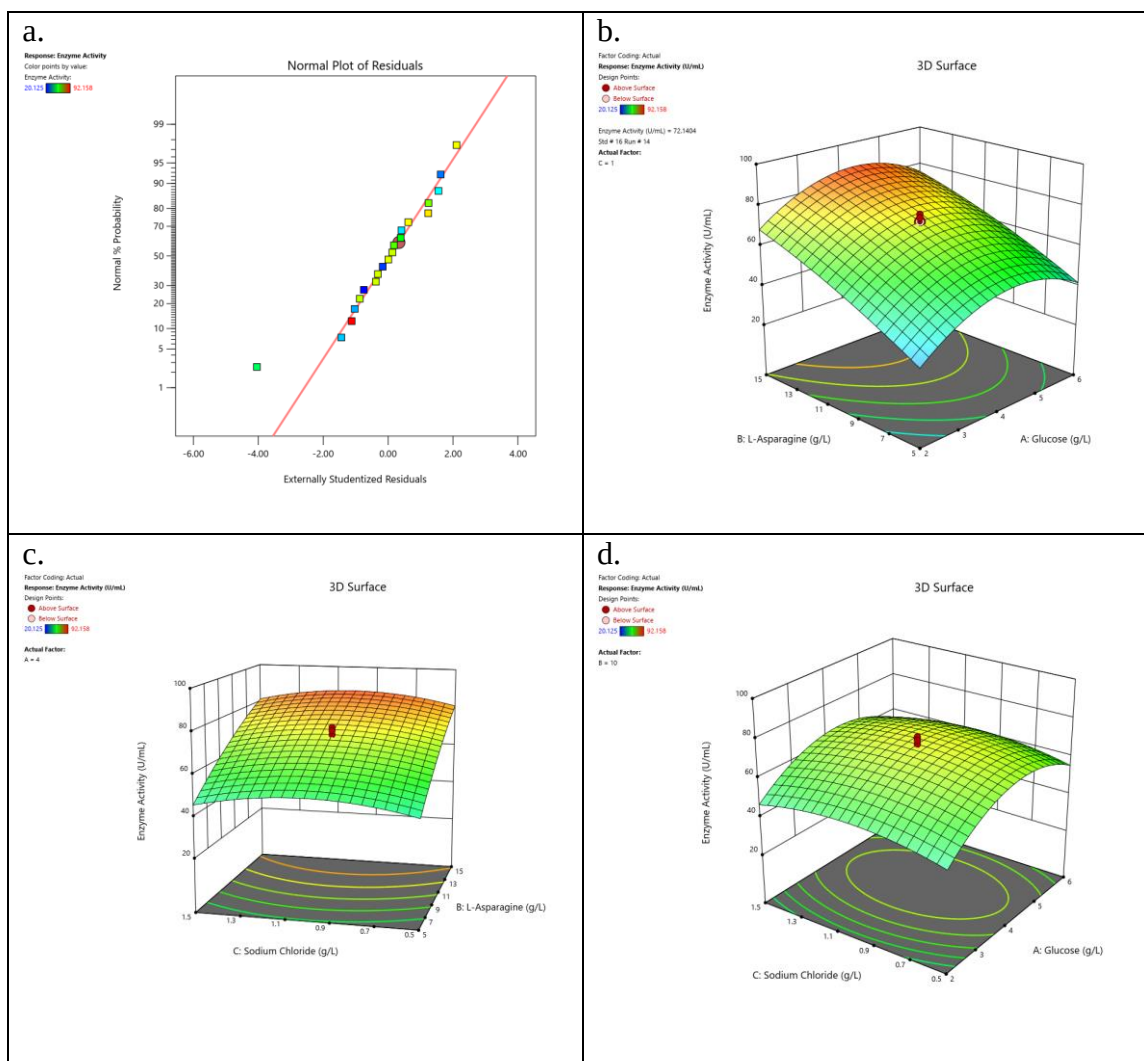


Figure 4.6: Three-dimensional response surface analysis depicting the influence of process variables on L-asparaginase production. a) Normal residual plot indicating the suitability of the model. b) Interactive influence of L-asparagine and glucose; c) Interaction between Sodium chloride and L-asparagine and d) Interaction between glucose and sodium chloride.

4.3.6 Partial purification of enzyme L-asparaginase

The cell free supernatant was used to obtain the enzyme by ammonium sulphate precipitation method. The protein precipitation was achieved at 25% of saturation and was further dialysed to free it from excess ammonia. The dialysed fraction was introduced on a Sephadex G-100 column for purification by size exclusion chromatography. The partially purified enzyme obtained from isolate MMS8 grown in a medium optimized by RSM, 120 fractions of 2 mL each were collected. Among the fractions that showed substantial enzyme activity, fraction number 66, as seen in Figure 4.7 a, showed a total activity of 482.67 U, having 0.402 mg of total protein and a purification fold of 3.14 depicted in Table 4.5. The SDS PAGE analysis of the partially purified enzyme presented in Figure 4.7 b showed bands observed near 43 kDa. The obtained SDS PAGE gel image was analysed using Gel analyzer software (version 19.1); the marker proteins were used to establish a molecular weight calibration curve and on basis of this curve the estimated molecular weight of the enzyme was 40kDa.

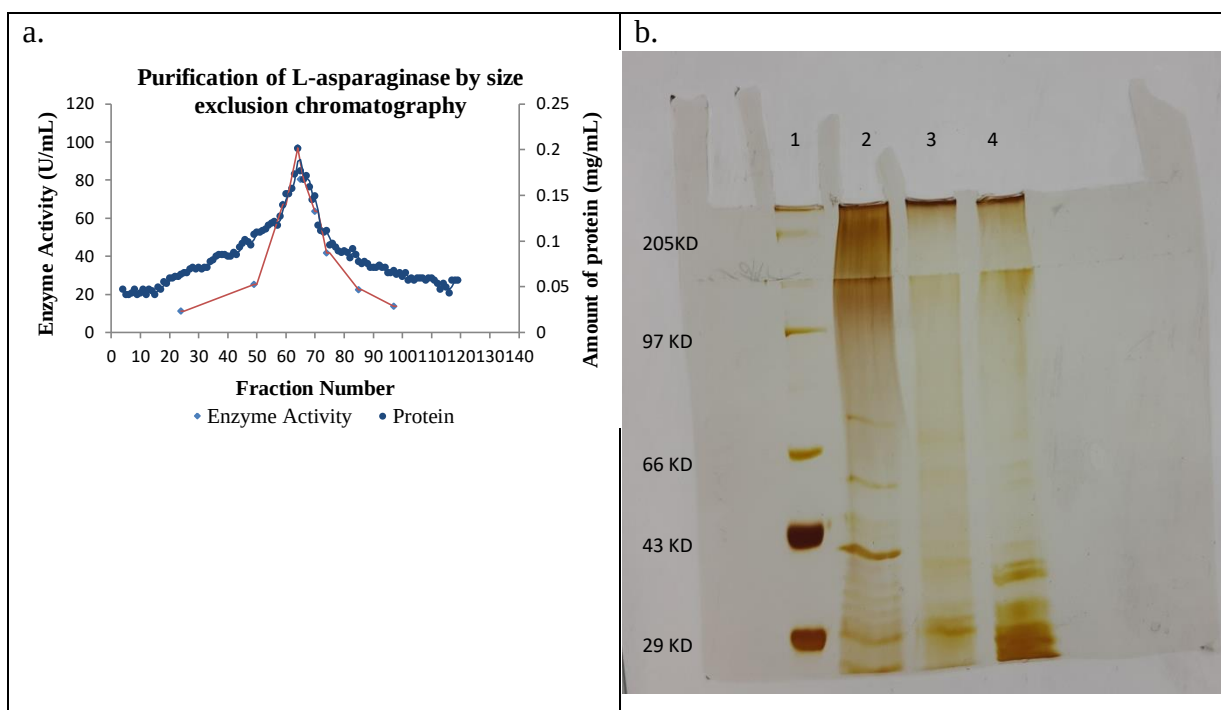


Figure 4.7: Partial purification of L-asparaginase produced by isolate MMS8: a. Size exclusion chromatogram; b. SDS PAGE analysis (left to right): Lane 1 Protein marker, Lane 2 Partially purified protein, Lane 3 Cell free supernatant and Lane 4 Dialyzed ammonium sulphate precipitate of protein.

Table 4.5: Purification profile of enzyme L-asparaginase obtained from isolate MMS8

Purification step	Volume of Fraction (mL)	Amount of Protein (mg/mL)	Total Protein (mg)	Enzyme activity (U/mL)	Total Activity (U)	Specific Activity (U/mg)	Yield (%)	Purification fold
Crude Extract (CFS)	100	0.4934	49.34	75.9641	7596.41	153.96	100.00	1.00
Ammonium sulphate precipitation	25	0.3474	8.69	84.7368	2118.42	243.92	27.89	1.58
Sephadex G-100	2	0.2010	0.40	97.0175	194.04	482.67	2.55	3.14

4.3.7 Characterization of partially purified enzyme

4.3.7.1 Effect of physical and reaction parameters on enzyme activity

4.3.7.1.1 Effect of incubation time on L-asparaginase activity

The effect of incubation period on enzyme activity of the partially purified enzyme was examined considering two time periods, as seen in Figure 4.8 a1 30-180 min and Figure 4.8 a2 from 15-90 min . The enzyme activity was highest at 60 min and gradually declined with the lowest at 180 min. It was notable that during the course of incubation from 30-75 min, almost 90% of the enzyme activity is unaffected and the lowest activity at 180 min has 50% residual activity. A significant change in activity as depicted in Figure 4.8 a2 was observed over 180 min of incubation ($p < 0.05$) compared to the values at 30 min and 60 min of incubation time. Similar values ($p > 0.05$) were recorded for the initial incubation period up to 60 min.

4.3.7.1.2 Effect of pH on L-asparaginase activity

Enzyme activity of partially purified L-asparaginase from selected isolate over a range of pH values was estimated. The reaction rate was maximum at pH 7.0 followed by pH 8.0 as seen in Figure 4.8 b indicating that the enzyme remains active within the pH range of 6.5 to 8.0. At both extreme acidic and alkaline conditions, enzyme activity reduced to 30%. The results indicate optimum pH range for the enzyme to obtain maximum reaction rate is pH 7.0 -8.0. Among the tested pH levels, significant distinction in enzyme activity was observed in comparison with pH 7.0 ($p < 0.05$), indicating that the optimum pH of 7.0.

4.3.7.1.3 Effect of incubation temperature on L-asparaginase activity

The optimum temperature for the partially purified enzyme was determined by varying the incubation temperature from 25 °C to 45 °C. It can be observed in Figure 4.8 c that the relative enzyme activity increased with increasing temperature from 25 °C to 35 °C with maximum activity observed at 37 °C. An increasing temperature above 37 °C showed a sharp decrease in the residual activity of the enzyme. At lower (25°C) and higher (45 °C) temperatures, the enzyme showed a loss of 70% of its original activity. Tukey's test results indicated notable differences in relative enzyme activity over the range of tested temperatures compared to 37 °C ($p < 0.05$). This implies that the optimum temperature for enhanced performance of the partially purified enzyme is 37 °C.

4.3.7.1.4 Effect of substrate concentration on L-asparaginase activity

The influence of substrate concentration (0.01 to 0.1 μM of L-asparagine) on the catalytic performance of the partially purified enzyme L-asparaginase was evaluated. An increase in the reaction rate proportional to the substrate concentration, with highest activity at 0.07 μM , was observed as depicted in the Figure 4.8 d. Subsequent increase in substrate concentration did not result in enhanced reaction rates, indicating saturation. Lineweaver–Burk plot analysis presented in Figure 4.8 e revealed that the partially purified L-asparaginase from MMS8 exhibited a K_m of 0.0323 μM and a V_{max} of 161.29 U/mL (0.802 U/ μg), reflecting its strong substrate affinity for L-asparagine, catalytic efficiency and potential for therapeutic applications.

Regression analysis of substrate concentration and reaction velocity using SPSS software revealed a strong positive relationship between the variables, with an R value of 0.941. The model explained 88.5% of the variation in velocity ($R^2 = 0.885$), and the adjusted R^2 (0.881) closely matched this value, indicating a reliable and well-fitted model. These results confirm that substrate concentration strongly influences the reaction rate.

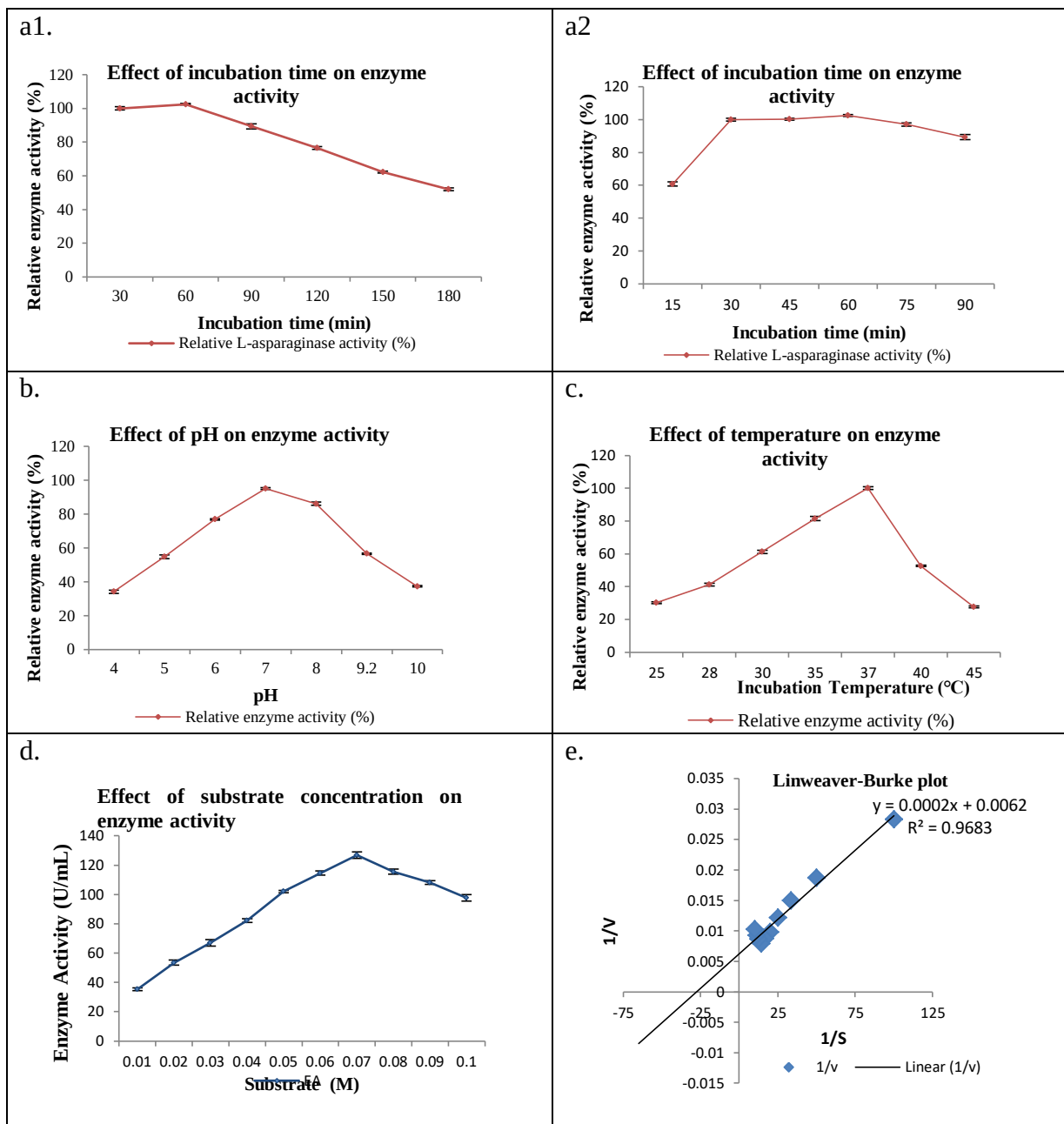


Figure 4.8: Characterization of the partially purified enzyme by studying the effect of factors on enzyme activity: a) Incubation time, b) pH of the reaction mixture, c) Incubation temperature, d) Substrate concentration and e) Lineweaver-Burke plot for determination K_m and V_{max} .

4.3.7.2 Enzyme stability

4.3.7.2.1 Effect of physicochemical parameters

The stability of the enzyme was assessed over 5 h at 37 °C and pH 7.2. A gradual decline in activity was observed (Figure 4.9 a), with about 50% loss between 1 and 3 h, after which no further decrease up to 5 h was observed. Statistical analysis was performed using the 30-minute incubation as the control. The results showed no significant difference in enzyme activity between 30 minutes and 1 h ($p > 0.05$) while a significant decline in activity was observed between 1 and 5 h ($p < 0.05$), with a 50% activity reduction indicating loss of enzyme stability over time.

Evaluation of enzyme stability across different pH conditions (Figure 4.9 b) revealed substantial activity loss at pH 5, 6, and 8, while complete inactivation occurred at pH 4, 9, and 10. Tukey's multiple comparison test was applied to assess differences in enzyme stability across pH 5, 6, 7, and 8, using pH 7.2 as the reference condition. The results indicated that enzyme activity at pH 5, 6 and 8 was significantly lower than at pH 7.2 ($p < 0.05$), while pH 7 showed a substantial reduction in activity. These findings confirm that the enzyme is most stable near physiological pH.

4.3.7.2.2 Effect of metal ions and inhibitors

The effect of the metal ions on the enzyme activity of partially purified enzyme was investigated and regular reaction conditions were considered as control. It was observed that the presence of ions Ca^{2+} , Fe^{2+} and Mg^{2+} results in a decline in the enzyme activity, as seen in Figure 4.9 c. Tukey's post hoc test indicated a significant reduction ($p < 0.05$) in enzyme activity on treatment with Ca^{2+} ions compared to the other ions tested. Inhibitors can cause a deleterious effect on the enzyme activity. The impact of inhibitors on the partially purified enzyme was estimated, including 2.5 mM Urea, 2 mM EDTA, 2% SDS, 1% Tween 20, and 1% Triton. From the inhibitors tested Triton, SDS and EDTA resulted in distinct loss in activity compared to the control conditions with no inhibitor as depicted in the Figure 4.9 d. Inhibitors Tween 80 and urea caused a minor enzyme activity loss. Statistical analysis revealed that Triton followed by SDS and EDTA showed a significant ($p < 0.05$) reduction in enzyme activity compared to the control and other inhibitors.

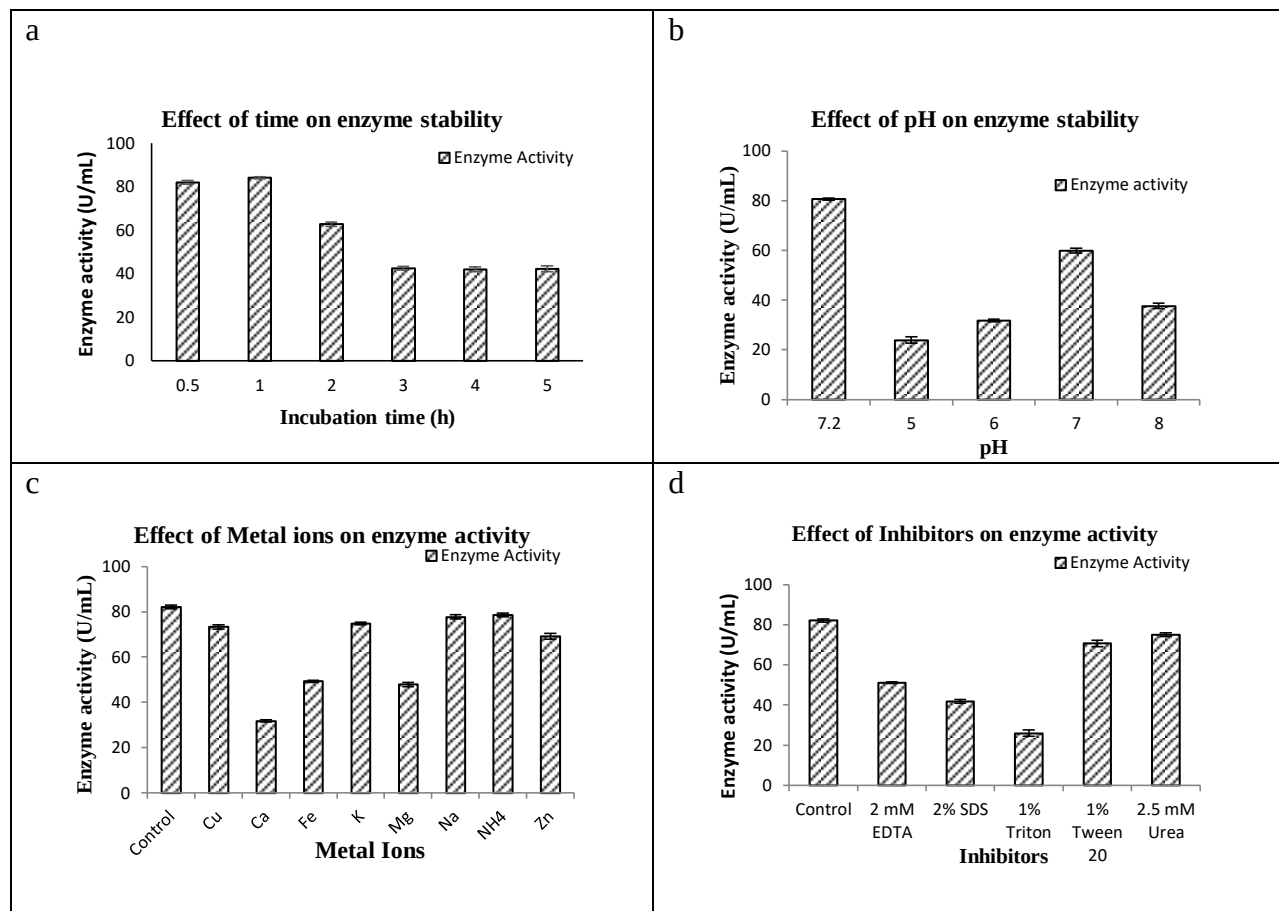


Figure 4.9: Determination of stability of the partially purified enzyme by studying the effect of factors on enzyme activity: a) Incubation time, b) pH of the reaction mixture, c) Metal ions and d) Inhibitors.

4.4 Discussion

The impact of growth parameters, including incubation time, incubation temperature, and pH of the medium, on enzyme production by the selected isolates CMS4 and MMS8 was determined. The study revealed that the optimum incubation temperature for maximum enzyme production is 28 °C, a finding supported by the results of a study conducted by Priya et al. (2011) on *Streptomyces* sp. isolated from the Western Ghats. Ashok et al. (2019) reported maximum enzyme activity at an incubation temperature of 30 °C based on a study conducted on L-asparaginase production by *Trichosporon asahii* IBBLA1. Further in a study by Baskar and Renganathan (2009) on L-asparaginase obtained from *Aspergillus terreus* MTCC 1782, it was reported that maximum enzyme activity was observed for growth conditions with a temperature of 30 °C and pH 6.2.

The influence of pH of the medium on enzyme production by CMS4 and MMS8 was studied. It indicated that maximum enzyme production is observed when pH of medium is 6.5 and 7.0, respectively. The results obtained in the current study are in agreement with those reported previously by Dunlop and Roon (1975) and Ferrara et al. (2004) where maximum L-asparaginase production by *S. cerevisiae* was observed at pH levels ranging between values 6.0 to 7.0. Similar results were reported by Ashok et al. (2019) based on a study conducted on L-asparaginase production by *Trichosporon asahii* IBBLA1 where maximum enzyme production was observed at pH 7.0.

The current study demonstrated that maximum enzyme production is observed when L-asparagine is used as the sole nitrogen source. These findings support that enzyme production is influenced by the phenomenon of nitrogen catabolite repression wherein, in the presence of a favored nitrogen source, microorganisms regulate the genes for the formation of enzymes needed to assimilate less preferred nitrogen compounds (Magasanik and Kaiser, 2002; Sanchez and Demain, 2008). However, the findings stated above differ from those reported previously in studies conducted by Baskar and Renganathan (2009); Friere et al. (2021); Moguel et al. (2022). These reports displayed that enzyme production was enhanced in the presence of nitrogen sources which included soyabean meal flour, yeast extract and L-proline.

Isolate CMS4 and MMS8 under optimal medium conditions displayed an enzyme activity of 52.57 U/mL and 57.54 U/mL, respectively, which is higher than previously reported enzyme activities from *Trichosporon asahii* IBBLA1 (20.57 U/mL) (Ashok et al. 2019)

and *Meyerozyma guilliermondii* (26.01 U/mL) (Ratuchne et al. 2023). Table 4.6 exhibits L-asparaginase enzyme activities of different yeasts reported in the literature.

Table 4.6: L-asparaginase production by different yeasts after optimization

Microorganism	Enzyme activity (U/mL)		Reference
	Initial	Final	
<i>Leucosporidium muscorum</i>	0.49	4.582	(Freire et al. 2021)
<i>Leucosporidium scotti</i> L115	0.057	2.85	(Moguel et al. 2022)
<i>Candida utilis</i>	-	245.6	(Moguel et al. 2022)
<i>Trichosporon asahii</i> IBBLA1	15	20.57	(Ashok et al. 2019)
<i>Meyerozyma guilliermondii</i> .	2.32	26.01	(Ratuchne et al. 2023)
<i>Yarrowia lipolytica</i> DSM3286	17.14	102.85	(Darvishi et al. 2018)
<i>Saccharomyces cerevisiae</i> X-2180-A2	-	3.506	(Moguel et al. 2022)
CMS4 (<i>Candida</i> sp.)	33.57	52.57	Present study
MMS8 (<i>Cyberlindnera</i> sp.)	29.77	57.54	Present study

In the present study the L-asparaginase production by isolate MMS8 was enhanced using RSM CCD. The enzyme activity showed a two-and-a-half-fold increase when optimized media consisting of 4.37 g/L glucose, 11.28 g/L L-asparagine and 1.28% sodium chloride was used. Studies on enhancing L-asparaginase production by microbes by altering media composition and parameters using RSM are conducted previously. The main focus of these studies is to optimize the levels of the factors to attain maximum enzyme production utilizing limited experiments by RSM.

The study on L-asparaginase production by yeast *Yarrowia lipolytica* suggests that media components glucose and L-asparagine are the most significant while the use of crude sources like palm kernel cake can also contribute in enzyme production enhancement. Precise conditions and media composition in case of *Y. lipolytica* DSM3286 lead to a 12-fold increase in enzyme production in a 5L bioreactor. The L-asparaginase production

by psychrotolerant yeast *Leucosporidium scotti* L115 reported by Moguel et al. (2022) showed a 50-fold increase when media optimized by RSM was used. The composition of the optimized media was sucrose (28.34 g L⁻¹) and glycerol (15.61 g L⁻¹) as carbon sources, supplemented with proline (6.15 g L⁻¹). In a study by Ratuchne et al. (2023) the L-asparaginase production exhibited a tenfold increase on implementation of the media optimized by RSM. Table 1 and 2 exhibits the strategies used to enhance L-asparaginase production by different microbes reported in the literature.

The present study aimed to optimize and characterize the clinically significant enzyme L-asparaginase from yeasts isolated from mangrove sediments of Goa. Isolates CMS4 and MMS8 were selected to obtain enhanced enzyme production. The enzyme activity exhibited by the selected isolates is comparable to that of other enzymes of bacterial and fungal origin, including yeast, while a high recombinant enzyme yield is observed, as seen in Tables 1.3, 1.5, 4.6, 4.7 and 4.9.

In a study on *Saccharomyces cerevisiae* X-2180-A2 Dunlop et al. (1978) reported a 250-fold purification of L-asparaginase type 2 enzyme with activity of 30.5 U/mg while the recombinant enzyme obtained from *Pichia pastoris* harbouring *Saccharomyces cerevisiae* ASP3 gene was reported to have an activity of 204.4 IU/mg by de Castro Girão et al. (2016). In a report by Lopes et al. (2019) the recombinant enzyme obtained from *Escherichia coli* containing *Saccharomyces cerevisiae* ASP3 gene showed an activity of 225.6 IU/g cells. Isolate MMS8 with its probable GRAS status and comparable enzyme yield can be a potential choice for future recombinant studies to obtain an enzyme with enhanced yield and purity.

Table 4.7: The optimized medium conditions for L-asparaginase production obtained from microbes

Microorganism	Optimization method	Optimized conditions	Enzyme activity (U/mL)	
			Initial	Final
<i>Nocardiopsis alba</i> NIOT-VKMA08 (Meena et al., 2015)	OFAT	L-asparagine and maltose (1.5 %), 144 h, 30–35 °C, pH 8.2	6.73	18.47
<i>Pseudomonas plecoglossicida</i> RS1 (Shakambari et al., 2015)	PBD and CCD RSM	KH ₂ PO ₄ (0.2 g% w/v), effluent (0.8 mL% v/v) and pH (6.5).	0.73	3.25
<i>Leucosporidium muscorum</i> (Freire et al. 2021)	OFAT and FD	20 g /L sucrose, 15 g /L yeast extract, 20 g /L proline, 12–15 °C, pH 5.5–6.5 and seawater >25% (v/v). Inoculum concentration seems not to interfere	0.49	4.582
<i>Leucosporidium scotti</i> L115 (Moguel et al. 2022)	CCD	Modified Czapek Dox medium with Sucrose (28.34 g L ⁻¹), glycerol (15.61 g L ⁻¹), proline (6.15 g L ⁻¹)	0.057	2.85
<i>Trichosporon asahii</i> IBBLA1 (Ashok et al. 2019)	Taguchi design	L-Asparagine concentration of 10 g L ⁻¹ and a Glucose concentration 2 g L ⁻¹ , 30 °C, pH of 7.0, 60 hours.	15	20.57
<i>Meyerozyma guilliermondii</i> (Ratuchne et al. 2023)	RSM CCRD	pH 4.0 1.0% l-asparagine and 0.5% glucose during 75 h of cultivation	2.32	24.75
<i>Yarrowia lipolytica</i> DSM3286 (Darvishi et al. 2018)	RSM CCD	8 glucose, 15 asparagine, 4.5 K ₂ HPO ₄ in g/L and pH 7, 20 h, 29 °C	17.14	102.85
CMS4 (<i>Candida</i> sp.) (Present study)	OFAT	2 glucose, 10 asparagine, 1.5 K ₂ HPO ₄ in g/L and pH 6.5/pH 7.2, 96 h, 28 °C	33.57	52.57
MMS8 (<i>Cyberlindnera</i> sp.) (Present study)	OFAT	6.5/pH 7.2, 96 h, 28 °C	29.77	57.54
	RSM CCD	4.37 glucose, 11.28 L-asparagine in g/L, 1.28% sodium chloride and pH 7, 96 h, 28 °C		74.21

In the current study, the purification and characterization of L-asparaginase produced by isolate MMS8 revealed a 3.14 fold purification of the protein having a specific activity of 483.67 U/mg and a molecular weight of 40kDa determined using SDS PAGE method. A low purification fold and high specific activity reflect the substantial enzyme activity of the crude enzyme and imply minimal contamination by other proteins. The molecular weight determined for the enzyme is similar to the values previously reported in the literature, as summarized in Table 1.5, 4.8 and 4.9. The partially purified enzyme exhibited an optimum activity at near neutral pH, physiological temperature and incubation of 60 minutes, indicating its potential suitability for medicinal preparations.

The partially purified enzyme was stable in the reaction mixture for 5 hours with loss in activity by 50% after 4 hours. The activity of the enzyme was unaffected in a buffer with near neutral pH, while a reduction was observed at pH 6 and 8, confirming the stability of the enzyme in a pH range from 6 to 8. Effect of the metal ions on the partially purified enzyme revealed that ions Ca^{2+} , Fe^{2+} and Mg^{2+} had a noticeable impact on enzyme activity. Inhibitors can negatively impact enzyme activity. A noteworthy loss in enzyme activity was observed on treatment with the inhibitors Triton, SDS and EDTA indicating probable disruption of enzyme protein structure.

In a study on characterisation of L-asparaginase from marine *Nocardiopsis alba* NIOT-VKMA08 Meena et al. (2015) reported optimum pH, time and temperature for the enzyme as 8, 30 min and 37°C, respectively. The enzyme was purified using ammonium sulphate precipitation followed by Q sepharose chromatography to obtain 58.44-fold purification. In a report on L-asparaginase enzyme from recombinant *Pichia pastoris* harbouring *Saccharomyces cerevisiae* ASP3 gene by de Castro Girao et al. (2016) it was revealed that the optimum pH and temperature for this enzyme is 46 °C and pH 7.2 respectively, having molecular weight of 48.6 kDa and 44.6 kDa estimated using SDS PAGE analysis. El-Naggar et al. (2016) from their study on L-asparaginase from *Streptomyces fradiae* NEAE-82 reported that the optimum parameters for enhanced enzyme action included pH 8.5, temperature 40 °C and time 30 min. The study of the molecular weight of this enzyme by SDS PAGE revealed a 53 kDa protein.

Table 4.8: The characteristics of purified L-asparaginase obtained from microbes

Microorganisms	Optimum conditions (pH, temperature)	Molecular weight (SDS)
<i>Saccharomyces cerevisiae</i> X2180-1A (L-asparaginase type 2) (Dunlop et al., 1978)	7, 35 °C	-
<i>Saccharomyces cerevisiae</i> X2180-1A (L-asparaginase type 1) (Dunlop et al., 1978)	8, 35 °C	-
<i>Rhodospiridium toruloides</i> CBS14 (Ramakrishnan & Joseph, 1996)	6.35, 37°C	87 kDa
<i>Pseudomonas plecoglossicida</i> RS1 (Shakambari et al., 2015)	8.5, 55°C	46 kDa
<i>Nocardiopsis alba</i> NIOT-VKMA08 (Meena et al., 2015)	8.0, 37 °C	-
<i>Streptomyces fradiae</i> NEAE-82 (El-Naggar et al., 2016)	8.5, 40 °C,	53 kDa
<i>Aspergillus oryzae</i> CCT 3940 (Dias et al., 2016)	8, 50 °C	115 kDa
<i>Leucosporidium scotti</i> L115 (Moguel et al., 2023)	-	53 kDa
MMS8 (<i>Cyberlindnera</i> sp.) (Present study)	pH 7.2, 37 °C	40 kDa

Table 4.9: The characteristics of recombinant L-asparaginase

Recombinant L-asparaginase	Molecular weight by SDS (kDa)	Specific activity (U/mg)
Recombinant <i>Pichia pastoris</i> harbouring <i>Saccharomyces cerevisiae</i> ASP3 gene (de Castro Girao et al. 2016)	48.6 and 44.6	204.4
Recombinant <i>Escherichia coli</i> containing <i>Saccharomyces cerevisiae</i> ASP3 gene (Lopes et al. 2019)	38	225.6
Recombinant <i>Escherichia coli</i> harbouring <i>Saccharomyces cerevisiae</i> ASP1 gene (Costa et al. 2016)	41.4	110

4.5 Conclusion

Isolates CMS4 and MMS8, was selected to evaluate the effects of physical parameters, carbon and nitrogen sources on enzyme production and the results indicated that the optimal conditions consisted of a medium having pH 6.5 and 7 respectively, incubated at 28 °C with glucose as carbon source and L-asparagine as nitrogen source. Isolate CMS4 showed a one and half fold increase in enzyme production on utilization of medium optimized by OFAT. Based on the experimental data obtained, a two-fold increase in enzyme production by MMS8 on optimization with OFAT, with a reduction in incubation time by half was observed. The medium optimized by RSM resulted in enhancing enzyme production by MMS8 by two and half fold.

It was noted that the isolate MMS8 does not grow at 37 °C; however, it produces an enzyme that is active at this temperature and pH 7.2. These conditions are similar to that of normal human body temperature and blood pH. This characteristic reveals the promising ability of the enzyme as an agent in cancer treatment. Isolate MMS8 with higher enzyme production and belonging to the genus *Cyberlindnera*, a yeast implemented in the food and beverage industry and generally regarded as safe, was chosen for the further experiments. L-asparaginase was partially purified 3.14 fold using salting out technique followed by size exclusion chromatography. The optimum parameters of the partially purified enzyme were pH 7.2, 37 °C and 30 min indicating its efficient and rapid action under physiological conditions.

In conclusion, the study demonstrated that altering the medium composition of the modified Czapek Dox medium significantly improves the L-asparaginase enzyme production. The L-asparaginase enzyme obtained from MMS8 after partial purification exhibits optimum activity at physiological conditions of blood pH and body temperature that is pH 7.2 and 37 °C confirming its suitability for use in medicinal formulations.

CHAPTER V

APPLICATION OF BIOACTIVITY OF

L-ASPARAGINASE AS ANTICANCER COMPOUND

5.1 Introduction

L-asparaginase amidohydrolase (L-asparaginase) catalyses the hydrolysis of L-asparagine into L-aspartic acid and ammonia. Normal cells possess the enzymatic machinery to synthesize L-asparagine endogenously. Cancer cells rely exclusively on extracellular L-asparagine as they are unable to synthesize L-asparagine for their metabolic requirements. In cancer therapy, administration of L-asparaginase into the bloodstream results in the depletion of circulating L-asparagine levels, thereby inhibiting protein synthesis in malignant cells and ultimately inducing their death (Cachumba et al. 2016).

The antineoplastic activity of enzyme L-asparaginase is the reason behind its chemotherapeutic application. The enzyme is administered along with other drugs to enhance the effectiveness in the treatment of cancers, including ALL (Acute lymphoblastic leukemia), Hodgkin's lymphoma and other types of leukaemia (Batool et al., 2016). The remission induction treatment of Acute Lymphoblastic Leukemia (ALL) and lymphoblastic lymphoma largely uses L-asparaginase as a chemotherapeutic agent. This treatment aims at reducing the cancer cells in the bloodstream using effective agents.

In a study conducted by Kidd (1953), it was observed that administration of guinea pig serum inhibited the growth of subcutaneous lymphosarcoma cells. Subsequently, Broome (1963) identified the serum factor responsible for this antileukemic activity as enzyme L-asparaginase. Consequent research efforts were directed toward isolating L-asparaginase from microbial sources, as it is easy to produce, genetically manipulate and purify compared to plant and animal sources. The ease of medium optimization to enhance enzyme production, along with the ability of microbes to utilize diverse substrates, that includes waste materials and highlights the effectiveness of microbial sources. The clinically approved bacterial L-asparaginase derived from *Escherichia coli* and *Erwinia chrysanthemi* was employed in the therapeutic management of acute lymphoblastic leukemia (ALL).

L-asparaginase of prokaryotic origin, *Escherichia coli* and *Erwinia chrysanthemi* are used in commercial treatment methods. The Type 2 L-asparaginase, periplasmic, having nitrogen-dependent expression (Wriston and Yellin., 1973; Coral-Medina et al., 2022) is preferred for therapeutic applications due to its higher affinity for L-asparagine, indicated by its low K_m value. This characteristic ensures effective hydrolysis of L-asparagine at

physiological concentrations implying superior antitumor activity (Batool et al., 2016; Cachumba et al., 2016).

The unfavorable effects caused by the enzyme of bacterial origin include acute allergic reactions, hypersensitivity, and immune inactivation. The efficiency of L-asparaginase as a chemotherapeutic agent diminishes on prolonged exposure during treatment, as circulating antibodies can cause clearance of the enzyme, resulting in the development of silent immunity (Cachumba et al., 2016; Teixeira et al., 2017).

L-asparaginase of eukaryotic origin can help avoid the undesirable effects of a prokaryotic enzyme as an anti-neoplastic agent. The primary focus of current research is exploring an alternative, preferably eukaryotic source of enzyme L-asparaginase exhibiting anti-tumor activity with least detrimental effects (Batool et al., 2016; Cachumba et al., 2016).

MTT assay is the standard method used in estimation of cytotoxicity. The cells on treatment with cytotoxic agents lose their ability to transform tetrazolium to formazan crystals, a characteristic feature possessed by live cells having functional enzymes. The resultant change in colour intensity is measured at 570nm. The colour formation is proportional to the presence of live cells. Different methodologies and cell lines utilized for the assessment of cytotoxic potential of L-asparaginase are summarized in Table 5.1.

Table 5.1: Methods implemented to estimate cytotoxicity of L-asparaginase enzyme on multiple cell lines

Method	Principle	Cell lines
Trypan Blue exclusion assay (Costa et al., 2016)	Manual cell counting under a haemocytometer where the dead cells are stained and live cells are not.	MOLT-4, REH
Tetrazolium reduction (MTT assay) (El-Naggar and El-Shweihy., 2020; Mazloun-Ravasan et al., 2021)	Metabolically active cells reduce tetrazolium to formazan product	MCF-7, A549, HeLa
Nuclear staining (DAPI staining) (Mazloun-Ravasan et al., 2021; Darnel et al., 2023)	Detection and staining of nuclei	K562, A549, MCF-7
Flow cytometry Annexin V/PI (Mazloun-Ravasan et al. 2021)	Detects the phase of apoptosis in cell and mode of cell death	MCF-7, A549
Western Blot (Song et al., 2015)	Detection of apoptotic markers including caspase 3	K562, KU812
JC1 assay, oxidative stress assay (Mazloun-Ravasan et al. 2021)	Measuring change in mitochondrial membrane potential, presence of increased ROS indicating apoptois.	MCF-7, A549

MOLT-4 – T-cell acute lymphoblastic leukaemia, REH – B-cell precursor acute lymphoblastic leukaemia, MCF-7 – breast adenocarcinoma, A549 – lung adenocarcinoma, HeLa – cervical carcinoma, K562 – chronic myelogenous leukaemia, KU812 – chronic myelogenous leukaemia

5.2 Methodology

The cytotoxic potential of crude and partially purified L-asparaginase enzyme was determined using the MTT assay and DAPI staining.

5.2.1 Quantitative estimation of cytotoxic potential of L-asparaginase enzyme by MTT assay method

The experiments were conducted at the cell culture facility in Birla Institute of Technology and Science (BITS), Goa. Freshly passaged HeLa cells were used to perform the experiments. They were grown in DMEM media containing 5% FBS and phenol red. Inoculum having a cell density of 0.05×10^6 was seeded into DMEM medium containing 50 μ L of the test enzyme sample in a 24 well plate and incubated for 24 h and 48 h at 37°C in 5% CO₂ incubator. On completion of incubation, the medium was discarded by aspiration, followed by the addition of 300 μ L MTT (4mg/mL) and incubation for 4 h at 37°C. Subsequently, DMSO was added and absorbance was measured at 595nm.

The cytotoxicity of two test enzyme samples was determined. Sample 1 consisted of cell free supernatant obtained after growing isolate MMS8 for 96 h in optimized MCDB and was incubated with HeLa cells for 48 h. Crude enzymes with activity values of 2 U/mL, 3 U/mL, and 4 U/mL were utilized. Sample 2 consisted of partially purified enzyme in phosphate buffered saline (PBS), having activities ranging from 2 U/mL to 10 U/mL, and the HeLa cells were exposed to it for 24 h and 48 h.

Untreated cells were used as controls. Control 1 consisted of DMEM media with cells, for control 2 cells were inoculated in DMEM media with the solvent in which the test enzyme was dissolved. The background control (blank) consisted of DMEM media and the respective solvent. The calculation of cell viability was carried out using the following formula (Mazloun-Ravasan et al. 2021):

$$\text{Cell viability (\%)} = \frac{(\text{absorbance of treated cells} - \text{absorbance of blank})}{(\text{absorbance of control1} - \text{absorbance of blank})} \times 100 \quad (5.1)$$

Statistical analysis

The experiments for determining cytotoxic potential of enzyme L-asparaginase were performed in triplicate. The findings were expressed as mean with their standard deviation. The statistical analysis of the data about cytotoxic effect of the CFS and partially purified enzyme was performed by a one-way analysis of variance (ANOVA) ($p \leq 0.05$), followed

by a Dunnett's test to determine significant differences between treated groups and the control, with $p < 0.05$ considered statistically significant. The statistical tests were performed using SPSS (version 31).

5.2.2 Qualitative estimation of cytotoxic potential of L-asparaginase enzyme by DAPI staining method

The staining method using 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) involves qualitative estimation of viable cells present in the treated sample reflected by the number of stained nuclei and aids in confirming the results observed during MTT assay. The cells exposed to the cytotoxic agent were monitored using the DAPI staining method to observe and confirm a loss in the number of viable cells. Changes in nuclear morphology following treatment were assessed using DAPI staining.

Cells were plated in 96-well plates at a density of 0.05×10^6 cells per well and treated with the L-asparaginase derived from isolate MMS8. The cultures were then incubated at 37 °C in a humidified atmosphere containing 5% CO₂ for 24 hours. After incubation, the cells were fixed with 4% paraformaldehyde for 30 minutes, washed with phosphate-buffered saline (PBS), and subsequently stained with DAPI (1 µg/mL) for 15 minutes in the dark. The nuclear morphological changes were then observed under a fluorescence microscope (Darnal et al., 2023).

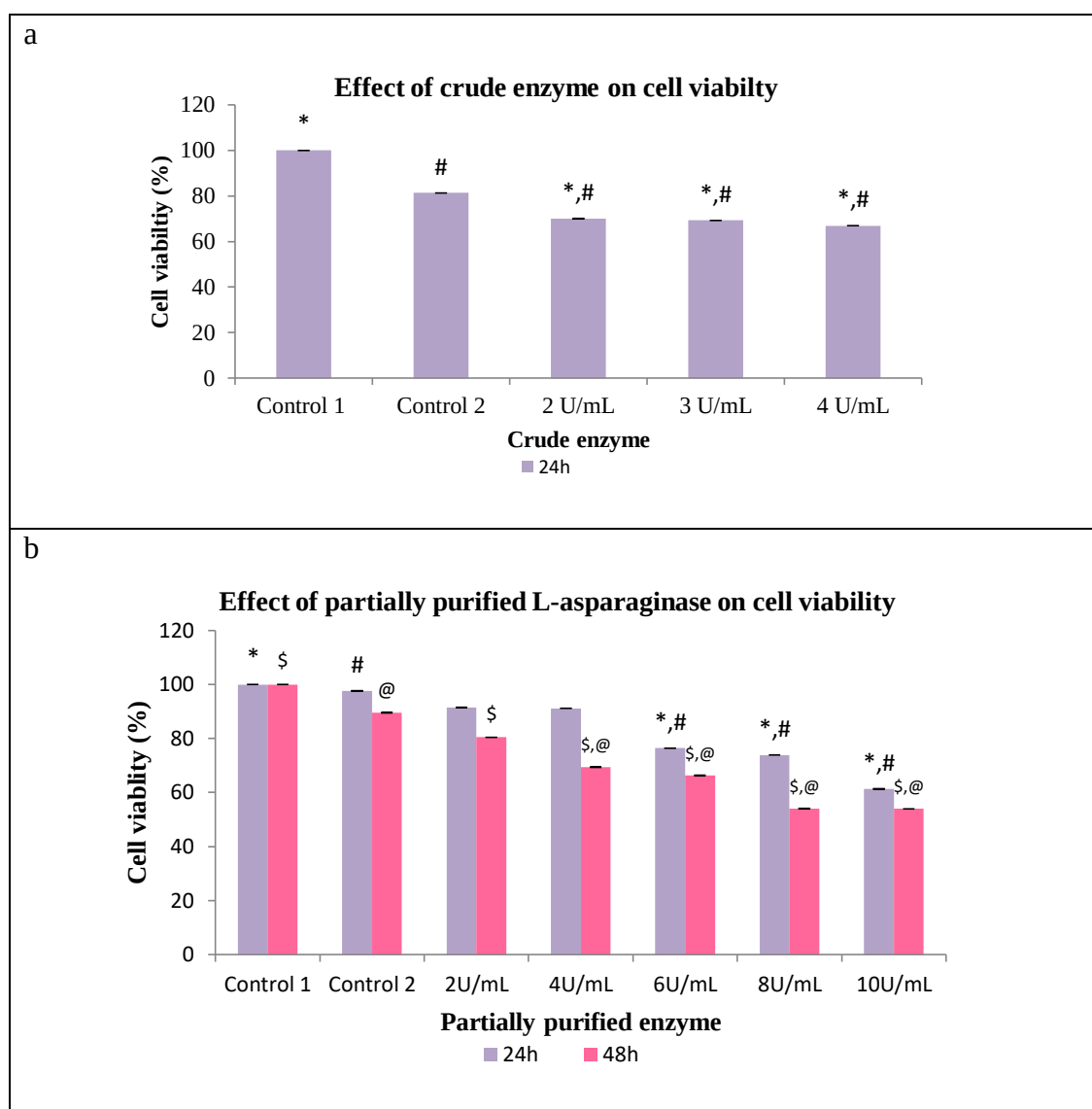


Figure 5.1: Effect of varying concentrations of L-asparaginase on percent cell viability. Control 1 consists of HeLa cells grown in DMEM and Control 2 consists of HeLa cells grown in DMEM with the solvent. a. Crude extract containing enzyme in MCDB. The symbol ‘*’ for Control 1 and ‘#’ for Control 2 is used to represent a comparatively statistically significant result; and b. partially purified enzyme in PBS. The symbol ‘*’ for Control 1 and ‘#’ for Control 2 at 24h; ‘\$’ for Control 1 and ‘@’ for Control 2 at 48h, is used to represent a comparatively statistically significant result.

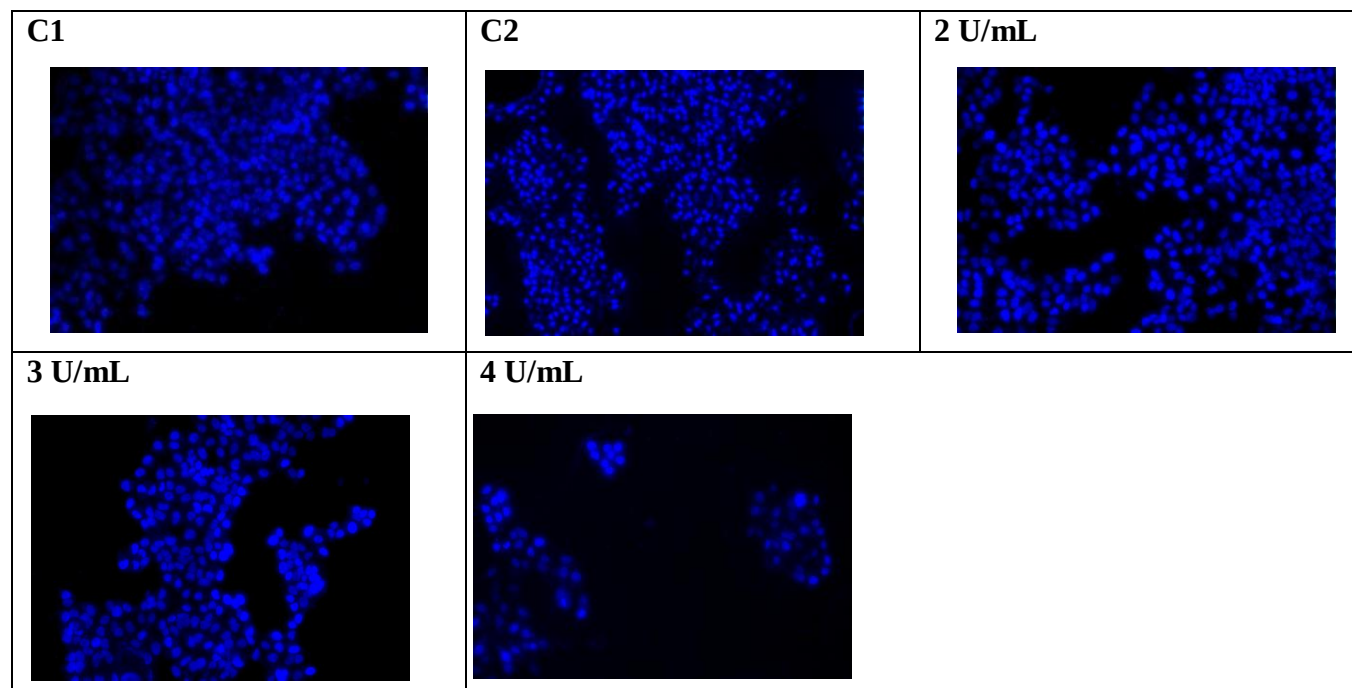


Figure 5.2: DAPI staining images of effect of crude enzyme (CFS) on viability of HeLa cells. Controls are C1 (DMEM medium+ HeLa cells) and C2 (DMEM +HeLa cells+MCDB). The tests include HeLa cells grown in DMEM medium with CFS ranging from 2U/mL to 4 U/mL

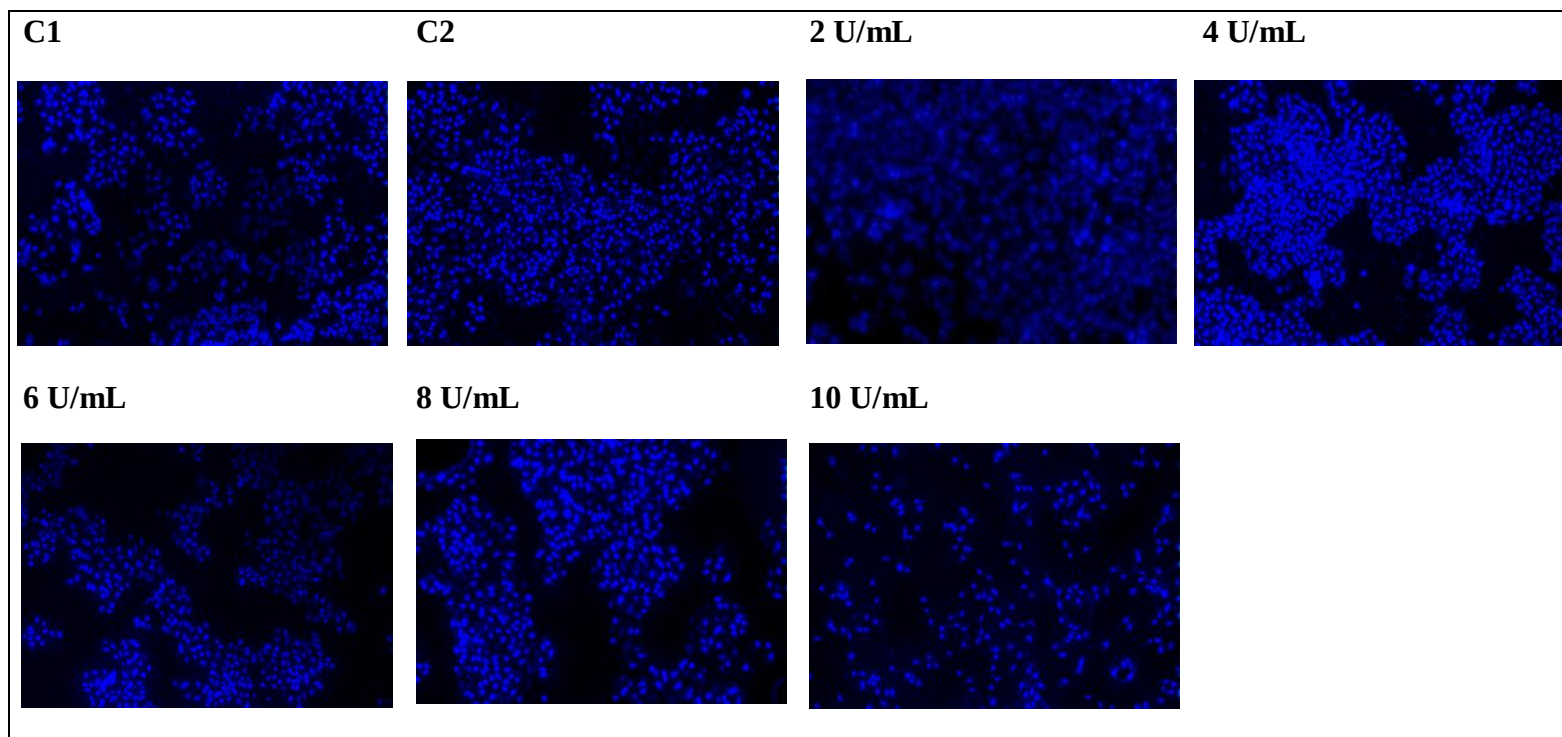


Figure 5.3: DAPI staining images of effect of partially purified enzyme on HeLa cells on incubating for 48h. Controls are C1 (DMEM medium+ HeLa cells) and C2 (DMEM +HeLa cells+PBS). The tests include HeLa cells grown in DMEM medium with partially purified L-asparaginase enzyme ranging from 2U/mL to 10 U/mL

5.3 Results

Cytotoxic potential of test enzyme samples against HeLa cells was determined by MTT assay for an incubation period of 24 h and 48 h. A decrease in cell viability for the crude enzyme was determined by the MTT assay, as displayed in Figure 5.1a. The enzyme solvent MCDB showed a decrease in cell viability, contributing to the overall cytotoxicity of the crude enzyme. The DAPI staining images in Figure 5.2 depicts the increase in the deleterious effect of the crude enzyme on the HeLa cells.

A decrease in percentage of viable cells on increasing enzyme concentration was observed when the partially purified enzyme was used, as seen in Figure 5.1 b and 5.3. Cells exposed to 8U/mL and 10U/mL of partially purified enzyme showed a 47% loss in cell viability as depicted in Figure 5.1 b.

The results indicate that the decrease in cell viability of the HeLa cell line is dependent on the concentration of the enzyme and the incubation time of treatment. Viability of the cells exposed to 4 U/mL, 6 U/mL, 8 U/mL and 10 U/mL of partially purified enzyme showed a statistically significant ($p < 0.05$) difference when compared to the two controls.

5.4 Discussion

The cytotoxic potential of enzyme L-asparaginase obtained from isolate MMS8 against HeLa cells was estimated using MTT assay and confirmed by the DAPI staining method. The cell free supernatant and partially purified enzyme was tested for its anti-cell proliferative abilities. The results indicate a concentration and time dependent reduction in cell viability, confirming the antineoplastic potential of the enzyme.

Mitochondrial metabolic activity of viable cells, as estimated by the MTT assay, revealed a pronounced decline in cell viability at high enzyme concentrations. Cytotoxic potential testing of crude enzyme samples showed a minor reduction in cell viability and with the partially purified enzyme, resulting in a 47% loss of viable cells compared to untreated controls. These findings demonstrate the impairment in the growth of HeLa cells due to the action of the enzyme L-asparaginase obtained from tropical mangrove yeast. Partially purified enzyme, free from other interfering molecules, showed a higher cytotoxic activity. In a study on recombinant L-asparaginase from *Saccharomyces cerevisiae*, Costa et al. (2016) reported cell death of 85% MOLT-4 cells when incubated with 10 U/mL of enzyme

for 72 h. Mazloun-Ravasan et al. (2021) demonstrated that L-asparaginase derived from *Yarrowia lipolytica* exhibited a significant, dose-dependent reduction in cell viability, comparable to the effect observed with commercial L-asparaginase. In a study on L-asparaginase obtained from *Streptomyces rochei*, El-Naggar and El-Shweihy (2020) reported a 79.6% inhibition of HeLa cells treated for 24 h with 10 U/mL of purified enzyme.

The DAPI stained cells were observed to understand the nuclear morphology of the cells and establish the cytotoxic potential of L-asparaginase obtained from MMS8 as determined by MTT assay. The untreated control cells showed a round shaped nucleus with uniform staining, representative of a viable cell. The treated samples depicted a decrease in the number of viable cells with an increase in enzyme concentration. The change in the number of viable cells was prominent for the partially purified enzyme, ascertaining the anti-neoplastic ability of L-asparaginase produced by MMS8. The credibility of the results demonstrating cytotoxic potential is strengthened through the combined use of morphological assessment (DAPI staining) and metabolic evaluation (MTT assay). In a study to determine cytotoxicity L-asparaginase obtained from *Y. lipolytica* by Mazloun-Ravasan et al. (2021) the DAPI staining images of treated A549 and MCF-7 cells showed nuclei with shrinkage and fragmentation. Nuclear fragmentation in treated K-562 cells, observed through DAPI staining, was reported by Darnel et al. (2023) during their study on the periplasmic L-asparaginase of *Pseudomonas* sp. PCH199.

5.5 Conclusion

The CFS, as a cytotoxic compound, showed a decrease in cell viability. A similar assay with partially purified enzyme as the cytotoxic agent revealed 47% loss in cell viability. The partially purified enzyme showed a higher decrease in cell viability compared with CFS. The decrease in cell viability on treatment with enzyme L-asparaginase obtained from isolate MMS8 exhibited the chemotherapeutic potential of the enzyme.

SUMMARY AND CONCLUSION

Summary

Mangroves are tropical forests with complex and dynamic properties. Their transitioning condition supports diverse microbial communities and boosts the production of bioactive compounds for survival. Understanding the importance of these unique forests they were chosen as the source for isolation of yeasts for present research study. Mangrove sediment samples were collected from nine sampling locations namely Adpai, Divar Island, Madkai Ribandar, Borim, Camorlim, Rachol, Raia and Shiroda. A total of 53 yeast isolates were obtained from these samples using the enrichment culture technique. The isolates were purified and maintained on YMA. Morphological features of the isolates were recorded in the form of colony characters and microscopy images.

Primary screening of isolates from all the sampling sites for L-asparaginase activity was conducted using a rapid plate assay method with two different indicator dyes namely Phenol red (PR) and Bromothymol blue (BTB). Out of the total 53 yeast isolates from nine sampling sites, eight isolates SMS1, RMS3, CMS4, MMS7, MMS8, MMS10, MMS11 and MMS12 showed a positive test for L-asparaginase production. Secondary screening included paper chromatography technique which revealed that these eight isolates showed spots on chromatogram with changing intensity of brown coloration for CFS collected at 96h of incubation. The position of the spots is more towards that of L-aspartate indicating conversion of L-asparagine to L-aspartate. The enzyme activity of the eight positive isolates was estimated using CFS as the crude enzyme extract. It was assessed every 24h for 120h. The eight isolates showed enzyme activity at 96h of incubation with isolates CMS4 having highest enzyme activity 35.03 U/mL followed by MMS8 31.52 U/mL. Isolates CMS4 and MMS8 showing highest enzyme activity were selected for further studies.

Biochemical and molecular identification of the selected isolates with subsequent phylogenetic analysis indicated that isolate CMS4 belongs to *Candida* sp., while MMS8 belongs to *Cyberlindnera* sp. *Candida tropicalis* is a recognized opportunistic pathogenic yeast and the study of enzyme L-asparaginase from isolate CMS4 may help in broadening the understanding of this enzyme of eukaryotic origin. In the food and pharmaceutical industries, the most preferred microorganisms are those categorized as generally recognized as safe (GRAS). Previous reports demonstrate the GRAS status of yeasts from the genus *Cyberlindnera* due to their application in the fermentation and cheese-making

industry. Isolate MMS8 was selected for further studies as it exhibited highest L-asparaginase activity and its likely GRAS status, making it a probable source for the development of future medical formulations.

Optimization of media conditions for maximum enzyme production was carried out by One Factor at a Time approach. In case of isolate CMS4 highest enzyme production was observed at an incubation period of 96 Hours with media conditions consisting of an incubation temperature of 28°C and pH 6.5, carbon source Glucose, nitrogen source L-Asparagine and inoculum size of 2%. In case of isolate MMS8 highest enzyme production was observed at an incubation period of 96 Hours with media conditions consisting of an incubation temperature of 28°C and pH 7.0, carbon source Glucose, nitrogen source L-Asparagine and inoculum size of 1%. The optimal levels for L-asparaginase production by isolate MMS8 were predicted by RSM CCD, consisting of medium containing glucose (4.09 g/L), L-asparagine (11.12 g/L) and sodium chloride (1.22%).

The CFS was used to obtain the enzyme by ammonium sulphate precipitation method. The protein precipitation was achieved at 25% of saturation and was further dialysed and introduced on a Sephadex G-100 column for purification by size exclusion chromatography. The molecular weight of partially purified enzyme was analyzed by SDS-PAGE. In the denaturing gel a prominent band was observed near 40 kD.

The optimal conditions of partially purified enzyme were determined to be an incubation period of 60 min, temperature of 37 °C, pH of 7.0. The enzyme is stable up to 3 h with 50% decrease in overall enzyme activity followed by no further reduction. The partially purified enzyme is stable at near neutral pH of 7 and 7.2, indicating its suitability for medical formulations as pH of blood is 7.2.

Effect of the metal ions on the partially purified enzyme revealed that ions Ca^{2+} , Fe^{2+} and Mg^{2+} had a noticeable impact on enzyme activity. Inhibitors can cause a deleterious effect on the enzyme activity. A noteworthy loss in enzyme activity was observed on treatment with the inhibitors Triton, SDS and EDTA indicating probable disruption of enzyme protein structure.

Cytotoxic potential of crude and partially purified enzyme against HeLa cells was determined by MTT assay for an incubation period of 48 h. Cell viability decrease was

observed for crude extract determined by both MTT assay and DAPI staining. A decrease in percentage of viable cells on increasing enzyme concentration is observed when partially purified enzyme is used. Cells exposed to 8U and 10U of partially purified enzyme showed nearly 47% loss in cell viability.

Conclusion

The present study demonstrates that yeasts isolated from the mangrove ecosystem are a promising source of the enzyme L-asparaginase with cytotoxic potential. This work presents the first report on L-asparaginase from tropical mangrove yeast belonging to the genus *Cyberlindnera*. The enzyme activity exhibited by isolate MMS8 is comparable to the previously reported activities for L-asparaginase of yeast origin. The partially purified enzyme showed nearly 47% loss in cell viability, indicating cytotoxic potential of the enzyme. The biochemical characteristics of the partially purified enzyme suggest its potential suitability for future use in medical formulations as an anti-tumour agent.

Future prospects

- Purification and characterization of the enzyme using more advanced techniques.
- Isolating the gene responsible for L-asparaginase production from the positive isolates.
- Incorporating said gene in an expression system to enhance enzyme production.
- Exploring the cytotoxic potential of the enzyme with other cell lines.

APPENDICES

Appendix A

Media Composition

A1. Czapek–Dox Medium (CDM)

Component	Quantity (g/L)
Sucrose	30.0
Sodium nitrate (NaNO_3)	2.0
Dipotassium phosphate (K_2HPO_4)	1.0
Magnesium sulfate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	0.5
Potassium chloride (KCl)	0.5
Ferrous sulfate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)	0.01
Agar (for solid medium)	15.0
Distilled water	1000 mL
Final pH	7.0 ± 0.2

A2. Sabouraud's Dextrose Medium (SDM)

Component	Quantity (g/L)
Dextrose	40.0
Peptone	10.0
Agar (for solid medium)	15.0
Distilled water	1000 mL
Final pH	5.6 ± 0.2

A3. Potato Dextrose Medium (PDM)

Component	Quantity (g/L)
Potato infusion (from 400 g potatoes)	4.0
Dextrose	20.0
Agar (for solid medium)	15.0
Distilled water	1000 mL
Final pH	5.6 ± 0.2

A4. Yeast Malt Medium (YM Medium)

Component	Quantity (g/L)
Yeast extract	3.0
Malt extract	3.0
Peptone	5.0
Dextrose	10.0
Agar (for solid medium)	15.0
Distilled water	1000 mL
Final pH	6.2 ± 0.2

A5. Modified Czapek–Dox Medium

Modified to enhance L-asparaginase enzyme production by altering carbon and nitrogen sources.

Component	Quantity (g/L)
Glucose	20.0
L-Asparagine	10.0
Dipotassium phosphate (K_2HPO_4)	1.0
Magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$)	0.5
Potassium chloride (KCl)	0.5
Ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$)	0.01
Agar (for solid medium)	15.0
Distilled water	1000 mL
Final pH	6.5–7.0

Appendix B

Reagents and Buffers

B1. Sodium acetate buffer pH 4 and pH 5

0.2 M Sodium acetate trihydrate ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$) 27.2g/L

0.2 M Acetic acid (CH_3COOH) 11.5mL of glacial acetic acid in 1L

x mL 0.2 M Sodium acetate and y mL 0.2 M acetic acid mixed

pH	x	y
4.0	18	82
5.0	70	30

B2. Potassium Phosphate Buffer (pH 6.0, 7.0 and 8.0)

0.1 M KH_2PO_4 (Monobasic potassium phosphate) 13.61 g/L

0.1M K_2HPO_4 (Dibasic potassium phosphate) 17.42 g/L

X mL of 0.1 M KH_2PO_4 and y ml of 0.1 M K_2HPO_4

pH	x	y
6.0	61.5	38.5
7.0	39.0	61.0
8.0	15.0	85.0

Volumes are from 0.1 M stock solutions; final volume adjusted to 1000 mL

Sterilize by autoclaving at 121 °C for 15 min.

B3. Bicarbonate Buffer (pH 9.0 and 10.0)

0.1 M Sodium Carbonate (Na_2CO_3) 28.62 g/L

0.1 M Sodium Bicarbonate (Na_2HCO_3) 8.40 g/L

x mL 0.1 M Na_2CO_3 and y mL 0.1 M Na_2HCO_3 mixed

pH	x	y
9.0	10	90
10.0	60	40

B4. Saline Preparation (0.85% NaCl)

Dissolve 8.5 g of Sodium chloride in distilled water to make a volume of 1000 mL. Sterilize by autoclaving at 121 °C for 15 min.

B5. Chloremphenicol

0.2g in 10mL ethanol. 1mL added to 100mL media to get 200mg/L concentration of the antibiotic.

B6. Phenol red 2.5 %

0.125 g of the indicator in 5 mL of ethanol. 300 μ L added to 100mL media to attain 0.009%

B7. Bromothymol blue 2%

0.1 g of the dye in 5mL ethanol. 350 μ L added to 100mL media to attain 0.007%

Appendix C

C1. DNA extraction protocol (Green and Sambrooke, 2018)

Freshly grown yeast colonies were pelleted and resuspended in 50 μ L STES buffer. Acid-washed glass beads, 20 μ L TE buffer (pH 7.6), and 60 μ L phenol:chloroform (1:1, v/v) were added. The mixture was vortexed vigorously for 1 min to achieve efficient yeast cell disruption.

The lysate was centrifuged at 10,000 rpm for 5 min at 20 °C, and the aqueous phase was transferred to a fresh microcentrifuge tube. Chilled absolute ethanol was added, and the mixture was incubated for 5 min to precipitate DNA. The sample was centrifuged at 10,000 rpm for 10 min at 4 °C.

The supernatant was discarded, and the DNA pellet was washed with 70% ethanol, followed by centrifugation at 10,000 rpm for 1 min at 20 °C. The pellet was air-dried for 15 min and finally resuspended in 40 μ L TE buffer for downstream applications.

1. Tris–Cl Buffer (1 M, pH 7.6)

Dissolve 121.1 g Tris base in ~800 mL distilled water. Adjust the pH to 7.6 using concentrated HCl. Make up the volume to 1000 mL with distilled water. Sterilize by autoclaving at 121 °C for 15 min and store at room temperature.

2. Sodium Chloride Solution (5 M)

Dissolve 292.2 g NaCl in ~800 mL distilled water. After complete dissolution, adjust the final volume to 1000 mL. Sterilize by autoclaving at 121 °C for 15 min and store at room temperature.

3. EDTA Solution (0.5 M, pH 8.0)

Dissolve 186.1 g disodium EDTA ($\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$) in ~800 mL distilled water. Adjust the pH to 8.0 using NaOH pellets to facilitate dissolution. Make up the volume to 1000 mL with distilled water. Sterilize by autoclaving and store at room temperature. EDTA does not dissolve completely until the pH approaches 8.0.

4. SDS Solution (10%, w/v)

Dissolve 100 g SDS in 800 mL distilled water with gentle heating if required. After complete dissolution, adjust the volume to 1000 mL. Sterilize by autoclaving or filter sterilization. Store at room temperature.

STES Buffer

Reagent	Final Concentration	Volume (mL)
1M Tris buffer pH 7.6	0.2M	20
5M NaCl	0.5M	10
0.5 M EDTA	0.01M	2
10% SDS	0.1%	1
Deionized water		67
Total volume		100

TE buffer (10X)

Reagent	Final Concentration	Volume (mL)
1M Tris Cl pH 7.6	100 mM	10
0.5 M EDTA	10 mM	2
Deionized water		88
Total volume		100

C2. Sodium dodecyl sulphate Polyacrylamide gel electrophoresis (SDS PAGE) (Sambrook et al., 1989)

Stock solutions for SDS-PAGE:

1. Acrylamide-bis-acrylamide solution (Monomer solution):

Acrylamide	29 g
N, N' methylene bis acrylamide	1 g
De-ionized water	100 ml

A stock solution containing 29%(w/v) acrylamide and 1%(w/v) N,N' methylene bis acrylamide was dissolved in warm de-ionized water. The pH of the solution was ≤ 7.0 . The solution was stored in dark bottle at room temperature and used within 3 months.

2. Stacking gel buffer (1.0M Tris, pH 6.8):

Tris-HCl	12.114 g
De-ionized water	100 mL

12.114g Tris was dissolved in 50ml deionized water. The pH of the solution was adjusted to 6.8 using 6N HCl and the volume was made up to 100ml with de-ionized water. The solution was stored at 4°C.

3. Resolving gel buffer (1.5M Tris, pH 8.8):

Tris-HCl	18.171 g
De-ionized water	100 mL

18.171g Tris was dissolved in 50ml deionized water. The pH of the solution was adjusted to 8.8 using 6N HCl and the volume was made up to 100ml with deionized water. The solution was stored at 4°C.

4. Ammonium per sulphate (APS, 10% w/v):

Ammonium persulfate	0.1 g
De-ionized water	1mL

0.1g APS was dissolved in 1ml deionized water and stored at 4°C. Fresh solution was prepared weekly.

5. Composition of 5X Tris-Glycine buffer:

Tris base	3.02 g
Glycine (pH 8.3)	18.8 g
SDS (10%)	10 mL
De-ionized water	200 mL

To prepare 1X tank buffer, 60 ml of 5X Tris-glycine electrophoresis buffer was made to 300 ml with de-ionized water.

6. Composition of 2X sample buffer (10 mL):

Tris-HCl (1 M, pH 6.8)	1 mL
Glycerol	2 mL
Bromophenol blue (1%)	2 mL
SDS (10%)	4 mL
β -Mercaptoethanol	284 μ L
Deionized water	716 μ L

7. Sodium dodecyl sulphate (SDS, 10% w/v):

Sodium dodecyl sulphate	10 g
De-ionized water	100 mL

10g SDS was dissolved in de-ionized water. Final volume was made up to 100ml with deionized water. The solution was stored at room temperature.

8. Hydrochloric acid (6N):

51ml conc. HCl was diluted to 100ml with de-ionized water.

9. Bromophenol blue (1% w/v):

Bromophenol blue	0.1 g
De-ionized water	10 mL

0.1g bromophenol blue powder was dissolved in 10ml de-ionized water.

Preparation of the gels:

Solution	8% Resolving gel (10ml)	5% Stacking gel (4ml)
Monomer	2.7	0.67
1.5M Tris, pH 8.8	2.5	-
1.0M Tris, pH 6.8	-	0.5
10% SDS	0.1	0.04
10% APS	0.1	0.04
Deionized water	4.6	2.7
TEMED	0.006	0.004

Preparation of sample:

Equal volume of protein sample and 2X sample buffer were mixed and subjected to 100 °C for 10 min. After cooling, the sample was loaded in the well of the gel.

C3. Silver staining of SDS-PAGE gels (Blum et al., 1987)**1. Fixative solution (100 ml):**

Methanol	50 mL
Glacial acetic acid	12 mL
Formaldehyde (37%)	0.05 mL
De-ionized water	37.95 mL

2. Na₂S₂O₃ stock solution (2ml):

Sodium thiosulfate (Na ₂ S ₂ O ₃ ·5H ₂ O)	50 mg
De-ionized water	2 mL

3. Pre-treatment solution (100ml):

Na ₂ S ₂ O ₃ stock solution	0.8 mL
De-ionized water	99.2 mL

4. Silver solution (100ml):

Component	Quantity
Silver nitrate (AgNO ₃)	0.2 g
Formaldehyde (37%)	0.075 mL
De-ionized water	99.92 mL

5. Developing solution (100ml):

Sodium carbonate (Na_2CO_3)	6 g
Sodium thiosulfate stock solution	0.016 mL
Formaldehyde (37%)	0.05 mL
De-ionized water	99.93 mL

6. Stop solution (100ml):

Methanol	50 mL
Glacial acetic acid	12 mL
De-ionized water	38 mL

Procedure:

To perform silver staining, the gel is first fixed in a solution of methanol and acetic acid to precipitate proteins and remove interfering substances, followed by sensitization to enhance protein reactivity to silver ions. Subsequently, the gel is impregnated with a dilute silver nitrate solution, allowing silver ions to bind selectively to protein residues. The bound silver is then reduced, typically using formaldehyde in an alkaline developer, to form insoluble metallic silver that renders the protein bands brown to black. The reaction is stopped with acetic acid, and the gel is washed before imaging or storage. This sequential fixation–impregnation–development process enables detection of protein bands in the low nanogram range, making silver staining a preferred method in proteomic and biochemical analyses requiring high sensitivity.

Appendix D

Methods for quantitative estimation

D1. Folin Lowry's method for proteins (Lowry et al., 1951)

Folin Lowry's method for protein estimation uses the Biuret reaction in addition to Folin-Ciocalteu chemistry. The peptide nitrogen[s] reacts with the copper [II] ions under alkaline condition giving dark blue colour and subsequently the Folin-Ciocalteu phosphomolybdic-phosphotungstic acid is reduced to heteropolymolybdenum blue by the copper-catalyzed oxidation of aromatic amino acids.

Reagents:

1. Reagent A (2% Na_2CO_3 in 0.1N NaOH)

Sodium hydroxide solution (0.1N)

Sodium hydroxide (NaOH) 0.4 g

De-ionized water 100 mL

Dissolve 2 g Na_2CO_3 in an aliquot of 0.1N NaOH solution and make volume to 100 ml with 0.1N NaOH.

2. Reagent B (0.5% CuSO_4 in 1% sodium potassium tartrate)

Sodium potassium tartrate (1%)

Sodium potassium tartrate 1 g

De-ionized water 100 mL

Dissolve 0.5 g CuSO_4 in an aliquot of 1% sodium potassium tartrate solution and make volume to 100 ml with 1% sodium potassium tartrate solution.

3. Reagent C (Alkaline copper solution)

Reagent A 50 mL

Reagent B 1 mL

Prepare fresh Reagent C solution by mixing Reagent A and Reagent B solutions in the ratio 50:1 prior to use.

4. Reagent D (Folin and Ciocalteu's phenol reagent)

Commercially available reagent diluted with equal volume of distilled water on the day of use. This reagent is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acids.

5. Standard bovine serum albumin solution (200 µg/ml)

BSA standard stock

BSA 0.2 g

De-ionized water 100 ml

BSA working stock

BSA standard stock 1ml

De-ionized water 9 ml

BSA working stock was prepared by mixing 1 ml BSA standard stock solution and 9 ml de-ionized water.

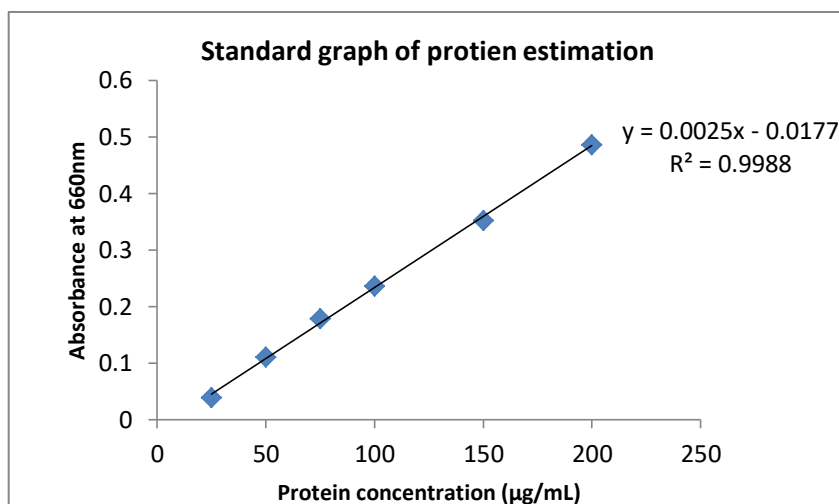
Procedure

To 1 ml of sample, 5 ml Reagent C was added and mixed. 0.5 ml of Reagent D was then added and tubes were kept standing in dark for 30 minutes. Absorbance was measured at 750 nm against reagent blank. Standard calibration curve was prepared using Bovine serum albumin as standard (50 - 200 µg/ml). Absorption values were converted into protein by using following equation:

$$\text{Protein concentration } (\mu\text{g/mL}) = \frac{\text{Absorbance at } 660 \text{ nm} + 0.0177}{0.0025} \quad (\text{D.1})$$

This is a linear regression equation of standard calibration curve prepared by using BSA.

Standard calibration curve for estimation of Protein



D2. Nessler's method for ammonia estimation (Skoog et al., 2014)

Reagents:

1. Stock solution (10 mM NH₄Cl)

Ammonium chloride 53.5 mg. Make up the volume to 100 mL with deionized water.

2. Working Standard (1mM NH₄Cl)

The working stock of ammonium chloride was prepared by mixing 1 mL of ammonium chloride standard stock solution with 9 mL of de-ionized water.

3. Nessler's reagent (Himedia)

A commercially available reagent was used.

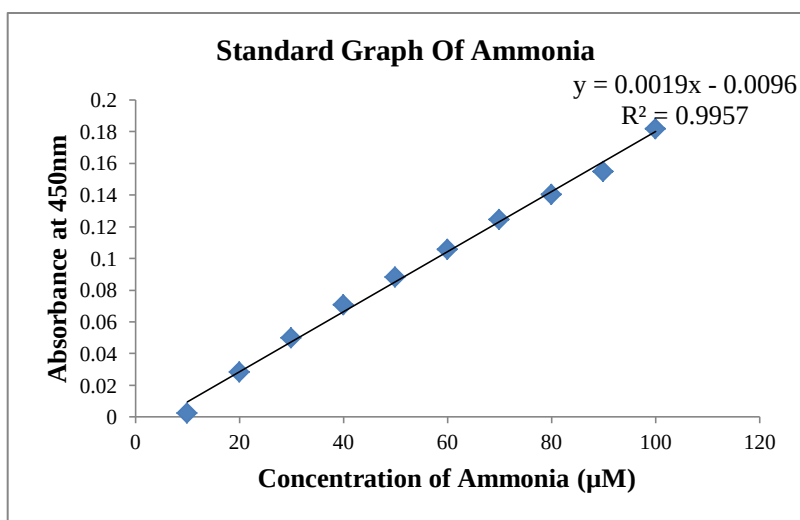
Procedure:

Ammonia standards were prepared using ammonium chloride for the colorimetric estimation of L-asparaginase activity by the Nesslerization method. A 10 mM ammonium chloride stock solution was prepared in distilled water and further diluted to obtain working standards in the range of 10–100 µM. For each standard, an appropriate volume of the working ammonium chloride solution was taken, and the volume was adjusted with distilled water to 3.8 mL. Subsequently, 0.2 mL of Nessler's reagent was added to each tube to obtain a final volume of 4.0 mL. The reaction mixture was mixed thoroughly and

incubated at room temperature for 10 min to allow color development. The absorbance was measured at 425 nm against a reagent blank. A standard calibration curve was constructed by plotting absorbance against ammonia concentration. The following equation was used for the calculation of ammonia released during the enzymatic reaction:

$$\text{Concentration of Ammonia in } \mu\text{M} = \frac{\text{Absorbance at 450nm} + 0.0096}{0.0019} \quad (\text{D.2})$$

Standard graph of ammonia estimation



D3. Estimation of L-asparaginase activity

Reagents for enzyme assay:

1. Tris Buffer (0.5 M, pH 7.2)

60.5 g of Tris base in 500 mL of distilled water. pH adjusted to 7.2 using concentrated HCl and volume to 1000 mL. Sterilized by autoclaving at 121 °C for 15 min.

2. L-Asparagine Solution (0.04 M)

5.28 g L-Asparagine make volume upto 1000mL. Filter sterilized using a 0.22 μm membrane filter.

3. Trichloroacetic Acid (TCA) Solution (1.5 M)

245.1 g of Trichloroacetic acid and distilled water to make volume of up to 1L.
Stored at 4 °C.

Procedure:

Experiments for enzyme assay were performed using the method described in section 3.2.5. the amount of ammonia in test sample was calculated on basis equation D.2. One unit of L-asparaginase is defined as the amount of enzyme required to liberate 1 µmol of ammonia per minute under the assay conditions. Enzyme activity was calculated using the formula given below (Murali T. S. et al.,2011):

$$Activity(U/mL) = \frac{A_C \times V_T}{(V_R \times T \times V_C)}$$

Where,

A_C = µmoles of Ammonia liberated

V_T = Total volume of Reaction mixture (2.5 mL)

V_R = Volume of Reaction mixture used in final reaction (0.1 mL)

T = Incubation time (30 min)

V_C = Volume of enzyme used (0.5 mL)

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