L-Asparaginase producing endophytic fungi isolated from medicinal plants

A thesis submitted in the partial fulfilment of the requirement

for the degree of

MASTERS OF SCIENCE

IN

BIOTECHNOLOGY

Submitted by:

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July, 2019

CERTIFICATE

This is to certify that the thesis entitled "L-Asparaginase producing endophytic fungi isolated from medicinal plants" submitted by Sonali Bedi in the partial fulfilment of the requirement of the award of the degree of Masters in Biotechnology, Thapar Institute of Engineering and Technology, Patiala, is a record of the student's own work carried out under my supervision and guidance. This report has not been submitted for the award of any other degree or certificate in this or any other University or Institute.

Vasundhara

Dr. M Vasundhara

Supervisor

DBT

TIET, Patiala

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I

DECELARATION

I hereby declare that the work that has been presented in the thesis **"L-Asparaginase producing endophytic fungi isolated from medicinal plants"** submitted by **Ms. Sonali Bedi** in the partial fulfilment of the requirement for the award of the degree of Masters of Science in Biotechnology, Thapar Institute of Engineering and Technology, Patiala, is an original record of my own work done during the period from January 2019 to July 2019, carried out under the guidance of **Dr. M. Vasundhara.** This dissertation report has not been submitted in part or full to any other university or institute for the award of any other degree.

Sonali Bedi

Sonali Bedi

Place: Patiala

Date: 19/08/19

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ACKNOWLEDGEMENT

I thank the almighty whose blessings have enabled me to undertake my dissertation work successfully. I am thankful to my parents and my family who supported me and motivated me during entire journey of my project.

I would also like to express my gratitude towards my supervisor **Dr. M. Vasundhara** who always supported and motivated me through the entire period of the project. She provided me with all the equipments whenever required.

I am fortunate enough to **Dr. Moushumi Ghosh,** HOD, Department of Biotechnology, Thapar Institute of Engineering and Technology, Patiala for giving me the opportunity to do the project.

I am thankful to **Dr. Anil Kumar,** Head of TIFAC CORE and **Dr. M.S. Reddy** for providing me with all the lab instruments.

I wish to thank **Mr. Babban, Mr Surinder and Mr. Lallan,** lab staff of Department of Biotechnology for providing all the lab equipments and other requisites to us.

I am thankful to laboratory helper of TIFAC CORE, **Mr. Soni.**

My sincere thanks to all those involved directly or indirectly in my project work.

Sonali Bedi.

Dated: 19/08/19 **Sonali Bedi**

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LIST OF ABBREVIATIONS

1	ALL	Acute Lymphoblastic Leukemia
2	g	Gram
3	BSA	Bovine Serum Albumin
4	h	Hour
5	L	Litre
6	M	Molar
7	mM	Milimolar
8	min	Minute
9	MCD	Modified Czapex Dox’s
10	nm	Nanometer
11	PDA	Potato Dextrose Agar
12	rpm	Rotations per minute
13	µg	Microgram
14	µmole	Micromole

ABSTRACT

Endophytic bioactive compounds from medicinal plants can be integrated in novel drug discoveries due to their wide variety of biological activities such as antibiotic, anticancer, antioxidant and anti-inflammatory agents (Jawahar et al. 2014). Acute Lymphoblastic Leukemia (ALL) is a cancer of the bone marrow in which early lymphoid precursors multiply and supplant the typical hematopoietic cells of the marrow. Larger part of these cases are in kids and youthful grown-ups. Asparaginases have been the foundation of ALL treatments for most recent 4 decades (Seiter et al. 2018). Medicinal plants are home of numerous proficient bioactive metabolites producing endophytic fungi that have incredible medicinal and therapeutic worth. L-Asparaginase is one of the enzymes produced by endophytic fungi.

The objectives of this study were to screen endophytic fungal isolates for L-Asparaginase activity, optimization of parameters of fermentation for the production of L-Asparaginase. Identification of endophytic fungus producing maximum amount of L-Asparaginase was also done.

In this study, pre-isolated fungal cultures of *Tinospora cordifolia* (Giloy), (GR, GR3, GS1, GS3, GS4, GL2), *Terminalia arjuna* (AL2, AL4, AJ4, AF2, AL3), and *Taxus baccata* (T6) were taken and analysed for L-Asparaginase activity. Out of 12 endophytic fungal cultures, 10 fungal cultures gave positive result in qualitative assay after 3 days of incubation at 26±2°C. These positive cultures were then subjected to quantitative analysis.

Out of the ten endophytic isolates tested for quantitative analysis, AL4 showed maximum L-Asparaginase activity of 16.407 U/mg/mL. Afterwards, optimization of fermentation parameters for maximum production of L-Asparaginase from AL4 was done. The specific enzyme activity was determined on 2^{nd}, 3^{rd}, 5^{th}, 7^{th} and 8^{th} day of incubation. Maximum enzyme activity of 137.80 U/mg/mL was recorded at pH 5 on the 5^{th} day. Optimum temperature was found to be 30°C with the specific enzyme activity of 31.58 U/mg/mL.

Identification of AL4 was done by using microscopic, macroscopic and molecular method. AL4 grown on PDA plate showed that the fungus was white, cottony and reached 8 cm diameter of the plate in ten days. Long branched hyphae were observed when seen under microscope. Flattened oval shaped spores were also observed. PCR amplification of ITS regions were done using ITS1 as forward primer and ITS4 as reverse primer. By running electrophoresis PCR product was evaluated. The ITS amplified sequence of 569 bp obtained

was run through blast n. Homologous sequences were obtained in FASTA format that were then aligned using Multalign. Evolutionary relationships between homologous sequences were found by neighbor-joining method with bootstrapping of 1000. MEGA 10 software was used to construct phylogenetic tree. It confirmed the clustering of AL4 with *Fusarium proliferatum.*

Endophytic fungi thus can be a novel source for the production of L-Asparaginase enzyme and can play a very important role in food, pharmaceuticals and other industries.

CHAPTER 1 INTRODUCTION

Living cells synthesize very selective biocatalysts called enzymes. The enzymes that can be used individually as well as in conjunction with other therapies to cure various diseases are therapeutic enzymes. L-asparagine amidohydrolase, E.C. 3.5.1.1 is an amidase group enzyme which catalyzes the hydrolytic cleavage of L-asparagine to ammonia and aspartic acid (Ghasemi et al. 2017). It is used as a therapeutic enzyme. This enzyme is extensively distributed and found in wide assortment of micro-organisms. For the first time, the fortunate revelation of L-Asparaginase was accounted as the antitumor activity of the guinea pig serum against rodents (Kidd, 1953). It has potential applications in food and medical industries.

L-Asparaginase has been utilized for curing acute lymphoblastic leukemia (ALL) for over 30 years (Ghasemi et al. 2017). It is a cancer of the bone marrow in which early lymphoid precursors multiply and supplant the typical hematopoietic cells of the marrow (Seiter et al. 2018). Larger part of these cases are in kids and youthful grown-ups. Asparaginases have been the foundation of ALL treatments for most recent 4 decades (Seiter et al. 2018). Likewise, L-Asparaginase is the potential natural additive in the mitigation of acrylamide formed at high temperature. Acrylamide in baked and fried starchy items can possibly cause harmful impacts in humans and animals (Baskar et al. 2018).

Asparagine is a dietary prerequisite of ordinary cells as well as malignant growth cells. L-Asparaginase enzyme as an anti-cancer agent breaks the L-asparagine present in circulating blood plasma that indirectly deprive cancerous cells of this amino acid and leads to cell death. Normal cells have enzyme asparagine synthetase for the synthesis of asparagine from aspartic acid though malignant cells do not express asparagine synthetase (Theantana et al. 2007). So, they acquire the required asparagine from circulating blood pool. Thus, L-Asparaginase is intravenously infused to diminish the L-asparagine concentration in blood, specifically denying the malignant cells of aspargine (Sarquis et al. 2004). Some commercially available L-Asparaginase injections are Elspar from *E. coli* (Ovation pharmaceuticals, US), Erwinase, kidrolase from *Erwinia* (EUSA pharma, UK), Leunase from *E.coli* (Sanofi-Aventies, France) (Pieters et al. 2011).

L-Asparaginase extraction from the mammalian cells is troublesome and costly. So, for efficient production of L-Asparaginase micro-organisms are better option, as they are proficient producers.

In spite of the fact that production and purification systems for L-Asparaginases have been developed for recovery from the bacterial species, the yields of L-Asparaginase have been low (Kenari et al. 2011). Likewise, cancer treatment with L-Asparaginases have been hindered by glutaminase activity for certain L-Asparaginases. Glutaminase action can cause neurological seizures, pancreatitis, leucopenia, liver dysfunction, and coagulation abnormalities leading to haemorrhage (Duval et al. 2002). Henceforth, it is attractive to discover approaches to produce L-Asparaginases in higher yields and to discover enzyme with low or no glutaminase action. Also, the problems associated with L-Asparaginase from bacterial sources make us switch to better sources.

Endophytic fungi are sources for potential novel drug. Endophytic fungi are the micro-organisms that reside in the living host plant without causing any harm to the plant building symbiotic relationships with plants. Medicinal plants have been perceived as an archive of endophytes with novel metabolites of therapeutic and pharmaceutical significance (Strobel et al. 2004). Endophytic fungi produce many bioactive compounds and enzymes such as lacase, paclitaxel, camptothecine, hypericin, esterases, diosgenin, hemicellulases, vinblastine, chitin deacetylase, podophyllotoxin. (Zhao et al. 2001; Robl et al. 2013; Suryanarayanan et al. 2012).

L-Asparaginase from endophytic fungi can be a promising source than bacteria as it is safe and non-allergic also, they are from eukaryotic organisms and more phylogenetically related. Further, the presence of extracellular L-Asparaginase in some endophytic fungi provide L-Glutaminase free L-Asparaginase. (Duval et al. 2002). These advantages make endophytic fungi the most dependable and strong hotspot for screening of L-Asparaginases and furthermore the ground of current studies.

CHAPTER 2 OBJECTIVES

1. Screening for L-Asparaginase producing endophytic fungi isolated from medicinal plants.
2. Optimization of parameters of fermentation for the production of L-Asparaginase
3. Identification of endophytic fungus producing L-Asparaginase.

CHAPTER 3 REVIEW OF LITERATURE

3.1 Enzymes and their role in therapeutics

Enzymes having greater affinity, specificity and good catalytic efficiency are required for many chemical transformations that maintain life and accelerate all the metabolic procedures. These whole qualities distinguish them from every single other sort of medications. Due to these, enzymes are generally been utilized for various therapeutic treatments and are securing much consideration. Therapeutic enzymes are either used individually or in conjunction with other treatments for curing many diseases such as digestive disorders, cancer, inflammation, ulcers etc. (Kaur and Sekhon, 2012).

Micro-organisms produce medically important enzymes that are being advantaged as being economical and reliable, as they are easy to modify, optimize and obtained yield is maximum. Some of the reported natural bacterial sources of L-Asparaginase are listed below (Table 1):

Table 1 Bacterial sources of L-Asparaginase

S.No.	SOURCE	REFERENCE
1	*Acinetobacter calcoaceticus*	Joner et al. (1973)
2	*B. mesentericus*	Tiul'panova et al. (1972)
3	*B. subtilus*	Fisher and Wray, (2002)
4	*Bacillus* sp.	Mohapatra et al. (1995)
5	*Corynebacterium glutamicum*	Mesas et al. (1990)
6	*E. aroideae*	Tiwari and Dua, (1996)
7	*E. cartovora*	Maladkar et al. (1993)
8	*Enterobacter aerogenes*	Mukherjee et al. (2000)
9	*Escherichia coli*	Netrval, (1977)
10	*Mycobacterium bovis*	Wriston and Yellin, (1973)
11	*Pectobacterium carotovorum*	Kumar et al. (2011)
12	*Pyrococcus furiosus*	Bansal et al. (2010)
13	*Rhodosporidium toruloides*	Ramakrishnan & Joseph, (1996)
14	*Saccharomyces cerevisiae*	Roon et al. (1982)
15	*Streptomyces gulbargensis*	Amena et al. (2010)

Enzymes help in the regulation of the growth and development of a single cell into a complete organism, it helps to convert complex food into simple for the production of energy that is required by the body. (Kaur and Sekhon, 2012). Some of the medically important therapeutic enzymes along with their uses are enlisted in Table 2

Table 2 List of therapeutic enzymes and their uses (Shrestha, 2010)

S.No.	Enzyme	Uses
1	Urokinase	Blood clots
2	Trypsin	Inflammation
3	β-lactamase	Penicillin allergy
4	Hyaluronidase	Heart attack
5	Streptokinase	Anticoagulant
6	Lysozyme	Antibiotic
7	Uricase	Gout
8	Glucosidase	Antitumour

3.2 Asparagine

Asparagine is an amino-acid that is synthesized in the body and is utilized in many metabolic functions. Asparagine synthetase (located in chromosome 7q21.3) catalyzes the formation of aspargine from aspartate and is utilized in the biosynthesis of proteins. Asparagine is important for liver and brain function (Ruzzo et al. 2013). In endoplasmic reticulum, oligosaccharyl transferase enzyme catalysis the expansion of N-acetylglucosamine to asparagine (Seidemann et al. 1999). This glycosylation is critical for both protein structure and function (Asselin et al. 1999). It is naturally found in animal and plant source such as soy, eggs, seafood, nuts, whey, seeds, whole grains. It is also a safe additive in food.

In normal cell, oxaloacetate is precursor of aspargine. Oxaloacetate is changed over to aspartate by utilizing transaminase enzyme. It exchanges the amino group from glutamate to oxaloacetate creating α-ketoglutarate and aspartate. From aspartate, glutamine and ATP the protein asparagine synthetase gives asparagine, glutamate, AMP and pyrophosphate (Figure 1).

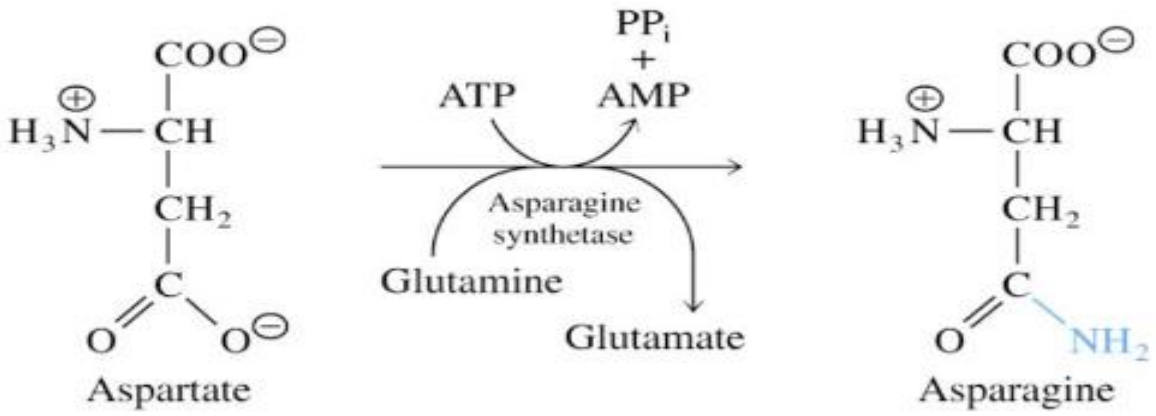


Figure 1 Biosynthesis of L-Asparagine.

Lymphoblastic leukemia cells do not have asparagine synthetase; therefore, they are unable to synthesize asparagine by their own (Asselin et al. 2000). They are dependent upon serum asparagine for their proliferation. Normal aspargine concentration in blood is between 40-80µM. L-Asparaginase selectively vanishes serum aspargine to 0.1-0.2 µM and deprives tumor cells of aspargine needed for protein synthesis hence these cells get arrested in G1 phase ultimately leading to apoptosis. (Boos et al. 1996; Zhang et al. 2014).

3.3 Mode of action of L-Asparaginase

Asparaginase was first recognized by Lang in 1904 in the beef tissues. Kidd in 1953 credited anti-tumor activity of guinea pig to asparaginase activity. Anti-tumor activity of *E.coli* isolated asparaginase was reported by Mashburn and Wriston in 1963. Work continued in a few research centers prompting clinical preliminaries made *E.coli* asparaginase II, the primary anti-leukemic to be utilized medically. Current studies with L-Asparaginase keeps on concentrating on its oncological significances, structure-function connections of L-Asparaginase from an assortment of sources are additionally right now under scrutiny.

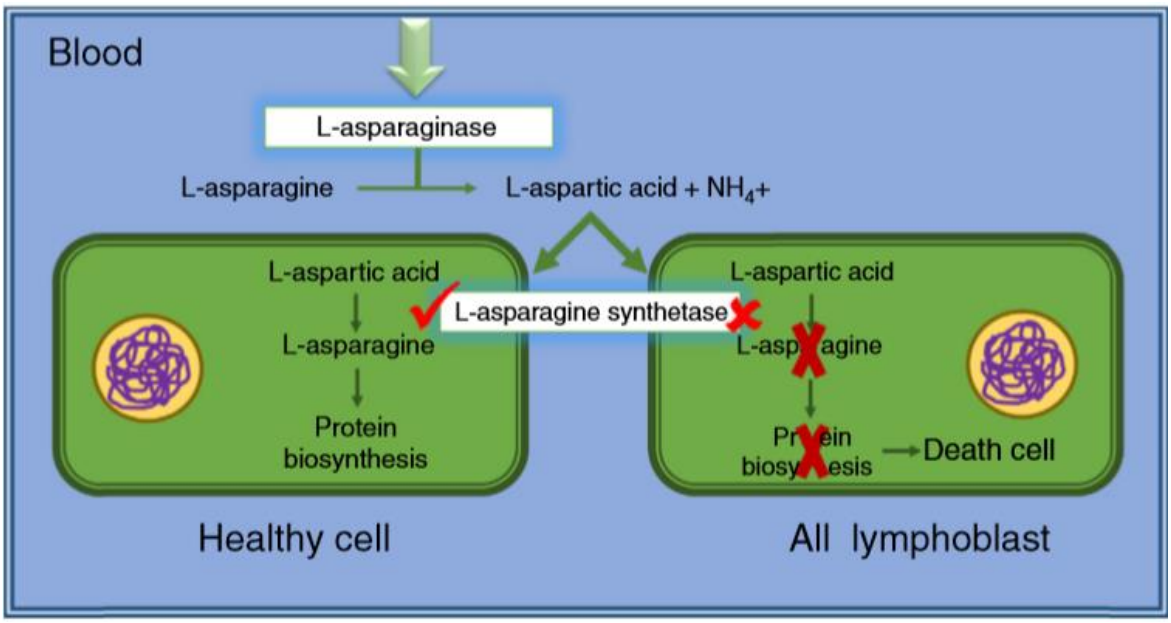


Figure 2 Antineoplastic action of L-Asparaginase (Van den Berg 2011; Jorge et al. 2016)

The use of L-Asparaginase as a chemotherapeutic specialist is fundamentally originated on its property of hydrolyzing L-asparagine. Infusion of L-Asparaginase into the circulatory system quickly drains the plasma pool of L-asparagine. The tumor cells have a bizarrely high necessity for the L-asparagine. Normal cells make up their own aspargine by using aspargine synthetase. (Figure 2). Tumors with moderate to low dimensions of asparagine synthetase rely on serum L-asparagine, and are sensitive to exhaustion of extracellular asparagine. In this manner, acceptability of L-Asparaginase treatment is identified with the failure of the susceptible cells to upregulate asparagine synthetase action to an adequate level, in contrast to the normal cells (Hutson et al. 1997). Richards & Kilberg in 2006 reported that response pathway of amino acid in mammalian cells is intended to recognize and react to amino acid shortage by increasing aspargine synthetase mRNA expression in normal cells.

On the other hand, in tumor cells, asparagine synthetase expression is not up regulated, it may be because of suppression of global translation initiation and phosphorylation of initiation factors (e.g. eIF4e and eIF-2a) which is aided by starvation-activated ribosome-associated kinase. L-Asparaginase treatment causes specific starvation of the tumor cells which leads to drop of asparaginyl tRNA synthesis, hence peptide synthesis is limited. S-phase of the cell cycle increases because of L-aspargine reduction that causes nucleotide formation. L-Asparaginase treatment captures cell cycle of the malignant cells at the G1 stage before DNA degradation. Diffusion of ammonium ions into the cytosol changes the pH, which leads to apoptosis of tumor cells (Ueno et al, 1997).

3.4 Endophytic fungi as producers of L-Asparaginase

Endophytes are micro-organisms colonizing the inner tissues of plants, under the epidermis of cell without making any obvious mischief or any negative impact on the host. They live inside the intercellular spaces of the tissues. It appears that they may infiltrate the living cells (Strobel 2003).

Endophytic fungi are valued for their ability to produce bioactive compounds (Chow et al. 2015). Endophytic bioactive compounds are integrated in novel drug discoveries due to their wide variety of biological activities such as antibiotic, anticancer, antioxidant and anti-inflammatory agents (Jawahar *et al.* 2014). L-Asparaginase is one of the enzymes produced by endophytic fungi.

Main applications of L-Aparaginase is in the food industries and clinically. It reduces acrylamide formation formed in the processing (frying and baking) of starchy foods due to Maillard's reaction between aspargine and reducing sugars. This property of asparaginase is used in medical to treat ALL in children and youthful grownups. Millions of people are suffering from ALL worldwide. It is considered as malignant tumor of bone marrow in which initial lymphoid precursors substitute typical bone marrow hematopoietic cells. It is an endemic disease which infects mainly children and young adults. In the past reports, it is well known that asparaginases have been used in the management of ALL for last 4 decades (Seiter et al. 2018).

Modified Czapek Dox media has been used to screen L-Asparaginase producing fungi where aspargine is used as sole nitrogen source. L-Asparaginase producing MCD agar plates turn yellow to pink which indicates the pH change because of conversion of L-aspargine into ammonia and aspartic acid (Theantana et al. 2007).

3.5 Qualitative estimation of L-Asparaginase

Modified Czapex Dox's (MCD) agar medium was used to assess the L-Asparaginase activity qualitatively. To carry out this activity an indicator, phenol red and substrate L-asparagine as were used. Control plates were maintained without adding asparagine. Cultures were inoculated on the MCD plates and incubated for 3 days at 26±2°C. Production of L-Asparaginase was indicated by pink coloured zone around the colonies (Gulati et al, 1997). These isolates were selected for determining L-Asparaginase activity.

3.6 Quantitative analysis of L-Asparaginase

The enzyme activity is determined by using modified method of Imada et al. 1973. In this technique optical density of released ammonia is measured at 450 nm, which further reacts with Nessler's reagent to give orange colour. Intensity of orange colour indicates the presence of total enzyme produced.

3.7 Shake flask culture method

A metabolic process in which complex organic compounds are broken down into simple compounds with the release of energy is known as fermentation. This breakdown takes place in anaerobic condition and in the presence of nutrients and metabolites. Submerged fermentation of L-Asparaginase from *Fusarium oxysporum* reported to give maximum enzyme activity of 182.5 U/ml on 5^{th} day of incubation (Yadav and Sarkar, 2014).

3.8 Protein assay

The important part of enzyme analysis is measuring the concentration of proteins. This is done by generally measuring the optical density with spectrophotometry in the UV range by adding dyes or interactions of copper with the protein. Protein content of centrifuged culture filtrates was done by Bradford's method (Kruger, 2009).

3.9 Optimization of parameters of fermentation for maximum enzyme production.

Production of efficient L-Asparaginase is influenced by different growth parameters, hence it is necessary to optimize different growth conditions in which fungi can produce maximum L-Asparaginase without any increase in cost. In the production of L-Asparaginase, parameters such as incubation period, pH, and temperature play a pivotal role and are optimized.

The accessible reports of optimization of fermentation conditions fit for producing maximum L-Asparaginases are referenced here:

Theantana et al. (2007), isolated 25 endophytic fungi from five Thai therapeutic plants – *Betula alnoides*, *Eupatorium odoratum*, *Adenanthera microsperma*, *Hiptage benghalensis*, and *Houttuynia cordata*; out of all isolates the maximum activity was shown by *Colletotrichum* sp. E1T9 from *Hiptage benghalensis* and when tried in vitro on CACO-2 (a cell line of Human Caucasian Colon Adenocarcinoma) totally diminished its survival rate in 48h; and furthermore

caused 70% abatement in the survival rate of HepG2 human Caucasian hepatocyte carcinoma cells in vitro.

From leaves and bark of *Aegle marmelos* (Linn), L-Asparaginase producing 12 strains of filamentous endophytic fungi were isolated (Patil et al. 2012). From marine algae, 64 L-asparaginase producing endophytic fungi were isolated. Maximum enzyme production was showned by *Fusarium* sp. 3 from *Sargassum wightii* thallus. Optimization of conditions such as pH and incubation time showed maximum enzyme production that was after 5 days and at pH 6.2. High concentration of aspargine in the media showed maximum enzyme production (Thirunavukkarasu et al. 2011).

25 endophytic fungi producing L-asparaginase from plants, *Oldenlandia diffusa, Murraya koenigii, Pereskia bleo,* and *Cymbopogon citratus* were isolated. Mostly *P bleo* isolates showed asparaginase activity ranging from 0.0069-0.025 µM mL^{-1} min^{-1} (Chow & Ting 2014)

Maximum enzyme activity by *Fusarium equiseti* and *Aspergillus terreus* 0.681 U mL^{-1} was estimated on the 6th day of incubation at pH 7.0 and temperature of 30°C at 120 rpm. Sucrose (0.2%) was found to be the best carbon source. Ammonium nitrate (0.4%) and sodium nitrate (0.4%) produced maximum amount of L-Asparaginase (2.08 U mL^{-1} and 2.01 U mL^{-1}). (Uzma et al. 2016).

Nagarajan et al. 2014 first reported fungal endophyte producing L-Glutaminase free L-Asparaginase after 96 h of incubation.

There is a conditional need to search for such endophytes, which may prompt the novel microbial strains isolation. Since plants are likewise eukaryotes, it would seem conceivable that the metabolites produced by the phytoendophytes have negligible lethality towards higher life forms.

CHAPTER 4 MATERIALS AND METHODS

4.1 Source of endophytes

Pre-isolated endophytic cultures isolated in CORE lab were used to carry out the research project. Table 3 shows the given culture isolates, their code and the host plant.

Table 3 Isolated cultures, their code and their host plants

S.No.	Host plant	Culture code
1	*Tinospora cordifolia*	GR
2	*Tinospora cordifolia*	GR3
3	*Tinospora cordifolia*	GS1
4	*Tinospora cordifolia*	GS3
5	*Tinospora cordifolia*	GS4
6	*Tinospora cordifolia*	GL2
7	*Terminalia arjuna*	AL2
8	*Terminalia arjuna*	AL4
9	*Terminalia arjuna*	AJ4
10	*Terminalia arjuna*	AF2
11	*Terminalia arjuna*	AL3
12	*Taxus baccata*	T6

4.2 Sub-culturing of endophytic fungi

In this study, pre-isolated fungal cultures of *Tinospora cordifolia* (Giloy), (GR, GR3, GS1, GS3, GS4, GL2), *Terminalia arjuna* (AL2, AL4, AJ4, AF2, AL3), and *Taxus baccata* (T6) were taken. These isolates were sub-cultured and screened for production of L-Asparaginase.

4.3 Qualitative screening for L-Asparaginase

The media used for plate assay was Modified Czapex Dox (MCD) media composing of L-asparagine (1%), KCl (0.052%), $MgSO_4.7H_2O$ (0.052%), KH_2PO_4 (0.152%), $FeSO_4.7H_2O$ (0.001%), glucose (0.2%), and agar (1.5%). A stock solution of 2.5 % phenol red was prepared (pH 6.2) with 95% ethanol and then 3 mL of this was added to 1000 mL of MCD medium. A

5 mm mycelia plug of an endophytic fungus was cut from the growing edge of the mycelia and was placed on the petri plate containing 25 mL of MCD medium. These plates were then incubated for 72 h at 26±2°C. MCD medium was initially yellow in colour but after the secretion of L-Asparaginase from mycelia it formed a pink coloured zone. (Gulati et al. 1997). Pink zone diameter of each plate was measured.

4.3 Quantitative analysis of L-Asparaginase

Nesslerization method was used to estimate L-Asparaginase activity. Shake flask method was used to carry out the fermentation process. A standard curve for protein estimation by Bradford's method and a standard curve of ammonium sulphate was made to estimate the amount of ammonium released by the crude enzyme.

4.3.1 Shake flask method for production of L-Asparaginase

L-Asparaginase producing endophytic fungal cultures were sub-cultured and incubated at 26±2°C for seven days. Optimization of L-Asparaginase was done in modified CD medium composing of L-asparagine (1%), KCl (0.052%), $MgSO_4.7H_2O$ (0.052%), KH_2PO_4 (0.152%), NH4SO4 (0.5%), $FeSO_4.7H_2O$ (0.001%), glucose (0.2%) and the pH was adjusted to 6.5 (Kumar et al. 2011; Thirunavukkarasu et al. 2011). Plugs of endophytic fungal cultures 7 days old were introduced into the medium and incubated at 120 rpm, 26±2°C for 5 days (Suryanarayanan et al. 2011). After an incubation period of 5 days, the mycelia was separated from the broth by muslin cloth. Centrifugation of the separated broth was done at 10,000 rpm for 15 min. The filtrate after separation of the mycelia was estimated for the enzyme L-Asparaginase by Nesslerization method (Imada et al. 1973).

4.3.2 Standard graph of ammonium sulphate

Ammonium sulphate (1.179 g) was weighed and dissolved in water and the volume was made up to 100 mL. Afterwards, solution was diluted with distilled water to make 1 µmole of ammonium per mL. Using this as a stock, various concentrations of ammonium sulphate was made as shown in table 4.

Table 4 Preparation of various concentrations of ammonium sulphate

Conc. of ammonium sulphate (µmole/mL)	**Stock (µL)**	**Distilled water (µL)**	**Nessler's reagent (mL)**
0.05	0.375	7.125	1
0.1	0.75	6.75	1
0.2	1.5	6.0	1
0.4	3.0	4.5	1
0.6	4.5	3.0	1
0.8	6.0	1.5	1
1.0	7.5	0.0	1
Blank	0	7.5	1

Absorbance was taken in spectrophotometer at 450 nm and the standard curve was made.

4.3.3 Standard curve for protein estimation (Bradford's method)

Coomassie brilliant blue G250 (100mg) was weighed and put into 50 mL of 95 % ethanol and followed by addition of 100 mL orthophosphoric acid. Thereafter, volume was made to 200 mL by distilled water and stored in refrigerated amber bottles. For use, 1 volume of concentrated solution was mixed with 4 volumes of distilled water.

BSA (1mg/mL) was used as standard protein. In 5 test tubes, different concentrations (0.1, 0.2, 0.3, 0.4, and 0.5) of BSA were made. To this, Bradford's reagent (5 mL) was added. Blank tube was prepared by adding distilled water and Bradford's reagent. In order to develop blue colour, the test tubes were vortexed and kept undisturbed for 5 min. Absorbance at 595 nm was recorded against blank tube. Standard curve was made considering X-axis as concentration of protein and Y-axis as absorbance at 595 nm. From this standard curve, the concentration of protein in the sample was calculated.

4.3.4 Estimation of L-Asparaginase by Nesslerization

Modified method of Imada et al. 1973 was used to analyse quantitatively the activity of L-Asparaginase in the medium. The culture filtrate was used as crude enzyme. A reaction mixture was set, containing 0.1 mL of 40 mM L-Asparagine, 0.5 mL of 0.5 M tris HCl buffer (pH 8.2), and 1 mL of culture filtrate (the reaction volume was made to 2 mL by adding distilled water) at 37°C for 30 min. After that 0.5 mL TCA was added to stop the reaction. Blank tubes were set by adding the crude enzyme after adding TCA.

After stopping the reaction, 0.1 mL of above reaction mixture was added to a new tube containing 3.7 mL of distilled water and 0.2 mL of Nessler's reagent and were incubated for 10 minutes at room temperature. Measuring the absorbance at 450 nm gives the amount of ammonium released during the reaction (Thirunavukkarasu et al. 2011). One international unit (IU) of L-asparaginase is the amount of enzyme required to release 1 μmol of ammonia in 1 min at 27°C (Imada et al. 1973).

$$\text{Specific activity of enzyme} = \frac{(\mu\text{mole of } NH_3 \text{ liberated}) \times (2.5)}{(0.1) \times (30) \times (1) \times \text{mg of protein per mL}}$$

2.5 = Initial volume of reaction mixture in mL

0.1 = Volume of reaction mixture used in final reaction in mL

30 = Incubation time in minutes

1 = Volume of enzyme used in mL

4.4 Optimization of fermentation factors for L-Asparaginase production

L-Asparaginase production from endophytic fungi depends mainly on factors such as pH, temperature, incubation period. In order to attain high yields these factors must be optimized.

4.4.1 Effect of Incubation time

The enzymatic activity of the filtrate was measured by inoculating 5 mm culture disc in 250 ml flasks having 50 mL of MCD medium (pH 6) with asparagine (1%) at various incubation periods from day 1 to day 8 at 30°C and 120 rpm. To determine the enzyme activity clear supernatant was used.

4.4.2 Effect of pH

The enzymatic activity of the filtrate was measured by inoculating 5 mm culture disc in 250 ml flasks consisting of 50 mL of MCD medium with asparagine (1%) at various pH ranges of 3, 5, 7 and 9 at 30°C for 5 days and at 120 rpm. Adjustment of pH was done using 1N sodium hydroxide and 1N hydrochloric acid. To determine the enzyme activity clear supernatant was used.

4.4.3 Effect of Temperature

The enzymatic activity of the filtrate was measured by inoculating 5 mm culture disc in 250 ml flasks having 50 mL of MCD medium (pH 6) with asparagine (1%) at various temperature ranges of 25°C, 30°C, 37°C and 45°C for 5 days. The flask was kept for shaking at 120 rpm. To determine the enzyme activity clear supernatant was used.

4.5 Identification of endophytic fungus through ITS screening method (white et al. 1990).

1. 100 mg of fungal tissue was crushed with liquid nitrogen and was collected into 2 mL sterilized eppendorf.
2. After that 600 µL of pre-heated extraction buffer was added to tube followed by incubation at 65°C for 1 hr.
3. Tubes were centrifuged at 13,000 g for 20 min at 4°C to remove debris.
4. Supernatant was transferred to fresh microfuge tube.
5. 800 µL of Phenol:Chloroform:Isoamyl (25:24:1) was added to the supernatant containing tube.
6. Thereafter, the microfuge tube was centrifuged at 13,000 g for 20 min at 4°C to obtain aqueous layer.
7. After collecting aqueous layer in fresh tube, 800 µL of isopropanol was added to it in order to precipitate DNA.
8. Tube was centrifuged at 13,500 g for 20 min at 4°C and pellet was collected.
9. 70% ethanol was added to centrifuged tube containing pellet followed by centrifugation 13,500 g for 20 min at 4°C.
10. Finally, supernatant was discarded and the DNA was dissolved in 25 µL of sterilized MQ water.

4.5.1 Polymerase chain reaction

The ITS region of DNA isolated from endophytic fungus was amplified by using forward primer ITS1 and reverse primer ITS4. 25 µL of reaction mixture of PCR was prepared by adding the following contents in Table 4.

Table 5 Reaction mixture for PCR

Taq buffer	2.5 µL
dNTP's	2 µL
Forward primer	1 µL
Reverse primer	1 µL
$MgCl_2$ (50 mM)	0.5 µL
Taq polymerase	0.3 µL
Template	1 µL
MQ water	16.7 µL

The conditional PCR (Amplified biosystems, Gene Amp PCR system 2700) was used. PCR consists of preliminary denaturation at 95°C of 2 min and 35 cycles at 95°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 1 min.

4.6.4 Agarose gel electrophoresis

0.8% agarose gel was prepared by adding 0.32g agarose in 40mL TE buffer to evaluate the PCR product. The gel was run in an electrophoresis unit at 80 V for 90 minutes. After this the gel was observed under UV transilluminator.

4.6.5 Purification of amplified DNA

Purification of amplified DNA was done by using GeneJET PCR purification kit (Thermoscientific). The procedure was followed as instructed by the instructor.

BioEdit Sequence Alignment Editor Version 7.0.5 software was used for sequencing. The consensus sequences were then compared with the other DNA sequences in GenBank using Basic Local Alignment Search Tool. It was then used to explore the genus and species of endophytic fungus and phylogenetic tree was constructed (Chow et al. 2014).

CHAPTER 5 RESULTS AND DISCUSSION

Medicinal plants are home of numerous proficient bioactive metabolites producing endophytic fungi that have incredible medicinal and therapeutic worth. In this study, pre-isolated fungal cultures of *Tinospora cordifolia* (Giloy), (GR, GR3, GS1, GS3, GS4, GL2), *Terminalia arjuna* (AL2, AL4, AJ4, AF2, AL3), and *Taxus baccata* (T6) were taken. These isolates were sub-cultured and were screened for L-Asparaginase production.

5.1 Qualitative analysis for L-Asparaginase

Using plate assay, qualitative test was done on Modified Czapex Dox's medium. Positive results gave pink zone in MCD agar plates. The diameters of pink zone were measured (Table 5). Out of 12 fungal cultures, 10 fungal cultures gave positive result after 3 days of incubation at 26±2°C.

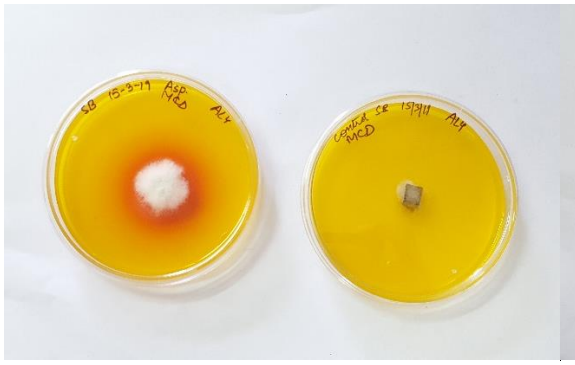
Table 6 Qualitative test for L-Asparaginase production

Isolate	**Result**	**Diameter of fungal colony (cm)**	**Diameter of pink zone (cm)**
GR	++	3.53	4.06
GR3	+++	4.16	7.36
GS1	+++	1.46	5.93
GS3	---	2.5	---
GS4	++	1.73	5.16
GL2	++	2.93	3.93
AL2	+	1.86	2.13
AL4	++	2.26	3.9
AJ4	+	1.36	2.03
AF2	++	3	4.63
AL3	---	2.76	---
T6	++	2.66	4.16

Note: (+) average activity; (++) good activity; (+++) excellent activity; (---) no activity

Average of 3 readings is given in the table.

A



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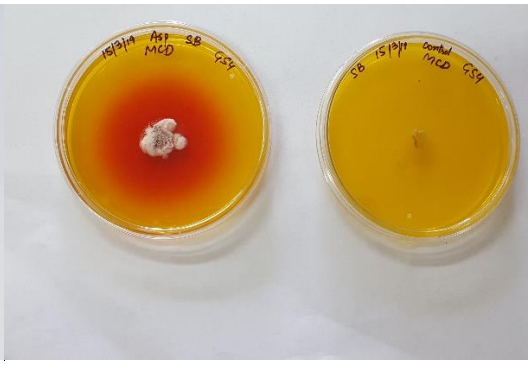

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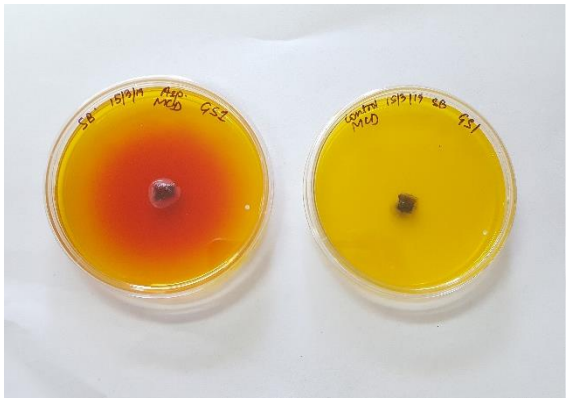

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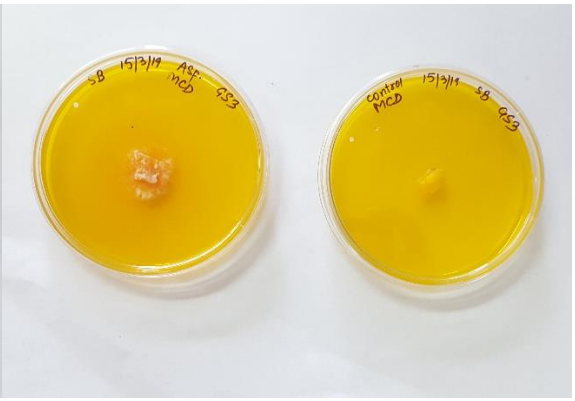


Figure 3 (A) AL4 plate showing positive result (B) GS4 plate showing positive result (C) GS1 plate showing positive result (D) GS3 plate showing negative result.

The isolates indicating positive test for L-Asparaginase were then used for further quantitative analysis.

5.2 Shake flask method for production of L-Asparaginase

Submerged fermentation of ten positive isolates was carried out for quantitative analysis to estimate the amount of L-Asparaginase. The flasks were placed at 120 rpm at 28ºC for 120 h in an incubator shaker. The fungal mycelium were separated by filtering through the muslin cloth. The separated broth was then centrifuged for 15 min at 10,000 rpm and at 4ºC. To determine enzymatic activity the filtered broth was used as crude enzyme.

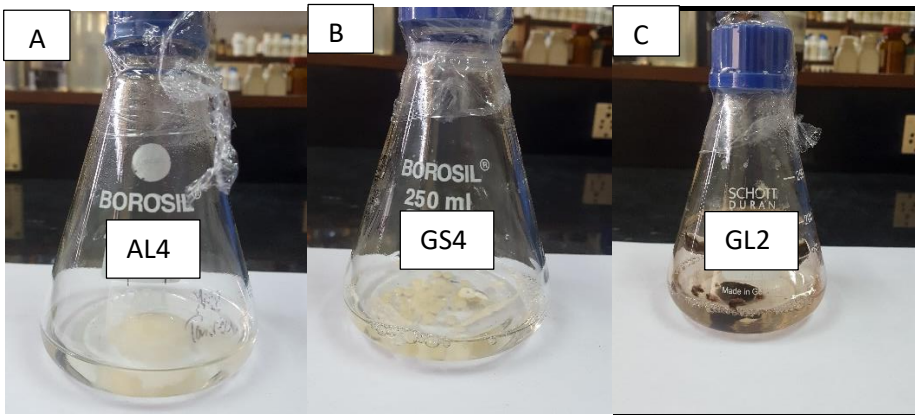


Figure 4 (A) fermentation broth of AL4 culture (B) fermentation broth of GS4 culture (C) fermentation broth of GL2 culture

5.2.1 Standard curve of Ammonium sulphate

To estimate the total ammonium released standard curve was plotted using ammonium sulphate as reference standard.

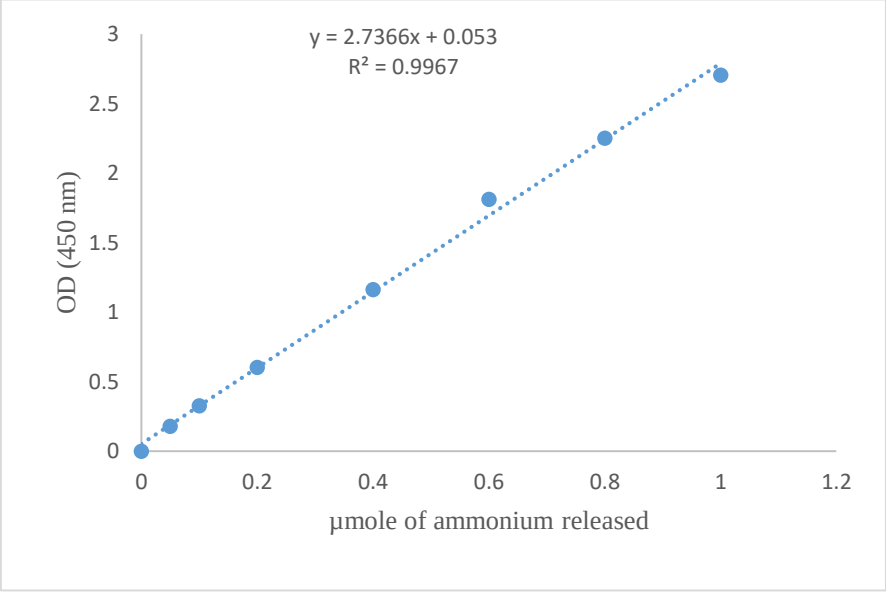


Figure 5 Standard curve of Ammonium sulphate

5.2.2 Standard curve for protein estimation

Standard curve was made to estimate the total protein. BSA was used as reference standard.

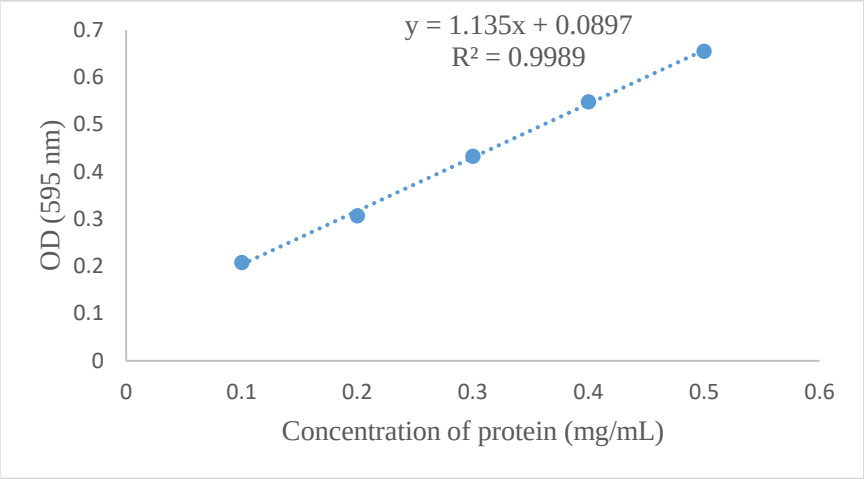


Figure 6 Standard curve for protein estimation

5.2.3 Estimation of L-Asparaginase by Nesslerization

After screening of L-Asparaginase, 10 cultures were selected for further estimation of enzyme by direct nesslerization. By calculating the total protein and ammonium released from the standard curves, specific enzyme activity was calculated by using the formula,

$$\text{Specific activity of enzyme} = \frac{(\mu\text{mole of } NH_3 \text{ liberated}) \times (2.5)}{(0.1) \times (30) \times (1) \times \text{mg of protein per mL}}$$

2.5 = Initial volume of reaction mixture in mL

0.1 = Volume of reaction mixture used in final reaction in mL

30 = Incubation time in minutes

1 = Volume of enzyme used in mL

The specific enzyme activity of 10 cultures were reported as follows:

Table 7 Specific enzyme activity (U/mg/mL) of endophytic fungal extracts

Culture	**Specific enzyme activity (U/mg/mL)**
AL2	0.3775
GS4	0.8426
GR3	0.7122
GS1	0.5354
AJ4	12.7155
T6	8.9479
AF2	1.2093
GR	2.2389
AL4	16.407
GL2	13.69
AL3	1.8956

Out of ten endophytic fungal cultures, one best L-Asparaginase producing culture was selected for further optimization.

5.4 Optimization of fermentation factors for L-Asparaginase production

Out of ten cultures, AL4 produced 16.407 U/mg/mL which was the maximum amount of L-Asparaginase produced and hence this endophytic fungal culture was chosen for further studies. Optimization of factors such as incubation temperature, incubation time and pH was done.

5.4.1 Influence of incubation period

For the maximum production of L-Asparaginase, the specific enzyme activity was determined by direct Nesslerization from Day 1 to day 8. The specific enzyme activity was calculated after Day 2, 3, 5, 7 and 8 to check for the L-Asparaginase produced into the medium.

On the 5^{th} day of incubation, maximum activity of enzyme was noted, after that gradual decrease in the enzyme production was observed up to 8^{th} day (Figure: 12). This might be because of change in pH, production of secondary growth inhibitors or due to nutrients depletion.

Thirunavukkarasu et al. (2011) reported that, from marine algae, 64 L-asparaginase producing endophytic fungi were isolated. Maximum enzyme production was showned by *Fusarium* sp. 3 from *Sargassum wightii* thallus. Optimization of conditions such as pH and incubation time showed maximum enzyme production that was after 5 days.

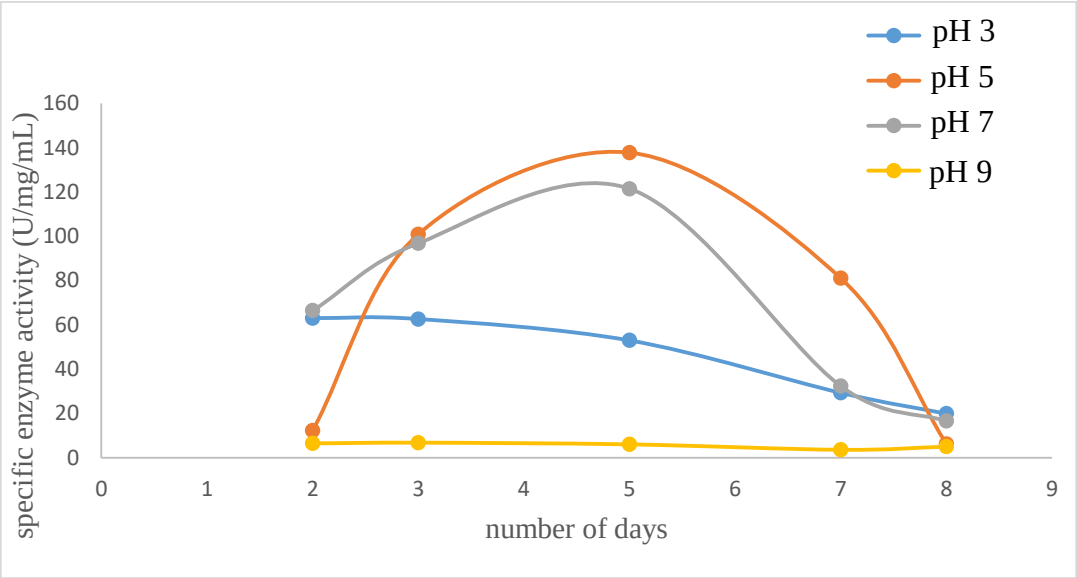


Figure 7 Effect of incubation period on L-Asparaginase

5.4.2 Influence of pH

Surface charges that are present on the amino acids are affected by the pH of the medium and hence influence the L-Asparaginase activity. The change in the pH can affect growth of micro-organisms and enzyme activity which can be inhibited or enhanced. Hence, it is important to select initial appropriate pH of the medium.

The maximum specific enzymatic activity of 137.80 U/mg/mL was noted at pH 5 by AL4 (Figure 13). A decrease in specific enzyme activity was noted before and after the optimal pH (pH 5). This can be because acidic and alkaline pH hinders the growth of endophytic fungus. Enzyme activity also decreases because acidic and alkaline pH cause partial denaturation of the enzyme which gives ionisable enzyme groups.

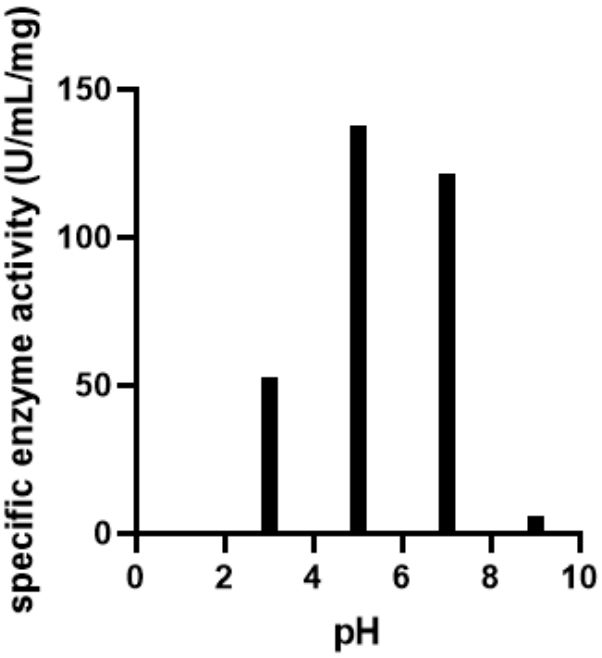


Figure 8 Effect of pH on L-Asparaginase

5.4.3 Effect of temperature

Temperature is an important fermentation factor for growth of endophytic fungus and L-Asparaginase production. The rate of reaction is affected by change in temperature, hence influences the enzyme activity rate also. The maximum specific activity of the enzyme was 31.58 U/mg/mL at 30°C (Figure 14). There was decline in the specific enzyme activity as the temperature increased. This can be due to the fact that increase in temperature ceases the growth of endophytic fungus, it also denatures the enzyme.

Uzma et al. (2016) reported that maximum enzyme activity by *Fusarium equiseti* and *Aspergillus terreus* 0.681 U mL^{-1} was estimated on the 6th day of incubation at temperature of 30°C at 120 rpm.

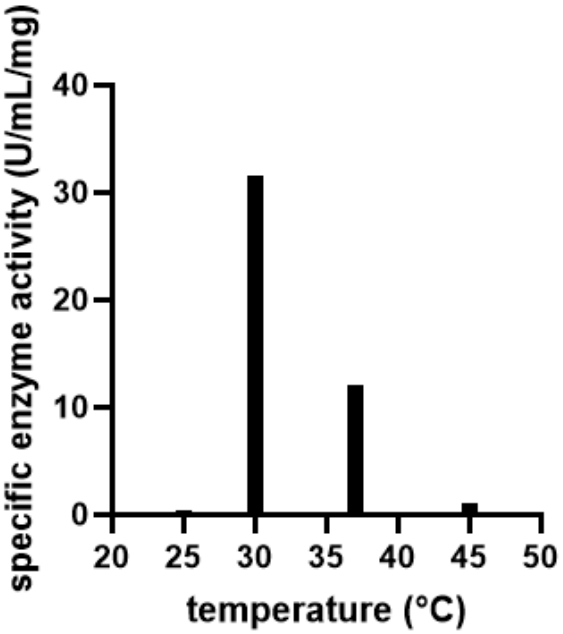


Figure 9 Effect of temperature on L-Asparaginase

5.5 Identification of endophytic fungus AL4

5.5.1 Identification based on morphology

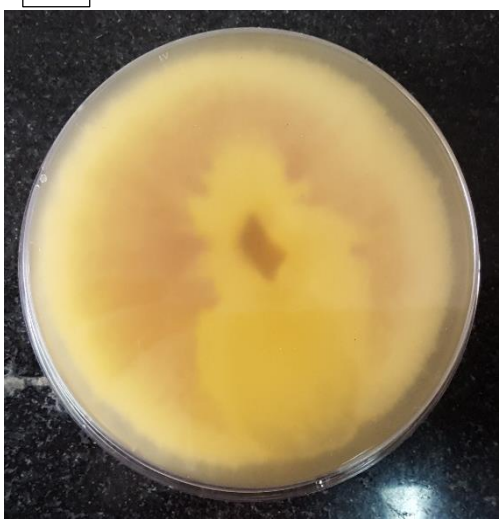
AL4 when grown on PDA plate showed that the fungus was white, cottony and reached 8 cm diameter of the plate in ten days. Long branched hyphae were observed when seen under microscope. Flattened oval shaped spores were also observed (Figure 10). The characteristics were similar to *Fusarium sp.* The classification of *Fusarium priliferatum* is given in table 8

Table 8 Classification of *Fusarium proliferatum*

Kingdom	Fungi
Division	Ascomycota
Class	Sordariomycetes
Order	Hypocreales
Family	Nectriaceae
Genus	*Fusarium*
Species	*F. proliferatum*

A

B



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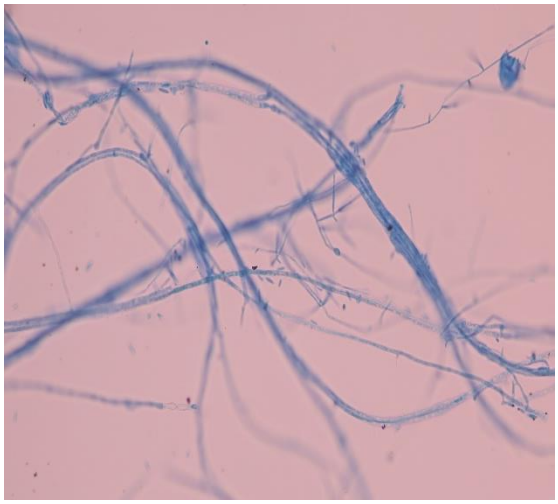

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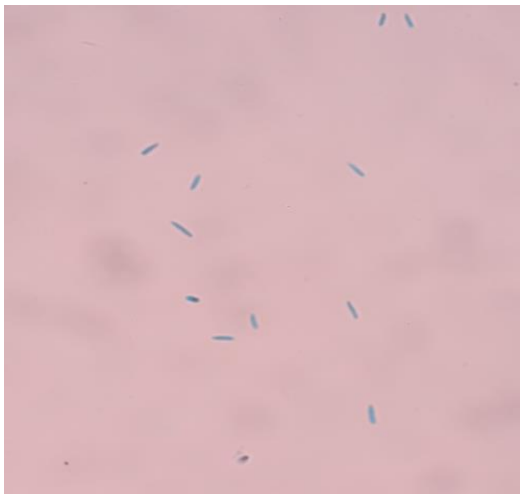


Figure 10 Morphological and microscopic view of AL4 (A) Front view of AL4 on PDA plate (B) Back view of AL4 on PDA plate (C) Hyphae of AL4 under 40X (D) Spores of AL4 under 40 X

5.5.2 Molecular based identification of endophytic fungi

Using CTAB method genomic DNA was isolated. PCR amplification of ITS regions were done using ITS1 as forward primer and ITS4 as reverse primer. By running electrophoresis PCR product was evaluated. Figure 11 shows the amplified DNA of AL4. 1 Kb ladder was loaded in lane 1 while amplified PCR products were in lane 3, 4 and 5. After this, the concentration using nanodrop was determined and was found to be 55.2 ng/µL. The A_{260}/A_{280} ratio was 1.90

and A_{260}/A_{230} was 1.14. The ITS amplified sequence of 569 bp obtained was run through blast n (Figure 12).

Homologous sequences were obtained in FASTA format that were then aligned using Multalign. Evolutionary relationships between homologous sequences were found by neighbor-joining method with bootstrapping of 1000. MEGA 10 software was used to construct phylogenetic tree (Figure 13). It confirmed the clustering of AL4 with *Fusarium proliferatum.*

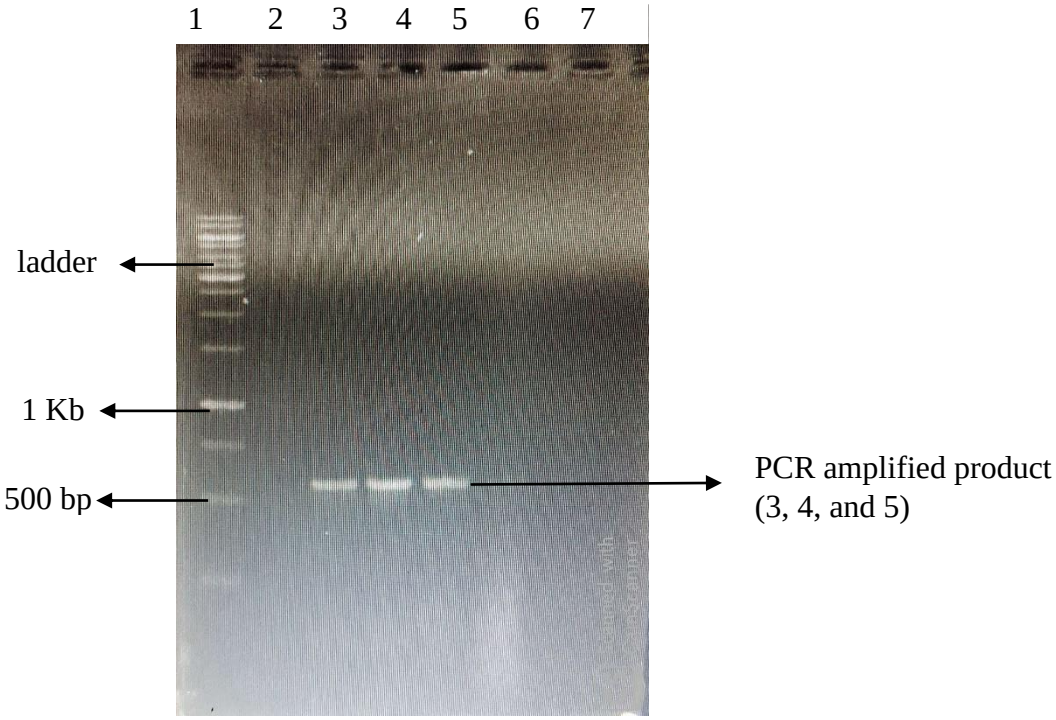


Figure 11 PCR amplified product of AL4

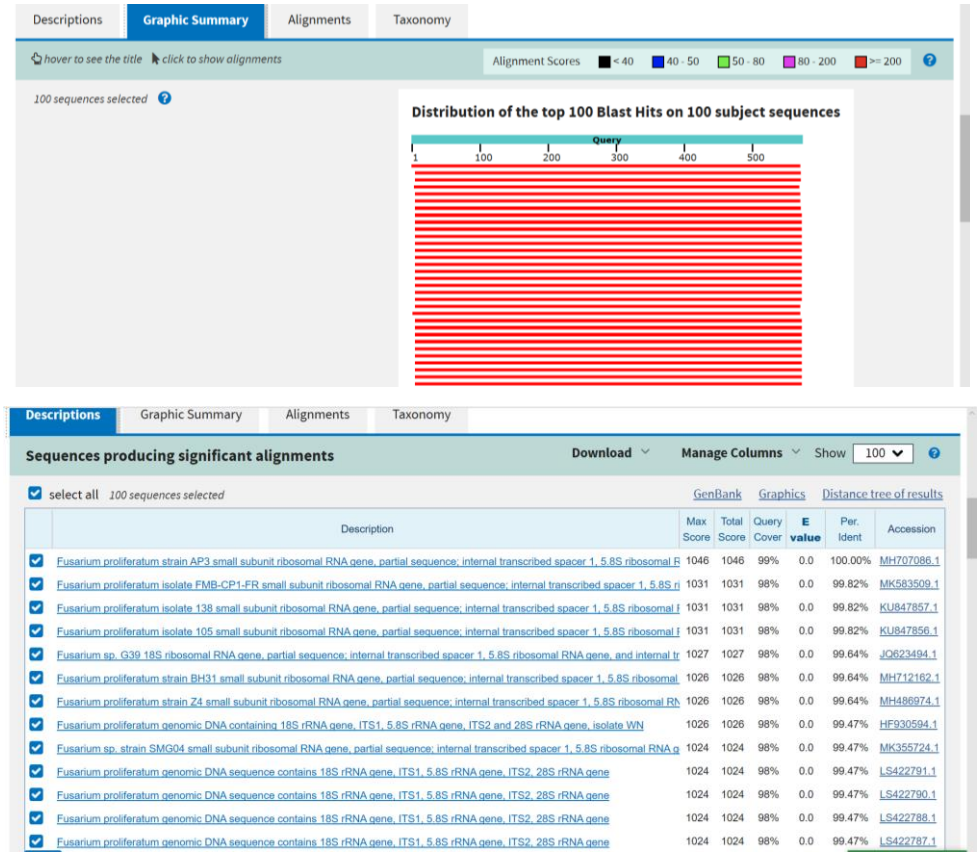
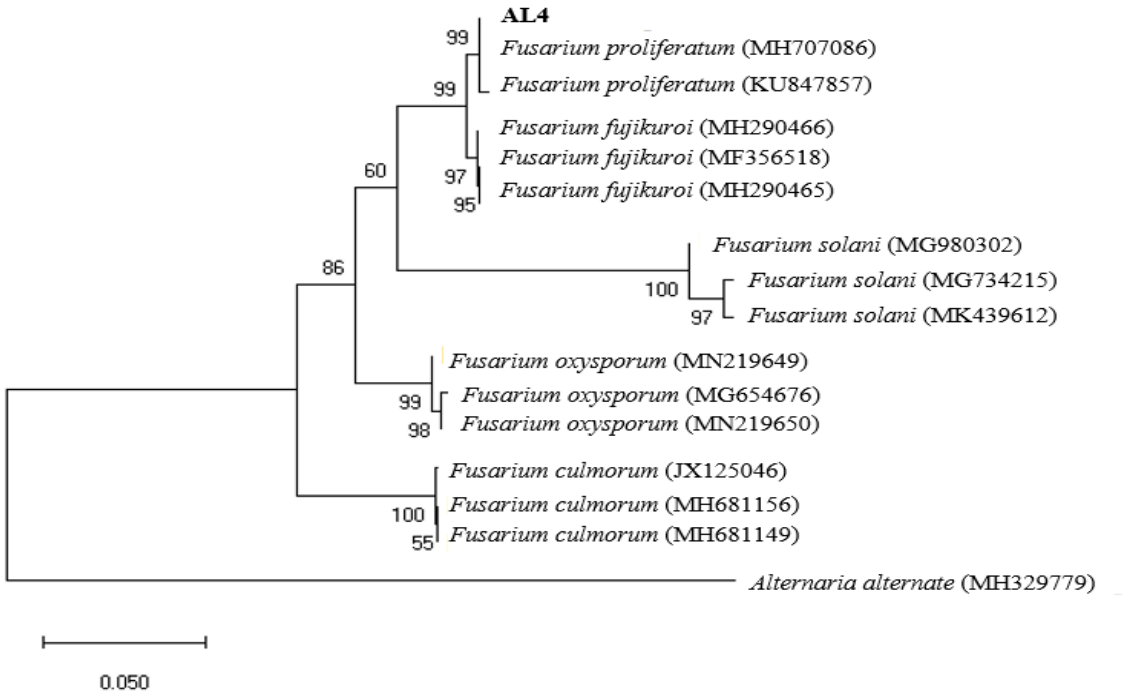


Figure 12 Blast n amplified sequence of AL4



Neighbour-Joining tree showing clustering of ITS amplified sequence AL4 with those related *Fusarium* sp.

CHAPTER 6 CONCLUSIONS

Endophytes are micro-organisms colonizing the inner tissues of plants, under the epidermis of cell without making any obvious mischief or any negative impact on the host. Endophytic fungi are valued for their ability to produce bioactive compounds.

The current study describes that endophytic fungi can be better source for the production of L-Asparaginase with low immunogenicity as they are eukaryotes and are more phylogenetically related.

Out of the ten endophytic isolates tested for quantitative analysis, AL4 showed maximum L-Asparaginase activity of 16.407 U/mg/mL. Afterwards, optimization of fermentation parameters for maximum production of L-Asparaginase from AL4 was done. The specific enzyme activity was determined on 2^{nd}, 3^{rd}, 5^{th}, 7^{th} and 8^{th} day of incubation. Maximum enzyme activity of 137.80 U/mg/mL was recorded at pH 5 on the 5^{th} day. Optimum temperature was found to be 30°C with the specific enzyme activity of 31.58 U/mg/mL.

PCR amplification of ITS regions were done using ITS1 as forward primer and ITS4 as reverse primer. By running electrophoresis PCR product was evaluated. The ITS amplified sequence of 569 bp obtained was run through blast n. Homologous sequences were obtained in FASTA format that were then aligned using Multalign. Evolutionary relationships between homologous sequences were found by neighbor-joining method with bootstrapping of 1000. MEGA 10 software was used to construct phylogenetic tree. It confirmed the clustering of AL4 with *Fusarium proliferatum.*

Endophytic fungi thus can be a novel source for the production of L-Asparaginase enzyme and can play a very important role in food, pharmaceuticals and other industries.

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