Assessment of optimal conditions for L- asparaginase production by endophytic *Lasiodiplodia pseudothreobromae*

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BIOTECHNOLOGY

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DECLARATION

I hereby affirm that this dissertation thesis titled "**Assessment of optimal conditions for L-asparaginase production by endophytic *Lasiodiplodia pseudothreobromae***" submitted to Thapar institute of Engineering and Technology, Patiala Punjab was granted out by me in the Department of Biotechnology, work carried out during the period of February 2022 to July 2022, under the valuable and efficient guidance of **DR. SANJAI SAXENA**, Professor and **DR. SIDDHARTH SHARMA**, Associate Professor. I also declared that the matter embodied in it is a sincere work and same has not formed the basis for the award of any degree.

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CERTIFICATE

This is to certify that this dissertation thesis titled **"Assessment of optimal conditions for L-asparaginase production by endophytic *Lasiodiplodia pseudothreobromae*"** submitted in partial fulfilment of the obligation for the award of degree of Master of Science in Biotechnology submitted to Thapar institute of Engineering and Technology, Patiala Punjab is bonafide work carried out by **Anindita Saha** and is a great work that was created with the guidance of **Dr. Sanjai Saxena** and **Dr. Siddharth Sharma.**

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LIST OF ABBREVIATION

S.NO.	ABBREVIATION	FULL FORM
1.	µL	Micro liters
2.	µg	Micro gram
3.	µM	Micro molar
4.	mM	Milli molar
5.	mL	Milli Liter
6.	mg	Milli Gram
7.	PDA	Potato dextrose agar
8.	PDB	Potato dextrose broth
9.	LA/ L-ASNase	L- asparaginase
10.	BSA	Bovine serum albumin
11.	U/ml/min	Unit / milli liter/minutes
12.	ALL	Acute lymphoblastic leukemia
13.	nm	Nanometer
14.	psi	Pounds per square inch
15.	MCD	Modified Czapek Dox
16.	ZA	Zone of activity
17.	TCA	Trichloroacetic acid
18.	M	Molar
19.	rpm	Revolutions per minute
20.	CTAB	Cetyltrimethylammonium bromide
21.	EDTA	Ethylenediamine tetra acetic acid
22.	UV	Ultra violet
23.	TBE	Tris-borate-EDTA
24.	V	Volt

EXECUTIVE SUMMARY

L-Asparaginase (LA) (E.C. 3.5.1.1) is a naturally occurring enzyme initiate in diverse microbes. LA catalyzes L-asparagine amidohydrolase an essential amino acid for leukemic cells, into aspartate and ammonia. L-asparaginase is the first line therapeutic enzyme that has been abundantly studied by researchers due to its antitumor properties. In Biopharmaceutical, L-asparaginase has been used in the treatment of acute lymphoblastic leukemia (ALL), acute myeloblastic leukemia (AML), and other lymphoid melanomas, in combination with drugs. In the medical field, endophytic bioactive compounds are playing a major role in the modern new medication because of their wide variety of biological activities as antibiotic, anticancer, antioxidant, and anti-inflammatory agents. Previous study suggested that the source of L-asparaginase supplied by recombinant enzymes from *Escherichia coli, Streptomyces albidoflavus* and *Erwinia chrysanthemi*. However, from bacterial sources proteins associated to immunogenicity problems such as anaphylaxis, hypersensitivity Type IV, hepatotoxicity, leukopenia, pancreatitis, thrombosis, neurological crises and which leads to the search for a better enzyme source. Thus, the use of endophytic fungi as substitute source which comes in trend. These fungi have antimicrobial activity and hence have a great role as biocontrol agent. The current study suggested that the L-asparaginase production and biochemical characterization pointing to increase the LA production. In the current investigation, endophytic fungi *Lasiodiplodia pseudotheobromae* isolated from *Aegle marmelos* was cultured in our laboratory and used to determine the potential of L-asparaginase production by both qualitative and quantitative assays. By screening LA production with the enzymatic zone of activity showed with the value of 0.56. The L-asparaginase activity of the crude protein showed in the range at 5.18 U/min/ml. The optimum temperature (25±2°C) and pH (7) were showed the maximum enzymatic zone of activity at 0.50 and 0.51 respectively. The maximum enzymatic substrate concentration shown at 0.9% (w/v).

Keywords: *Aegle marmelos*, L-asparaginase, endophytic fungi, *Lasiodiplodia pseudotheobromae*, Nesslerization.

CHAPTER 1

INTRODUCTION

1.Introduction

L-Asparaginase (EC 3.5.1.1; L-asparagine amidohydrolase) is an enzyme that catalyzes the hydrolysis of L-asparagine into L-aspartic acid and ammonia (Yadav et al., 2014). L-asparagine is a non-essentially amino acid required for the survival of both normal and cancer cells (Schrek et al., 1973). Normal cells can synthesize L-asparagine for their growth by utilizing a transaminase enzyme that converts oxaloacetate into an intermediate L-aspartate, which later transfers an amino group from glutamate to oxaloacetate producing α-ketoglutarate and aspartate. So finally in healthy cells aspartate is converted to L-asparagine by enzyme asparagine synthetase (Cooper et al., 2015).

Previous studies reported that Guinea-pig serum showed antitumor properties, later credited to asparaginase activity (Broome, 1961). Mashburn and Wriston (1964) found that *Escherichia coli* culture retained antitumor activity, which was then found to be *E. coli* asparaginase II, the first L-asparaginase to be used clinically. However, for tumor cells, L-asparagine is an essential amino acid. Tumor cell cannot synthesize the asparagine due to the absence of the L-asparagine synthetase enzyme, hence are dependent on the exogenous supply of L-asparagine (Kebeish et al., 2016).

Recently, the asparaginase enzyme has received great attention because of its medicinal uses and application as a promising acrylamide-mitigating agent in food industries (Jia et al., 2013). L-asparaginase is also used to develop a biosensor to investigate asparagine levels in leukemia or the food industry (Verma et al., 2012). The mechanism of action of the biosensor is based on asparaginase activity. The ammonium ions produced by the hydrolysis of asparagine cause a change in pH resulting in a color change and absorption (Kumar et al., 2013). Various spectroscopy techniques such as XRD, XPS, SEM, and TEM are presently used for L-asparagine analysis (Zubavichus et al., 2004). L-asparaginase has been a vital chemotherapeutic agent used to treat lymphoproliferative and lymphoma diseases. Particularly, it has huge importance in chemotherapeutic protocols for acute lymphoblastic leukemia (ALL) and Hodgkin's lymphomas (Schrappe et al., 2016).

Previous studies suggested that the source of L-asparaginase enzyme is algae, yeast, bacterial, actinomycetes and fungi (Baghdadi et al., 2014). But due to the difficulties in extraction of L-

asparaginase from mammalian cells, microbes are better sources for production of L-asparaginase (Kebeish, 2016). Asparaginase enzyme has been characterized from many bacterial genera, including *Erwinia carotovora* (Abakumova, 2012), *Streptomyces albidoflavus* (Narayana, 2008), *Pseudomonas aeruginosa* (Kuwabara, 2015), *Helicobacter pylori* (Gladilina, 2009), *Escherichia coli* (Sajitha, 2015). In recent times L-asparaginase of bacterial origin is used in treatment of Acute Lymphoblastic leukemia (ALL). But, in 60% of patients these came up with serious clinical complications even leading to their death (Kaur, 2014). In bacterial cell shown adverse reaction and toxicity associated with the use and enzyme from prokaryotes including anaphylaxis, coagulation disorder, hypersensitivity Type IV, hepatotoxicity, leukopenia, pancreatitis, thrombosis, neurological crises (Duval, 2002).

So, use of endophytic fungi as an alternative source that comes in trend. Endophytic fungi are the microorganisms which reside inside the plant tissues without giving rise to any disease symptoms (Hyde et al., 2008). Endophytes are ubiquitously found in all plants and are valued for their ability to synthesize various useful bioactive compounds (Kaul et al., 2012). Fungal endophytes have been recognized as repository of novel secondary metabolites. Many researchers hypothesize that endophytes from anticancer plants have potential to synthesize compounds with anticancer properties. This prompted investigations on the study of anti-cancer compounds produced by various endophytes, such as, Maytansine (Chow and Ting, 2015), Cajanol (Zhao et al., 2013), *Aegle marmelos* (Patil et al., 2012). Fungal endophytes have been recognized as repository of novel secondary metabolites. *Aspergillus*, *Penicillium* and *Fusarium* spp. have been reported to produce L-asparaginase (Kaur et al., 2014)

L-asparaginase (L-ASN) derived from endophytic fungi have many benefits over bacterial L-ASN preparation (Doriya et al., 2016). The first and most important benefit is fungal L-asparaginases are non- immunogenic as they are phylogenetically related and post-translational modification are present in their system, being the eukaryotic microbes and residing inside the plant body so the stability issues are also tackled (Agnello et al., 2016). The presence of extracellular LA in fungi also focused the way into depth investigations of this enzyme among the various genera of fungi (Nagarethinam et al., 2012). There are only few studies on L-asparaginase production by fungi (Lapmak et al., 2009), these benefits made endophytic fungi the most reliable and potent source

for screening and optimization of L-asparaginase enzyme under in-vitro conditions and also the basis of current study. The interest is to source for L-asparaginase-producing endophytes. Endophytic fungi *Lasiodiplodia pseudothreobromae* (#1048AMSTITYEL) were isolated from *Aegle marmelos* which was already preserved in the laboratory. This study was conducted for screening, optimization and cloning of L-asparaginase enzyme from endophytic fungi.

CHAPTER 2

REVIEW OF LITERATURE

2. Review of literature

2.1 L-Asparagine

Asparagine is neutral polar amino acid, which encodes and synthesized from Aspartate by amidohydrolase in the presence of asparagine synthetase (Oikawa, 2006). In asparagine biosynthesis and catabolism, asparaginase enzyme play a significant role (Borek et al., 2001). Asparagine synthesis is essential for the development and function of the brain (Ruzzo et al., 2013). Its play an important role in food industry also as an acrylamide- mitigating agent, which catalyze the hydrolysis of L-asparagine into L-aspartate and ammonia (Chand et al., 2020; Munner et al., 2020; Sharma et al., 2018).

The potential use of L-asparaginase was discovered as an anti-cancer drug started in 1953, when Kidd observed lymphomas in rat and mice relapsed after treatment with guinea pig serum (Kidd, 1953). Now, L-asparaginase is broadly used in the treatment of acute lymphoblastic leukemia (ALL) (Husain et al., 2016). Though, interpretation of this protein represents 40% of the total enzyme necessities worldwide and one-third portions need for anticancer agents as therapeutic enzymes (Izadpanah et al., 2018).

2.2 Mechanism of L-asparaginase in Normal cells and Tumor cells

In normal cells L-asparagine can synthesize by utilizing transaminase enzyme which converts oxaloacetate into an intermediate aspartate, which later om transfers an amino group from glutamate to oxaloacetate producing alpha-ketoglutarate and aspartate (Ratnikov et al., 2015). Finally in healthy cells, aspartate is converted into asparagine by enzyme asparagine synthetase (Fig. 2.1).

The human asparagine synthetase gene is located in the chromosome region 7q21.3 derived from long arm of human chromosome 7 (Balasubramanian et al., 2013). Tumor cells lack the ability to synthesize the asparagine due to the absence of L-asparagine synthetase enzyme and need exogenous supply of L-asparaginase, which is based on the ability to cleave L-asparagine to ammonia and L-aspartic acid for their proliferation (Naggar et al., 2014).

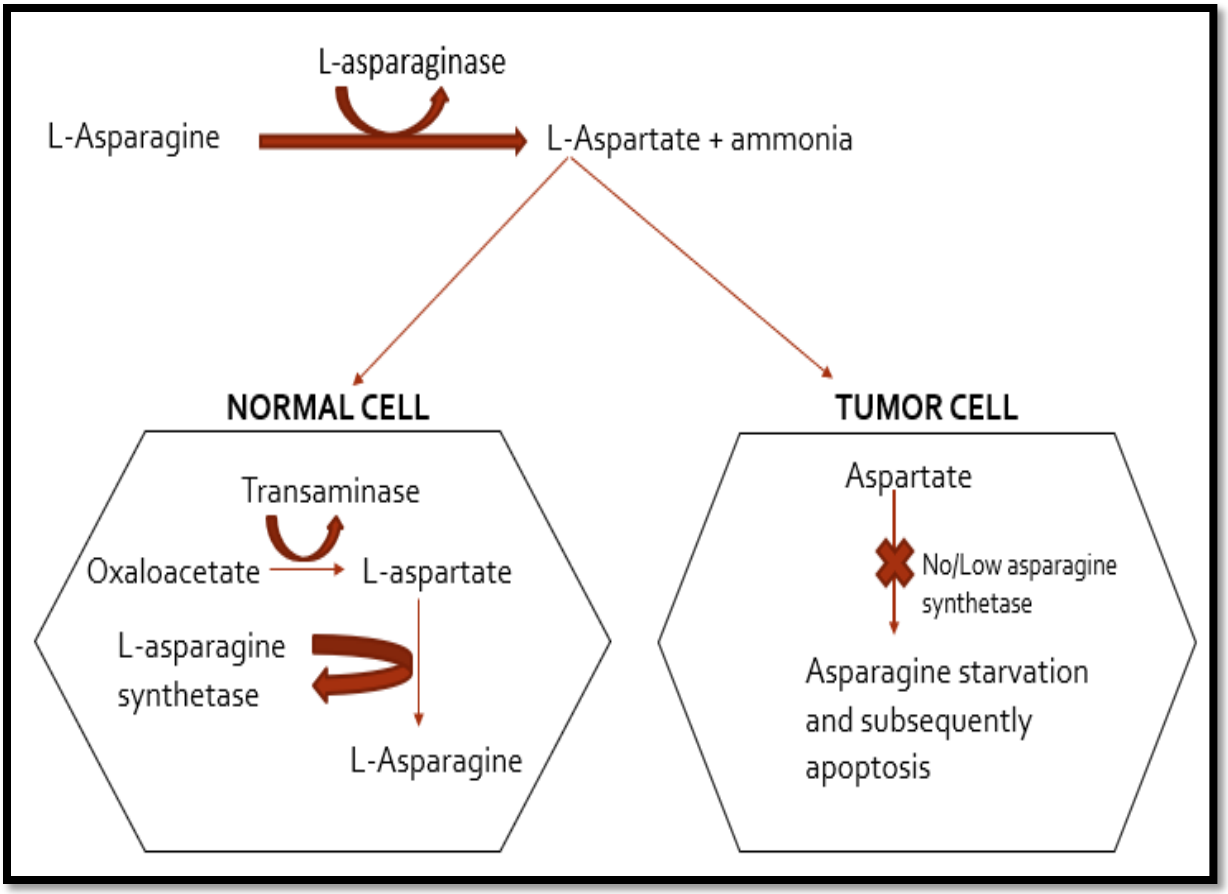


Figure 2.1- Biosynthetic pathway of normal cell and tumor cell

2.3 Sources of L-asparaginase

L-asparaginase has been investigated across a wide-ranging of organisms such as bacteria, yeast, filamentous fungi, algae, higher plants and animals. Previous studies suggested that the source of L-asparaginase from bacteria such as *Bacillus* sp. (Mohapatra, 1995), *Corynebacterium glutamicum* (Mesas, 1990), *Staphylococcus* sp. (Mikueki, 1977), *Erwinia aroideae* (Tiwari and Dua 1996) showed enzyme activity. The enzyme from bacterial sources is limited by antibody response and by various side effects, which are attributed to the L-glutaminase activity of the enzyme (Derst et al. 2000; Pieters et al., 2011). Bacterial cell shown adverse reaction and toxicity associated with the use and enzyme from prokaryotes including anaphylaxis, coagulation disorder, hypersensitivity Type IV, hepatotoxicity, leukopenia, pancreatitis, thrombosis, neurological crises (Duval, 2002).

2.4 Endophytic fungi as a source of L-asparaginase

The term "endophyte" was introduced by De Bary. Heinrich Friedrich first described endophytes in 1809. Endophytes are defined as an organism, mostly bacteria and fungi which colonize the

internal plants tissues without causing any negative effects (Kumar and Kaushik, 2013). Interaction between plant and endophytic fungi includes hostility, mutualistic symbiosis, interdependence, virulent and pathogenicity (Hallman et al., 1997). Endophytic fungi produce several secondary metabolites such as terpenoids, alkaloids, steroids, isocoumarins and chromones, phenolics and phenolic ester, peptides, flavonoids, quinines, volatiles and other compounds, which are used as therapeutic agent and have medicinal value against multifarious diseases (Deshmukh et al, 2018). Endophytic fungi produce several enzymes such as amylases, pectinases, proteases, cellulases, lipases, which are used in pharmaceuticals, medical therapy, molecular biology, food industry, leather industry, tissue industry (Nielsen, 1999; Correa et al., 2014).

Table 2.1: List of endophytic fungi produced L-asparaginase enzyme

S. No.	Culture name	Host plant	References
1	*Lasiodiplodia pseudothreobromae*	*Aegle Marmelos*	Kapoor and Saxena, 2014
2	*C. siamense* F272	*Pereskia bleo*	Chow and Ting 2015
3	*Fusarium Proliferatum*	*Anthemis altissima*	Hatamzadeh et al., 2020
4	*Plenodomus tracheiphilus*	*A. altissima*	Hatamzadeh et al., 2020
5	*Torula herbarum*	*Cichorium intybus*	Hatamzadeh et al., 2020
6	*Fusarium avenaceum*	*A. altissima*	Hatamzadeh et al., 2020
7	*Fusarium subglotinans*	*T. eriocalyx*	Masumi, 2014
8	*Colletotrichum sp.*	*A.microsperma*	Theantana et al., 2009
9	*Alternaria alternata*	*W. somnifera*	Moharram et al., 2016
10	*Fusarium graminearum*	*T. heyneana*	Manasa and Nalin, 2014
11	*Chaetomium sp.1*	*P. tetrastromatica*	Thirunavukkarasu et al., 2011
12	*L. theobromae*	*T. polium*	Balbool and Azeem, 2020

The first reporting of L-asparaginase fungal endophytes for *Cymbopogon citratus, Pereskia bleo, Murraya koenigii, Oldenlandia diffusa* (Chow and Ting., 2015). The distinct numbers of endophytes residing in leaf and stem tissues observed in *P. bleo, C. citratus, M. koenigii, O. diffusa* was

consistent with observations in other important medicinal plants where frequency of endophytes was highest in leaves, followed by stems and roots (Ting, 2020).

2.5 Endophytic fungi associated with anticancer properties

The endophytic fungi with good L-asparaginase activities, an essential tumor-controlling enzyme. Endophytes positive for L-asparaginase activity were predominantly of the genus *Collectotrichum*, followed by the species of *Fusarium, Penicillium* and *Phoma* (Thirunavukkarasu, 2011). *Collectotrichum, Fusarium*, *Penicillium* and *Phoma* spp. are more well known as producers of a variety of bioactive compounds such as alkaloids and antibiotics (Nisa et al., 2015). Hence from this study, L-asparaginase production, particularly for *Fusarium oxysporuman* and *P. simlicissimum* from the Malaysian plants has shown potential host to L-asparaginase producing endophytes. *P. bleo* hosted the highest number of L-asparaginase producing endophytes, mostly in leaf tissues (Chow and Ting 2015). These endophytes can be cultured in the laboratory and L-asparaginase produced can be harvested for development as anticancer compounds.

2.6 Screening for L-asparaginase activity

Previous studies suggested that bacterial isolates were screened for L-asparaginase activity using two methods (Farahat et al., 2020).

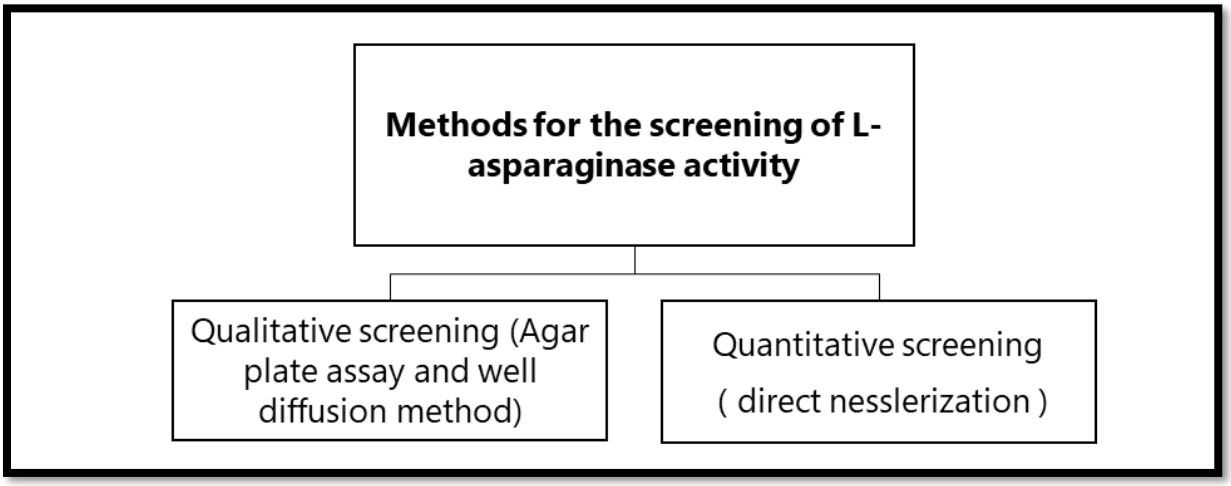


Figure 2.2: Methods used to evaluate L-asparaginase activity

The first method of qualitative screening is the plate assay method. In this method, modified Czapek Dox's (MCD) agar medium containing phenol red was used to screen fungal endophytes for L-asparaginase activity. After incubation pink color zone around the colonies were reflected L-

asparaginase activity (Chow and Ting 2015). The second method is agar well diffusion which used to evaluate extracellular L-asparaginase enzyme activity in Modified Czapek Dox (MCD) medium (Fazeli et al. 2021).

Quantitative analysis of enzyme production is detected by determining the amount of released ammonia derived by the hydrolysis of L-asparagine using Nessler's reagent. In direct nesslerization technique the ammonia released is detected by estimating the optical density at 450 nm. The ammonia released in the reaction reacts with Nessler's reagent to give a colored complex, which is yellow to brown depending upon the amount of enzyme present (Imada et al., 1973).

2.7 Parameters affecting the L-asparaginase enzyme activity

2.7.1 Effect of pH in L-asparaginase activity

In order to assure the greatest possible performance of an enzyme activity, biochemical optimization concerning pH are vital factors to define its function (Krishnapura et al., 2016). Different studies have been performed in order to estimate the effect of pH on activity of LA by performing different concentrations of pH. In general, the LA shows maximum enzyme activity ranges from acidic to alkaline pH values (Chand et al., 2020). The pH affects not only the enzyme configuration but also its affinity for the substrate. The optimum pH or the LA must lie in the physiological range for therapeutic use, but for the food industry, the LA must retain sufficient activity even at acidic pH (Krishnapura et al., 2016). LA produced by bacteria such as *E. coli, Bacillus megaterium, and Pseudomonas* fluorescens presents optimum activity at pH of 6.0, 7.0, and 7.5, respectively (Borah et al. 2012; Sindhu and Manonmani 2018b; Zhang et al., 2015). According to Jeyaraj et al., (2020), a pH value close to 8.0 is needed for a maximum activity for L-ASNase from *B. subtillis* (Jeyaraj et al., 2020). the Gram-negative bacteria *Pseudomonas aeruginosa PAO1 and Rhizobium etli* produce enzymes with maximum activity in acidic and alkaline conditions, 5.5 and 9.0, respectively (Angélica et al., 2012; Dutta et al., 2015).

2.7.2 Effect of temperature in L-asparaginase activity

Temperature also plays a crucial role in stability of the enzyme activity (Daniel et al., 2010). Temperature tolerance and stability of LA differs from species to species as showed in the Table 2. Nevertheless, the enzymes activity has an optimal temperature range in between 25 °C and

45 °C (Chand et al., 2020). Nevertheless, the extreme thermophiles *Thermococcus kodaka* produce LA with optimal activity at 85 °C (Chohan et al., 2020; Muneer et al. 2020). Previous study performed by Kumar et al. (2017), they concluded that the L-asparaginase produced by *B. subtillis* shows an optimal activity at 37°C. Additionally, they showed that the enzyme is also functional in an extensive range of temperature from 30 °C to 75 °C (Kumar et al., 2017). Similarly, Patro and Gupta (2012) obtained a LA from *Penicillum sp*. with optimal activity at 37 °C. They determined the optimal temperature for the enzyme using a range of temperatures between 30 °C and 50 °C. The study performed by Borah et al., (2012) showed the production of LA from *E. coli*, whereas the optimal enzyme activity was reached at 55 °C.

Table 2.2: Optimum temperature of microbial L-asparaginase

Microorganism	**Temperature (°C)**	**Reference**
Escherichia coli	55.0	Borah et al., 2012
Aspergillus niger	40.0	Sharma et al., 2018
Bacillus subtillis	37.0	Kumar et al., 2017
Thermococcus kodaka (TK1656)	85.0	Muneer et al., 2020
Penicillum sp.	37.0	Chand et al., 2020
Aspergillus oryzae (CCT 3940)	40.0	Dias et al., 2016
Cobetia amphilecti AMI6	60.0	Farahat et al., 2020

2.7.3 Effect of substrate concentration in L-asparaginase activity

The enzyme characteristics including L-asparaginase activity and stability is to contain as high substrate selectively (Kotzia et al., 2007). L-asparaginase activity was assayed by using L-asparagine as a substrate (Campbell et al., 1969). Substrate specificity was checked at different concentration of L-asparagine. Enzyme kinetics parameters were determined by incubating the purified enzyme with various concentrations of L-asparagine and enzyme activity was assayed by Nesslerization. In the previous study (Farahat et al., 2019) they conclude that the substrate

specificity of the enzyme was extremely vigorous toward L-asparagine and didn't show any activity regarding L-glutamine.

2.8 Application of L-Asparaginase

In biopharmaceutical, LA has been used as the treatment of acute lymphoblastic leukemia (ALL), acute myeloblastic leukemia (AML), and other lymphoid malignancies. The current ALL therapy involving L-asparaginase is performed with intravenous injection of the enzyme from *E. coli and E. chrysanthemi* in combination with other drugs, radiotherapy or chemotherapy (Ebrahiminezhad et al., 2014; Shrivastava et al., 2016). In commercial market L-asparaginase formulations for therapeutic use are accessible under altered trade names namely Kidrolase, Crisantaspase, Oncaspar (Pegaspargase), Elspar and Erwinase contains L-asparaginase derived from *E. coli*. But the intravenous administration of Pegaspargase increased the risk of allergic reactions (Galindo-Rodriguez et al., 2017).

Asparaginase-based biosensors are auspicious and advanced technology that can be used both to detect and monitor the level of L-asparagine in blood serum samples of leukemia patients and in different food samples (Prabhu et al., 2017). Asparaginase-based biosensor was intended for ammonia detection via the combination of asparaginase in garlic tissue cells, responsible for L-asparagine conversion into ammonia, subsequently detected by an ammonia gas electrode. This electrode-based, ammonia-liberating asparaginase assay system enabled the quick and accurate concentration analysis of L-asparagine in blood serum samples (Nunes et al., 2021). In food industry, it also plays an important role as an acrylamide mitigation agent. After frying, high level of acrylamide was produced during frying process. To reduce the liter of acrylamide was due to decrease of L-asparagine, which is catalyzed by L-asparaginase. In baking, frying and roasted foods, the use of L-asparaginase has been scrutinized as a promising approach (Jia et al., 2013).

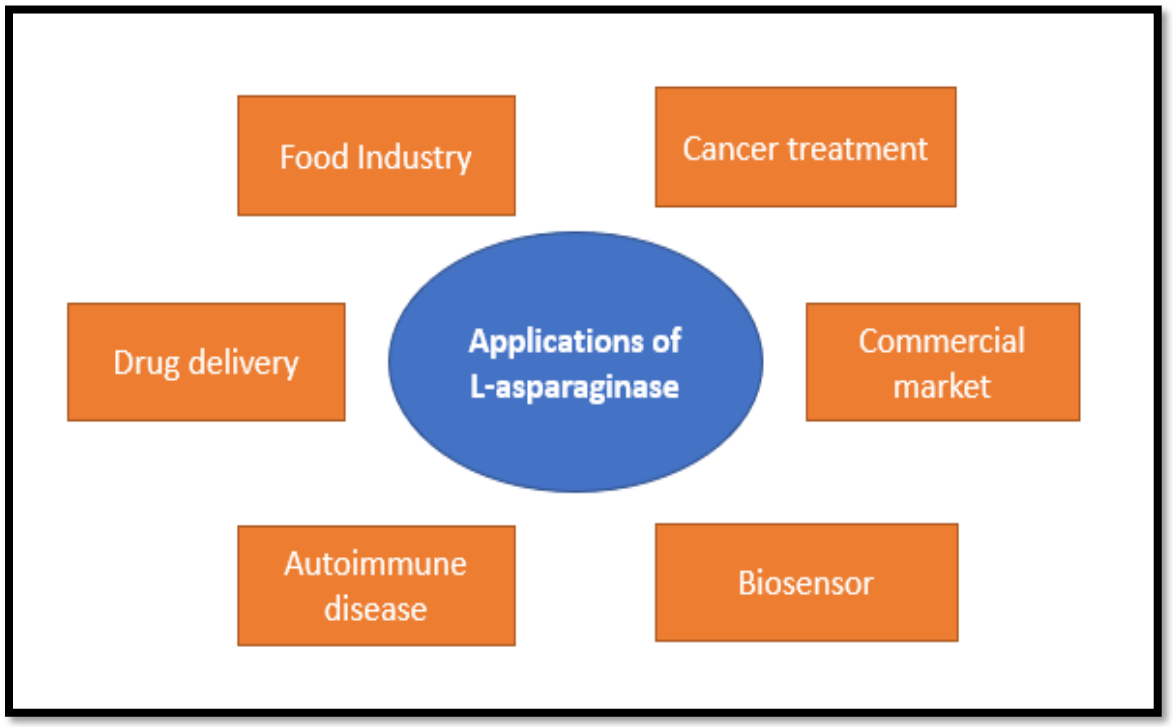


Figure 2.3: Applications of L-asparaginase enzyme

2.9 Protein Quantitation using Bradford method

Bradford method is rapid and accurate method for protein estimation. This technique is faster, simpler and sensitive than the Lowry method (Kruger et al., 2009). Extracellular protein content in crude enzyme and culture broth can be determined using Bradfords method using Bovine serum albumin as the standard curve (Mala et al., 2015).

Bradford assay is for protein determination involving the bonding of Coomassie Blue G-250 dye to proteins (Bradford, 1976). The dye used in assay exist in 3 forms; cationic is red, neutral is green have absorbance maximum at 470nm and 650nm and anionic is blue in color have absorbance maximum at 590 nm (Compton and Jones 1976). Under acidic conditions Coomassie brilliant dye exist in doubly protonated red cationic form, but when it binds to protein, it gets converted to unprotonated blue form. The protein-dye complex formed has absorption maximum at 595 nm, this complex is detected in the assay using spectrophotometer or micro plate reader (Reisner et al., 1975). Practical advantage of this method is that reagent is simple to prepare and the color is developed rapidly and is stable.

2.10 Isolation of genomic DNA

The genus of the fungi *Lasiodiplodia* that belongs to the family Botryosphaeriaceae**.** This fungus is the source of L-asparaginase. With the application of molecular technique, the isolation of genomic DNA becomes an important pre-requisite. Although several protocols are used for isolation of genomic DNA of endophytic fungi described by Elies et al. (2004), based on CTAB extraction procedure, although several successful DNA extraction protocols for species containing phenolics and polysaccharides compound has been developed, none of these are applicable for all species (Borges et al., 2009). Previous protocols are also limited because of degradation of DNA by DNases and other nucleases (Sharma and Sharma, 1980). Therefore, researchers often modify a protocol more different way to get DNA of the desired quality (Varma et al., 2007). The common procedure is to grind the tissue in liquid nitrogen and transfer to CTAB buffer (Dellaporta et al., 1983; Mohapatra et al., 1992). In this study, we had done Genomic DNA isolation from endophytic fungi that can be stored for a longer duration, lasting for several PCR reactions.

CHAPTER 3

AIM OF THE STUDY

3. Aim of the study

The present study is subjected towards the production and optimization of L-asparaginase from endophytic fungi.

The objectives of current study involve:

- Preliminary screening for the production of L-asparaginase enzyme by Endophytic fungi (#1048AMSTITYEL)
- Biochemical optimization of L-asparaginase
- Isolation of genomic DNA from endophytic fungi, Plasmid DNA from PUC19

CHAPTER 4

MATERIALS AND METHODS

4.0 Material and Methods

4.1 Chemicals and microorganism

Nessler's reagent was obtained from S D Fine chemical Limited (India) and all other chemicals used in the experiments were come from Hi-Media, Ranbaxy fine chemicals limited (India)**.** The fungal strain used in this work was *Lasiodiplodia pseudothreobromae* (#1048AMSTITYEL) isolated from *Aegle marmelos,* which was preserved in our laboratory, Thapar institute of engineering and technology, Patiala, Punjab.

4.2 Maintenance and preservation of endophytic fungi

Potato Dextrose Agar (PDA) was used to revive the fungal isolates from the preserved master stock cultures. Then maintenance of the fungal culture was done by sub-culturing and preserved the pure cultures in PDA slants (Agarwal and Hasija, 1986).

4.2.1 Preparation of Potato Dextrose Agar (PDA)

250 g of peeled potatoes were cut into small pieces and boiled in distilled water, till the potato starts to mesh (Rinaldi, 1982). Passed the extract through 8 folds muslin cloth, till it became clear. Further, 15g/l dextrose (Hi-media, India) was added in the potato extract followed by the pH analysis (pH-5.0±0.2). Then, 15g/l Agar (Hi-media, India) were added into medium. The final volume (1litre) of the medium was made up using distilled water. The prepared media was autoclaved at 121° C at 15 psi for 15 minutes. The autoclaved media was aseptically poured in sterile petri dishes of dimensions 90 ×15 mm in laminar air flow hood (LAF) (Thermodyne Pvt Ltd., India).

4.2.2 Culture revival and preservation of pure culture

From the master slants, #1048AMSTITYEL culture was inoculated on the PDA plates using sterile loop followed by the incubated at 26± 2° C for 7-8 days. After the incubation, plate morphology of the culture was noted. The fungal culture was maintained on PDA slants containing 10% glycerol and stored at 4° C for long term preservation (Ana et al., 2016).

4.3 Morphology and culture characteristics

The potent endophytic fungus #1048AMSTITYEL was cultured on potato dextrose agar (PDA) and water agar (WA) medium. The petri plates were incubated for 7-8 days at 26± 2° C (Chow and Ting., 2015). Micro slides of fungal culture were prepared using lactophenol-cotton blue dye and

their various microscopic features like hyphal characteristics were observed under a Nikon optical microscope (Nikon, ECLIPSE E100, Japan) (Sreekanth et al., 2011).

4.4 Qualitative screening of #1048AMSTITYEL endophytic fungi for L-asparaginase-production

Screening of endophytic fungi was done for L-asparaginase production using modified protocols described by Chow and Ting, 2015. For this assay, L-asparagine was used as substrate and phenol red was used as an indicator. To determine the ability to produce L-asparaginase enzyme, #1048AMSTITYEL endophytic fungus was inoculated on Modified Czapek Dox (MCD) agar medium. The composition of Modified Czapek Dox (MCD) medium was mentioned in the Table 4.1.

Table 4.1: Compositions of Modified Czapek Dox (MCD) Agar medium

S. No.	Components	Concentration (g/l)
1.	Dextrose	2
2.	Agar	20
3.	L-asparagine	10
4.	Di-potassium phosphate	1.52
5.	Potassium chloride	0.52
6.	Magnesium sulphate	0.52
7.	Copper nitrate	0.001
8.	Zinc sulphate	0.001
9.	Ferrous sulphate	0.001

After mixing all the components, 0.3ml of 2.5% phenol red was added to the media and then autoclaved at 121° C, 15 psi for 15 min. After the autoclaving of media, pouring of the media is done into sterile plates under sterile conditions. After the solidification, 7 days old culture was inoculated on MCD agar plates using sterile loop. The inoculated plates were kept at 26±2° C for 5-7 days. Pink color zone around the colonies indicates the L-asparaginase activity. The diameter of halo zone and fungal colony was measured for 7-8 day of incubation to determine the

enzymatic zone of activity (Theantana et al., 2009). The enzymatic zone of activity (ZA) was calculated using the following formula:

Enzymatic zone of activity (ZA) = colony diameter/colony diameter+ Halo zone

Here, ZA- 0.9 to 1 was interpreted as very weak enzyme production;
ZA ranging from 0.89 to 0.80 as weak enzyme producers,
ZA ranging from 0.79 to 0.70 as strong enzyme producers,
and ZA lesser than 0.69 as very strong enzyme producers.

4.5 Preparation of culture filtrate of #1048AMSTITYEL isolate

For screening of endophytic fungi, 5mm mycelial plug of 7 days old culture was inoculated in the Modified czapek Dox (MCD) broth in Erlenmeyer flask and were incubated in rotatory shaker incubator at 120 rpm at 28° C for 7-8 days (Imada et al., 1973). After the incubation, the fungal mycelium was separated from broth through filtration using Whatman filter paper followed by centrifugation at 10,000 rpm for 15 min to get cell free culture filtrate. The culture filtrate then used for further experiments.

4.6 Agar well diffusion method for qualitative assay

Culture filtrate of the selected fungi #1048AMSTITYEL were screened for the production of L-asparaginase using plate assay. L-asparagine agar plates containing phenol red as an indicator were prepared in similar way as mentioned previously. The plates were divided into four quadrants and 5mm well were made in each quadrants using cork borer. 20µl of culture free filtrate was poured in 3 wells and fourth well served as control in which sterile un-inoculated broth was added. The plates were incubated at 28° C for 2-3 days. After the incubation, change in color around the wells indicates the positive results. The size of pink zone formed around the wells was measured.

4.7 Quantitative analysis of #1048AMSTITYEL endophytic fungus for L-asparaginase production

4.7.1 Preparation of standard curve of ammonium chloride

A standard curve was prepared using ammonium chloride to determine the activity of L-asparaginase enzyme produced by potent fungus.

Stock solution: 2 mg of ammonium chloride was dissolved in 50 ml of double distilled water.

Working solution: The working solution of ammonium chloride (1µm/ml) was prepared by pipetting out 1 ml of stock solution and final volume was made up to 50 ml with sterile distilled

water. Using working solutions, different concentration of ammonium chloride was prepared as shown in Table 4.2.

Composition of Nessler reagent (1litre): Potassium iodide: 50gm in 50 ml of cold water; Mercuric chloride: 22 gm in 350 ml water; 5N NaOH in 200ml**.**

Table 4.2: Different concentration of ammonium chloride used for preparation of standard curve

S.no.	Conc. (µm/ml)	Amount of working solution used (µl)	Amount of distilled water used (µl)	Final volume (µl)	Amount of Nessler reagent added (µl)	Incubated at room temperature for 10 min and OD was taken at 425nm
1	0.2	20	480	500	500	
2	0.4	40	460	500	500	
3	0.6	60	440	500	500	
4	0.8	80	420	500	500	
5	1	100	400	500	500	

4.7.2 Estimation of ammonia production by potent #1048AMSTITYEL isolate

Activity of L-asparaginase was estimated by determining the amount of released ammonia derived from hydrolysis of L-asparagine using Nessler's reagent. Briefly, the enzyme reaction mixture containing 40mM L-asparagine, 50mM Tris-HCl (pH- 8.0) and 500 µL of crude enzyme extract was mixed thoroughly and incubated at 37° C for 30 min. After the incubation, 1.5M trichloroacetic acid (TCA) was added into it. Further, centrifugation at 15000 rpm for 5 min was done to collect the supernatant. 500µL of supernatant, 3.5mL of distilled water and 1ml of Nessler's reagent was mixed in a test tube.

Further, reaction mixture was incubated at room temperature for 10 min. After incubation, absorbance of developing color was measured using spectrophotometrically at 425 nm. Blank reaction was carried out as the enzyme assay reactions, except that TCA was added prior to enzyme addition. Using ammonium chloride as standard, specific activity of enzyme was

calculated and expressed in term of U/ml was determined. A yellow coloration indicates the amount of ammonia present in higher concentration (Ashok et al., 2019). Enzyme activity was calculated as given below:

$$\textit{Enzyme Activity(U/ml)} = \frac{\textit{amount of ammonia produced (µM) X volume of initial mixture (ml)}}{\textit{incubation time X used crude extract (ml) X enzyme used in final reaction}}$$

Where, initial volume of mixture (ml) =2.5 ml

Incubation time= 30 min

Enzyme taken= 0.5ml

vol. of enzyme used in final=0.5 ml

one international unit of enzyme (1U) is defined as the amount of enzyme needed to liberate one micromole of ammonia from the L-asparaginase enzyme under a particular set of conditions.

4.8 Analysis of parameters that affects the L-asparaginase enzyme activity

4.8.1 Effect of temperature on enzyme activity

Temperature is one of the most vital factors for the enzyme production and play an important role in fungal growth. The effects of incubation temperatures on L-asparaginase production by #1048AMSTITYEL isolate were investigated. The optimum temperature was examined in czapek dox agar plates, keeping the plates in various temperature (15° C, 25° C, 35° C, 45° C) and results were observed after 7-8 days (Jia et al. 2013). The diameter of halo zone and fungal colony was determined to calculate the enzymatic zone of activity (Freitas et al., 2021).

4.8.2 Effect of pH on enzyme activity

pH value of culture medium plays a beneficial role for improving L-asparaginase productivity. The optimum pH was analyzed in MCD agar plates (pH 3, 5, 7, 9). The pH of L-asparaginase was determined by incubating culture plates at different pH levels for 7-8 days at 26± 2° C. After the incubation, enzymatic zone of activity was determined (Freitas et al., 2021).

4.8.3 Effect of substate concentration on enzyme activity

L-asparagine was used as a substrate for the determination of L-asparaginase activity. Substrate specificity was checked using different concentrations of L-asparagine in the medium. Czapek dox broth media containing different concentrations of substrate (0.1-1%) were prepared and culture

was inoculated. After the inoculation, flasks were incubated at 26± 2° C for 7-8 days under shaking conditions (120 rpm). Further, enzyme activity was assayed by nesslerization as described above (Farahat et al., 2020).

4.9 Protein estimation using Bradford method

Total protein content was estimated by using Bradford's method (Bradford, 1976).

4.9.1 Preparation of standard curve of BSA

Firstly, the standard curve of Bovine serum albumin (BSA) was made.

Solutions required:

Solution I: 2% alkaline sodium carbonate ($Na_2CO_3.10H_2O$) + 0.1 N sodium hydroxide (NaOH)

Solution II: 1% Sodium potassium tartrate ($C_4H_4O_6Na.4H_2O$)

Solution III: 0.5% Copper sulphate ($CuSO_4$)

Different dilutions of BSA ranging from 0.2- 1 mg/ml were prepared (Table 4.3) by adding adequate volume of BSA stock solution (1mg/ml) and distilled water in test tubes.

Table 4.3: Different concentration of BSA protein used for preparation of standard curve

Sample conc. (mg/ml)	Protein (µl)	Water (ml)	Bradford reagent (ml)	Incubated at room temperature for 10 min and OD was taken at 595 nm
Blank	0	50	2.5	
0.2	10	40	2.5	
0.4	20	30	2.5	
0.6	30	20	2.5	
0.8	40	10	2.5	
1	50	-	2.5	

4.9.2 Estimation of protein concentration in fungal culture extract

The potent culture was inoculated into czapek dox agar broth and incubated at 26± 2° C for 7-8 days. After the incubation, culture filtrate was centrifuged at 10,000 rpm for 10 min at 4°C. The culture free supernatant was used to estimate the protein. Similar way, the absorbance of the culture filtrate of #1048AMSTITYEL isolate was noted using Bradford reagent and standard curve of BSA was used to calculate the concentration of the sample.

4.10 Isolation of genomic DNA of #1048AMSTITYEL isolate

For the isolation of genomic DNA, the protocol of CTAB extraction method was followed (Borges et al., 2009).

1. 25 mg of fungal mycelium was crushed and converted into a fine powder using liquid nitrogen.
2. Powder was then placed in 2 ml microtubes containing 1ml of CTAB buffer (1M Tris-HCL pH-8.0, 0.5M EDTA, 5M Nacl, 2% CTAB, 0.4% β-mercaptoethanol,1% polyvinylpyrrolidone) and vortexed for 15 min.
3. Then tubes were incubated at 65° C for 30 min, mix gently after 15 min for adequate homogenization.
4. Centrifugation was done for 10 min at 12000 rpm
5. After the centrifugation, 1µL RNAase A was added and incubated at 37° C for 30 min.
6. Phenol: chloroform: isoamyl (25:24:1) was added in reaction mixture in equal volume and inverted for sometimes.
7. Centrifugation of the reaction mixture was done for 10 min at 12000 rpm and aqueous layer was transferred into fresh tube
8. Chloroform: isoamyl (24:1) was further added into the tube followed by centrifugation for 10 min at 12000 rpm.
9. Aqueous layer was transferred into new Eppendorf. Then, 0.08 volume cold 7.5M ammonium acetate was added into it.
10. Further, 0.54 volume of cold isopropanol was added into the reaction mixture followed by incubation at -20° C for 10 min.
11. After the incubation, centrifugation was done for 5 min at 12000 rpm and pellet was dissolved in 80% ethanol.
12. Again, centrifugation was done at 12000 rpm for 10 min, pellet was mixed in 50 µL TE buffer.

DNA Quantification by Agarose Gel Electrophoresis

1. DNA was quantified in 0.8% Agarose gels.

2. 1µl of 6X gel loading dye and 3µL of DNA sample were mixed together before loading into the wells of the gel.
3. Electrophoresis was conducted in a 1 X TBE buffer [100 mL 10X TBE (0.89M Tris Base, 0.89M Boric acid, 20mM EDTA pH 8.) and 900 mL distilled water]. The gel was the stained in EtBr.
4. Electrophoresis gel was run at 60V till the dye has migrated 1/3rd of the distance of the gel.
5. DNA bands was visualized using a UV transilluminator.

4.11 Isolation of Plasmid DNA from PUC19

This method is efficient and rapid method for the isolation of highly purified plasmid DNA from PUC19 (Feliciello and Chinali, 1993).

Requirements for plasmid DNA isolation:

Solution 1: 50mM glucose, 10mM EDTA, 25mM Tris-HCl pH-8.0

Solution 2: 0.2 M NaoH, 1.0% SDS (needs to be freshly prepared)

Solution 3: 3.0M with respect to potassium & 5.0M with respect to acetate

(For 100ml solution III 60 ml of 5.0M potassium acetate, 11.5 ml of glacial acetic acid and water to a final volume of 100ml: pH-5)

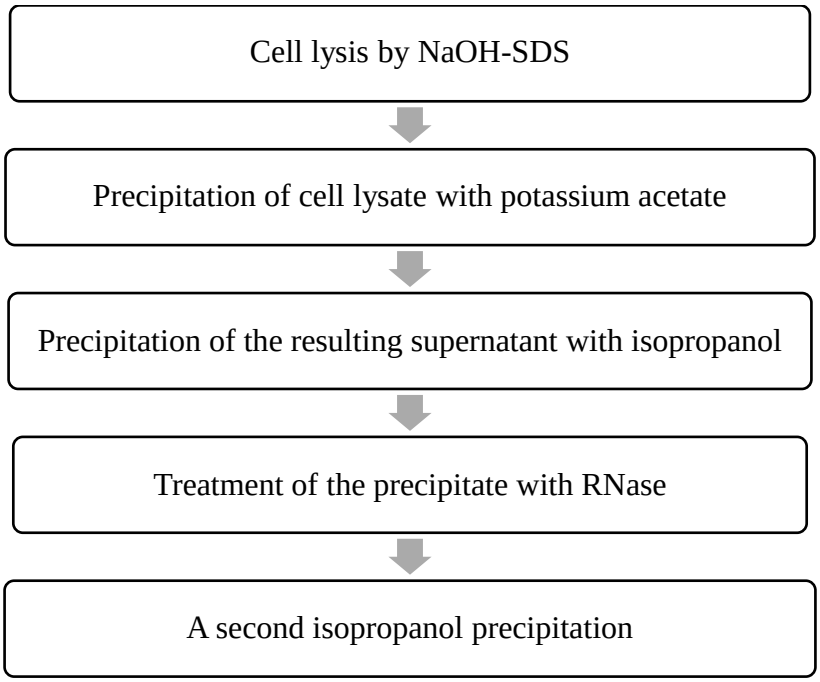


Figure 4.1: Steps of Plasmid DNA isolation

Procedure:

1. A single transformed PUC19 was inoculated in LB medium containing ampicillin in a 250ml conical flask and incubated at 37° C with vigorous shaking.
2. After the incubation, 2ml of culture broth was transferred into a microfuge tube and centrifugation was done at 8000 rpm for 5 min.
3. Supernatant was discarded and bacterial pellet was dried as possible.
4. 2 ml of solution I was mixed with pellet by vigorous vertexing.
5. Further, 2 ml of freshly prepared solution II was added and mixed by gentle inversion of the tubes 5-10 times.
6. After the proper mixing, tubes were incubated on ice for 10-15min.
7. Then, 3 ml of ice-cold solution III was added into the tubes followed by the incubation on ice for 5-10 minutes.
8. Centrifugation was done at 12000 rpm for 10 min at 4° C in a microfuge. Supernatant was transferred into a fresh tube.
9. Equal volume of isopropanol was mixed with supernatant and mixture was kept at room temperature for 5-10 minutes resulting in a DNA precipitation.

10. Again, Centrifugation was done at 10000 rpm for 10 min at 4° C in a microfuge.
11. Supernatant was discarded and tubes were kept in an inverted position on a paper towel to allow all of the fluid to drain away.
12. Pellet was air dried and dissolved in 50ml of TE buffer.
13. DNase free RNase solution (20mg/ml) was added and incubated at 37° C for 30 min.
14. Extraction was done with equal volume of Phenol: Chloroform: isoamyl alcohol (25:24:1).
15. Again, extraction was done using equal volume of Chloroform: isoamyl alcohol (24:1).
16. Upper aqueous layer was transferred into a fresh microfuge tube followed by the addition of $1/10^{th}$ volume of 3M sodium acetate.
17. Double volume of ethanol was added and incubated at -20° C for overnight.
18. Centrifugation was done at 12000 rpm for 10 min at 4 ° C in a microfuge tube. Supernatant was removed and pellet was mixed with 1ml of 70% ethanol.
19. Centrifugation was done at 10000 rpm for 10 minutes.
20. The supernatant was discarded and the pellet was air dried
21. The pellet was dissolved in 30 µl of TE buffer. The DNA was stored at -20° C for further use.
22. Visualization of Plasmid DNA bands by agarose gel electrophoresis.

CHAPTER 5

RESULTS

5.0 Results

5.1 Re-culturing of endophytic fungus #1048AMSTITYEL

Endophytic fungi used in the current study was regularly sub-cultured on PDA plates and maintained at 28°C. For long term preservation, the culture was transferred to PDA slants containing 10% glycerol and maintained at 4°C. The culture was isolated from *Aegle marmelos,* which was preserved in our laboratory. These endophytic fungi associated with the production of L-asparaginase, which can be used as potential treatment for acute lymphoid leukemia (ALL).

5.2 Morphology and culture characteristics of the fungus #1048AMSTITYEL

#1048AMSTITYEL culture was grown over PDA medium and their plate morphology was observed.

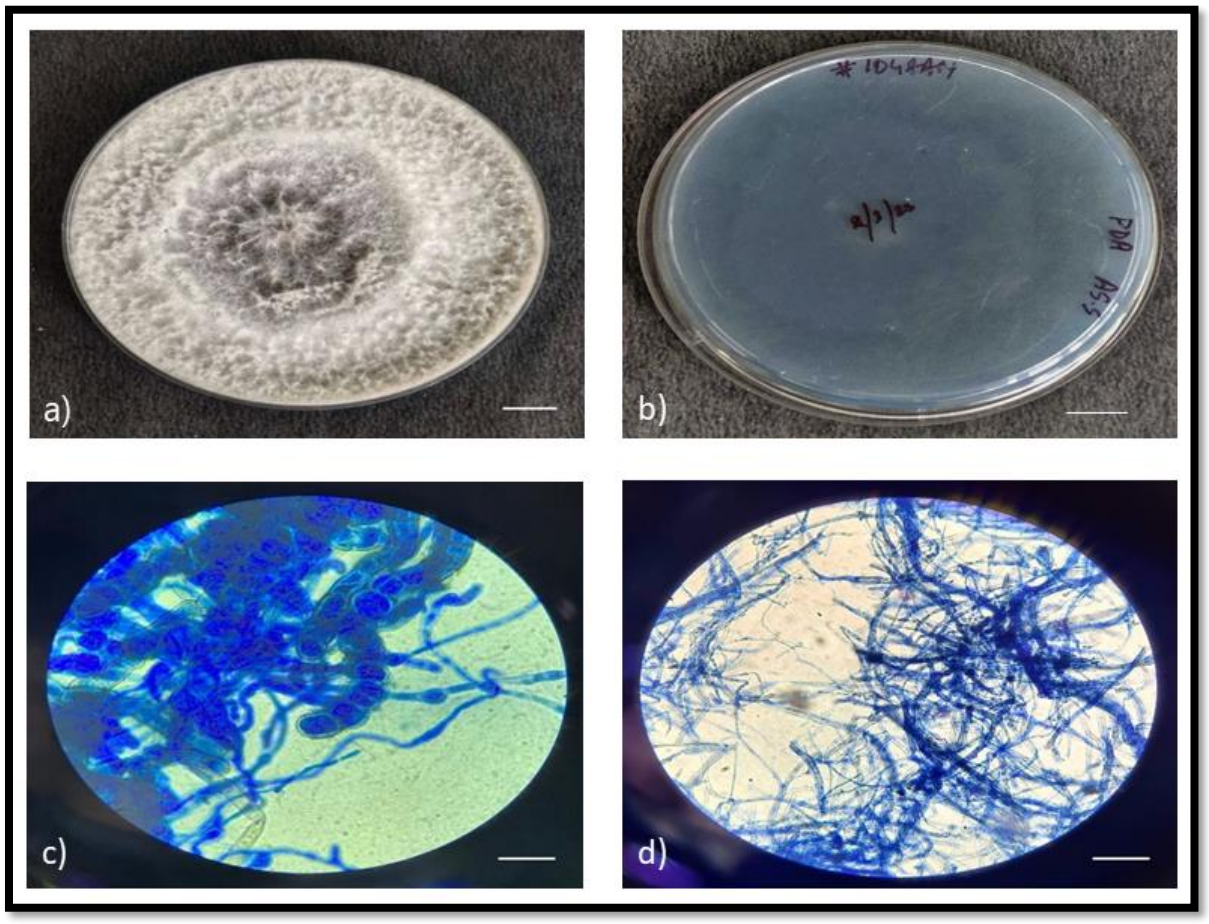


Figure 5.1: Plate and microscopic analysis of endophytic fungi #1048AMSTITYEL on PDA plates

On PDA plates, culture showed sparse, circular, cottony to fairly fluffy with sparse aspect from above side and dark-blackish from the reverse side. Under microscopic, conidial shape with hyaline, cylindrical and holoblastic hyphae was observed. Conidia with oval, both ends round and blunt, wider in the middle, initially transparent and aseptate or not partitioned (Fig. 1).

5.3 Qualitative screening of L-asparaginase producing by endophytic fungi #1048AMSTITYEL

5.3.1 Qualitative screening for L-asparaginase production

The culture was subjected for preliminary screening. The culture when grown on MCD agar medium with phenol red as a pH indicator on the basis of their ability to convert yellow plates to pink under alkaline conditions. The changed color specified the accumulation of ammonia which resulted as L-asparagine converted to L-aspartic acid. So, the culture found to be positive for asparaginase production with the enzymatic zone of activity with the value of 0.56 (Fig. 1). Maximum enzymatic zone of activity was observed after the 7 days of incubation (Graph 1).

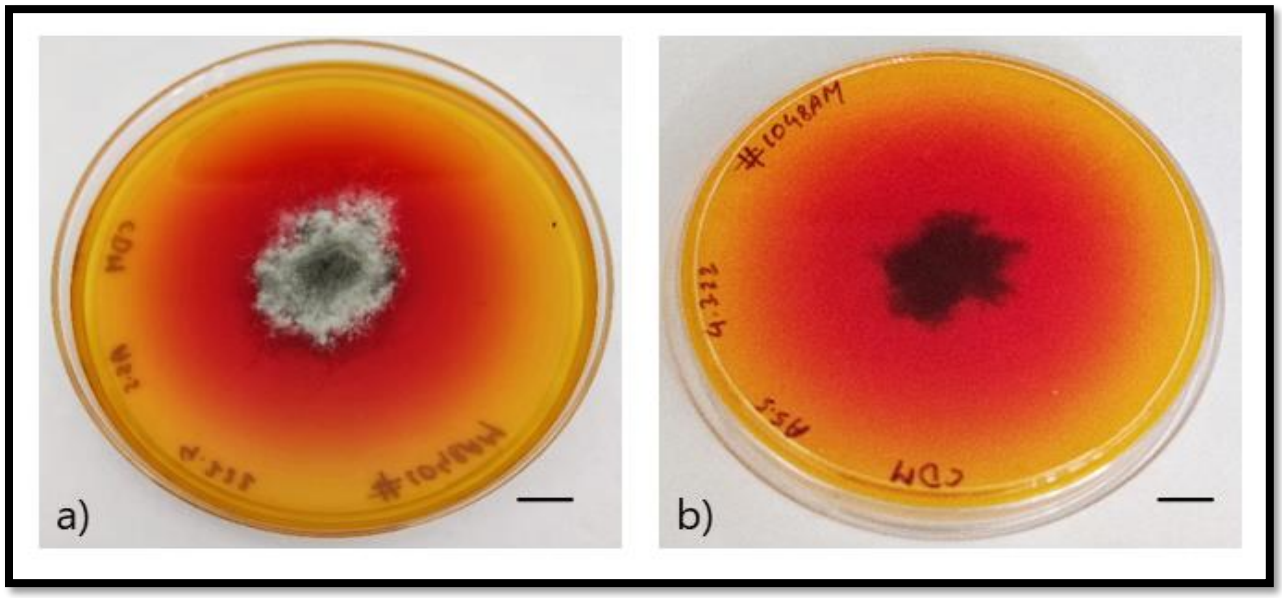
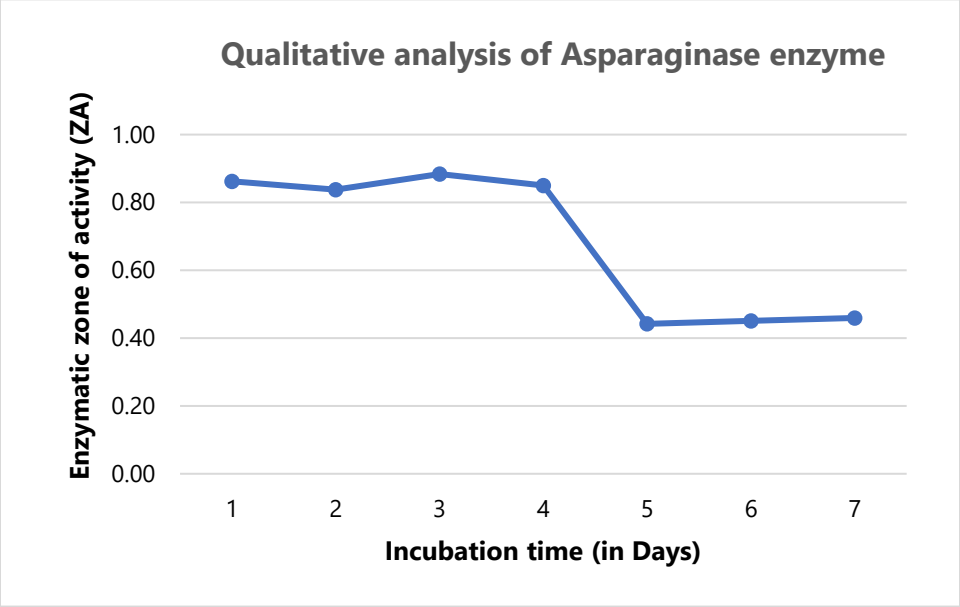


Figure 5.2: Qualitative analysis of L-asparaginase producing by endophytic fungi #1048AMSTITYEL of Enzyme activity on MCD plates

Graph 5.1: showing enzymatic zone of activity of L-asparaginase producing by endophytic fungi #1048AMSTITYEL after different interval of time



5.3.2 Agar well diffusion (Qualitative assay)

The culture filtrate of the endophytic fungi **#**1048AMSTITYEL was assessed for L-asparaginase production by agar well diffusion assay. **#**1048AMSTITYEL showed zone diameter of 1.86 cm after the incubation of 3 days. Control well containing uninoculated broth did not showed any zone.

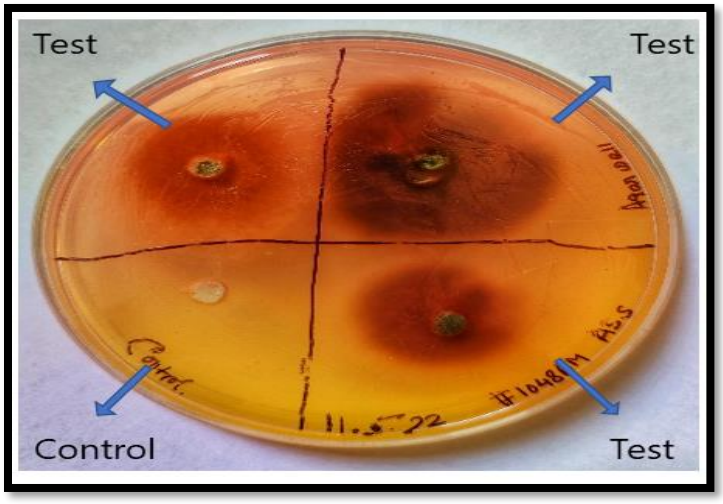


Figure 5.3: Agar well diffusion assay to determine asparaginase activity

5.3.3 Quantitative evaluation of L-asparaginase by Nesslerization

The activity of L-asparaginase was evaluated when the accumulation of ammonia resulted in the conversion of L-asparaginase to L-aspartic acid as a result pink color appeared, which was next evaluated for L-asparaginase production in liquid conditions by direct nesslerization (Imada et al.,1973). To find the activity of L-asparaginase by quantifying the amount of ammonia released, standard curve of Ammonia was constructed via. Equation obtained was Y= 0.1519x + 0.0408 with R^2=0.998. Then, from this graph the ammonia released in the test sample was calculated using the following equation:

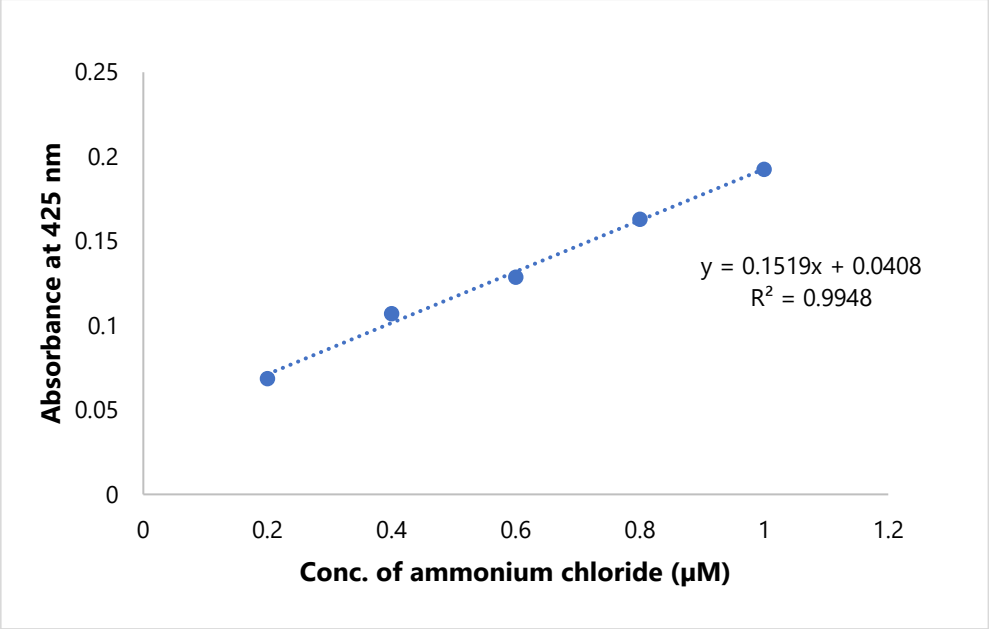
$$Y=Mx+C \quad \text{..... (1)}$$

$$Y= 0.1519x + 0.0408 \quad \text{..... (2)}$$

$$x= Y - 0.0408/0.1519 \quad \text{..... (3)}$$

Where, Y= Absorbance at 425 nm, x= concentration of ammonia liberated (µmol/ml)

Graph 5.2: Standard curve of ammonium chloride



Finally, activity of the L-asparaginase from the test fungal was calculated in (Units/ml) using the formula:

Enzyme Activity(U/ml) = amount of ammonia produced (µM) X volume of initial mixture (ml)
incubation time X amount of crude extract (ml) X vol. of enzyme used in final reaction

Where, initial volume of mixture (ml) =2.5 ml

Incubation time= 30 min

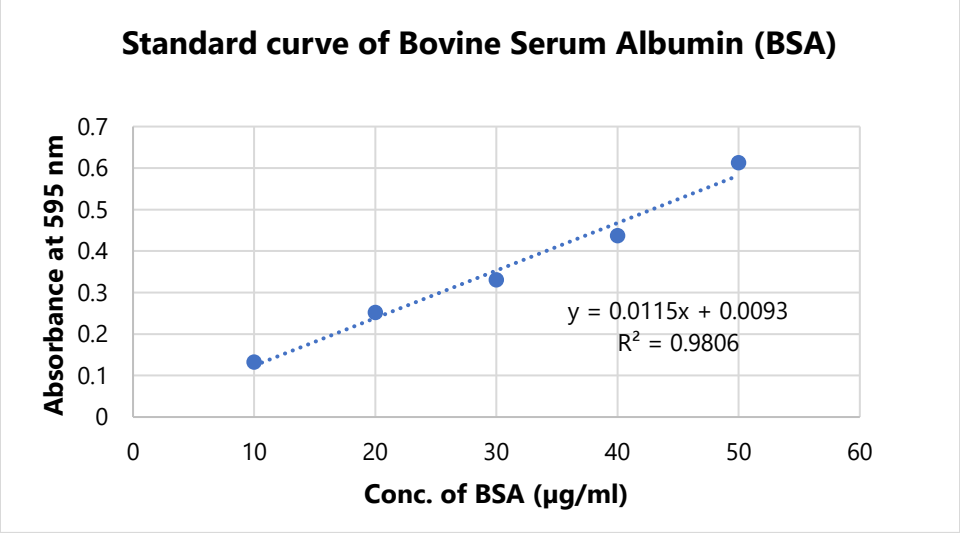
Enzyme taken= 0.5ml

vol. of enzyme used in final=0.5 ml

one international unit of enzyme (1U) is defined as the amount of enzyme needed to liberate one micromole of ammonia from the L-asparaginase enzyme under a particular set of conditions.

5.3 Estimation of protein content of the crude extract

Bradford assay standard curve of concentration versus absorbance. In a standard reaction 30µL of each BSA concentration with 2.5ml of Bradford reagent and allowed to stand at room temperature for 10 min and then absorbance was measured at 595nm. The calibration curve is needed to calculate samples concentrations. The concentration of protein (mg/ml) was determined using the equation y=0.0115x + 0.0093 with in an R^2 value of 0.9806, where y is absorbance and x is concentration as shown in the graph (5.3).

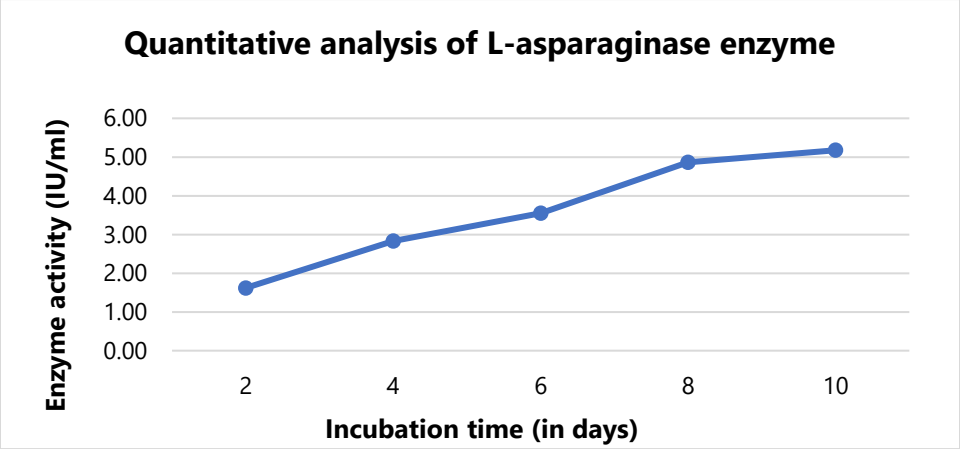


Graph 5.3: Standard curve of Bovine Serum albumin

Table 5.1: L- asparaginase activity of #1048AMSTITYEL isolated at different interval of time (days) in U/ml of crude protein

Incubation time (In days)	Absorbance± Standard deviation	Protein conc. (mg/ml)	Enzyme activity (U/ml of crude protein)
2	0.41±0.01	0.04	1.62
4	0.68±0.02	1.09	2.83
6	0.85±0.03	1.66	3.55
8	1.15±0.02	2.13	4.87
10	1.22±0.04	2.53	5.18

Graph 5.4: Estimation of L-asparaginase showed maximum activity on the 10th day



5.5 Parameters that affect the Asparaginase enzyme activity

5.5.1 Effect of temperature on enzyme activity

Fungal isolate's capacity for LA activity was tested on modified MCD supplemented with L-Asparagine, as a sole source of nitrogen. The primary assay was achieved by using phenol red as indicator. For presence of corresponding enzymes, the color changes from yellow to pink. The presence of a pink color region around the colonies on MCD plates because of released of enzyme. The enzyme showed maximum activity at 25°C. The optimum temperature of L-asparaginase for

high stability at 25°C and showed zone of enzyme activity at 0.50 after 7 days incubation in 25°C. And at 15°C and 45°C no zone formed.

Table 5.2- Effect of different temperature on enzyme activity

Temperature (°C)	Plate observations after 7 days of incubation	Enzymatic zone of activity (ZA)
15	minor fungal growth with no halo zone	-
25	Pink halo zone formation with maximum fungal growth	0.50
35	A small halo zone formation with minor fungal growth	0.83
45	No fungal growth	-

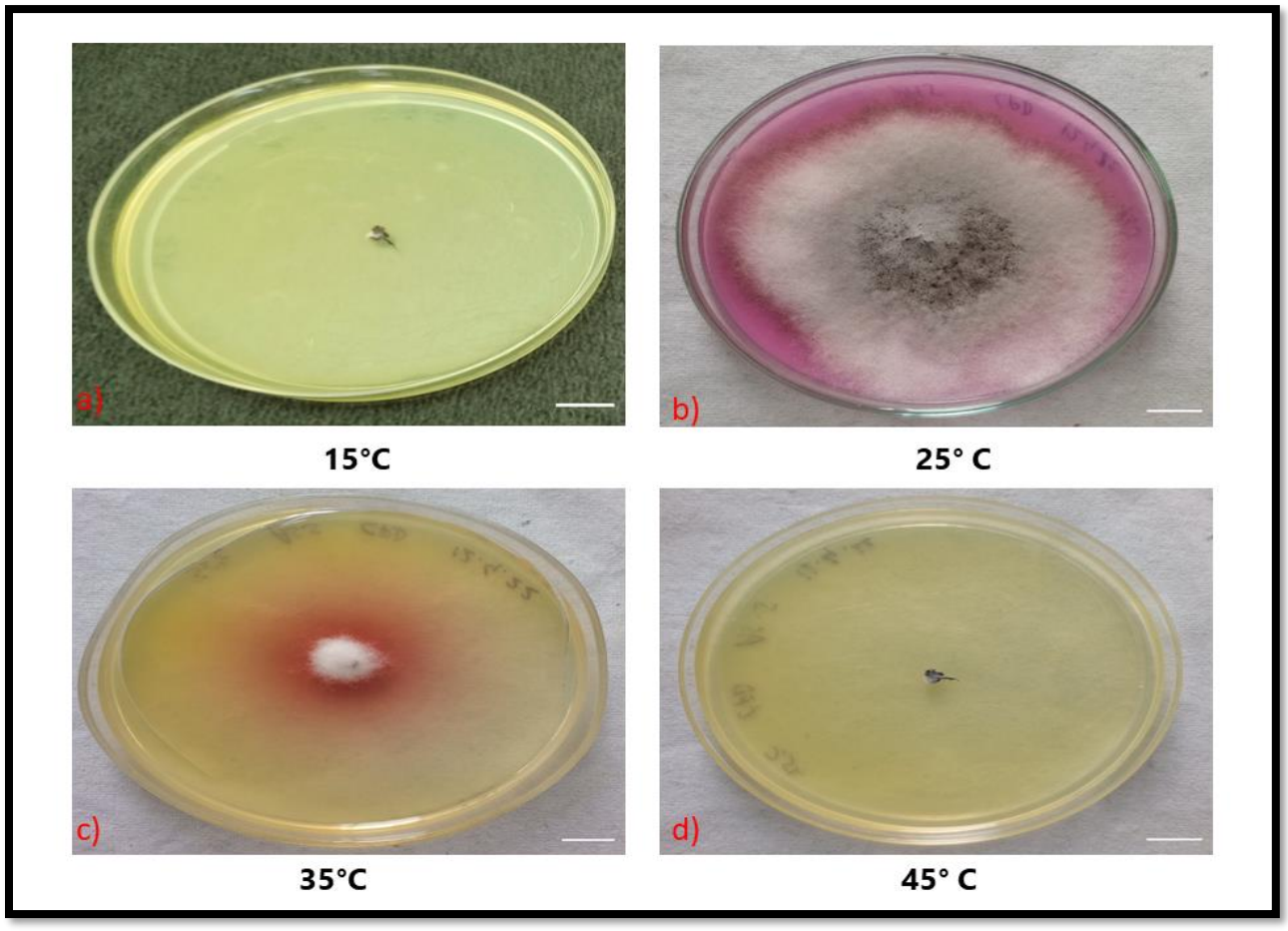


Figure 5.4: Effect of different temperature on enzyme activity

5.5.2 Effect of pH on enzyme activity

As same the fungal isolated was tested on MCD supplemented with L-Asparagine. The assay was done by using phenol red as indicator, phenol red dye is yellow at acidic pH and changes to pink at alkaline pH. So, the existence of pink color around the plates indicates corresponding enzyme. The optimum pH of L-asparaginase for hydrolyzing L-Asn was pH 7. The maximum enzyme activity retained after incubation at pH 7 for 7 days with zone of activity 0.51

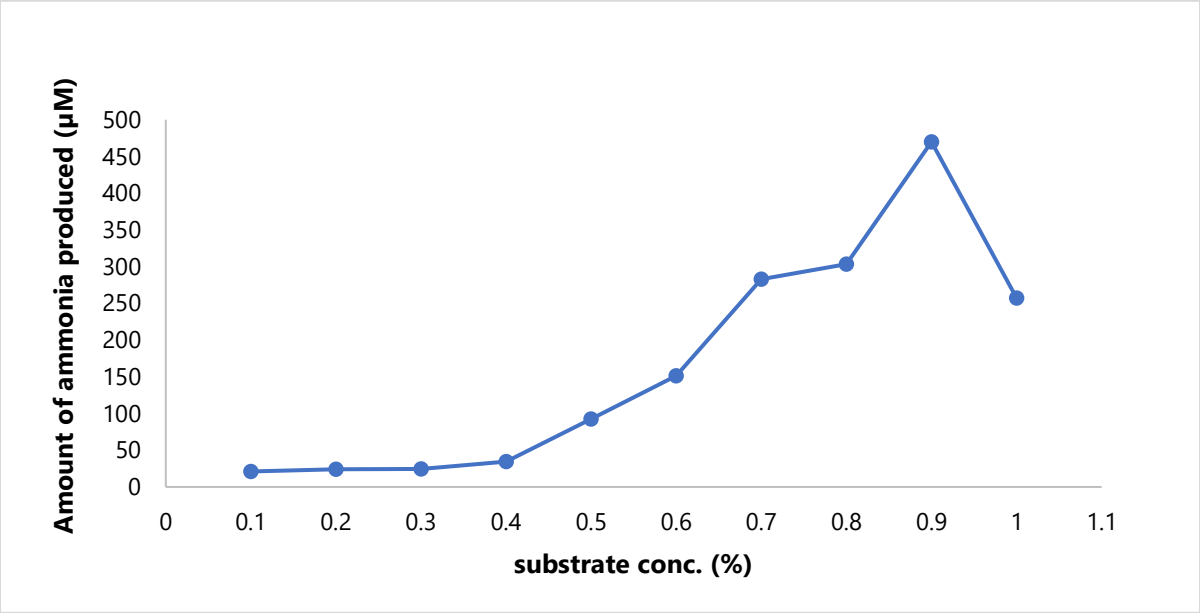
Table 5.3- Effect of different pH on enzyme activity

pH	Plate observations after 7 days of incubation	Enzymatic zone of activity (ZA)
3	Light halo zone with minor fungal growth	0.95
5	Halo zone formation with good fungal growth	0.56
7	Halo zone formation with good fungal growth	0.51
9	No fungal growth	-

Figure 5.5: showed highest enzyme activity at pH-7

5.5.3 Effect of substate concentration on enzyme activity

The L-Asparaginase activity was assayed by using Nesslerization method. Higher the concentration of the substrate, more ammonia liberated. At the concentration of 0.9%, highest ammonia was produced as shown in the graph 5.5.



Graph 5.5: showed the L-asparaginase activity under different substrate concentrations

5.6 Isolation of genomic DNA of #1048AMSTITYEL isolate

The DNA isolated from the endophytic fungi were carried out using CTAB method. The quality of the extracted genomic DNA was observed on 0.8% gel electrophoresis (Fig 5.6). Sharp molecular DNA band was observed in the lane (2,3 and 4) as shown in the figure without any smear. The extracted DNA sample was suitable enough for PCR based methods.

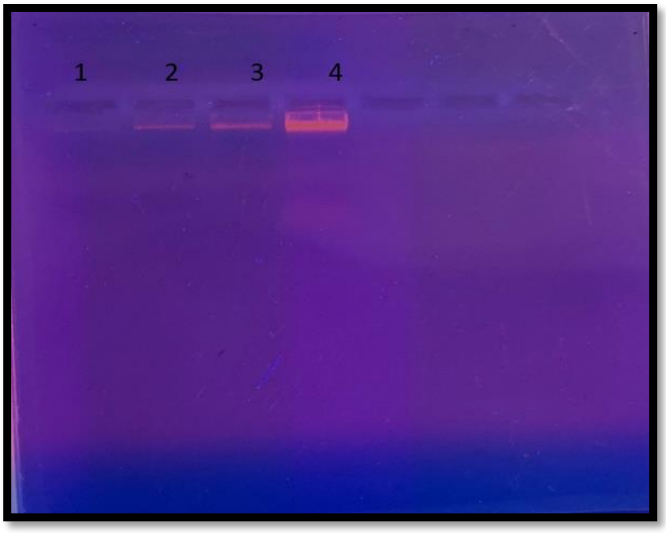


Figure 5.6: Results of Gel- electrophoresis after DNA isolation

5.7 Isolation of Plasmid DNA from PUC19

Plasmids have been found to be extensive distribution in bacteria. They are self-replicating extrachromosomal DNA which are not essential for the growth of their host cells. For further cloning purpose, these PUC19 recombinant plasmid have to be isolated from the transformed hosts in order to characterize by restriction analysis and sequencing. Gel electrophoresis using agarose has been used in the detection of plasmid DNA fragments. Plasmid DNA isolation was positively done (Fig 5.7). As a reference we used 1kb ladder (lane 1). And lane 2,3,4,5 contains plasmid DNA.

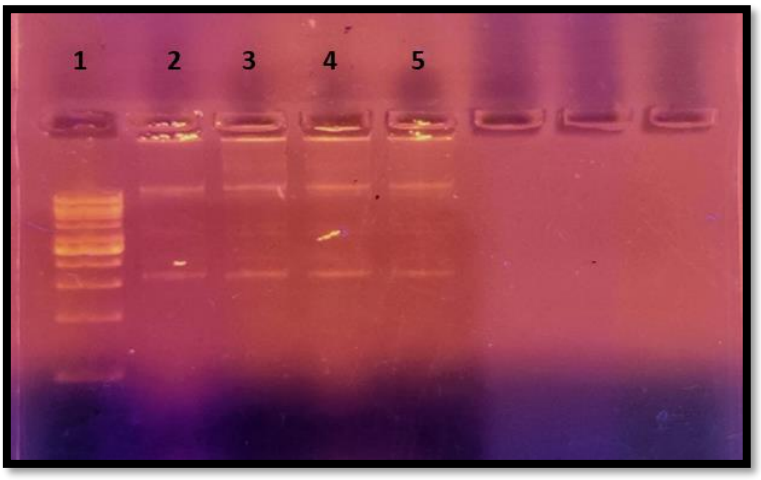


Figure 5.7: Plasmid DNA was visible in agarose gel electrophoresis

CHAPTER 6

DISCUSSION

6. Discussion

L-asparaginase is the first line therapeutic enzyme that has been abundantly used as anti-tumor properties. It is also used to treat Acute lymphoblastic leukemia (ALL). Asparaginase enzyme has been characterized from many bacterial genera. But prokaryotic cells shown adverse reaction and toxicity associated with the use and enzyme, which also attributed to the L-glutaminase activity of this enzyme (Duval 2002; Jia et al., 2013). Thus, using alternative method using endophytic fungi. The fungi that colonize the internal plant tissues without causing any negative effect on host plant are known as endophytic fungi. Fungal endophytes released secondary metabolites. In the medical field, these endophytic bioactive compounds are playing a major role in the biological activities as antibiotic, anticancer, antioxidant, and anti-inflammatory agents. Previous study suggested that the other source of LA was *C. siamense* F272 isolated from *Pereskia bleo* are potential host of endophytes with medicinal properties (Chow and Ting 2015), From endophytic fungi it also showed that there is no contamination of L-glutaminase activity (Kaur and Saxena., 2014).

Endophytic fungi were proven to excellent source for the production of l-asparaginase without any difficulties. Benefit from the fungal isolated L-asparaginase as it has lowered immunogenic of the L-asparaginase. Fungi and humans are both eukaryotic in origin and the immunogenicity of fungal L-asparaginase may be reduced due to the post translational modification of protein in fungi. In this view, the present study was carried out using fungal endophytic fungi *Lasiodiplodia pseudothreobromae* isolated from *Aegle Marmelos* produce LA. Previous study also suggests that *Fusarium proliferatum isolated from Anthemis altissima (*Hatamzadeh et al., 2020), *Fusarium subglotinans* isolated from *T. eriocalyx* (Masumi, 2014) also showed LA production. Previous study reported that the isolated *Colletotrichum sp.* from *Adenanthera microsperma* shows enzyme activity within the range of 1.523 U/ml, whereas in this study isolated *L. pseudothreobromae* showed maximum L-asparaginase activity with 5.18 U/ml.

In this study, the optimum temperature for maximum L-asparaginase production was determined at 7 days incubation. Temperature is the important variable in enhancing L-asparaginase production in *L. pseudothreobromae*. *L. pseudothreobromae* is not selected in (15°C, 35°C, 45°C) tested. LA production and fungal growth were ceased at high temperature and low temperature

as metabolic reactions were exaggerated, resulting in slower growth and cellular damages. The optimum incubation temperature is 25°, but it was able to withstand the increase temperature up to 27°C for *L. pseudothreobromae*, while in other endophytic fungus such as *F. proliferatum* reported 35°C as optimum temperature for L-asparaginase production (Yap et al., 2021). Thus, include for different fungus or species temperature vary differently.

Another parameter that is well known to the effect of LA production is the pH of culture media. In this study, pH differences of the growth medium pH-3, pH-9 verified no significant growth on L-asparaginase activity. *L. pseudothreobromae* was able to adapt to the endurable pH range (pH-5, pH-7). In this study consistent with Basu et al., 2015, thus penetrable to its ability to produce L-asparaginase, while other studies suggested that most fungi are able to tolerate a varied range of pH (pH 4 to 9) but sporulation and maximum growth observed in pH that is slightly acidic to neutral pH (5-7) (Papagianni., 2004). No significant result shown any enzyme activity at the range of pH-9 due to the growth inhibition as *L. pseudothreobromae* was unable to endure high alkaline culture condition, thus distressing fungal metabolism and enzyme production.

In this study, the optimum incubation period for highest L-asparaginase activity was resolute at 12 days. The log phase of *L. pseudothreobromae* was from day 3 and reached the stationary phase at day 12 as the production of L-asparaginase. This is due to the reduction of fungal growth caused by nutrient limitation, changes in pH value in the culture media or inhibitor end product such as ammonia, which may have affected the growth of *L. pseudothreobromae* and production of LA (Papagianni., 2004). Enzyme production will be inhibited after reaching the peak due to nutrient exhaustion stress resulting in inactivation of enzyme production (Krishnapura and Belur. 2016). In this study, substrate concentration is also an important factor for the enzyme activity, *L. pseudothreobromae* shows substrate affinity at 469.93mM. For instance, Asha and Pallavi (2012) reported an enzyme from *Fusarium sp*. shows substrate affinity 443.98 mM. This was further proved by the comparison with crude enzyme produced by another fungal isolate which demonstrated a lower L-asparaginase activity. Overall, this study supports the production of fungal L-asparaginase. Nevertheless, from isolated fungus *L. pseudothreobromae* there are improvements in the production of L-asparaginase to be considered by optimization. This study suggesting that there is potential concentrating the crude enzyme in order to produce L-

asparaginase with higher purity. We expect better cytotoxicity effect from the use of purified enzyme produced from endophytic *L. pseudothreobromae* in the near future, as it is cost effective and it decreases the degradation of L-asparaginase.

CHAPTER 7

CONCLUSION

7. Conclusion

The current study describes that the production of L-asparaginase from the endophytic fungi #1048AMSTITYEL. This potent culture was screened by qualitative and quantitative assay and showed L-asparaginase yield from the particular fungus *Lasiodiplodia pseudothreobromae*. The qualitative analysis was done by i) Agar well diffusion and ii) by using indictor in MCD agar. The quantitative analysis was done by Nesslerization method.

Various parameters such as different temperature, pH and substrate concentrations was considered to determine the optimum conditions for enzyme activity. The maximum enzyme activity was observed at Temperature (25°C), pH (pH-7) and substrate concentrations (0.9%). For further cloning purpose, isolation of the genomic DNA from the endophytic fungi and isolation of the plasmid DNA from PUC19 was done.

CHAPTER 8

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